Food Chemistry 117 (2009) 1–8

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Potential of hydroxytyrosol-rich composition from olive mill wastewater as a natural disinfectant and its effect on seeds vigour response

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ARTICLE INFO

Article history:
Received 18 November 2008
Received in revised form 18 February 2009
Accepted 16 March 2009

Keywords:
Olive mill wastewater
Hydroxytyrosol
Agronomic seed vigour response
Seed disinfectant

ABSTRACT

Hydroxytyrosol-rich olive mill wastewater (HROMW) and hydroxytyrosol-rich composition (HRC) were prepared from olive mill wastewater using hydrolysis and post-hydrolysis purification processes. The HROMW and HRC showed powerful bactericidal and fungicidal activities against phytopathogens, and their minimal inhibition concentrations for fungi and bacteria were 7.18–57.4 mg l^{-1} and 7.18–14.4 mg l^{-1}, respectively. After 5 min of contact time, the disinfectant properties of the HROMW and HRC added at concentrations of 1.25% (dw/v) allowed for a reduction in bacterial viability by greater than 5 log units. However, a higher concentration of 1.5–3% (dw/v) or a longer contact time of 30 min were needed to achieve values for fungal viability reduction that were higher than the 4 log units recommended by EN 1275 [EN 1275 (1997a). Chemical disinfectants and antiseptics. Basic fungicidial activity. Test method and requirements (phase 1)]. HROMW and HRC were less effective with the most ubiquitous fungi, *Fusarium* spp., which needed 10% dw/v. The addition of HRC at 10% (dw/v) showed that the composition was a potent exogenous enhancer of growth that stimulated the seedling vigour of tomato and muskmelon, according to the conventional agronomic parameters for seed vigour. Compared to the control, the germination percentage, shoot weight, shoot height, and root length were all significantly enhanced in the HRC-treated seed plants. HRC was found to have effective disinfectant properties against seed-borne diseases. In treated seeds, the composition had significant effects on the control of damping-off disease groups at the pre-germination stage. HRC was also equally effective in the control of root rot diseases caused by *Fusarium sambucinum* and *Alternaria solani* as well as of wilts and even bacterial seed-borne pathogens. HROMW was also found to be as effective as HRC in terms of its efficacy against the three seed-borne diseases mentioned above.

1. Introduction

Root rots are reputed to be among the most devastating plant diseases. They are caused by a complex of fungal pathogens resident in the soil and have often been considered a major constraint to the establishment and development of plants in nurseries, glass houses and crops worldwide (Agrios, 2005). They are often associated with significant yield losses or decline in productivity (Tu, 1992). Studies have often reported that those fungi, either individually or associated, have severe effects on seed germination and young seedlings; observations of antagonism and synergism phenomena would then follow (Davet, Ravise, & Baroduy, 1980). Reports have also emphasised that those pathogens constitute the major factors behind the low productivity rates in many plantation fields. Based on the plant organ or growth stage that they affect, fungal disease pathogens can be divided into three groups: damping-off, root rot and wilts. Damping-off cause seed rot in pre-germination or germination stages or plant death after germination. Root rots cause rot and/or destruction of the root, restricting the absorption of water and nutrients. Wilts appear after flowering, and rarely at the juvenile stage of growth. Accordingly, many seed phytopathogenic bacteria are able to survive on dried seeds for extended periods (Ciafardini & Zullo, 2003). They, therefore, do not influence the establishment of plants but are the major means for the long-term spread of the disease. Rapid emergence can reduce root rot (Phillips, 1989); hence the presence of root rot can be directly related to the use of different sowing techniques, particularly those that support a favourable microclimate for their development (Valenciano, Casquero, & Boto, 2004). The disinfection of seeds enhances seed germination rates, increases plant growth, improves plant emergence and reduces damping-off (Gupta, Mathew, Shyam, & Sharma, 1999).

The application of pesticide seed treatment on various crops results in the improvement of plant emergence because it reduces plant mortality and losses caused by damping-off and root rot

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doi:10.1016/j.foodchem.2009.03.069

(Tu & Zheng, 1993), thus improving the overall yield. Nevertheless, disinfectants, such as sodium hypochlorite, or fumigants, such as methyl bromide, can cause serious toxicity problems in young plants, as well as present risks to handlers and the environment (Soriano, Porras-Piedra, & Porras-Soriano, 2006). They can also cause irreparable damage to the metallic structure of green houses (Ciafardini & Zullo, 2003). Physical methods, such as heat treatment, are not adequately appropriate for application and often produce large amounts of unviable seeds (Soriano et al., 2006).

During the last few decades, the severe limitations of the use of methyl bromide prior to its complete phase-out seem to have triggered immense interest among researchers in the search of alternative solutions. In fact, the use of natural substances as alternative substitutes to synthetic chemical pesticides has often been recommended, particularly because they are less persistent and are known to have fewer non-targeted toxic impacts than traditional agrochemical aggregates. More pertinent to the aims and objectives of the current study, olive mill wastes, particularly olive mill wastewaters, have recently been reported to offer promising opportunities to overcome the problem at hand.

Olive mill wastewaters (OMW) are known to contain a number of biologically-active substances with promising potentials. The phytotoxic and antimicrobial properties of these residues have been extensively investigated and are associated with the presence of phenolic compounds (Mekki, Dhouib, Aloui, & Sayadi, 2006; Obied et al. 2005). Several investigators have reported on the inhibition of plant and microbial growth by low-molecular-weight phenols present in OMW (Fiorentino et al., 2003). Hydroxytyrosol has been identified as one of the major natural phenolic monomers present in OMW that has powerful antimicrobial activity (Fiorentino et al., 2003). Many compounds, however, still remain unidentified and controversy still persists over the exact type and amounts of phytotoxic components in OMW. Raw OMW exhibits a broad spectrum of toxicity against bacteria, fungi, plants, animals and human cells (Capasso et al., 1995; Obied, Bedgood, Prenzler, & Robards, 2007). However, OMW fractional extracts and isolated biophenols demonstrate selective or minimal toxicity (Capasso et al., 1995). For instance, Gonzalez, Moreno, Quevedo-Sarmiento, and Ramos-Cormenzana (1990) found that the antimicrobial activity of OMW phenolic acids (tested separately) did not coincide with the inhibitory effect of OMW. Some investigators have evaluated the recovery of the biological activity in soil after its treatment with OMW. Piotrowska, Iamarino, Rao, and Gianfreda (2006) observed a complete recovery of seed germination 42 days after OMW had been applied at 40 m^3 ha^{-1}. Yangui, Rhouma, Gargouri, Triki, and Bouzid (2008a, 2008b) reported on the phytopathogen suppression capacity of OMW. This helped demonstrate that OMW could be safely used as a pre-plant or seed disinfectant in plantation fields with no phytotoxic effect on plant development and crop yield.

This being so, the present study was undertaken to evaluate the potential that natural phenolic aggregates taken from olive mill wastewater might have as a biobased pesticide against a variety of seed infections. It aimed to evaluate the hypothesis that a series of biochemical compounds that are naturally-occurring in OMW could act against various seed infections. Treatment with purified and unpurified hydroxytyrosol-rich composition, and vigour-related growth parameters were also evaluated.

2. Materials and methods

2.1. Plant material

Seeds of tomato (*Lycopersicon esculentum*) and muskmelon (*Cucumis melo*) were obtained from Agricultural Garden Seeds (Sfax, Tunisia) in 11/2007 and were also obtained from the "National Institute of Agronomic Research of Tunisia (INRAT), under organic conditions and stored under normal conditions. Seeds were washed five times with sterile water, surface sterilised with 10% (v/v) commercial bleach for 15 min, and subjected to 3–4 further washes in sterile distilled water.

2.2. Preparation of hydroxytyrosol-rich composition

2.2.1. Preparation of hydroxytyrosol-rich OMW (HROMW)

A sample of about 20 l of fresh olive mill wastewater (FOMW) was taken in February 2006 from a three-phase continuous extraction factory located in Sfax, Tunisia. A fraction of 500 ml of this FOMW sample was used for physico-chemical characterisation. The pH and electrical conductivity (EC) were determined according to the standard of Sierra, Marti, Montserrat, Cruanas, and Garau (2001). Total organic carbon was determined by dry combustion (TOC Analyser multi N/C 1000). Total nitrogen was determined by Kjeldahl (1883) method. Chemical oxygen demand (COD) was determined according to Knechtel (1978) standard method. Phosphorus, iron, magnesium, potassium, sodium, calcium and copper were determined by atomic absorption (AAnalyst 200, Perkin–Elmer, Waltham, MA). The main characteristics of FOMW are: Total C measured 18.2 g l^{-1}, $N_{Kjeldahl}$: 0.5 g l^{-1} with a C:N of 36.4, P: 36.1 mg l^{-1}, K: 1.45 mg l^{-1}, Fe: 2.58 mg l^{-1}, Mg: 1.08 mg l^{-1}, Ca: 11.51 mg l^{-1}, Cu: 0.15 mg l^{-1}, Na: 1.59 mg l^{-1}, pH: 5.2, EC: 7.8 dS m^{-1}, and chemical oxygen demand was 63.5 g l^{-1}. The remainder of the FOMW sample was used to produce HROMW, according to the methods of Bilter et al. (2005) and Crea and Mateo (2008), by acidification with acetic acid to pH 3 and incubation for 6 months (from February to July) in the dark and at room temperature (varying between a minimum of 8–10 °C by night in February and a maximum of 38–40 °C by day in July). The supernatant was ultrafiltered using a 100 kDa pore size.

2.2.2. Preparation of hydroxytyrosol-rich composition (HRC)

In order to achieve the deodorisation, decolourisation and, above all, the removal/recovery of the hydroxytyrosol-rich composition, the filtered HROMW was passed through a series of XAD4, XAD7HP, and XAD16 adsorbent resins according to the procedure of Agalias et al. (2007). The hydroxytyrosol-rich composition obtained was concentrated 10 times to honeyed liquid under vacuum at 45 °C using a Büchi Rotavapor (Büchi Laboratories, Flawil, Switzerland).

2.2.3. Characterisation of fresh OMW (FOMW), hydroxytyrosol-rich OMW (HROMW) and hydroxytyrosol-rich composition (HRC)

Total phenols, total flavonoids and total flavonols were determined using Folin–Ciocalteu reagent according to the methods of Miliauskas, Venskutonis, and Van Beek (2004), Singleton and Rossi (1965) and Zqhishen, Mengcheng and Jimming (1999), respectively. Results were expressed on a dry weight basis as gallic acid equivalents (GAE), catechin equivalents(CE) and rutin equivalents (RE), respectively.

The identification and quantification of phenolic monomers were carried out by HPLC and LC–MS analysis as described by Bouaziz, Fki, Jemai, Ayadi, and Sayadi (2007). The main characteristics of fresh OMW (FOMW), hydroxytyrosol-rich OMW (HROMW) and hydroxytyrosol-rich composition (HRC) are given in Table 1 and Fig. 1.

2.3. Microorganisms

Fusarium sambucinum, *Verticillium dahliae*, *Alternaria solani* and *Pseudomonas syringae* pv tomato were originally isolated in 2003 from tomato plants that exhibited the main characteristic symp-

Table 1
Physicochemical characteristics of fresh OMW (FOMW), hydroxytyrosol-rich OMW (HROMW) and hydroxytyrosol-rich composition (HRC).

Characteristics	FOMW	HROMW	HRC
pH (25 °C)	5.2 ± 0.2	4.5 ± 0.2	6.5 ± 0.2
Total solids (%) (w/v)	6.28 ± 0.9	2.87 ± 1.9	13.1 ± 0.2
Mineral matter (%) (w/v)	0.68 ± 0.3	1.14 ± 0.1	5.7 ± 0.2
Volatile solids (%)(w/v)	5.7 ± 0.5	1.81 ± 0.2	7.4 ± 0.3
Total phenols (PyE) (%, w/dw)	5.45 ± 0.3	7.39 ± 0.38	27.5 ± 1.8
Flavono (CaE) (%, w/dw)	1.13 ± 0.06	2.30 ± 0.11	20 ± 0.8
Flavonols (RuE) (%, w/dw) 0.05	± 0.01	0.06 ± 0.01	0.8 ± 0.3
Hydroxytyrosol (%, w/dw)	0.3 ± 0.1	29.3 ± 3.4	52.7 ± 4.3
K (%, w/dw)	0.44 ± 0.02	0.28 ± 0.02	ND
Fe (%, w/dw)	0.26 ± 0.03	0.07 ± 0.01	0.41 ± 0.05
Ca (%, w/dw)	1.15 ± 0.1	0.21 ± 0.02	ND
Cu (%, w/dw)	0.02 ± 0.002	0.01 ± 0.002	0.01 ± 0.002
Mg (%, w/dw)	0.11 ± 0.01	0.11 ± 0.01	ND

Values are mean ± standard deviation of three repetitions.
CaE: catechin equivalent.
ND: not detected.
PyE: pyrogallol equivalent.
RuE: rutin equivalent.

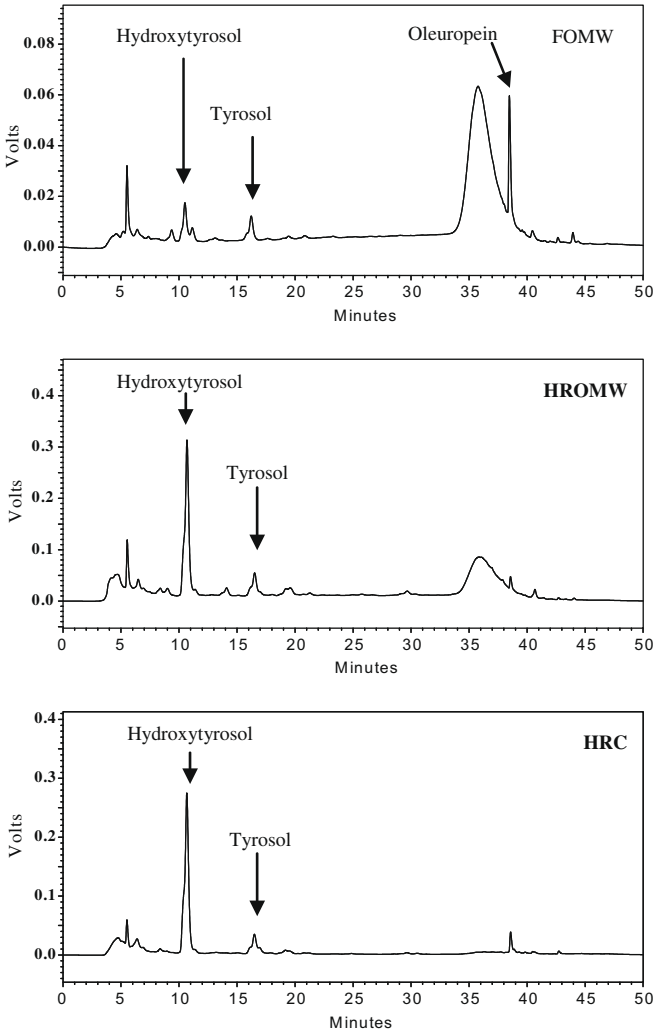

Fig. 1. HPLC chromatogram (UV 280 nm) of fresh OMW (FOMW), hydroxytyrosol-rich OMW (HROMW) and hydroxytyrosol-rich composition (HRC).

toms and were identified and provided by Institut de l'Olivier de Sfax, Tunisia. *Xanthomonas campestris* seed-borne phytopathogenic bacterium producing leaf spot symptoms in cruciferous plants was kindly provided by Pr. Maria Lopez from IVIA, Spain. For long-term conservation, the isolates were deposited at the Centre of Biotechnology of Sfax culture collection. For short-term conservation, bacteria were subcultured on nutrient agar (Difco; BD, Franklin Lakes, NJ) and fungi on malt extract agar (Difco) slant tubes at 25 °C for 72 h, then stored at 4 °C. Prior to use, the bacterial strains were cultured overnight on nutrient broth (Difco) and inocula were prepared by adjusting the turbidity of each bacterial culture to reach an optical comparison to that of a 0.5 McFarland standard, corresponding to approximately $1–5 \times 10^6$ CFU ml^{-1}. Fungi were cultured on malt extract agar plates for 7 days until sporulation, then the spore suspension of each fungal culture was prepared by pouring sterile buffered dilution water (SBDW) containing 0.1% of Tween 80 over the individual fungal cultures. The concentration of spore suspensions was determined by using a haematocytometer (Thoma cell) and adjusted to $1–5 \times 10^6$ spores per ml.

2.4. Bioassays

2.4.1. Antimicrobial activity

Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC).

MICs, MBCs and MFCs were determined by NCCLS (2000) broth dilution method. The HROMW and HRC were 2-fold serially diluted for bacteria in nutrient broth or for fungi in malt extract broth. MIC was taken as the interval of concentration between the highest dilution of HROMW and HRC that showed no detectable growth and the subsequent dilution. MFC was determined by sub-culturing all tubes that showed no visible growth.

2.4.2. Bactericidal and fungicidal activities

The bactericidal and fungicidal activities of the HROMW and HRC as natural disinfectants were evaluated according to the European Standard methods EN 1276 (1997b) and EN 1275 (1997a) under dirty conditions (3 g l^{-1} bovine albumin, 300 mg kg^{-1} $CaCO_3$) and the method of choice was the dilution-neutralisation method with 3% Tween 80, 3% saponin, 0.1% histidine, 0.1% cysteine as neutraliser and sterile hard water (300 mg kg^{-1} $CaCO_3$) as diluents. The HROMW and HRC-test concentrations were 0.5, 0.75, l.0, and 1.25% (w/v) for bactericidal activity and 1.25, 1.5, 3.0, and 6.0% (w/v) for fungicidal activity. The contact time and test temperature were: t = 5 min ± 10 s and θ = 20 °C ± 1 °C, respectively.

2.4.3. Phytotoxicity

Phytotoxicity was assessed by the determination of the germination index at two times, 36–72 h for tomato and 25–50 h for muskmelon, according to the standard method of Zucconi, Forte, Monac, and Beritodi (1981). Post-germinated seeds were transplanted in a sterile potting mix, as described in Section 2.4.4 below. Traditional seed vigour biomarkers were determined based on the total numbers of seedlings that fully emerged, shoot height, shoot weight and root length.

2.4.4. Seeds disinfection bioassays

Seed disinfection was carried out in two steps. The first step consisted of assessing all microorganisms in Petri dishes; the second involved the transplantation of the germinated seeds of fungi that exhibited symptoms of damping-off, root rot and wilt at the juvenile stage of tomato growth in plug trays.

For each microorganism, 50 g of bleached seeds were infected with 1% (v/w) of the microbial suspension which contained approximately 10^6 spores ml^{-1} for phytopathogenic fungi or 10^8 cfu ml^{-1} for phytopathogenic bacteria, and the infected seeds were then dried at room temperature for about 8 h. The contaminated seeds were divided into four parts. The first part was disinfected by placing the seeds in contact with a 10% (dw/v) sodium

hypochlorite solution for 30 min and then rinsed three times with sterile distilled water. The second part was mixed with HROMW at the ratios of 10% and 1% (dw/v) for fungi-infected and bacteria-infected seeds, respectively. The third part was mixed with HRC, using the same dosage rate used for HROMW. The fourth fraction was used, untreated, as control. After 24 h of incubation at room temperature, the seeds were transplanted into Petri dishes (10 seeds/Petri dish of 9 cm in diameter) containing sterile potting mix (twice autoclaved). The Petri dishes were then incubated at 27 °C for 52 and 72 h for muskmelon and tomato seeds, respectively. After that, the contaminated seeds were counted and the germination index was calculated, according to the method of Zucconi et al. (1981). This step was assessed to determine whether HROMW and HRC had disinfestation or disinfection properties. Accordingly, post-germinated tomato seeds were planted in a sterile potting mix (twice autoclaved) and grown in seedling plug trays (plug size 3 cm × 3 cm × 5 cm, 120 plugs per tray). The plug trays were placed under greenhouse conditions and monitored for a growing period of 4 weeks. The plants showing characteristic symptoms of the disease were noted.

2.5. Statistical analysis

The trial was established according to the randomised plots experimental design with three triplicates, including 30 plants in each replicate. Data were subjected to analysis of variance using SPSS software (Version 11; SPSS Inc., Chicago, IL). Mean values among treatments were compared by the Duncan's multiple range test at the 5% ($p = 0.05$) level of significance.

3. Results

3.1. Concentration of hydroxytyrosol in FOMW, HROMW and HRC

The analysis (Table 1 and Fig. 1) showed the effect of hydrolysis of complex phenols, such as oleuropein, into simple phenols, such as hydroxytyrosol, which was observed as a reduction in the peak height at 34–40 min and the amplification of the hydroxytyrosol peak at 10.5 min of retention. Moreover, an almost complete reduction in complex phenols detectable at 280 nm was observed for HRC with the phenols removed by separation on resin. Hydrolysis occurred during the storage of acidified FOMW for 6 months, and purification steps was also observed in the analyses. As the stages of processes progressed a significant increase in the content of hydroxytyrosol, total phenols, flavonoids and flavonols was observed (Table 1). After hydrolysis, the hydroxytyrosol concentration shifted from 0.2 g l^{-1} corresponding to 0.3% (w/dw) in FOMW to 8.4 g l^{-1} corresponding to 29.3% (w/dw) in HROMW, and the hydroxytyrosol yield of this step was 0.82% (w/v) (Table 1). After decantation, filtration and separation on resins and 10-fold concentration by water evaporation, the hydroxytyrosol concentration increased to 69 g l^{-1}, corresponding to 52.7% (w/dw) in the HRC.

3.2. Fungicidal and bactericidal potential of HROMW and HRC

Table 2 shows MICs, MBCs or MFCs of HROMW and HRC determined on the five microorganisms tested. Both HROMW and HRC inhibited the two bacteria tested, with MIC and MBC values ranging from 7.18 to 14.4 mg l^{-1}. For fungal strains, the HROMW MIC values ranged from 7.18 to 28.7 mg l^{-1} and the HRC MIC values ranged from 14.4 to 57.4 mg l^{-1}. As for those of HROMW and HRC, the MIC values were equal to the MFC values for *A. solani* and *V. dahlia* but only half those of the MFC values for *F. sambucinum*.

HROMW and HRC were bactericidal against both types of phytopathogens, *Pseudomonas* and *Xanthomonas*. Indeed, more than 5 log units of reduction in viability of *P. syringae* were achieved at a dosage rate of at least 1%, even after 5 min (Table 3). But, higher HROMW and HRC concentrations (1.25%) were actually needed to obtain 5 log units of reduction in viability for *Xanthomonas* spp. (Table 3). In the case of phytopathogen fungi, the antifungal activity shown by HROMW and HRC was moderately significant when compared to bactericidal activity. The fungicidal activity of HROMW and HRC against *A. solani* was obtained at the concentration of 3% after 5 min of contact. When the lowest dose recommended by the standard test method EN 1275 1.5% (w/v) was employed, the fungicidal activity against *V. dahlia* was achieved with the two products tested after 30 min of contact time (Table 4). *F. sambucinum* was not sensitive to the two extracts tested. In fact, a dosage rate (10%) that was higher than the one recommended by the standard test method EN 1275, 6% (w/v) was necessary to achieve the 4 log units of reduction in the viability of this fungus (Data not shown).

3.3. Phytotoxicity effect of HROMW and HRC on plant species

Seed germination was conducted on both HROMW (one dose was applied, 10%, which was used for the disinfection treatment) and HRC, and were used in comparison to those treated with water (negative control) and with sodium hypochlorite (positive control). The results showed that seed germination was strongly inhibited for the two species studied when treated with both sodium hypochlorite and HROMW (Fig. 2). HRC did not show any inhibitory effect on seed germination and the two crops represented close ratios of germination when compared to the control subjected to water treatment (Fig. 2). The application of HRC improved the germinative energy of the seeds. The index of germination at the half-period of germination was 36 and 25 h for tomato and muskmelon, respectively, and was significantly higher than that of the negative controls (Fig. 2). Treatments with HROMW and sodium hypochlorite, however, have yielded a germinative energy that was lower than the optimum (ratio <50%).

Initial vigour response was measured using traditional agronomic parameters, such as germination percentage, root length and shoot weight and height. Results indicated that vigour response was, indeed, higher in response to HRC than the other treatments (Fig. 3). HROMW, on the other hand, negatively affected the

Table 2
Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC) of HROMW and HRC using NCCLS (2000) method.

Microorganisms	HROMW		HRC	
	MIC (mg l^{-1})	MBC/MFC (mg l^{-1})	MIC (mg l^{-1})	MBC/MFC (mg l^{-1})
Alternaria solani	7.18–14.36	7.18–14.36	14.36–28.72	14.36–28.72
Fusarium sambicinum	14.36–28.72	28.72–57.44	28.72–57.44	57.44–114.88
Verticillum dahliae	7.18–14.36	7.18–14.36	14.36–28.72	14.36–28.72
Pseudomonas syringae	7.18–14.36	7.18–14.36	7.18–14.36	7.18–14.36
Xanthomonas campestris	7.18–14.36	7.18–14.36	7.18–14.36	7.18–14.36

Table 3
Evaluation of bactericidal activity of HROMW and HRC using European Standard EN 1276 (1997b) method in dirty conditions and 5 min of contact time.

Bacteria test	N: Bacterial test suspension (cfu ml^{-1})	HROMW		HRC	
		Test procedure at concentration % (dw/v) .			
		1.0	1.25	1.0	1.25
		Reduction in viability (cfu ml^{-1})			
Pseudomonas syringae	N: 2.1×10^8	1.5×10^5	2.6×10^5	1.3×10^5	1.9×10^5
Xanthomonas campestris	N: 1.3×10^8	$<10^5$	1.3×10^5	$<10^5$	1.1×10^5

Table 4
Evaluation of fungicidal activity of HROMW and HRC using European Standard EN 1275 (1997a) method in dirty conditions and 30 min of contact time.

Fungi test	N: Spore test Suspension (cfu ml^{-1})	HROMW			HRC		
		Test procedure at concentration % (dw/v)					
		1.5	3.0	6.0	1.5	3.0	6.0
		Reduction in viability (cfu ml^{-1})					
Fusarium sambicinum	N: 5.5×10^6	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$
Alternaria solani	N: 1.1×10^6	$<10^4$	1.4×10^4	6.9×10^4	$<10^4$	1.1×10^4	2.7×10^4
Verticillum dahliae	N: 2.0×10^6	2.0×10^4	3.5×10^4	1.2×10^4	1.5×10^4	2.3×10^4	2.3×10^5

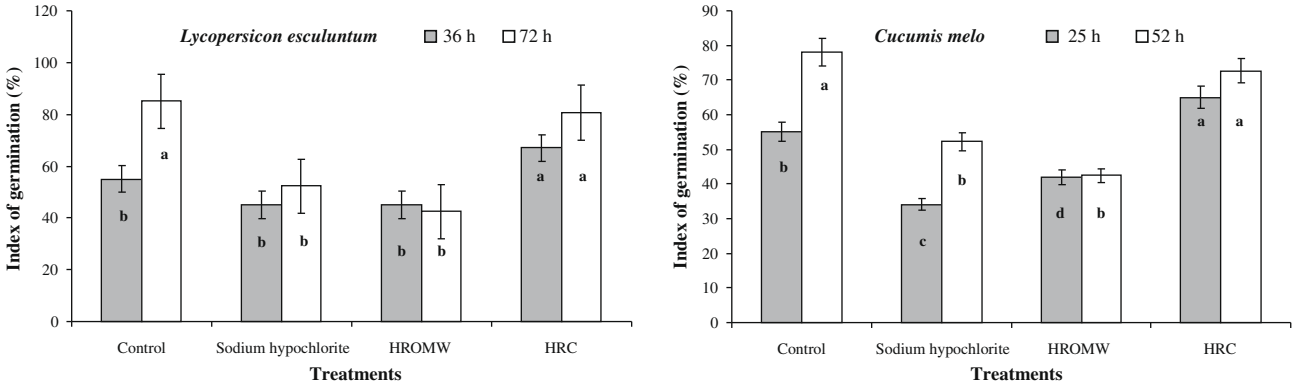

Fig. 2. Germination index of *Lycopersicon esculuntum* and *Cucumis melo* determined on distilled water (control), sodium hypochloride, HROMW, and HRC. Histograms followed with different letters are significantly different according to the test of Duncan ($p = 0.05$).

vigour response of seeds with a significant reduction in root heights as well as in shoot heights and weights. These results were comparable to those of sodium hypochlorite treatment, where the seed vigour biomarkers were drastically affected.

3.4. Disinfectant property

The trials carried out in Petri dishes demonstrated that HRC was able to inhibit the growth of the seed-borne diseases used. In fact, HRC allowed for a highly significant control of the damping-off disease groups on the treated seeds in the pre-germination stage (Figs. 4 and 5). With regard to the application of HROMW, highly significant differences were observed with the treated seeds, as opposed to the inoculated seeds (Figs. 4 and 5). A higher damping-off control was observed in the seeds subjected to HRC + *X. campestris* when compared to those subjected to HRC + *P. syringae* pv tomato (Fig. 4).

Furthermore, the trials carried out in plug trays demonstrated that HRC was able to control root rot caused by *F. sambucinum* and *A. solani*. Under greenhouse conditions, HRC also allowed for a highly significant control of this group of seed diseases (Fig. 5). Wilts were also significantly decreased in seeds subjected to HRC + *V. dahliae* treatment (Fig. 5). Likewise, HROMW was noted to be as effective as HRC against the three seed-borne diseases under investigation.

4. Discussion

Given the fact that OMW is generated for only 3–4 months a year, and to assure a continuous industrial activity of hydroxytyrosol production, a sufficient quantity of OMW must be provided during the year. In a previous survey Feki, Allouche, Bouaziz, Gargoubi, and Sayadia (2006) demonstrated that the storage of OMW is doubly beneficial; first, hydroxytyrosol concentration increased from 0.98 to 3.5 g l^{-1} during 5 months of OMW storage. As well, OMW storage resulted in the abolition of the centrifugation step necessary for the elimination of suspended matter. Bilter et al. (2005) and Crea and Mateo, (2008) also demonstrated the addition of acid to OMW in an amount effective to produce a pH between 2 and 4, and incubating OMW for a period of 6–12 months until at least 75–90% of the oleuropein originally present in OMW has been converted to hydroxytyrosol. The HROMW remained black-coloured; consequently, further purification steps through resin separation were deemed necessary to recover the HRC from the HROMW.

The antimicrobial effect of HRC was revealed by MIC and proved through basic method against most phytopathogenic fungi and

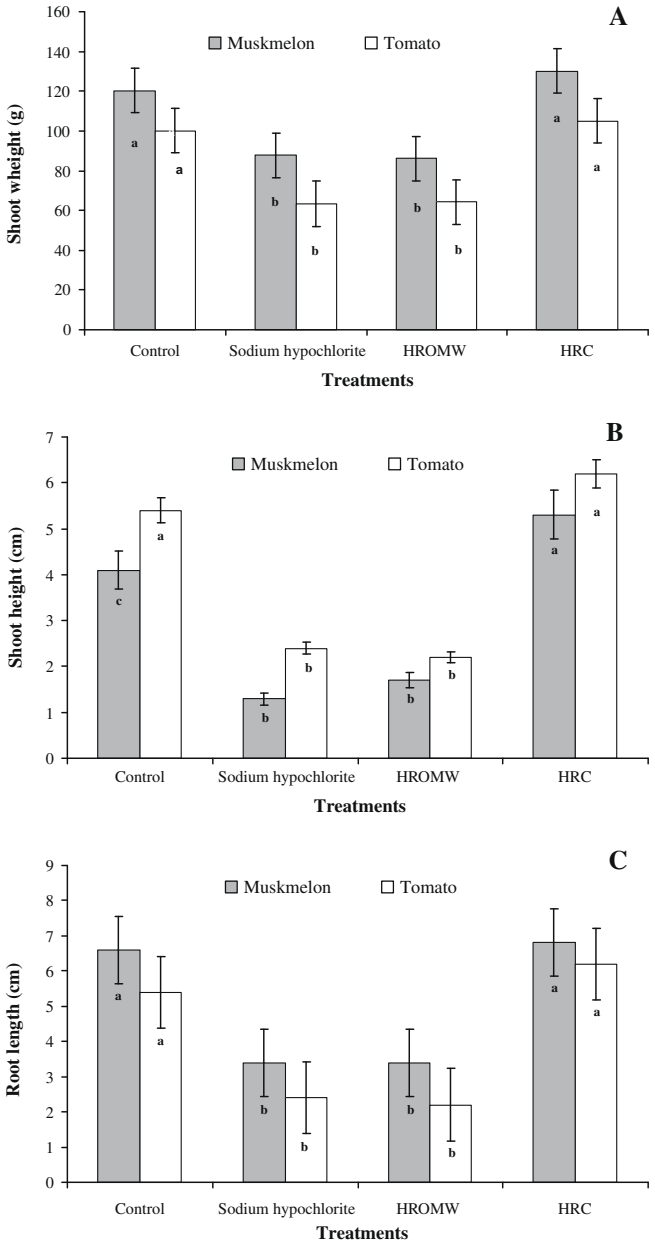

Fig. 3. The effect of germination index of distilled water (control), sodium hypochloride, HROMW, and HRC on (A) shoot weight; (B) shoot height; and (C) root length at 2 months after transplantation of tomato (*Lycopersicon esculuntum*) and muskmelon (*Cucumis melo*) plants. Histograms followed with different letters are significantly different according to the test of Duncan ($p = 0.05$).

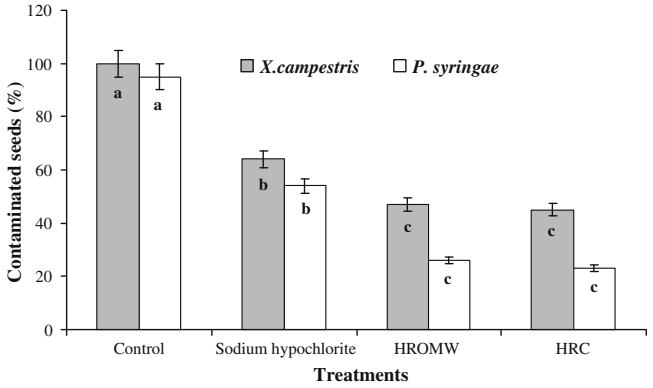

Fig. 4. Disinfection effect of water (control), sodium hypochloride, HROMW, and HRC on percentage of contaminated seeds by *Xanthomonas campestris* and *Pseudomonas syringae* pv. Tomato. Histograms followed with different letters are significantly different according to the test of Duncan ($p = 0.05$).

bacteria. Hence, most plant extracts show activity against Gram-positive bacteria. Activity against Gram-negative bacteria and fungi is also considered a critical measure of success (Ezzoubeiri et al., 2005). It was pointed out in previous studies, however, that Gram-positive bacteria are more susceptible to antimicrobial extracts of plants than the Gram-negative ones (Ezzoubeiri et al., 2005). The different activities against Gram-negative and Gram-positive bacteria may be rationalised by considering differences in cell wall composition. Gram-negative bacteria have a lipopolysaccharide component in their outer membrane that makes them more resistant to antibacterial compounds. As far as the current study is concerned, the hydroxytyrosol-rich composition inhibited the growth of Gram-negative bacteria within the recommended doses of application. The effectiveness of HRC could be attributed to the chelating of transition metals by polyphenols. Wong and Kitts (2006) reported that phenolic compounds are capable of chelating transition metals and lowering the reactivity of metal iron by forming an inert metal–ligand complex. Chelation of transition metals, such as iron and copper, reduces bioavaibility for microbial growth.

The control of tested phytopathogenic fungi is a very difficult process, even with the application of pre-harvest fungicides (Polashock, Ehlenfeldt, Stretch, and Kramer, 2005). Sampedro, D'Annibale, Ocampo, Satazi, and Gargia-Romera (2005)) demonstrated the capability of a saprophytic *Fusarium* to grow on a medium rich with phenolic compounds. This can explain the inefficacy of hydroxytyrosol-rich solutions against the soil-borne *Fusarium* strain. The effectiveness of HRC against other phytopathogenic fungi, namely *Verticillium* and *Alternaria*, can be related to the altering of microbial permeability, which can permit the loss of cytoplasmic macromolecules.

It is widely acknowledged, and as is found in numerous references, products that are rich in oleuropein demonstrate antimicrobial activity against a variety of viruses, bacteria, yeasts and fungi (Aziz, Farag, & Mousa, 1998; Bisignano et al., 1999; Fleming, Walter, & Etchells, 1973; Tassou & Nychas, 1995; Tassou, Nychas, & Broad, 1991). Since higher antibacterial effects were observed using HRC, the latter should be examined in terms of its powers as a new antimicrobial intensifier. The present *in vitro* study demonstrated that the experimental antiseptic and/or disinfectant were undeniably effective in inhibiting plant pathogens. This could be of particular interest to the nurserymen whose plants have always suffered from fungal disease pathogens. It is common for the nurserymen in Tunisia to reuse their propagation trays, which may contain the remains of contaminated substrates. Several approaches have been tried to remove the pathogens from those trays (Soriano et al. 2006), none of which is fully satisfactory. Disinfectants, such as sodium hypochlorite or formalin, can cause serious toxicity problems in young plants and create severe risks to handlers and the environment (Ciafardini & Zullo, 2003; Soriano et al., 2006). HRC can, therefore, be considered a strong alternative candidate for the current inadequate methods involving the removal of the harmful disinfectants in both seed and propagation trays.

HRC did not exhibit any inhibitory effects on seed germination and two crops presented a high germination ratio (>50%) even at half-period of germination. It can be inferred that the germination inhibition is basically due to the phenolic compounds that are present at high levels in HROMW. This is in line with previous findings by Mekki, Dhouib, Feki, and Sayadi (2008), Komilis, Karatzas, and Halvadakis (2005) and Piotrowska et al. (2006), which demonstrated that the germination inhibitory effect is partly due to the

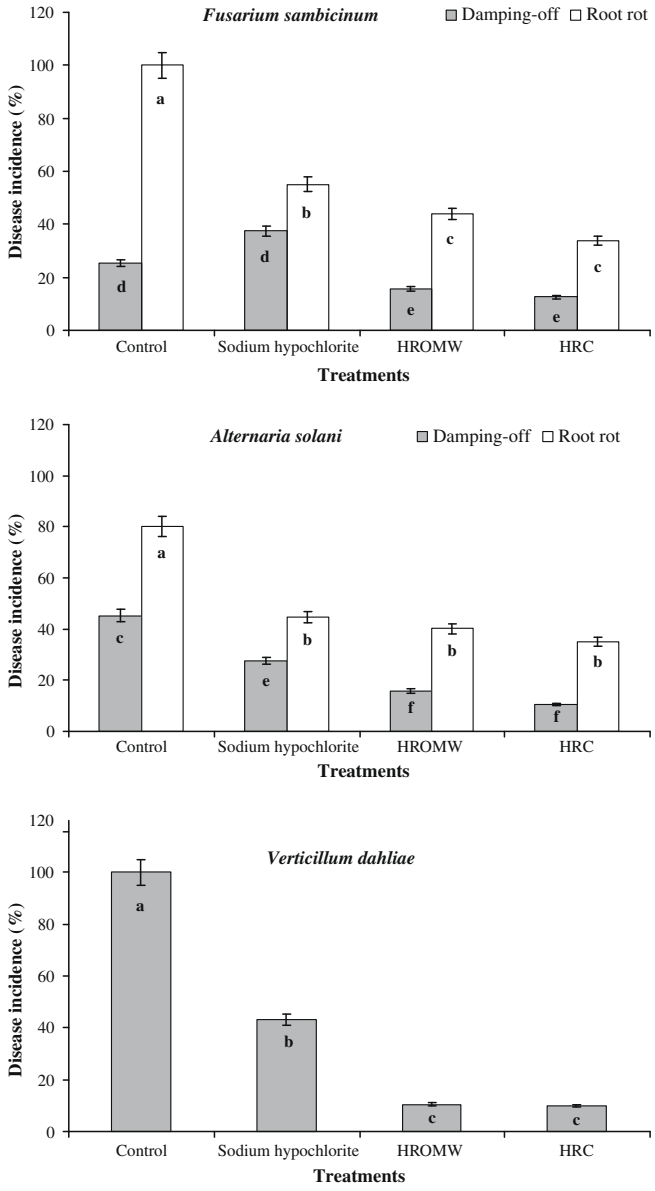

Fig. 5. Effect of treatments by distilled water (control), sodium hypochloride, HROMW, and HRC on the percentage of damping-off and root rot incidence of tomato (*Lycopersicon esculuntum*) plants caused by *Fusarium sambicinum* and *Alternaria solani* and on the percentage of *Verticillum* wilt incidence of tomato (*Lycopersicon esculuntum*) plants caused by *Verticillum dahliae*. Histograms followed with different letters are significantly different according to the test of Duncan ($p = 0.05$).

high amount of phenolic compounds and partly to the high molecular weight of phenolics present in untreated OMW. Gonzalez et al. (1990) reported that the antimicrobial activity of phenolic acids (tested separately) did not coincide with the inhibitory effect of OMW. Obied et al. (2007) also reported that crude OMW has a broad spectrum of toxicity against bacteria, fungi, algae, plants, animals and human cells and added that fractionated OMW extract and isolated biophenols, on the other hand, demonstrate selective or minimal toxicity. This could explain the reason why HRC had an antimicrobial effect with no phytotoxic effects (Table 1). In addition, the relatively high salinity of the HROMW solution may affect the germinability of the crops. This seems similar to the findings of Ramana, Biswas, Kundu, Saha, and Yadava (2002) who reported that in some crop species, such as tomato, cucumber, chilli and onion, the increase in the concentration of the highly saline effluent was paralleled by a similar decrease in the percentage of germination. The germination rate, in particular, is immensely important, for the increase in germination is usually followed by an improvement in overall seedling performance (Parera & Cantliffe, 1991). The findings of the current study indicated that the tomato and muskmelon seedlings treated with HRC similarly showed higher and average germination rates, when compared to the HROMW solution and control seedlings, respectively. Root lengths and shoot weights and heights are all vital indices of seeds' vigour. Results indicated that vigour response, in terms of those factors, is a useful approach to meeting productivity of crops (Perry, 1978). With regard to improving seed vigour through the stimulation of phenolic synthesis, it has often been suggested that exogenously applied phenolic antioxidants may be able to stimulate the endogenous phenolic content in plants (Randhir & Shetty, 2003). This stimulation is hypothesised to involve the concurrent activation of antioxidant enzyme metabolites which are dependent upon reductants (NADPH) obtained as a by-product of carbon flux through the pentose phosphate pathway, which is, itself, the source of sugar phosphate precursors required for phenolic synthesis (Randhir & Shetty, 2003).

Quick seedling emergence and even stands are essential in maximising the yield of all crops. The use of high quality, disease-free seed is the first step to producing stands. The application of HRC treatment improved the germinative energy measured at half-period of germination. Germinative energy can play an important role in achieving quick and uniform seedling emergence and in reducing damping-off incidence, thus improving the yield. Gupta et al. (1999) reported that the use of fungicides is effective in enhancing germination, emergence and growth as well as in reducing damping-off. In addition, accelerated germination is reported to help improve stress resistance and enhance overall plant growth and productivity (Pattan, Gothkar, Joshi, Chivasa, & Nyamudeza, 2001). This could account for the usefulness of the HRC solution for seed disinfection.

A further transplantation of post-germinated seeds demonstrated the compelling disinfection effect of both HRC and HROMW, and not a mere surface disinfestation of seeds. Yields were higher when seeds were treated by HRC, which led to earlier and better emergence and to improved control of both damping-off/root rot and wilt caused by *Fusarium*/*Alternaria* and *Verticillium*, respectively. Valenciano et al. (2004) demonstrated that the use of pesticides for the protection and/or disinfection of seed improved yields because it increased the number of established plants. A negative correlation exists between yield and the presence of disease in the soil and/or in the seeds because the loss of plants from root rot has a huge impact on yield (Pedroza, Teliz, de la Torre, & Campbell, 1994). Thus treatment is a fundamental operation because the initial stage in plant development is the most susceptible to adverse environmental conditions (Valenciano et al., 2004). Sodium hypochlorite is the most used solution for the disinfection of seeds as well as of the equipment used in the production of plants (Soriano et al., 2006). Nevertheless, and due to its highly corrosive nature, sodium hypochlorite must be used with extreme caution. As a matter of fact, it can seriously hurt the seeds and reduce germination. This justifies the urgent need for research on efficient alternative substitutes for the current disinfestations, disinfections or protections used for seed and propagation equipment. The results obtained demonstrate that HRC represents a class of natural products that offers an efficient substitute for the currently used commercial corrosive disinfectants such as sodium hypochlorite. In fact, it can be used to control the most resistant fungal structure (chlamydospores) produced by the species of *Fusarium* (Mavrogianopoulos, Frangoudakis, & Pandelakis, 2000), *Alternaria*, *Verticillium*, and even *Xanthomonas* and *Pseudomonas*, without causing any damage to the germination ability of crops.

In short, HRC has proven to be a solution that: (1) has high element content; (2) promotes early vigour and improves plant health; (3) provides protection against pathogens which attack germinating seeds and emerging seedlings; (4) aids in the production of healthy vigorous crops and higher yields; (5) promotes quick emergence and crop uniformity; (6) is easy to use and simple to clean up with water; and (7) is environmentally safe and not harmful for plants, animals and humans.

Acknowledgements

The present research study was supported by the MESRST of Tunisia under Contract Program of the Bioprocesses Laboratory, ASTF and AUF, (PER-LBP) projects.

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Food Chemistry 117 (2009) 9–14

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

The effect of heat and light on the composition of some volatile compounds in wine

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ARTICLE INFO

Article history:
Received 7 November 2008
Received in revised form 12 February 2009
Accepted 16 March 2009

Keywords:
Wine
Volatile compounds
Solid phase microextraction
Thermal treatment
Irradiation

ABSTRACT

HS-SPME–GC–MS analysis of Aglianico del Vulture red wine from Southern Italy showed the presence of ethyl acetate, 3-methyl-1-butanol, 3-methylbutyl acetate, ethyl hexanoate, diethyl butanedioate, ethyl octanoate, and ethyl decanoate. Thermal treatment for 24 h in the 20–60 °C range showed that for most of the compounds analysed the simple transition from 20 to 30 °C induces a significant modification on the composition of volatile compounds. A sharp decrease was observed for ethyl acetate, diethyl butanedioate, and 3-methylbutyl acetate; a moderate decrease was observed for ethyl hexanoate, while an increase was observed for 3-methyl-1-butanol, ethyl octanoate, and ethyl decanoate. The temperature of 40 °C induces the maximum evolution of this type of compound. Higher temperatures induce a reduction of volatile compounds in wine. Irradiation of Barolo (red wine, Northern Italy), Amarone (red wine, Northern Italy), and Brunello di Montalcino (red wine, Central Italy), at 20 °C was performed with a 13 W neon lamp. 3-Methyl-1-butanol was affected by the irradiation. In the case of Barolo and Amarone, we observed a moderate increase, while, for Brunello di Montalcino, a decrease was observed. In the same way, esters were degraded during irradiation. In the red wine one or more components (phenols?) are able to protect esters from photodegradation. In diethyl butanedioate and ethyl hexanoate, octanoate, and decanoate we observed an initial increase of the concentration of the esters in some cases.

1. Introduction

Temperature changes can modify the amounts of volatile compounds in wine (Benítez, Castro, Natera, & Barroso, 2006; Cutzach, Chatonnet, & Dubourdieu, 2000; Pérez-Coello, Gonzáles-Viñas, García-Romero, Díaz-Maroto, & Cabezudo, 2003). Some years ago the analysis of sherry wine exposed to UV–vis radiation showed that an increase in the amount of volatile compounds can be observed (Benítez, Castro, & Barroso, 2003; Benítez et al., 2006). Esters and acids showed a sharp tendency to increase during irradiation; with the exception of 2-methyl-1-butanol, hexanol, and 2-methyl-1-propanol, the same tendency was found in alcohols present in wine. Also aldehydes and ketones increased during irradiation. This result was not in agreement with our report where solid phase microextraction was used in order to estimate the modifications occurred in volatile compounds in champagne after UV irradiation (D'Auria, Emanuele, Mauriello & Racioppi, 2003). In fact, while most of the alcohols found did not change their concentration after irradiation, a sharp reduction was observed in the amounts of esters (ethyl acetate, ethyl butanoate, ethyl 2-hydroxypropanoate, 3-methyl-1-butanol acetate, diethyl butanedioate, ethyl octanoate, and ethyl decanoate).

The esters in wine are important constituents of the aroma. In fact, the high variability of the ester fraction has an effect on the intensity of fruity notes in the aroma profile (Belitz, Grosch, & Schieberle, 2004). Furthermore, the results reported by Benítez et al. (2003, 2006) showed that the increase of the ester fraction was higher in topaz glass (cut-off near 600 nm, but 20% transmittance at 350 nm) than in transparent glass (cut-off at 300 nm). Also, the nature of the glass bottles can influence the organoleptic properties of the wine. Most of the wine is sold in supermarkets where the wine bottles are exposed for a large period to artificial light. This exposure can modify the aroma profile of the wine. We have to know the nature of all the modifications occurring in the wine after light irradiation in order to find how to prevent the loss of quality due to light irradiation.

In this report our results obtained irradiating some important Italian red wines (Barolo, Amarone, Brunello di Montalcino). We tested the temperature effect on the amount of volatile organic compounds on a red wine typical of Southern Italy (Aglianico del Vulture). We performed the irradiation of wine with a neon lamp in the original bottles in order to simulate the real situation of exposure of wine to light.

2. Materials and methods

2.1. General

Aglianico del Vulture is a typical red wine of Basilicata (Southern Italy). It is produced by using only Aglianico grapes, which are

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doi:10.1016/j.foodchem.2009.03.070

of Greek origin. The sample we used was made in 2002, showed an alcoholic grade of 13.5% and a volatile acidity of 0.40 g/l.

Barolo is produced in Cuneo's province, south–west of Alba, within the region of Piemonte. The wine is produced from the Nebbiolo grape variety. The Lampia, Michet, and Rosè types are authorised. The grapes mature at the end of September. The clusters are dark blue and greyish with an abundant wax that dresses the grapes. The form of bunches is lengthened, pyramidal, with small, spherical grapes with substantial peel. The leaves are of average size with three or five lobes. The bottle we used contained wine of 2001 and showed an alcoholic grade of 14%.

Amarone della Valpolicella is produced from Corvina, Rondinella, and Molinara desiccated grapes, Grapes are harvested perfectly ripe in the first two weeks of October, by carefully choosing bunches having fruits not too close to each other, to let the air flow. Grapes are allowed to dry, traditionally on straw mats. This process is called *rasinate* (to dry and shrivel) in Italian. The length of the drying process is typically 120 days but varies according to producer and the quality of the harvest. The most evident consequence of this process is the lost of weight: 35–45% for Corvina grapes, 30–40% for Molinara and 27–40% for Rondinella. Following drying, at the end of January/beginning of February, the grapes are crushed and go through a dry low temperature fermentation process which can last up to 30–50 days. The reduced water content can slow down the fermentation process, increasing the risk of spoilage and potential wine faults, such as high volatile acidity. After fermentation, the wine is then aged in barrels made from either French or Slovenian oak. The sample we used was bottled in 2003, showed an alcoholic grade of 15.5% and a volatile acidity of 0.92 g/l.

Brunello di Montalcino must be made 100% from the Brunello clone of Sangiovese. The current ageing requirements were established in 1998 and dictate that Brunellos are to be aged in wood for 2 years and at least 4 months in a bottle before release. Traditionally, the wines are aged 3 years or so in barrel, large oak casks that impart little oak flavour but allow for the controlled softening of the wine. Modernists use small barrique which impart a more pronounced oak flavor. There is a middle ground where the wine is aged in small barrique for a short time and then spends a longer sojourn in the traditional botte. The sample we used was dated 2002 and showed an alcoholic grade of 13.5%.

2.2. SPME analysis of wine

An SPME fibre coated with 100 μm of poly(dimethylsiloxane) (PDMS) phase (Supelco 57300-U, mounted on a Supelco 57330 support) was conditioned for 1 h at 250 °C in a stream of helium. A single fibre was used for the complete study. A blank run was performed after the analysis in order to confirm that no residual compound was polluting the fibre or the column. The headspace was generated from 10 ml samples placed in a 20 ml flask. The flask was sealed and heated for 20 min in an aluminium block maintained at 45 °C (40 °C in the flask). During this time, the fibre was maintained over the sample. The fibre was then introduced into the injection port of an HP6890 Plus gas chromatograph equipped with a Phenomenex Zebron ZB-5 MS capillary column (30 m × 0.25 mm ID × 0.25 μm film thickness; Phenomenex, Torrance, CA). An HP5973 mass selective detector (mass range: 15–800 amu; scan rate: 1.9 scans/s; EM voltage: 1435) was used, with helium at 0.8 ml/min as carrier gas. The injection port, equipped with glass insert (internal diameter 0.75 mm) was at 250 °C; the injection port was maintained in splitless node for all of the chromatographic analysis. A desorption time of 1.0 min was used. The interface was maintained at 230 °C. The oven was maintained at 40 °C for 2 min, then the temperature increased to 250 °C (at 8 °C/min); finally, this temperature was maintained for 10 min. All the analyses were performed in triplicate. The chromatograms obtained from the total ion current (TIC) were integrated without any correction for coelutions and the results were expressed in arbitrary surface units (asu). All the peaks were identified from their mass spectra by comparison with spectra in Wiley6 N and NIST98 libraries and by comparison with an authentic standard for the seven compounds reported below.

2.3. Calibration curves

Calibration curves were determined by using known amounts of every compound dissolved in ethanol/water 1:9 using the methodology described above. The tested compounds were dissolved in ethanol:water in order to obtain solutions containing 2, 10, 50, 100, 300 and 500 mg l^{-1} of the analytes. The area count obtained after the analysis is plotted against the concentration. The linear regression was determined by using least squares method. All the analyses were performed in triplicate. Results: ethyl acetate: slope = 0.1103 [area count $\times 10^{-6} \times l \times mg^{-1}$], $r^2 = 0.9834$; 3-methyl-1-butanol: slope = 0.0209 [area count $\times 10^{-6} \times l \times mg^{-1}$], $r^2 = 0.9974$; 3-methylbutyl acetate: slope = 0.0063 [area count $\times 10^{-9} \times l \times mg^{-1}$], $r^2 = 0.9880$; ethyl hexanoate: slope = 0.0062 [area count $\times 10^{-9} \times l \times mg^{-1}$], $r^2 = 0.9883$; diethyl butanedioate: slope = 0.0083 [area count $\times 10^{-8} \times l \times mg^{-1}$], $r^2 = 0.9977$; ethyl octanoate: slope = 0.0096 [area count $\times 10^{-9} \times l \times mg^{-1}$], $r^2 = 0.9993$; ethyl decanoate: slope = 0.0131 [area count $\times 10^{-9} \times l \times mg^{-1}$], $r^2 = 0.9884$.

2.4. Thermal treatment of wine

Wine (10 ml) was maintained in a 25 ml Pyrex sealed vial at each selected temperature (20, 30, 40, 50, and 60 °C) for 24 h. Each experiment was performed in triplicate.

2.5. Irradiation of wine in the bottles

The red wines were in topaz bottles. Before irradiation, analysis of the wines was performed drawing samples from the bottles using a syringe. The bottles were irradiated with a Lyvia ESL 13 W 2700 K 800 lm for 7 days. The bottles were maintained at 20 °C. The lamp was at a distance of 20 cm from the bottle. A sample was analysed every 24 h using a syringe from the same bottle every day.

3. Results and discussion

The object of our work is to verify the changes in the volatile compounds in wines exposed to the light present in a standard supermarket. First of all, as preliminary work, we tested the behaviour of a red wine when it is exposed to a thermal treatment. In wines sold in tropical countries the temperature of wine could increase, modifying the volatile compounds' composition. We used an Aglianico del Vulture wine, a typical wine of Southern Italy. We tested seven volatile compounds found in the wine at 20 °C. We analysed the presence of volatile compounds by using SPME. The analyses were performed on samples maintained at the selected temperature for 24 h. The results are reported in Figs. 1–7. The amount of ethyl acetate decreased until 30 °C, and then increased to reach a maximum at 50 °C (Fig. 1). The behaviour of diethyl butanedioate (Fig. 2) and ethyl hexanoate (Fig. 3) was similar. The amount of these esters decreased at 30 °C, reached a maximum at 40 °C and, then, decreased until disappearance. Also the behaviour of ethyl octanoate (Fig. 2) and ethyl decanoate (Fig. 2) was similar. The amount of these esters increased until 40 °C, and then decreased until disappearance. The amount of 3-

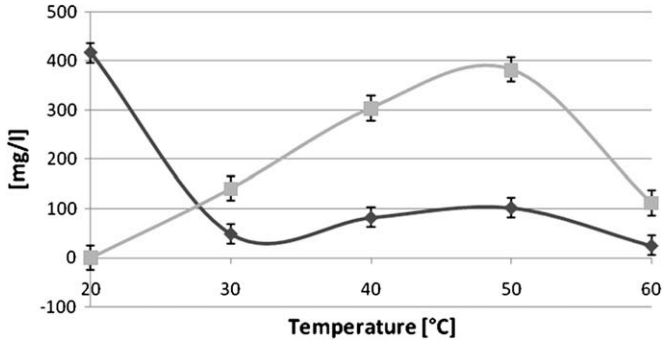

Fig. 1. Evolution of ethyl acetate [♦] and 3-methyl-1-butanol [■] in red wine (Aglianico) at different temperatures for 24 h.

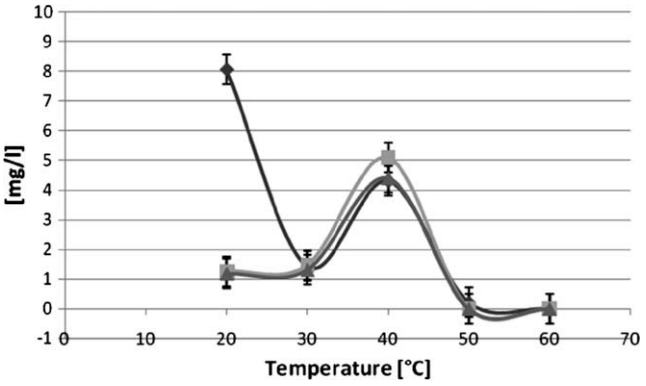

Fig. 2. Evolution of diethyl butanedioate [♦], ethyl octanoate [■] and ethyl decanoate [▲] in red wine (Aglianico) at different temperatures for 24 h.

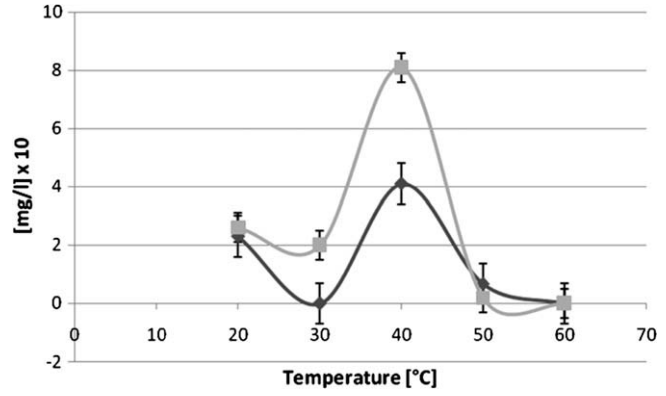

Fig. 3. Evolution of 3-methyl-1-butanol acetate [♦] and ethyl hexanoate [■] in red wine (Aglianico) at different temperatures for 24 h.

methyl-1-butanol increased until 50 °C, then decreased (Fig. 1). Finally, 3-methylbutyl acetate decreased at 30 °C, reached a maximum at 40 °C, then decreased (Fig. 3). For most of the compounds analysed in this study, the simple transition from 20 to 30 °C induces a significant modification on the composition of volatile compounds. The temperature of 40 °C induces the maximum evolution of this type of compounds. Higher temperatures induce a reduction of volatile compounds in wine.

Zoecklein, Hackney, Duncan, and Marcy (1999) found significant decreases in the concentration of most esters, after a treatment at 45 °C in Riesling (*Vitis vinifera* L.) wines. Rapp and Marais (1993) noted that acetates decreased in concentration with time and high temperature. Rapp and Mandery (1986) found that ethyl esters hydrolyse more slowly than acetate esters. Significant decreases in these types of volatile compounds were found by various authors for white wines stored at high temperature (De la Presa-Owens & Noble, 1997). Marais, Van Wyk, and Rapp (1992) observed more significant decreases in the concentration of some acetates and alcohols in wines stores at 30 °C than those stored at 15 °C. Ferreira, Escudero, Fernández, and Cacho (1997) explained the increases in the concentrations of some alcohols in wines stored under oxygen on the basis of the degradation of some of the precursors present in the wine.

We have also to note that the increase of the temperature induced the formation of compounds not present in the wine at 20 °C. In Table 1 we reported the area per cent of some compounds found at higher temperature and not quantified in this study. We can see, in agreement with the above reported results, that the maximum expression of volatile compounds was observed at 40 °C.

On the basis of these results, we performed the irradiation of some red wines at 20 °C. This temperature was selected because we showed that the temperature used can modify the aroma profile of the wine, and because this temperature is, probably, that nearest to the actual temperature in a supermarket store. We used three important Italian wines: Barolo from Piemonte, Amarone from Veneto, and Brunello from Toscana. The bottles were irradiated with a 13 W neon lamp, whose emission is showed in Fig. 4. The lamp showed emissions at λ 364, 404, 435, and 545 nm. These emissions were filtered but not cut by the glass of the bottles. As an example, we report in Fig. 4 (red line) the result obtained with the glass of Barolo wine. The emissions at 364 and 404 nm were almost completely filtered (99.1 and 98.6%, respectively); however, the emission at 435 nm remains (91.7% of the emission was filtered) as well as that at 545 nm (the emission showed 25.3% reduction). In our experiments we analysed the wine every day for a week, withdrawing the sample from the intact bottle with a syringe.

Fig. 5 represents the photochemical behaviour of ethyl acetate. In Barolo wine, after one day's irradiation, we observed an increase of the concentration of ethyl acetate and, after a week, the concentration of this compound was higher than that present at the beginning of the experiment. In Amarone wine we observed a sharp increase of the concentration of ethyl acetate after two days. After a week the concentration was more or less the same as that observed at the beginning of the experiment. In the case of Brunello wine, after a week's irradiation we observed a sharp decrease of the amount of ethyl acetate.

Fig. 6 reports the data relative to the photochemical behaviour of ethyl hexanoate. In Barolo wine it was not present at the beginning of our experiments; however, after one day's irradiation it was present in the wine and its concentration reached a maximum. In Amarone wine we observed a sharp reduction of the amount of this ester after a week irradiation; we have to note a relative maximum after two days' irradiation. The amount of ethyl hexanoate in Brunello wine showed a maximum after two days' irradiation, while, after a week, we observed a sharp decrease of its concentration in wine.

Extensive degradation after irradiation was observed also in the amounts of diethyl butanedioate (Fig. 7): however, in the case of Barolo wine, at the beginning of the experiments it was present in a very low amount and the concentration increased after a week's irradiation with a maximum after two days' irradiation. On the contrary, in Amarone wine, the concentration of this ester increased, reaching a maximum after two days' irradiation, but showing a sharp decrease after a week. Finally, in Brunello wine, we observed a sharp degradation of diethyl butanedioate during the irradiation with a relative maximum after three days.

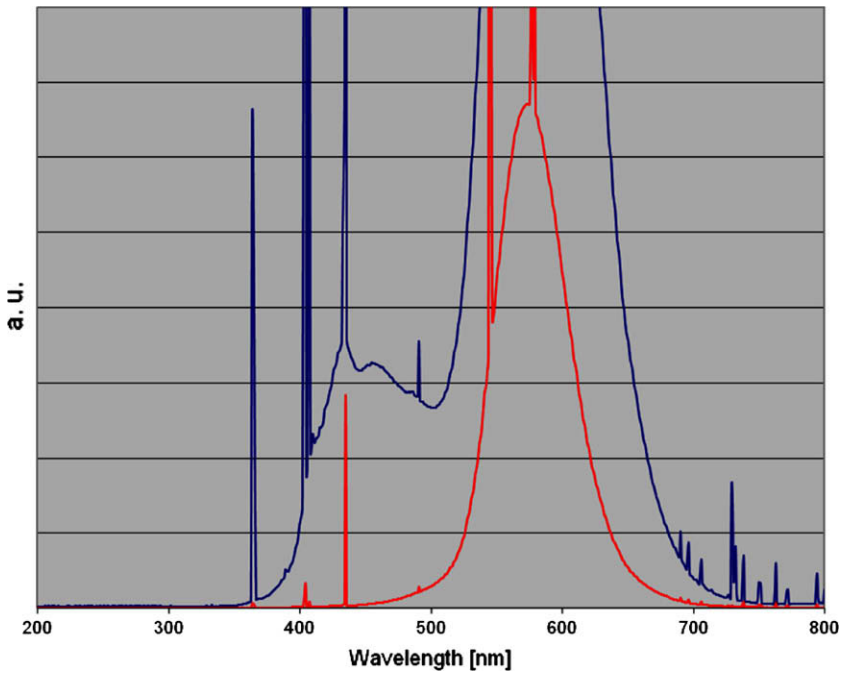

Fig. 4. Emission of the lamp used in this study (blue line) and emission filtered by the glass of bottle containing Barolo wine (red line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

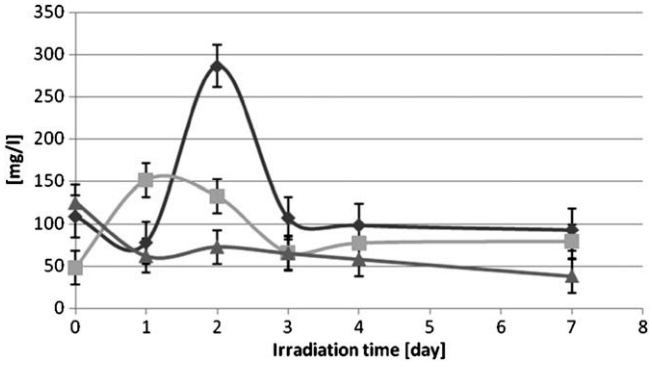

Fig. 5. Photochemical behaviour of ethyl acetate under irradiation. [■]: Barolo; [♦]: Amarone; [▲]: Brunello.

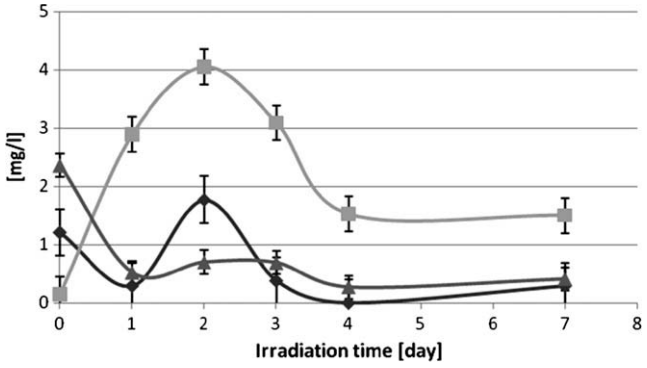

Fig. 7. Photochemical behaviour of diethyl butanedioate under irradiation. [■]: Barolo; [♦]: Amarone; [▲]: Brunello.

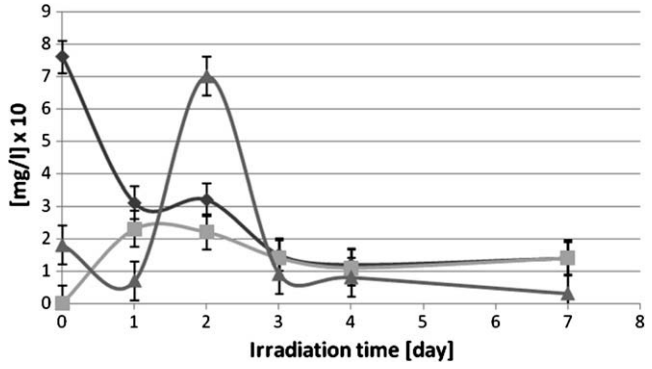

Fig. 6. Photochemical behaviour of ethyl hexanoate under irradiation. [■]: Barolo; [♦]: Amarone; [▲]: Brunello.

Table 1
Unquantified compounds found in the thermal treatment of Aglianico wine.

Compound	Temperature (°C)				
	20	30	40	50	60
	Area%				
Ethyl dodecanoate	0.00	0.10	0.79	0.00	0.00
Ethyl hexadecanoate	0.00	0.09	0.26	0.00	0.00
Ethyl 2-methylpropanoate	0.00	0.00	0.19	0.15	0.00
Ethyl 2-methylbutanoate	0.00	0.00	0.09	0.00	0.00
Ethyl 3-methylbutanoate	0.00	0.00	0.14	0.00	0.00
Ethyl 9-decanoate	0.00	0.00	0.18	0.00	0.00
Ethyl tetradecanoate	0.00	0.00	0.41	0.00	0.00
Ethyl pentadecanoate	0.00	0.00	0.13	0.00	0.00
2-Methyl-1-propanol	0.00	0.00	0.00	0.44	0.38
Ethyl propanoate	0.00	0.00	0.00	0.036	0.00
Ethyl butanoate	0.00	0.00	0.00	0.036	0.00

In Barolo wine, the ethyl octanoate concentration (Fig. 8) showed a maximum after two days' irradiation while, after a week, a sharp degradation was observed: the initial concentration was 0.11 mg l^{-1} and it was reduced to 0.02 mg l^{-1} at the end of the experiment. In Amarone wine we observed a clear decrease of

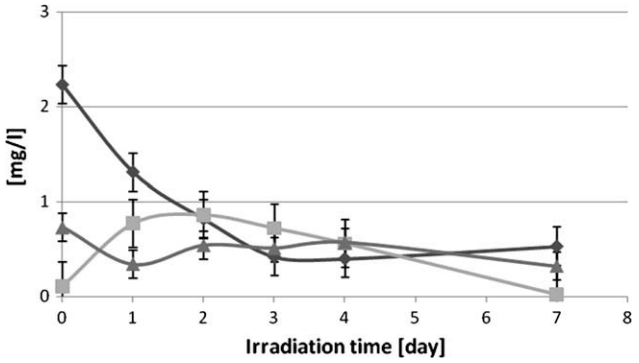

Fig. 8. Photochemical behaviour of ethyl octanoate under irradiation. [■]: Barolo; [♦]: Amarone; [▲]: Brunello.

the concentration of this ester. In Brunello wine, after a week's irradiation, we observed that the concentration of ethyl octanoate decreased from the initial value of 0.73–0.32 mg l^{-1}, with a relative maximum after four days irradiation. The same trend was observed also for ethyl decanoate (Fig. 9).

Fig. 10 reports our results obtained after irradiating the wines and analysing the photochemical behaviour of 3-methyl-1-butanol. In Barolo wine, we observed a maximum after one day irradiation and, at the end of the experiment, the concentration of this alcohol showed a moderate increase. In Amarone wine, we observed a maximum after two days' irradiation and, also in this case, a moderate increase after a week. On the contrary, the concentration of 3-methyl-1-butanol decreased in Brunello wine from the initial value of 206 mg l^{-1} to the final value of 139 mg l^{-1}.

Finally, Fig. 11 showed the fate of 3-methylbutyl acetate in the wines. In Barolo wine it was absent at the beginning of the experiment and it increased, reaching a maximum after one day's irradiation. In Amarone wine, the concentration of this ester decreased during the irradiation with a relative maximum after two days' irradiation. After a week it was absent in the wine. In Brunello wine a sharp decrease of the concentration of 3-methylbutyl acetate was observed.

Also in this case some other compounds, not quantified in this study, were found during the irradiation. The compounds were identified on the basis of their mass spectra. The area per cent is reported in Table 2.

On the basis of the above reported results we can express some comments. The only alcohol determined in this study, 3-methyl-1-butanol, is affected by the irradiation. For Barolo and Amarone, we observed a moderate increase, while for Brunello, a decrease was observed. This is a new result. Our previous reported results on champagne (D'Auria et al., 2003) showed that the alcohols were

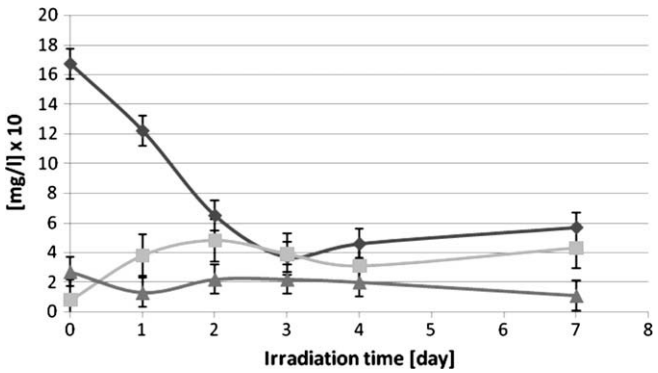

Fig. 9. Photochemical behaviour of ethyl decanoate under irradiation. [■]: Barolo; [♦]: Amarone; [▲]: Brunello.

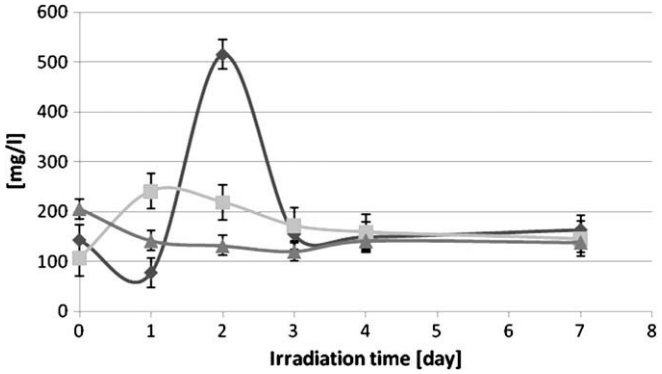

Fig. 10. Photochemical behaviour of 3-methyl-1-butanol under irradiation. [■]: Barolo; [♦]: Amarone; [▲]: Brunello.

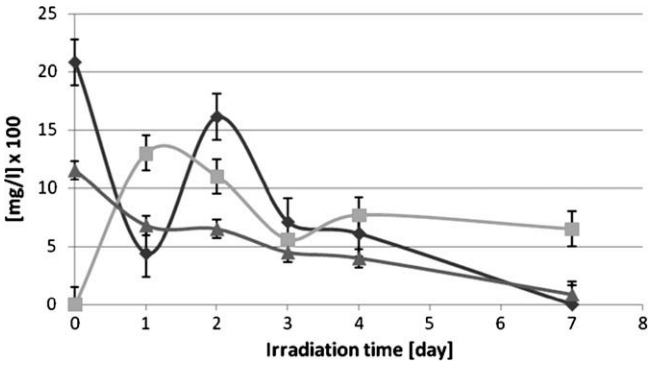

Fig. 11. Photochemical behaviour of 3-methylbutyl acetate under irradiation. [■]: Barolo; [♦]: Amarone; [▲]: Brunello.

not affected during the irradiation. Other authors reported that 2-methyl-1-propanol and 2-methyl-1-butanol decreased in wines during irradiation (Benítez et al., 2003). In the same way, esters were degraded during irradiation. These results are in agreement with our previous reported data on the photodegradation of champagne (D'Auria et al., 2003). On the contrary, Benítez et al. (2003) found an increase of the amount of esters in a red wine.

In red wine one or more components (phenols?) are able to protect esters from photodegradation. In diethyl butanedioate and ethyl hexanoate, octanoate, and decanoate we observed an initial increase of the concentration of the esters in some cases. This initial increase of the amount of esters is in agreement with the results reported in the work of Benítez et al. (2003). These authors noted that "these results are not surprising and can be explained on the basis of their hydrolysis–esterification equilibria". Other authors have reported these increases in wines stored under oxygen (Ramey & Ough, 1980). These results can be explained assuming that the photodegradation in the red wine is inhibited by some components, allowing the increase of the amount of the esters in the wine. When these components are destroyed by light, degradation starts, as in the white wine. On the other hand, the initial increase of the ester amounts could be an effect of the irradiation: light can increase the amount of esters through a Norrish Type II reaction from suitable precursors (Herrmann, 2007). In this case, white wines do not contain those suitable precursors.

The degradation process of the esters is probably an oxidative process catalysed by riboflavin. The riboflavin absorption spectrum in aqueous medium exhibits four structureless peaks centred at 446, 375, 265, and 220 nm with high molar extinction coefficients (>10^4 M^{-1} cm^{-1}). The absorption at 446 nm can occur in our samples, considering that the emission of the lamp at 435 nm is not

Table 2
Unquantified compounds found during the irradiation of Barolo, Brunello, and Amarone wines.

Wine	Compound	Irradiation time (day)					
		0	1	2	3	4	7
		Area (%)					
Barolo	Ethyl hexadecanoate	0.02	0.00	0.01	0.02	0.00	0.00
	Ethyl butanoate	0.00	0.07	0.00	0.00	0.00	0.00
	Ethyl pentadecanoate	0.00	0.02	0.04	0.00	0.00	0.00
	Ethyl 4-methyloctanoate	0.00	0.00	0.03	0.00	0.00	0.00
	Ethyl nonanoate	0.00	0.00	0.00	0.02	0.00	0.00
	1-Propanol	1.68	0.00	0.00	0.00	0.00	0.00
	2-Methyl-1-propanol	0.84	0.00	0.00	0.00	0.00	0.00
Brunello	Ethyl 4-methyloctanoate	0.00	0.00	0.00	0.00	0.00	0.05
	Ethyl 2-methylbutanoate	0.05	0.00	0.00	0.00	0.00	0.00
	Ethyl 3-methylbutanoate	0.07	0.00	0.04	0.00	0.04	0.00
	2-Methyl-1-butanol	0.00	0.00	0.00	0.63	0.00	0.00
	2-Hexanol	0.00	0.00	0.00	0.06	0.00	0.00
	Ethyl nonadecanoate	0.00	0.00	0.00	0.00	0.07	0.00
Amarone	Ethyl butanoate	0.20	0.00	0.12	0.00	0.00	0.00
	Ethyl 2-methylbutanoate	0.05	0.00	0.03	0.00	0.00	0.00
	Ethyl nonanoate	0.11	0.00	0.00	0.00	0.00	0.00
	Ethyl pentanoate	0.04	0.00	0.00	0.00	0.02	0.00
	Ethyl 2-methylpropanoate	0.00	0.09	0.19	0.00	0.00	0.00
	Ethyl hexadecanoate	0.00	0.01	0.08	0.00	0.00	0.05
	1-Propanol	0.00	0.00	0.57	0.00	0.00	0.00
	2-Propanol	0.00	0.00	0.10	0.00	0.00	0.00
	2-Methyl-1-propanol	0.00	0.00	0.40	0.00	0.00	0.00
	Ethyl propanoate	0.00	0.00	0.08	0.00	0.00	0.00
	Diethyl decanedioate	0.00	0.00	0.01	0.00	0.00	0.00
	Ethyl octadecanoate	0.00	0.00	0.02	0.00	0.00	0.00
	Ethyl 3-methylbutanoate	0.00	0.00	0.00	0.00	0.00	0.03

completely cut by the glass bottles. Riboflavin is particularly sensitive to UV and visible light and induces both Type I and Type II photosensitised oxidation mechanisms. The former involves the formation of free radicals through hydrogen or electron transfer between riboflavin triplet excited state and the substrates. The semi-oxidised substrate can undergo further oxidation in the presence of oxygen. The Type II process involves the formation of singlet oxygen by energy transfer from triplet excited riboflavin to molecular oxygen. Riboflavin is able to induce decomposition of the esters (D'Auria et al., 2003): this reaction probably occurs through a Type I photosensitised mechanism. In fact, in our knowledge, singlet oxygen is not able to attack aliphatic esters.

In conclusion, we have shown that UV–vis irradiation is able to induce a rapid photodegradation of the volatile compounds in wines. While others authors reported that 2-methyl-1-propanol and 2-methyl-1-butanol decreased in wines during irradiation (Benítez et al., 2003), the degradation of esters was reported only in a previous article on the degradation of champagne (D'Auria et al., 2003). In particular, Benítez et al. (2003) found an increase in the amount of esters in a red wine after irradiation. Red wines seem to be more protected against photodegradation, in particular in the case of aliphatic esters. In fact, our results showed that the amount of esters increases in red wine after 2 h irradiation. Longer irradiation times allowed the degradation of these components. These results can be explained assuming the presence of a protecting agent in the red wine that is destroyed by light and assuming that light can increase the amount of esters through a Norrish Type II reaction from suitable precursors. UV irradiation can act in the bottle: the use of coloured bottles protects the red wine against photodegradation but not sufficiently.

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Food Chemistry 117 (2009) 15–19

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Isolation and identification of steroidal hydrocarbons in soybean oil deodorizer distillate

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ARTICLE INFO

Article history:
Received 3 October 2008
Accepted 17 March 2009

Keywords:
Soybean oil deodorizer distillate
Steroidal hydrocarbons
Modified Soxhlet extraction
Modified silica gel column chromatography

ABSTRACT

In the isolation of squalene from soybean oil deodorizer distillate (SODD), ca. 30% of nonpolar lipid fraction (NPLF) is hydrocarbons (HCs), which can be divided into three main groups, namely aliphatic HCs, steroidal HCs and terpene HCs. The steroidal HCs content of vegetable oil can be used as a means of identifying the oil and therefore, for detecting adulteration. The objective of this study was to isolate the steroidal HCs contained in SODD and identify the individual steroidal HCs. Isolation of steroidal HCs can be achieved in three steps: modified Soxhlet extraction, modified silica gel column chromatography, and distillation. Identification of individual steroidal HCs was accomplished by TLC, GC and GC–MS analyses. The GC chromatogram of steroidal HCs showed five major peaks. Most of the major peaks were identified as derivatives of free sterols. Special attention was needed in order to identify isomers which differ in the position of double bonds and compounds which have three double bonds in the ring system.

1. Introduction

Steroidal hydrocarbons (HCs) are formed in vegetable oils as the dehydration products of Δ^5-phytosterols, because of bleaching with acidic earth and deodorization at high temperature during the refining process. Steradienes and steratrienes are steroidal HCs that have two double bonds and three double bonds, respectively. Their contents in vegetable oils can be used as a means for identifying the oil and therefore, for detecting adulteration (Wretensjö & Karlberg, 2002). The application limit of the steroidal HCs in the virgin olive oil is between 0.01 and 4 mg/kg and that is regulated by the International Olive Oil Council. The limit of 3,5-stigmastadiene and the total steroidal HCs content in virgin olive oil have been fixed at 0.15 and 0.3 mg/kg, respectively and have become a European Economic Community regulation (Toschi, Bendini, & Lercker, 1996).

Soybean oil deodorizer distillate (SODD) is a byproduct of the refining process in obtaining refined soybean oil. Depending on the sources, SODD usually has significantly different characteristics, uses and value. It can be a good raw material for the production of tocopherols, phytosterols and fatty acids, (Nagao et al., 2005; Torres, Torrelo, Seňorans, & Reglero, 2007; Watanabe, Nagao, Hirota, Kitano, & Shimada, 2004; Winters, 1986) and squalene (Gunawan, Kasim, & Ju, 2008). Little information about detailed steroidal HCs in SODD is available in the literature. Cert, Lanzón, Carelli, and Albi (1994) and Verleyen et al. (2002) reported the presence of 3,5-stigmastadiene in olive oil and compared its quantitative analyses using GC and HPLC. Bortolomeazzi, Pizzale, Novelli, and Conte (1996) and Bortolomeazzi, Zan, Pizzale, and Conte (2000) detected the existence of steradienes and steratrienes in olive oil from the occurrence of ions at *m*/*z* 255 and 213 and at *m*/*z* 253 and 211 in the gas chromatography–mass spectrometry (GC–MS) chromatogram, respectively. Mennie, Moffat, and McGill (1994) detected the presence of steroidal HCs at different stages in the refining process of vegetable oils (maize oil, sunflower oil, rapeseed oil, palm oil and soybean oil) and succeeded in identifying 3,5-, 2,5-, 4,6-, 3,5,22-, and -4,6,22- steroidal HCs using thin-layer chromatography (TLC), gas chromatography (GC), and GC–MS. Gunawan et al. (2008) reported that, in the isolation of squalene from SODD, ca. 30–40% of nonpolar lipid fraction (NPLF) is HCs. Their preliminary results suggest that the HCs contained in NPLF can be divided into four main groups, which are aliphatic, steroidal, sesquiterpene and triterpene (squalene) HCs.

A better knowledge of the separation, purification, and chemical composition of steroidal hydrocarbons in SODD will help researchers finding a better utilization of this byproduct. The American Oil Chemists' Society (AOCS) method Cd27-96 (1997) and the International Union of Pure and Applied Chemistry (IUPAC) method pp. M7–M11 (1994) are used to analyze the steradienes content in a vegetable oil by GC. Steradienes were isolated from the unsaponifiable matters by silica gel column chromatography (Toschi et al., 1996). The weak point of these methods is the interferences of steradienes with other hydrocarbons in the chromatogram and this makes it difficult to quantify the exact steradiene amount. In order to determine the exact content and composition of steroidal HCs in

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doi:10.1016/j.foodchem.2009.03.066

SODD, enrichment, followed by separation of these lipid classes prior to analysis, is important. The objective of this study was to isolate the steroidal HCs contained in SODD and identify the individual steroidal HC.

2. Materials and methods

2.1. Materials and reagents

SODD was donated by Taiwan Sugar Company (Tainan, Taiwan). Stigmasterol (95%) and cholesta-3,5-diene (approx. 95%) standards were obtained from Sigma Chemicals Company (St. Louis, MO). Reagents used in the isolation of steroidal HCs were of industrial grade and those used in the analyses (GC, TLC, GC–MS) were of analytical grade. Silica gel (pore size: 60 Å, 70–230 mesh) was purchased from Silicycle (Quebec, Canada). Analytical TLC silica gel plates (0.25 mm thickness) were obtained from Merck KGaA (Darmstadt, Germany). The silica gel was first activated by heating in the oven at 150 °C for 1–2 h prior to use.

2.2. Isolation of steroidal hydrocarbons from SODD

Fig. 1 shows the flow chart for the isolation of steroidal HCs from SODD. About 20 g of SODD were subjected to a modified Soxhlet extraction (Gunawan et al., 2008) to obtain a nonpolar lipid fraction (NPLF) which is rich in fatty acid steryl esters (FASEs), squalene and other HCs. The NPLF was then subjected to a modified silica gel column chromatography (MSC) to obtain the HCs fraction. Detail of the MSC was described by Gunawan, Ismadji, and Ju (2008). During MSC elution, the HCs fraction was obtained in the first 4 h. Squalene was obtained in the next two and half hours. It is important to obtain the first HCs fraction that is free of any squalene, because squalene and steroidal HCs have similar molecular weight and boiling points. If present together, it is difficult to separate them by distillation. The HCs fraction was distilled at 270–280 °C and 3–5 mTorr to obtain pure steroidal HCs in the residue. The distillate contained aliphatic, sesquiterpene, and a small amount of steroidal HCs. The steroidal HCs obtained in the residue were analyzed by GC, TLC, and GC–MS to identify the individual steroidal HC.

2.3. Gas chromatography (GC) and GC–mass spectrometry (GC–MS) studies

A Shimadzu GC-17A equipped with a split–split injector and a flame ionization detector (FID) was employed for analyzing steroidal HC. The fused silica column used was a DB-5HT, 15 m × 0.32 mm i.d., 0.1 μm film thickness (Agilent Tech. Palo Alto, California, USA). The initial column temperature was 80 °C, which then was raised to 365 °C at 15 °C/min and held for 8 min. The temperature of the injector and detector was 370 °C. For GC–MS analysis, a GC Shimadzu model GC-2010 coupled with MS model QP2010 was used. The fused silica column was a DB-5 ms, 30 m × 0.25 mm i.d., 1 μm film thickness (Agilent Tech. Palo Alto, California, USA). The initial column temperature was 80 °C, which was then raised to 320 °C at 15 °C/min and held for 14 min. The injection was in split mode (split ratio was 1:50) with helium as the carrier gas and with a linear velocity of 30.6 cm/s. The temperature of injector, interface, and ion source were, 250, 250, and 200 °C, respectively. Electron ionization was carried out at 70 eV. The mass spectra were recorded at 1250 scans/s.

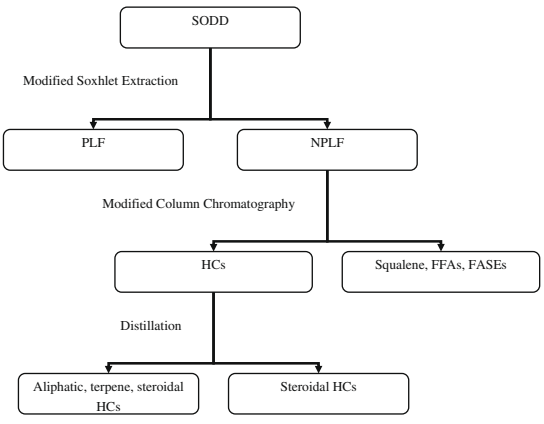

Fig. 1. Schematic diagram of steroidal HCs isolation.

2.4. Thin-layer chromatography (TLC)

The specific reagent used to detect the steroidal HCs spot was made by dissolving 50 mg $FeCl_3.6H_2O$ in 90 ml H_2O, along with 5 ml acetic acid and 5 ml sulfuric acid. The desired spot yields a red–violet colour (Fried, 2003). The mobile phase used in the TLC analyses was HPLC grade *n*-hexane (95% purity). The spot was observed at 366 nm for analytical TLC. The reagent was sprayed onto the TLC plate, and the plate was heated at 90–100 °C until the desired spot appeared.

3. Results and discussion

3.1. Isolation of steroidal HCs

Based on IUPAC and IUBMB recommendations of steroid nomenclature (1989), steroidal HCs and free sterols have similar structures. For free sterols, there is an OH group at position 3. For steroidal HCs, there is no OH group on the skeleton; however, there are double bonds in the ring system. In the bleaching process, activated (acid) earth is added to refined oil to remove colour, odour, other impurities, and residual soap. Deodorization is a high temperature, high vacuum steam-distillation process that is necessary for the removal of volatile flavour and odour compounds to transform the oil into a bland-tasting clear liquid desirable to consumers (Pryde, 1995). These processes may cause the free sterol in soybean oil to undergo isomerization reactions, such as the shifting of the double bond position and also the dehydration of sterol, to produce steroidal HCs (Biedermann, Grob, Mariani, & Schmid, 1996). The reaction scheme is shown in Fig. 2. The dominant products of this dehydration reaction are products B. This is because π-bonding in products B are the strongest bond among other products.

SODD in Taiwan typically contains about 40% FFAs and 17% triacylglycerols (TAGs) (Gunawan, 2008). The elimination of these compounds is crucial for enriching steroidal HCs due to their high contents in SODD. By using modified Soxhlet extraction, we were able to isolate all HCs in the NPLF (5 g NPLF from 20 g of SODD). TLC and HTGC analyses of the polar lipid fraction (PLF) showed the absence of HCs. More than 90% of TAGs and other polar components were concentrated in the PLF after modified Soxhlet extraction. NPLF (3 g), which contains 43.7% HCs, was subjected to modified silica gel column chromatography, using hexane as the mobile phase. All aliphatic, steroidal and sesquiterpene HCs (1.3 g) were obtained in the first fraction after eluting the column with hexane for 4 h. Analysis of this fraction showed that it contained only HCs. The first fraction had a steroidal HCs content of 11.9% (100% recovery). Modified Soxhlet extraction and modified silica gel column chromatography were efficient for the extraction of HCs using hexane as the solvent. Advantages of the current method are: a large sample to silica gel ratio, easy recovery and

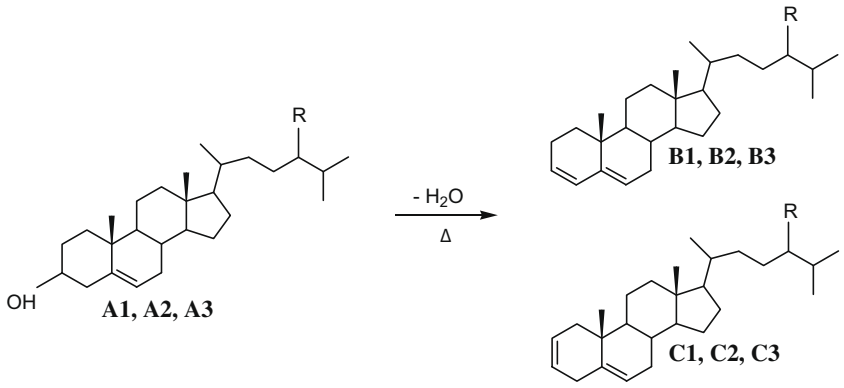

Fig. 2. Reaction scheme for isomerization during the dehydration of free sterol to become steroidal HCs, where 1: R = CH_3; 2: R i = C_2H_5; 3: R = C_2H_5 and one hydrogen removed from carbon 22 and another hydrogen from carbon 23.

reuse of solvents, and simple operational procedure. Distillation was employed for the purification steroidal HCs due to the large differences in the boiling point of steroidal HCs and other HCs. Steroidal HCs (purity 90.8%, recovery 81.3%) were obtained as residue. The quantification of the steroidal HCs is based on external calibration of cholesta-3,5-diene. The chromatogram of the steroidal HCs obtained from distillation showed that there was a small hump, as seen in Fig. 4. The calculation of steroidal HCs content is based on the summation of all resolvable steroidal HC peaks. Although the five resolvable peaks made up only 70.0% of the steroidal HCs, the steroidal HCs obtained had very high purity. This was confirmed on TLC analysis (Fig. 3). Based on the steroidal HCs content obtained in the distillation residue, we can calculate the steroidal HCs content in SODD (Gunawan, 2008), which is (2.46 ± 0.12) wt% of SODD.

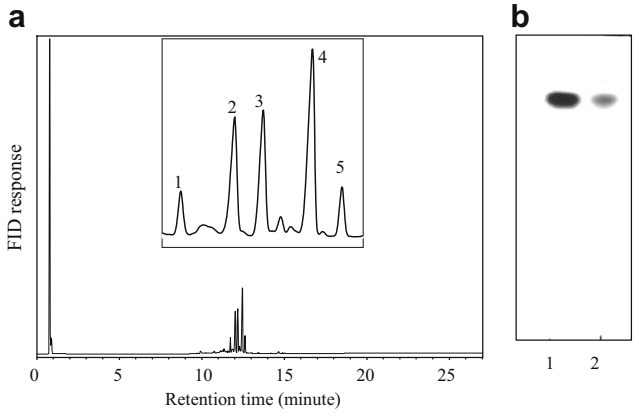

Fig. 3. Results of HTGC and TLC analyses: (a) GC chromatogram of residue containing mainly steroidal HCs; (b) TLC plate of cholesta-3,5-diene standard (lane I) and the residue (lane II).

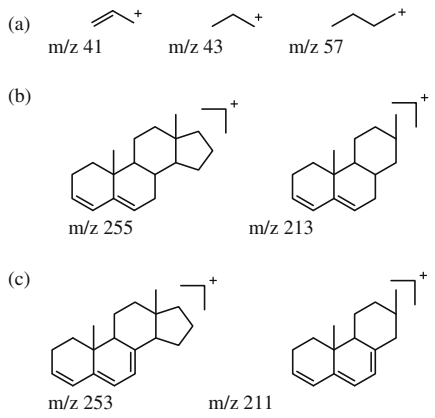

Fig. 4. Common fragmentations of steroidal HC compounds: (a) of its side chains, (b) of its ring system with two double bonds, and (c) of its ring system with three double bonds.

3.2. Identification of steroidal HCs

The identification of steroidal HCs was achieved by comparing results from GC, TLC, and GC–MS analyses. The separation between peaks in the chromatograms of both GC and GC–MS analyses is based on differences in molecular weight and boiling point of the compound that each peak represents. For GC–MS, the fragmentation of certain functional groups can be detected. By applying a standard, that has the same or similar structure as the target compound, on a TLC plate, together with the target compound, and detecting them using a specific reagent; the target compound structure or its functional group can be revealed. In this study, we used these three methods of analysis to identify the individual steroidal HCs in the mixture which was obtained from SODD.

The TLC chromatogram of the residue from distillation shows absence of aliphatic and sesquiterpene HCs (Fig. 3b). The fact that the peaks represent steroidal HCs was supported by the TLC chromatogram (Fig. 3b). TLC analysis showed that the Rf value of the residue spot (lane 2) was 0.75–0.81, which has about the same Rf value of the spot of cholesta-3,5-diene standard (lane 1). When the TLC plate was treated with a ferric chloride reagent, both spots gave a violet colour which is a specific character of compounds with sterol ring structures. The relative retention times (RRT) of the five major peaks, as well as their weight percentages, which are presented in parentheses, in Fig. 3a were 0.94 (5.21%), 0.97 (16.80%), 0.98 (16.05%), 1.00 (25.56%), and 1.01 (5.37%), for peak numbers 1 until 5, respectively. The RRT of a peak was calculated, based on the retention time of that peak divided by the retention time of peak number 4.

From the results of GC and TLC analyses of the sample, as shown in Fig. 3, there are several major peaks with different retention times in the GC chromatogram with each peak representing one particular steroidal HC. However, in the TLC chromatogram, there is only one spot appeared, which means that all steroidal HCs in the sample have almost the same polarity. Further isolation of individual steroidal HCs from the residue of distillation is extremely difficult, if not impossible. The residue was applied for GC–MS analysis to extract information on the structure of individual steroidal HCs. Table 1 shows names of the five major peaks, their

Table 1
GC–MS fragmentations of the five major peaks in Fig. 3a.

HTGC Peak No.	Name and M_W	m/z (relative intensity, %)
1	24-ethylcholesta-2,5-diene (**C2**); 396	41(50%), 43(98.10%), 55[a](100%), 57(35.71%), 67(47.62%), 69(35.71%), 71(28.57%), 79(47.62%), 81(71.43%), 83(56.19%), 91(63.33%), 93(46.67%), 95(47.62%), 105(64.76%), 121(21.43%), 135(22.86%), 147(26.19%), 161(13.81%), 213(14.29%), 255(29.52%), 382(40.48%), 396(14.29%).
2	24-methylcholesta-3,5-diene (**B1**); 382	41(34.29%), 43(76.19%), 55(41.90%), 57(33.33%), 67(34.29%), 81(71.43%), 91(47.62%), 105(52.38%), 121(26.19%), 133(23.81%), 147(57.14%), 159(14.29%), 213(16.67%), 255(19.05%), 261(18.57%), 274(23.81%), 367(28.57%), 382[a](100%).
3	24-ethylcholesta-3,5,22-triene (**B3**); 394	41(33.33%), 55(83.33%), 67(41.90%), 69(47.62%), 81[a](100%), 91(40.48%), 105(42.86%), 119(19.05%), 133(28.57%), 145(28.57%), 159(19.05%), 213(11.90%), 255(38.10%), 267(0.45%), 282(0.93%), 295(0.40%), 309(0.10%), 323(<0.10%), 337(<0.10%), 351(0.95%), 365(0.10%), 379(0.95%), 394(95.24%).
4	24-ethylcholesta-3,5-diene (**B2**); 396	41(40.94%), 43(86.55%), 55(52.63%), 57(49.71%), 67(40.94%), 81[a](100%), 91(55.56%), 105(71.93%), 121(38.01%), 133(32.16%), 147(86.55%), 213(23.98%), 255(26.90%), 275(22.81%), 288(27.19%), 381(35.09%), 396 (87.13%).
5	24-ethylcholesta-2,4,6-triene; 394	41(30%), 55[a](100%), 67(25.71%), 69(52.38%), 81(38.10%), 97(30%), 105(28.57%), 121(10%), 213(5.71%), 253(4.76%), 281(15.71%), 296(61.90%), 394(21.43%).

[a] The base peak.

molecular weight (M_W), and mass fragmentations with their relative intensities.

Steroidal HCs have a strong π-bond in the ring system. As ions in mass spectrometry bombard steroidal HCs, side chains will be cleft first, followed by cleavage of the ring system. The mass spectrum of the cholesta-3,5-diene standard can be seen in the NIST database (2007). From this mass spectrum we were able to analyze some common fragmentations. The fragmentation of steroidal HC side chain results in groups, such as CH_3 at m/z 15, $C_3H_5^+$, at m/z 41, $C_3H_7^+$, at m/z 43, $C_4H_9^+$, at m/z 57, Fig. 4a (Johnsson & Dutta, 2003). The breakdown of the ring system in the steroidal HCs results either in groups such as fragmentations at m/z 255 and at m/z 213 (Fig. 4b), which show that the compounds have two double bonds in the ring system or in groups such as fragmentations at m/z 253 and at m/z 211 (Fig. 4c), which show that the compounds have three double bonds in the ring system (Bortolomeazzi, Zan, Pizzale, & Conte, 2000).

HTGC peak 2 shows a base peak at its molecular ion peak (Table 1). This indicates that the structure of the corresponding compound is very stable. Fragmentations at m/z 255 and 213 show that there are two pairs of double bonds inside the ring system. The compound of peak 2 has a M_W of 382; it is derived from the dehydration of campesterol (**A1** in Fig. 2, M_W = 400). Based on its fragmentations at m/z 55, 67, and 81 (Fig. 5), this compound may have double bonds at positions 3 and 5 or 2 and 5. The amount of isomer with double bonds at positions 3 and 5 is much larger than that with double bonds at positions 2 and 5, due to the fact that double bonds at positions 3 and 5 are more stable that those at 2 and 5, (Bortolomeazzi, Pizzale, Novelli, & Conte, 1996; Grob, Biedermann, Artho, & Schmid, 1994; Mennie et al., 1994). The double bonds located at positions 3 and 5 are in a conjugated system, which is why this isomer is more stable than other isomers. There are unique fragmentations at m/z 261 and 274, which are present because of side chain effect (Mennie et al., 1994). Hence, this compound is identified as **B1** in Fig. 2.

The compound of peak 4 has a molecular ion peak at m/z 396, fragmentations at m/z 255 and 213, and high relative intensities of fragmentations at m/z 55, 67, and 81 (Table 1). These show that the compound of peak 4 is **B2** (Fig. 2), which is derived from β-sitosterol (**A2** in Fig. 2, M_W = 414). There are unique fragmentations at m/z 275 and 288, which are present because of the side chain effect (Mennie et al., 1994).

The compound of peak 3 has a molecular ion peak at m/z 394. Fragmentation patterns of its side chain occur at m/z 255, 267, 282, 295, 309, 323, 337, 351 and 365. These fragmentations exist because of one double bond at the side chain that easily moves along the side chain and has a tendency to move to the end of the side chain. High relative intensities occur at fragmentations of m/z 55, 67 and 81 (Table 1). The fragmentations of the side chain appeared in reduced intensity because the side chain in this compound is not stable, due to the double bond effect in the side chain. These facts suggest that the compound of peak 3 is **B3** (Fig. 2), which is derived from stigmasterol (**A3**, M_W = 412).

The compound of peak 1 has a mass spectrum very similar to that of **B2** (Table 1). Their most favourable fragmentations are the same, namely at m/z 43, 55, 67, 81, 213 and 255. The double bond positions of compound of peak 1 and **B2** are in the same ring system. They differ at the positions inside the ring system. The compound of peak 1 is suggested as **C2** (Fig. 2). The fragmentations at m/z 67 and 81 show that the double bond is at position 5. From the fragmentation at m/z 55, the first cyclohexane ring has only one double bond, but its position has yet to be determined. Based on the area percentage ratio of **C2** and **B2**, and the isomerization scheme (Fig. 2), **B2** is more abundant than its isomer **C2**. The fragmentation patterns of **C2** will be quite similar to those of **B2** because the only difference between the structures of **C2** and **B2** are that **C2** and **B2** have double bonds at positions 2 and 3, respectively. The fragmentation of the compound of peak 1 at m/z 55 is the base peak. This fragmentation is the most favoured one because it will result in a stable butadiene fragmentation (Roderbourg & Kuzdal-Savoie, 1979). Isomers with double bounds at positions 2 and 5 may occur, but their percentages are much smaller than those of the 3,5 isomers. Hence GC–MS analyses of minor peaks in Fig. 3a were not carried out in this study.

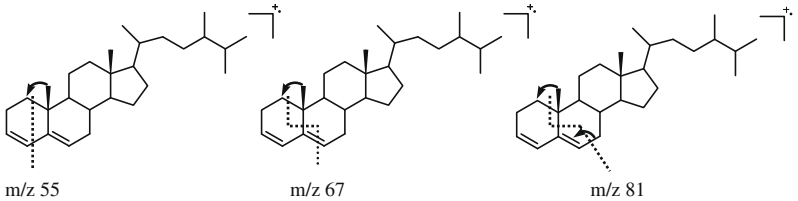

Fig. 5. Unique fragmentations which show double bond at positions 3 and 5, where (...) indicate locations where bonds can be cleft.

The compound of peak 5 has the same molecular ion as that of **B3**, at *m*/*z* 394 (Table 1). However, the compound of peak 5 has fragmentations at *m*/*z* 253 and 211 while **B3** has fragmentations at *m*/*z* 255 and 213. This compound has three double bonds inside the ring system. From the molecular ion and the fragmentations, this compound is derived from the hydroxy derivative of **A2**, (Bortolomeazzi, Zan, Pizzale, & Conte, 1999), not derived from **A3**. The fragmentations at *m*/*z* 55, 67, 81, 93, and 105 suggest that the double bonds are located at positions either 2,4,6, or 3,5,7. Fragmentations at *m*/*z* 55, 95, 107, 119, 131 and 145 suggest that the double bonds are located at positions 3, 8, and 11. This may happen because the impact energy of EI is high enough to cleave bonds in the compound and give enough energy to the compound to undergo isomerization. This is why intramolecular isomerization reactions can occur. Based on the relative intensities of fragmentations mentioned above, the 2,4,6 or 3,5,7-isomer is more abundant than is the 3,8,11-isomer. The 2,4,6- or 3,5,7-isomer forms a conjugated system, so it will result in a more preferable isomer product. Hence, the compound of peak 5 is identified as 24-ethylcholesta-2,4,6-triene.

4. Conclusion

From the analyses of steroidal HC mass spectra, it can be concluded that there are five major steroidal HCs in SODD, which are 24-ethylcholesta-3,5-diene, 24-ethylcholesta-3,5,22-triene, 24-methylcholesta-3,5-diene, 24-ethylcholesta-2,5-diene, and 24-ethylcholesta-2,4,6-triene. Their content in steroidal HCs are 25.6%, 16.0%, 16.8%, 5.21% and 5.37%, respectively, based on their weight percentages in the GC chromatogram. Most of these compounds are derived from the dehydration of free sterol in SODD. There are several minor peaks in the GC chromatogram insert of Fig. 4a. Because the amount of each minor peak is very small, it is more difficult to identify these minor peaks by GC–MS. These minor peaks are believed to be the products of unfavourable dehydration reaction of free sterol or hydroxy derivatives of free sterol.

Acknowledgements

The authors wish to thank Dr. Jiang, Jyh-Chiang for technical assistance and to the National Science Council of Taiwan for funding the project (NSC94-2214-E011-004).

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Food Chemistry 117 (2009) 20–27

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

The kinetics of inactivation of pectin methylesterase and polygalacturonase in tomato juice by thermosonication

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ARTICLE INFO

Article history:
Received 31 July 2008
Received in revised form 12 February 2009
Accepted 17 March 2009

Keywords:
Tomato
Pectin methylesterase
Polygalacturonase
Ultrasound
Heat
Thermosonication

ABSTRACT

The ultrasonic inactivation kinetics of polygalacturonase (PG) and pectin methylesterase (PME) in tomato juice were studied at a frequency of 20 kHz, amplitude of 65 μm and temperatures between 50 and 75 °C. Thermal treatments at the same temperatures were conducted to separate the effects of heat and ultrasound. The thermal inactivation of PG was described by a fractional conversion model with PG 1 remaining stable, whereas the inactivation of PG by combined ultrasonic and heat treatment (thermosonication) was best described by first order biphasic kinetics, with both PG1 and PG2 inactivated at different rates. The thermal and thermosonication inactivation of PME was described well by first order kinetics. The inactivating effect of combined ultrasound and heat was synergistic. Thermosonication enhanced the inactivation rates of both PME and PG. The inactivation rate of PME was increased by 1.5–6 times and the inactivation rate of PG2 by 2.3–4 times in the temperature range 60–75 °C, with the highest increase corresponding to the lowest temperature.

1. Introduction

One of the most important quality characteristics of tomato-based products is consistency, which is mainly determined by the pectic substances that serve as a continuous phase in which other particles are suspended (Vercet, Sanchez, Burgos, Montanes, & Buesa, 2002). The rheology of tomato-based products is affected by the modification of the pectin structure during processing, either by chemical conversion (β-elimination) or by the action of endogenous pectin-degrading enzymes, namely pectin methylesterase (PME; E.C. 3.1.1.11) and endopolygalacturonase (PG; E.C. 1.2.1.15) (Verlent, Loey, Smout, Duvetter, & Hendrickx, 2004). Following tomato crushing during processing, degradation of pectin by the synergistic action of PME and PG commences, resulting in a large decrease in viscosity. PME catalyses the de-esterification of pectin to pectic acid, with a lower degree of esterification. PG catalyses the hydrolytic cleavage of the α-1,4-hydrolytic bonds in pectin, leading to loss of viscosity and phase separation during storage. The actions of these two enzymes are synergistic as demethylated pectin is the preferred substrate for the catalytic action of PG (Fachin et al., 2003). In conventional tomato processing, these enzymes are fully or partially inactivated by thermal treatment. Two types of processes are commonly employed: hot break and cold break. In the hot break process, the pulp is rapidly heated to temperatures between 95 and 102 °C immediately after or during crushing, leading to complete inactivation of the enzymes and high consistency products. However, the severe heat treatment causes degradation of the colour, flavour and nutritional quality of the juice. In a cold break process, the pulp is heated to 60–71 °C resulting only in partial inactivation of the enzymes and consequently a low consistency product and syneresis during storage since both enzymes are relatively heat-resistant. On the other hand, quality degradation due to the thermal treatment is minimised. In addition, the low viscosity of the juice makes pumping and evaporative concentration easier and reduces fouling of heat exchangers (Vercet et al., 2002).

Increased awareness by consumers of the relationship between diet and health has in recent years created greater impetus and effort for the exploration of alternative food processing technologies, which use minimal heat and preservatives and result in fresh-like products of superior nutritional quality. In this regard, emerging technologies, such as high pressure processing, pulsed electric field and ultrasound, are being investigated by several groups around the world. The application of ultrasound in the food industry has been studied for many years. Ultrasound has two main application areas in the food industry: high frequency (1–10 MHz), low power ultrasound as an analytical tool and low frequency (20–100 kHz) high power ultrasound in food processing. Currently, high-frequency ultrasound is used in non-destructive measurements of a wide range of food process and quality attributes, such as the level in tanks, flow rate and flow profile, composition, particle size,

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doi:10.1016/j.foodchem.2009.03.067

emulsion stability and state of aggregation, molecular properties and phase transitions. Low frequency ultrasound can be used in a wide range of applications, such as enhancing oxidation processes, as in the fermentation of wines and spirits, controlling enzymatic reactions (inactivation and activation), stimulation of living cells, as in the fermentation of yoghurt, increasing the efficiency of emulsification and extraction processes, and surface decontamination (Mason, Paniwnyk, & Lorimer, 1996). Inactivation of enzymes by ultrasound is mainly attributed to the mechanical and chemical effects of cavitation, which is the formation, growth and implosion of bubbles (Raviyan, Zhang, & Feng, 2005). Collapse of bubbles is accompanied by extreme localised increase in pressure (50 MPa) and temperature (5000 K) on a micro scale (Sala, Burgos, Condon, Lopez, & Raso, 1995). In addition, ultrasound makes stable cavitating bubbles vibrate, creating shock waves that cause strong shear and microstreaming in the adjacent liquid. Under these extreme conditions, sonication could cause the breakdown of hydrogen bonding and Van der Waals interactions in the polypeptide chains of proteins, including enzymes, leading to the modification of the secondary and tertiary structures. With changes in the secondary and tertiary structure, the biological activity of the enzyme is lost (Zhong, Ming, Su, & Ju, 2004). The extreme localised increase in pressure and temperature also leads to homolytic cleavage of water molecules, generating high energy intermediates, such as hydroxyl and hydrogen free radicals. The free radicals formed may react with some amino acid residues that participate in enzyme stability, substrate binding or catalytic function, with a consequent change in biological activity (Barteri, Diociaiuti, Pala, & Rotella, 2004). Low frequency ultrasound is more effective for enzyme inactivation, in combination with other inactivating methods, such as mild heat and mild pressure. The term thermosonication refers to the combination of ultrasound with mild heat whilst manothermosonication refers to the combination of ultrasound with mild heat and moderate pressure (Sala et al., 1995). The objective of this work was to study the thermosonication inactivation kinetics of pectin methyesterase (PME) and polygalacturonase (PG) in tomato juice so as to determine the potential of using this technology as a milder alternative to 'hot break' processing of tomatoes.

2. Materials and methods

2.1. Tomato juice

Ripened tomatoes (Heinz 3402 variety) were washed with chlorinated water (50 ml of Milton disinfectant solution (Probiotec, VIC, Australia), containing 0.95% w/w sodium hypochlorite added to 4 l water), to remove dirt and other extraneous matter adhering to the fruit. The extraction of tomato juice and separation of the seeds and peel from the pulp were performed using a laboratory scale juice homogeniser (Nutrifaster Australia, Model Ruby 2000, Ruby Co., USA). The juice was transferred to plastic bags in 20 ml portions and was kept frozen at −25 °C for up to 4 weeks prior to thermal and sonication experiments. Thawing was carried out by keeping the samples overnight at 4 °C. The activities of both enzymes were not affected by the freezing and the frozen storage steps.

2.2. Thermosonication and thermal inactivation experiments

The sonication experiments were carried out using a Branson 450 sonifier equipped with a horn with a diameter of 10 mm and length of 60 mm. The treatments were carried out at 20 kHz frequency and 65 μm (acoustic power input = 40 W, with a nominal energy conversion efficiency of 80%) at temperatures ranging from 50 to 75 °C. Twenty kilohertz was used as the ultrasonic treatment frequency since it is the most effective for enzyme inactivation, based on literature (Sala et al., 1995) and experience. The amplitude (65 μm) was the maximum possible with the sonifier used in the study. Samples were pre-heated to the experimental temperature by immersing in a thermostatted water bath maintained at 90 °C for 30–50 s, depending on the experimental temperature, and they were immediately transferred to the water bath in which the experiments were conducted. The time to get to the experimental temperature at the centre of the sample (the slowest heating point) was taken as the temperature equilibration time. The tip of the horn was immersed (40 mm) into 20 ml of tomato juice in a custom-made stainless steel cylindrical container (ID = 22 mm, L = 103 mm). The temperature in the samples during the sonication treatment was maintained at the experimental temperature, using a recirculating water bath at a temperature 3 °C below the experimental temperature. Thermal treatments at the same temperatures were performed to distinguish between the effects of ultrasound and heat treatment, with samples taken at suitable time intervals, depending on the experimental temperature. Timing started after the samples reached the experimental temperature. Following each treatment, the samples were cooled in ice-water for up to 10 min and were immediately analysed or kept at −18 °C until analysis. Frozen samples were thawed by keeping them overnight at 4 °C. Freezing and subsequent thawing did not have any measurable impact on the activity of the enzymes. All experiments were performed in duplicates.

2.3. Enzyme extraction

The extraction of the enzymes was performed using the method of Verlent et al. (2004) after slight modification to use it for tomato juice. The pH of the juice was adjusted to 3.0 using 0.1 M HCl, stirred for 15 min and centrifuged at 8000g and 4 °C for 20 min. The supernatant was discarded and the pellet obtained was suspended in cold distilled water (1:1 [w/v]), its pH adjusted to 3.0 with 0.1 M HCl, stirred for 15 min and centrifuged at 8000g and 4 °C for 20 min. The supernatant was discarded. The pellet was re-suspended in 1.2 M NaCl (1:1 [w/v]) solution to provide the necessary ionic strength for the extraction of PME and PG. The pH of the suspension was adjusted to 6.0 using 0.1 M NaOH. The extraction of the enzymes was conducted by stirring the suspension for 3 h at 4 °C, maintaining the pH constant by the addition of 0.1 M NaOH. This was followed by centrifugation at 10,000g and 4 °C for 20 min. The supernatant obtained was used in PME and PG activity measurements.

2.4. Pectin methylesterase assay

The activity of PME was determined by measuring the release of carboxylic acid per unit time, at 30 °C, titrimetrically. A 200–400 μl (depending on the residual PME activity) portion of crude enzyme extract was mixed with 0.35% apple pectin solution (pH = 7.5, 70–75% degree of esterification, Fluka, Australia) containing 0.125 M NaCl, which was equilibrated to 30 °C. During the hydrolysis of pectin at 30 °C, the pH was maintained at 7.5 by addition of 0.01 N NaOH solution, using an automatic pH-stat titrator (TIM854, Radiometer analytical, France). The consumption of NaOH per unit time was recorded for a 15 min reaction period. The activity of PME is proportional to the rate of NaOH consumption (dV_{NaOH}/dt). Thus, the activity of PME was calculated according to Eq. (1):

$$\text{PME (U/ml)} = \left(\frac{\left(\frac{dV_{NaOH}}{dt}\right) \cdot N \cdot 1000}{V_s} \right) \quad (1)$$

where dV_{NaOH}/dt is the rate of NaOH consumption (ml/min), N is the concentration of NaOH (Normality) and V_s is the sample volume (ml). The activity unit (U) is defined as the amount of enzyme required to release 1 μmol of carboxyl group per minute under the assay condition. All the assays were done in triplicate.

2.5. Polygalacturonase assay

The activity of PG in the samples was analysed according to the method of Gross (1982), which is based on the spectrophotometric determination of reducing sugars formed during the PG-catalysed hydrolysis of polygalacturonic acid at 35 °C. In the PG assay, the reaction mixture consisted of 100 μl of the crude enzyme extract and 300 μl of 0.3% (w/v) polygalacturonic acid solution (Sigma, Australia) in 40 mm sodium acetate buffer (pH = 4.4). The reaction mixture was incubated for 10 min at 35 °C. The reaction was stopped by adding 2 ml of 0.1 M borate solution (pH = 9.0) and 400 μl of 1% cyanoacetamide solution (Aldrich, Australia), which was followed by a 10 min incubation at 100 °C and cooling in ice-water. Following equilibration to room temperature, the absorbance of the sample was measured at 276 nm against a blank, using a UV–visible spectrophotometer (UV-1700 pharmaspec, Shimadzu). The blank consisted of the polygalacturonic acid solution and inactivated enzyme (inactivated by 5 min heating in boiling water bath) treated in the same way as the sample. The amount of reducing sugar produced was calculated from a standard curve prepared using monogalacturonic acid (Fluka, Australia), assuming that the concentration of monogalacturonic acid is proportional to the concentration of reducing sugars. All the assays were performed in triplicate.

2.6. Data analysis

The following equations were used in the analyses of the experimental kinetic data. The inactivation kinetics of enzymes is usually described by a first order reaction (Eq. (2))

$$A = A_o \exp(-kt) \tag{2}$$

where A is enzyme activity at time t, A_o is initial enzyme activity and k is the first order inactivation constant in [min^{-1}].

Eq. (1) can be linearised by logarithmic transformation into

$$\ln\frac{A}{A_o} = -kt \tag{3}$$

The temperature dependence of the inactivation rate constant can be described by the Arrhenius equation, the linearised form of which is given in Eq. (4)

$$\ln(k) = \ln(k_{ref}) - \frac{E_A}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right) \tag{4}$$

where T and T_{ref} are the experimental and reference temperatures (K), E_A is the activation energy (J/mol), R is the universal gas constant (8.314 J/mol K) and k and k_{ref} are the inactivation rate constants (s^{-1}) at T and T_{ref}, respectively.

In food processing, first order reactions are commonly described by D and z values. The decimal reduction time D is the time for 90% reduction in activity A under defined temperature conditions. It can be estimated from the slope of the $\log(A/A_o)$ versus time curve as described in Eq. (5)

$$\log\left(\frac{A}{A_o}\right) = -\frac{t}{D} \tag{5}$$

For first order reactions, the D value is related to the first order reaction rate constant, k, by Eq. (6)

$$D = \frac{2.303}{k} \tag{6}$$

The z value is defined as the temperature increase required for a 10-fold decrease of the D value. The z value is calculated from the negative reciprocal of the slope of $\log D$ versus temperature curve (Eq. (7))

$$\log D = \log D_{ref} - \frac{T - T_{ref}}{z} \tag{7}$$

where D_{ref} is the D value at a reference temperature, T_{ref}.

In cases when several isoenzymes of different stabilities exist together, the inactivation kinetics may be described by either the biphasic model or the fractional conversion model, which are special forms of the first order kinetic model (Fachin et al., 2003). If two isozyme fractions with different stabilities exist together, the inactivation kinetics are described by the biphasic model (Eq. (8))

$$A = A_s \exp(-k_s t) + A_L \exp(-k_L t) \tag{8}$$

where A_s and A_L are activities of the stable and the labile fractions, respectively, and k_s and k_L are the inactivation rate constants of the stable and the labile fractions, respectively.

If there is a residual enzyme fraction whose activity remains constant after a prolonged treatment time, enzyme inactivation kinetics follow a fractional conversion model (Eq. (9))

$$A = A_o + (A_o - A_\infty)\exp(-kt) \tag{9}$$

where A_∞ is the residual activity after prolonged treatment time, *i.e.* the activity of the stable fraction.

All data analysis was performed using table curve 2D software (version 3, Systat software Inc., USA).

3. Results and discussion

3.1. Thermal and thermosonication inactivation kinetics of PME

Fig. 1 shows the thermal inactivation curves of PME. As can be seen, thermal treatment of tomato juice at 50 °C did not cause significant inactivation of PME. At temperature higher than 50 °C, significant inactivation of tomato PME was observed. The thermal inactivation kinetics of tomato PME was described reasonably well by first order inactivation kinetics, as confirmed by visual inspection of the model fitting, as well as the correlation coefficients

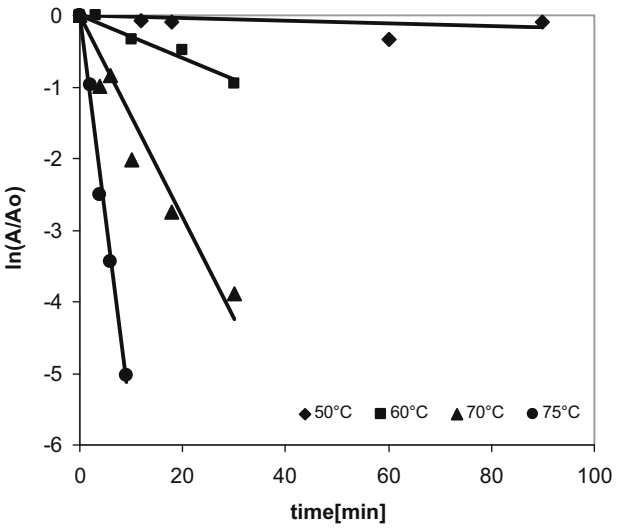

Fig. 1. Thermal inactivation curves of tomato pectin methylesterase in tomato juice.

obtained, which were in the range 0.963–0.998. The inactivation rate constants, estimated by linear regression analysis, varied from 0.026 min^{-1} at 60 °C to 0.57 min^{-1} at 75 °C, with the corresponding *D* values of 89.0 min and 4.04 min, respectively. The activation energy for the thermal inactivation of PME was determined (from the slope of the natural logarithm of the inactivation rate constant versus the reciprocal of the absolute temperature) to be 193 kJ/mol (R^2 = 0.980). The *z* value estimated from the slope of the logarithm of the *D* value versus temperature is 11.4 °C (Table 1).

The thermal inactivation kinetics of tomato PME are well studied. However, there is significant variation in the reported inactivation parameters (Anthon, Sekine, Watanbe, & Barret, 2002; Nath, Rao, & Gupta, 1983; Raviyan et al., 2005; Wu, Gamage, Vilkhu, Simons, & Mawson, 2008). For instance, *D* values ranging from 1.5 to 36.4 min (estimated from the data of Raviyan et al. (2005)), have been reported for inactivation of tomato PME at 70 °C. Our value of 16.2 min falls within this range. The reported *z* values for the thermal inactivation of tomato PME vary from 4.5 to 32 °C (Anthon et al., 2002; Nath et al., 1983; Raviyan et al., 2005). Our *z* value of 11.4 °C is in close agreement with the value of Nath et al. (1983) and Raviyan et al. (2005) who reported *z* values of 9 and 12.3 °C, respectively, for the inactivation of PME. In contrast, Anthon et al. (2002) reported a *z* value of about 5 °C, which indicates much higher temperature sensitivity of tomato PME. Several factors may be responsible for the observed discrepancies in kinetic parameters. Some of the studies were on the inactivation of PME in tomato juice and homogenates (Anthon et al., 2002; Nath et al., 1983; Wu et al., 2008), whilst others were on purified enzyme suspended in buffers at different pH values (Raviyan et al., 2005). In general an enzyme is more stable in an intact tissue or in a homogenate where it is protected by the presence of other materials, such as proteins, carbohydrates and pectins, than in its purified form (Whitaker, 1972). In addition, there are varietal differences in the tomatoes used for the different experiments. Anthon et al. (2002) reported D_{70} values of 7.2 and 10.4 min for the thermal inactivation of PME in tomato juice for cultivars BOS 3155 and CXD-199, respectively, which shows the influence of varietal differences.

The curves for ultrasound-induced inactivation of tomato PME at different temperatures are presented in Fig. 2. In contrast to thermal treatment alone, thermosonication caused significant inactivation of tomato PME at 50 °C. As can be seen in Fig. 2, the inactivation is well described by first order kinetics. The estimated first order kinetic parameters, as well as the activation energy and *z* values, are presented in Table 1. Comparing the kinetic parameters for thermal and thermosonication inactivation, it can be seen that thermosonication substantially enhanced the inactivation kinetics of pectin methylesterase in tomato juice. At 60 °C, an approximately six times increase in the inactivation rate was observed compared to the thermal inactivation rate at the same temperature. This decreased to 1.5 times as the temperature increased to 75 °C. Clearly the observed inactivation effect of heat and ultrasound on PME in this study is synergistic rather than additive. The inactivation rate constants for thermosonication are much higher

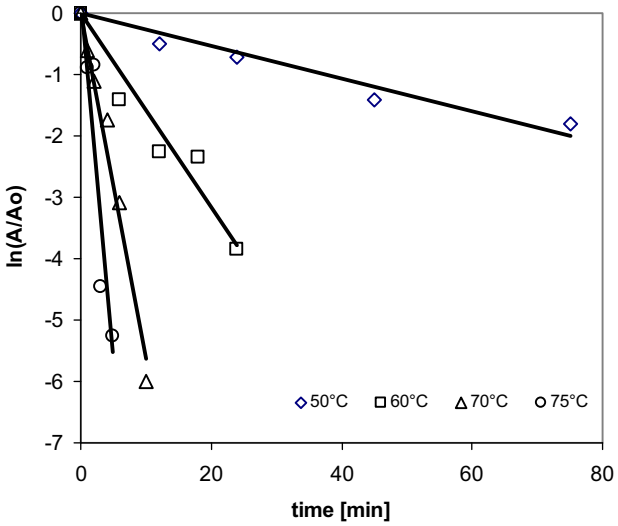

Fig. 2. Ultrasound (20 kHz, 64 μm) inactivation curves of pectin methylesterase in tomato juice at different temperatures.

than what can be obtained from the sum of the thermal inactivation constant and the ultrasound inactivation constant (the inactivation at 50 °C can be considered to be solely due to ultrasound as thermal inactivation at that temperature is insignificant) at a given temperature. For instance, the thermosonication inactivation constant at 60 °C is 0.16 min^{-1}, whilst the additive rate constant would be 0.053 min^{-1} (obtained from the sum of the thermal inactivation rate constant at 60 °C (0.026 min^{-1}) and the ultrasonic inactivation rate constant at 50 °C (0.027 min^{-1}), which shows that the effects of heat and ultrasound are synergistic, with the thermosonication rate constant about three times that of the additive rate constant. This is illustrated in Fig. 3.

A few studies have been reported on the effects of thermosonication on tomato PME (Raviyan et al., 2005; Wu et al., 2008). In all cases, the application of ultrasound resulted in significant enhancement of the rate of tomato PME inactivation, with the degree of enhancement dependent on the media in which the enzyme is suspended and the ultrasound processing conditions. Raviyan et al. (2005) reported a 39- to 374-fold increase in the inactivation rate of crude tomato PME extract under thermosonication (20 kHz, 20 μm, 100 W) compared to just thermal treatment at 61 °C. The effect was dependent on sample volume. The maximum increase in the inactivation rate was observed in the sample with the smallest volume (50 ml), where the acoustic power density (~2 W/ml) and hence the cavitation intensity (0.012 mg/l H_2O_2 generation/min) were the highest. The lowest effect was observed in the sample with the largest volume (200 ml). The efficacy of ultrasound was less at 72 °C with 36- to 84-fold increases in the rate of inactivation for sample volumes ranging from 50 to 200 ml (Raviyan et al., 2005). Significantly less pronounced effects were

Table 1
Estimated kinetic parameters for the inactivation of tomato pectin methylesterase by thermal and thermosonication treatments.

Temperature (°C)	Thermosonication			Thermal treatment		
	k (min^{-1})	D (min)	R^2	k (min^{-1})	D (min)	R^2
50	0.027 ± 0.002[a]	86.5 ± 6.1	0.980	–	–	
60	0.16 ± 0.01	14.6 ± 1.1	0.978	0.026 ± 0.003	89 ± 10.1	0.963
70	0.56 ± 0.02	4.1 ± 0.2	0.990	0.14 ± 0.01	16.2 ± 1.2	0.975
75	0.83 ± 0.12	2.8 ± 0.4	0.905	0.57 ± 0.01	4.04 ± 0.1	0.998
E_A (kJ/mol K)	130 ± 10.5		0.987	193 ± 27.7		0.980
z (°C)	16.6 ± 1.6		0.982	11.4 ± 1.5		0.983

[a] Standard error.

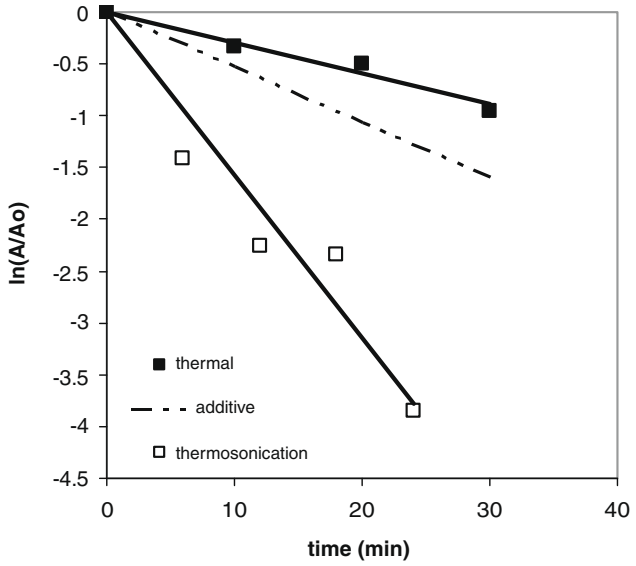

Fig. 3. Inactivation curves for tomato pectin methylesterase at 60 °C illustrating the synergistic effect of heat and ultrasound (determined at 50 °C where no thermal effect was observed).

reported for the inactivation of this enzyme in tomato juice under thermosonication (24 kHz, 25–75 μm) with about 2.3- and 2.1-fold decreases in *D* value at 55 and 65 °C compared to thermal treatment at the same temperatures, whilst the effect of ultrasound diminished as the temperature increased to 70 °C (Wu et al., 2008). As in the case of Wu et al. (2008), we observed much less enhancement in the inactivation of PME in tomato juice compared to what was reported by Raviyan et al. (2005) for the inactivation of tomato PME in buffer. One factor may be the much higher viscosity of tomato juice compared to aqueous buffer, resulting in less cavitation in the juice compared to the buffer and less pronounced effect of ultrasound on the enzyme (Vercet et al., 2002). The different components of the juice, including pectin, may also have a protective effect on the enzyme (Whitaker, 1972). Another important factor is difference in acoustic power input density and sonotrode geometry (Sala et al., 1995). The differences in acoustic power input density and sonotrode geometry may also account for the difference between our result and that of Wu et al. (2008). The power input density in our study was about 1.6 W/ml compared to approximately 0.48 W/ml in the case of Wu et al. (2008).

The combined effect of ultrasound, heat and mild pressure (manothermosonication) on the activity of tomato PME has also been investigated in a buffer system (Lopez, Vercet, Sanchez, & Burgos, 1998) as well as in tomato juice (Vercet et al., 2002). Lopez et al. (1998) studied the inactivation kinetics of purified tomato PME in citrate buffer (pH = 4.0) during ultrasonication (20 kHz, 117 μm) in a continuous system performed at a pressure of 200 kPa and temperature of 62.5 and 37 °C. Manothermosonication at 37 °C inactivated the enzyme (*D* = 4.3 min), whereas heating under the same conditions did not affect the activity of the enzyme. At 62.5 °C, a synergistic effect was observed between heat and ultrasound with the *D* value reduced 52.9 times compared to thermal inactivation (45–0.85 min). Vercet et al. (2002) compared the effects of a manothermosonication (20 kHz, 200 kPa, 117 μm, 70 °C) treatment of tomato juice for 1 min on tomato PME with a thermal treatment at the same temperature. Complete inactivation of PME was observed following manothermosonication, whereas only 38% inactivation was achieved by thermal treatment. In our study, only about 43% inactivation of PME was observed by thermosonication after a 1 min treatment at 70 °C (calculated from the kinetic data) and atmospheric pressure. The higher pressure and ultrasound amplitude (114 μm compared to 64 μm) may have contributed to the observed higher inactivation of PME in the study of Vercet et al. (2002). High pressure, of the order of magnitude that is commonly applied in manothermosonication studies (⩽300 kPa), does not inactivate enzymes. In general, a pressure higher than 300 MPa is required for irreversible inactivation of enzymes (Hendrickx, Ludikhuyze, Vanden Broeck, & Weemaes, 1998). However, high pressure improves the efficiency and violence of bubble implosion and free radical production, since the vapour pressure of water in the bubbles is reduced (Sala et al., 1995; Vercet, Lopez, & Burgos, 1998).

The inactivation effect of ultrasound over and above thermal treatment, in this study, decreased as the temperature increased from 60 to 75 °C. This is also manifested in the lower activation energy (130 kJ/mol) and higher *z* value (16.6 °C) compared to the thermal treatment alone (see Table 1). Similarly, a higher *z* value (40 °C) was estimated for the inactivation of tomato PME in buffer by manothermosonication than for thermal treatment alone (*Z* = 5 °C), indicating that the synergistic effect between sonication and heat decreases with temperature (Lopez et al., 1998). This is attributed to the increased vapour pressure of water making the collapse of bubbles and cavitation less violent at higher temperature. Increased temperature results in increased formation and growth of bubbles as vapour pressure increases and tensile strength decreases. However, the violence of collapse decreases as high vapour pressure acts as a cushion. In addition, increased temperature results in decreased viscosity, resulting in decreased violence of implosion (Sala et al., 1995). Such decrease in the efficacy of ultrasound with increase in temperature has also been observed in the studies cited earlier. For instance, in the study of Raviyan et al. (2005), the increase in the rate of inactivation of tomato PME decreased to 84-fold at 72 °C compared to 374-fold at 61 °C for the 50 ml sample. Wu et al. (2008) did not observe any increase in the inactivation rate of PME in tomato juice by ultrasonication at 70 °C.

Low frequency ultrasound causes the inactivation of enzymes through various effects, including the localised extreme increase in temperature and pressure accompanying the collapse of cavitation bubbles, strong shear force and shock waves created by stable cavitating bubbles and the generation of free radicals that oxidise amino acid residues that participate in the stability and catalytic activity of enzymes (Barteri et al., 2004; Sala et al., 1995; Zhong et al., 2004). Cavitation-induced high shear stress and pressure have been reported to cause the dissociation of oligomeric enzymes, such as glucose-6-phosphate dehydrogenase (Rachinskaya, Karasyova, & Metelitza, 2004) and human butyrylcholinesterase (Froment, Lockridge, & Masson, 1998) and breakdown of polypeptide chains in enzymes such as trypsin (Zhong et al., 2004). Moreover, several studies suggest that free radicals, generated during sonication, play a major role in enzyme inactivation. Barteri et al. (2004) studied the inactivation of fumarase by ultrasound. They observed progressive oxidation of the cysteine residues and aggregation of the enzyme, from which they concluded that the inactivation of the enzyme was due to the formation of disulfide-linked aggregates, formed as a result of the oxidation of the cysteine residues by the free radicals generated during sonication (Barteri et al., 2004). The involvement of free radicals has also been reported in the ultrasonic-induced inactivation of trypsin (Zhong et al., 2004), horse radish peroxidase (Grintsevich, Adzerikho, Mrochek, & Metelitza, 2001), catalase (Potapovich, Eremin, & Metelitza, 2003), glucose-6-phosphate dehydrogenase (Rachinskaya et al., 2004), urease (Tarun, Adzerikho, & Metelitsa, 2003) and α-amylase (Liu, Chen, & Chou, 2003). The creation of large interfacial area by ultrasound has also been shown to contribute to the inactivation of trypsin (Zhong et al., 2004) and lactate dehydrogenase (Niven, Ip,

Mittelman, Prestrelski, & Arakawa, 1995), since that disturbs hydrophobic interaction and hydrogen bonding which play major roles in the stability of proteins (Zhong et al., 2004). The inactivation of tomato PME by ultrasound in this study may have been caused by one or more of the above-mentioned mechanisms. Amino acids, such as tryptophan, tyrosine, histidine and cysteine, are particularly susceptible to degradation by hydroxyl and superoxide free radicals (Davies, Delsignore, & Lin, 1987). Tryptophan and tyrosine residues are present at the active site of tomato PME (D'Avino, Camardella, Christensen, Giovane & Servillo, 2003). Moreover, tomato PME is stabilised by cysteine residues which are involved in disulfide bridges in the native enzyme (Markovic & Jornvall, 1992). Thus, free radical-mediated mechanisms may play significant roles in the ultrasonic inactivation of tomato PME.

3.2. Thermal and thermosonication inactivation kinetics of polygalacturonase

As in the case of PME, thermal treatment at 50 °C did not have a significant effect on the activity of polygalacturonase in tomato juice (see Fig. 4). At temperatures between 50 and 75 °C, thermal treatment resulted in partial PG inactivation. The inactivation kinetics was well described by a fractional conversion model, indicating that part of the tomato PG remained stable after prolonged treatment under these conditions. The existence of thermolabile and thermostable PG fractions in tomato is well documented. Tomato PG is reported to exist in three isoforms: PG1, PG2A and PG2B. PG2A and PG2B differ mainly in their degree of glycosylation and are usually grouped together as PG2 since they have the same thermostability. PG2 is heat-labile and is totally inactivated after a 5 min treatment at 65 °C. PG1 is a dimer formed from a structural association of PG2 with a heat-stable glycoprotein β-subunit, which confers heat-stability on it (Fachin et al., 2003; Pogson & Brady, 1993). Thus, only the labile form of PG (PG2) was inactivated under the conditions in this study. Other studies have also reported fractional conversion of polygalacturonase under mild temperature conditions. The thermal inactivation kinetics of polygalacturonase in tomato juice in the temperature range between 55 and 70 °C are described by a fractional conversion model (Fachin et al., 2003). Under higher temperature conditions, biphasic inactivation curves are commonly observed, whereby the two isozyme fractions are inactivated at different rates (Anthon et al., 2002; Fachin et al., 2003; Pogson & Brady, 1993).

The kinetic parameters for the thermal inactivation of tomato PG, estimated from non-linear regression analysis of our data, are presented in Table 2. The inactivation rate constants ranged from 0.085 min^{-1} at 60 °C to 0.57 min^{-1} at 75 °C, which is equivalent to a D value ranging from 11.7 to 1.75 min. As in the case of tomato PME, there is a wide variation in the estimated kinetic parameters for the inactivation of tomato PG in the literature. A D value of 3.99 min has been reported for the thermal inactivation rate of the labile faction of partially purified PG in sodium acetate buffer (pH = 4.4) at 70 °C (Fachin, Loey, Indrawati, Ludikhuyze, & Hendrickx, 2002), whilst a D value of 0.78 min was observed in tomato juice (Fachin et al., 2003). This is not unusual, since the stability of enzymes depends on several factors, including their source, pH and medium composition. The activation energy for the thermal inactivation of PG2 was estimated from linear regression of the natural logarithm of the inactivation constant versus the reciprocal of the absolute temperature (R^2 = 0.954) and is presented in Table 2. Our value of 116 kJ/mol is less than most published values. In the literature, activation energy values of 208–228 kJ/mol have been reported for the thermal inactivation of PG2 in tomato juice (Anthon et al., 2002; Fachin et al., 2003) whilst an activation

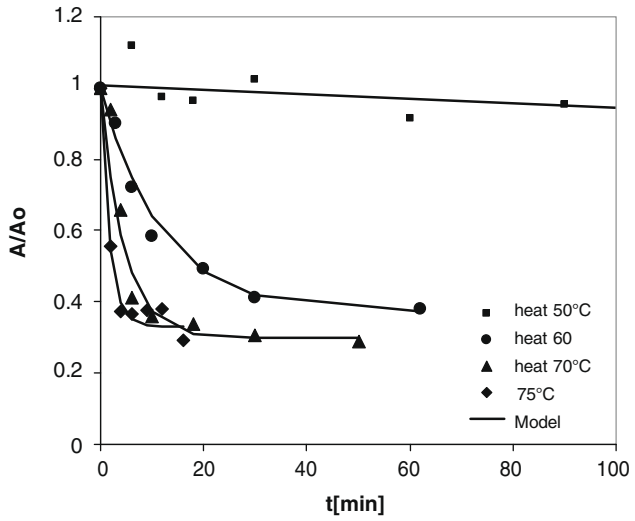

Fig. 4. Thermal inactivation curves of polygalacturonase in tomato juice at different temperatures.

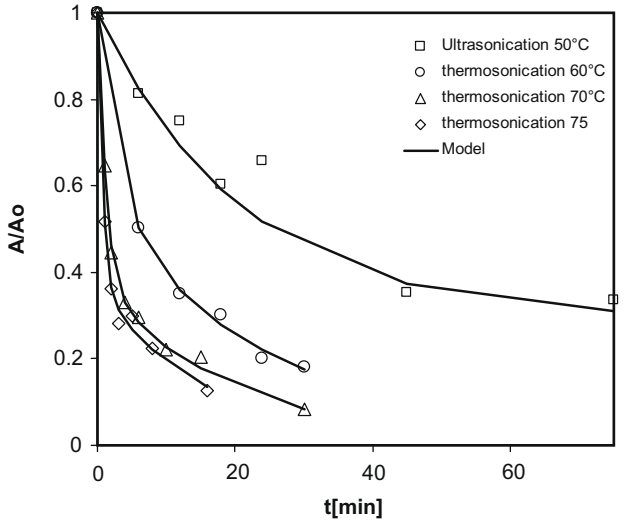

Fig. 5. Ultrasound (20 kHz, 64 μm) inactivation curves of polygalacturonase in tomato juice at different temperatures.

Table 2
Estimated kinetic parameters (k: inactivation rate constant, A_∞: % stable fraction, E_A: activation energy) for the thermal and ultrasonic inactivation (at 50 °C) of PG in tomato juice.

Temperature (°C)	k (min^{-1})	A_∞ (%)	R^2	E_A (kJ/mol)
50 (Ultrasonication)	0.047 ± 0.0078[a]	28.9 ± 7.5	0.994	
60	0.085 ± 0.024	37.1 ± 5.7	0.986	116 ± 25.5 (R^2 = 0.954)
70	0.22 ± 0.034	29.8 ± 2.6	0.931	
75	0.57 ± 0.08	32.9 ± 2.1	0.983	

[a] Standard error.

Table 3
Estimated kinetic parameters (k_L, k_s: inactivation rate constants of labile and stable fractions, respectively, A_L, A_s: % labile and stable fractions, respectively) for the inactivation of tomato PG by thermosonication (20 kHz, 65 μm).

Temperature (°C)	k_L (min^{-1})	k_s (min^{-1})	A_L (%)	A_s (%)	R^2
60	0.34 ± 0.16[a]	0.039 ± 0.008	56.3 ± 10.7	43.7 ± 10.7	0.998
70	0.82 ± 0.09	0.050 ± 0.009	62.5 ± 4.2	37.5 ± 4.2	0.998
75	1.31 ± 0.26	0.061 ± 0.02	64.0 ± 7.0	36.0 ± 6.5	0.997
E_A (kJ/mol)	70.0 ± 7.5 (R^2 = 0.988)	28.0 ± 3.5 (R^2 = 0.984)			

[a] Standard error.

energy value of 407 kJ/mol has been reported for the inactivation PG2 in partially purified PG (Fachin et al., 2002). The thermostable PG1 fraction (A_∞) was estimated to be about 30% of the total PG activity (see Table 2). Pogson and Brady (1993) observed 21% and 35% PG1 in Heinz 4236 and Heinz 4148 tomato varieties, which are close to our estimated value for Heinz 3402 tomatoes. Published data for the relative proportion of PG1 vary between 14% and 55% (Fachin et al., 2003; Pogson & Brady, 1993). According to Pressey (1986), the proportion of PG1 depends on the pH of the extraction solution, the concentration of sodium chloride, the amount of active pectin methylesterase and whether ammonium sulphate precipitation is used for protein concentration. If active PME is present in the system, demethylation of pectin occurs during the extraction. Therefore, some of the cationic PG1 will be bound to the anionic demethylated pectin, leading to lower proportions of PG1 in the final extract (Pressey, 1986).

The curves for the ultrasonic inactivation of PG at various temperatures are presented in Fig. 5. Ultrasound treatment caused inactivation of PG in the temperature range 50–75 °C. At 50 °C only the labile PG fraction (PG 2) was inactivated, as may be deduced from the curve. As in the case of thermal inactivation, the inactivation rate was described by a fractional conversion model. The kinetic parameters for ultrasound inactivation of PG at 50 °C are presented in Table 2, together with the thermal inactivation parameters. As in the case of thermal treatment, the stable PG 1 fraction was estimated to be about 30%. At higher temperatures, biphasic inactivation was observed, with both PG1 and PG2 inactivated at different rates. The biphasic inactivation kinetics parameters estimated from non-linear regression analysis of the data are given in Table 3. Thermosonication enhanced the inactivation kinetics of PG2 4-fold at 60 °C (compare Tables 2 and 3). The enhancement decreased to 2.3-fold at 75 °C. As in the case of PME, a lower activation energy was observed for the thermosonication inactivation of PG2 (70 kJ/mol) compared to that of thermal inactivation (116 kJ/mol). The proportion of the stable fraction PG1 was estimated to be around 40%.

The biphasic inactivation of PG can be attributed to the synergistic action of heat and ultrasound, as neither treatments alone [heat (50–75 °C) or ultrasound (50 °C)] caused inactivation of PG1 under the studied conditions. The synergy between the two is also clear from the thermal and thermosonication inactivation h rate constants for the inactivation of the thermolabile fraction, PG2. Under all temperature conditions, the inactivation rate constants for thermosonication are much higher than those that can be obtained from simple additive effects of the two treatments (see Tables 2 and 3). As mentioned in the discussion on PME, sonication can cause the breakdown of oligomeric proteins into their subunits. This would make it particularly effective for the inactivation of PG1, which is stabilised by its structural association with the heat-stable β-subunit. During thermosonication, sonication may have caused the dissociation of PG1 into the heat-labile PG2 and the heat-stable β-subunit, followed by the inactivation of PG2 by the synergistic effect of heat and sonication.

There are no reports in the literature on the effects of thermosonication on the inactivation kinetics of tomato polygalacturonases. A study on the effect of manothermosonication reported synergistic effects of heat and ultrasound for the inactivation of purified tomato PG1 and PG2 in buffer (Lopez et al., 1998). Manothermosonication (20 kHz, 117 μm, 200 kPa) enhanced the inactivation of PG1 85.8 times at 86 °C whilst the inactivation of the heat-labile PG2 was enhanced 26.3 times at 52.6 °C compared to thermal treatment. Manothermsonication also caused substantial inactivation of the two isozymes at 37 °C (D = 3.17 min and 2.23 min for PG1 and PG2, respectively), whereas thermal treatment did not have any effect (Lopez et al., 1998). Another study on the effect of manothermosonication on the activity of PG in tomato juice showed that manothermosonication (20 kHz, 117 μm, 200 kPa) at 70 °C caused 62% inactivation of PG in 1 min whilst thermal treatment under comparable conditions did not have any effect on the enzyme (Vercet et al., 2002).

4. Conclusions

Our result showed that thermosonication (20 kHz, 75 μm) processing of tomato juice at 75 °C causes almost complete inactivation of PME and about 72% inactivation of PG after 4 min. Under these conditions, the residual PG activity is unlikely to cause substantial pectin degradation as the preferred substrate for PG is demethylated pectin, which would be less available as PME is almost completely inactivated. The inactivation effect of sonication can also be further enhanced by optimisation of the processing conditions, such as by increasing the ultrasound power intensity, changing the sonotrode geometry and applying pressure in a batch or continuous system, which will be the subject of our future research work. Thermosonication has already been shown to significantly improve the rheological properties of tomato products (Wu et al., 2008). Our current work shows the separate effects of temperature, sonication and thermosonication on PME and PG, the enzymes which determine the rheological characteristics of tomato products. This provides scientific and technological bases to further develop thermosonication as an alternative to the tomato 'hot break' process.

Acknowledgments

We thank the Victorian Department of Innovation Industry and Regional Development for funding the infrastructure through the science and technology infrastructure (STI) grant. We would also like to thank Piotr Swiergon for his help with the experimental device.

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Food Chemistry 117 (2009) 28–34

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Degradation compounds of carotenoids formed during heating of a simulated cashew apple juice

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ARTICLE INFO

Article history:
Received 17 December 2008
Received in revised form 6 February 2009
Accepted 17 March 2009

Keywords:
Carotenoids
Degradation compounds
Cashew apple juice
HPLC–PDA–MS/MS
Anacardium occidentale L.

ABSTRACT

The influence of organic acid and heating treatments on carotenoid degradation on a simulated cashew apple juice was assessed by high performance liquid chromatography coupled with a photodiode array and mass spectrometry detectors. A total of nineteen *carotenoids* were separated in unheated simulated cashew apple juice, with all-*trans*-β-cryptoxanthin and all-*trans*-β-carotene as the major ones. As a consequence of heating, five xanthophylls disappeared, whereas two new *cis* isomers and five epoxide or furanoid-derivatives were formed and the levels of all *cis* isomers increased. In addition, 12′-apo-β-carotenal was formed at 90 °C. Two oxidation compounds (12′-apo-β-carotenal and 5,6-epoxy-β-cryptoxanthin) were formed after β-cryptoxanthin heating at 90 °C in an aqueous-based system. In all systems, the amounts of total carotenoids lost were not compensated by those formed. These facts indicated that isomerisation and oxidation to both coloured and non-coloured compounds were the main reactions occurring during heating of carotenoids in aqueous-based and juice systems.

1. Introduction

In the last decade, the Brazilian juice industry showed rapid and intensive growth, mainly due to the increasing national and international interests in fruit juices, since these products have been strongly associated with health benefits. The global juice market registered a growth of about 8%, which is the equivalent of US$ 2 billion in sales in 2006–2007 (ACNielsen, 2008; Cooper, 2004), with Brazil producing 472,187,000 litres of fruit juice in 2007 (Abir, 2008).

The cashew apple (Fig. S1 from Supplementary data) is a tropical fruit native to Brazil, principally grown in the North and Northeast regions. The pseudo-fruit, known as the cashew apple, is the part of the tree that connects it to the cashew nut, the real fruit, a well-known product worldwide. The pseudo-fruit is commercially exploited mainly as frozen pulp, juice, and nectar. Carotenoids are the main pigments responsible for the colour of cashew apple fruits and juices, the major ones being β-cryptoxanthin in red and yellow cashew apple fruits from the hottest Northeast region of Brazil, and β-carotene in the same fruits from the Brazilian Southeast region (Assunção & Mercadante, 2003a, 2003b).

Carotenoids are highly susceptible to degradation by external agents, such as heat, low pH, and light exposure, promoting changes of colour due to the rearrangement or formation of degradation compounds such as *cis*-isomers, epoxides, short chain products, and in some cases, volatile compounds (Mercadante, 2008a). Besides colour fate, carotenoid oxidation compounds are also supposed to have detrimental effects *in vivo*, through the induction of oxidative stress, exertion of cytotoxic and genotoxic effects, and inhibition of gap junction intercellular communications (Caris-Veyrat, 2008; Hurst, Saini, Jin, Awasthi, & van Kuijk, 2005; Siems et al., 2005).

References to thermal instability of carotenoids are found in the pioneering work of Zechmeister (1944), with emphasis on the *trans*- to *cis*-isomerisation. This topic has been intensively investigated and striking examples are found in literature (Aman, Schieber, & Carle, 2005; Dhuique-Mayer et al., 2007; Doering, Sotiriou-Leventis, & Roth, 1995; Kuki, Koyama, & Nagae, 1991; Rios, Borsarelli, & Mercadante, 2005).

On the contrary, the effect of acid on carotenoids was reported in fewer studies. Carotenoids were found to be mono and diprotonated by nitric acid and moderately strong acids, such as trichloroacetic and trifluoroacetic acids (Mortensen & Skibsted, 2000). Organic acids liberated during the processing of fruit juices are strong enough to promote rearrangements of 5,6-epoxide groups to 5,8-furanoid groups of carotenoids (Assunção & Mercadante, 2003a; Dhuique-Mayer et al., 2007; Meléndez-Martínez, Vicario, & Heredia, 2007a).

In a food system, the mechanisms involved in the nutrient degradation are complex, and therefore, insights into the mechanism of carotenoid thermal degradation can be derived from model systems, which are more easily controlled than foods and the formation of initial, intermediate, and final compounds can be

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.03.071

more easily monitored (Mercadante, 2008a). In addition, considering that *cis* isomers are expected to be formed, analysis should be done by high performance liquid chromatography (HPLC) connected at least to a photodiode array detector (PDA). The carotenoid separation should be carried out on a C_{30} column, since among reversed-phase columns, the C_{30} is the only one capable of resolving peaks of geometrical isomers at the same position but at opposite ends of asymmetrical carotenoids, such as β-cryptoxanthin (Emenhiser, Sander, & Schwartz, 1995; Mercadante, 2008b).

Thus, the objectives of this study were threefold: (1) to separate and identify, by HPLC–PDA connected to mass spectrometry (MS/MS), the non-volatile degradation compounds derived from carotenoids during the heating of a simulated cashew apple juice; (2) to verify the influence of organic acids, at the same concentration as that found in a juice, on the carotenoid degradation; and (3) to indicate the main carotenoid thermal degradation pathways in such systems. In addition, for the first time, thermal degradation of β-cryptoxanthin in an aqueous-based system was evaluated.

2. Materials and methods

2.1. Samples and standards

Red cashew apple fruits (*Anacardium occidentale* L.), from the Petrolina region (Pernambuco State, Northeast region, Brazil), were acquired in a supermarket in Campinas city (São Paulo State, Brazil), during their harvest season (October, 2006) and kept frozen at −18 °C until carotenoid extraction.

Standards of all-*trans*-lutein, all-*trans*-zeaxanthin, all-*trans*-β-cryptoxanthin, all-*trans*-β-carotene, 9-*cis*-β-carotene, 13-*cis*-β-carotene, all-*trans*-α-carotene, β-apo-8′-carotenal, β-apo-10′-carotenal, and β-apo-12′-carotenal were donated by DSM Nutritional Products (Basel, Switzerland), with purities ranging from 95.0% to 99.9%, as determined by HPLC–PDA.

2.2. Simulated cashew apple juice

The carotenoids were extracted with acetone, transferred to petroleum ether/diethyl ether, saponified overnight at room temperature with 10% methanolic KOH, followed by alkali removal and concentration on a rotary evaporator (<35 °C). Due to the high oil content in the cashew apple peel, it was necessary to physically remove the oil, as follows: prior to ether transference, the carotenoid extract was kept in the freezer at −18 °C for 2 h, followed by filtration using cold glassware and washing with cold acetone (De Rosso & Mercadante, 2007a). The dried cashew apple carotenoid extract was flushed with nitrogen (99.0%) and kept at −35 °C.

The cashew apple extract, containing about 200 μg carotenoid in 5 ml of ethanol, was added to 25 ml of Milli-Q water (Millipore, Billerica, USA) acidified at pH 3.8 with *ca.* 300 μl of 0.5 M citric acid (simulated juice). The acid concentration allowed the addition of less than 1% of the total system volume to achieve the pH value found in commercial cashew apple juice.

The simulated cashew apple juice was distributed into glass tubes, sealed and heated for 1, 2, and 4 h at 60 °C and 90 °C in a water bath. After each time, tubes were taken from the bath and immediately cooled in current water.

In order to confirm whether the thermal degradation compounds were derived from β-cryptoxanthin or β-carotene, 100 μg of synthetic all-*trans*-β-cryptoxanthin was used to elaborate systems as described above, with and without acid. These systems were monitored by HPLC–PDA–MS/MS and by a UV–visible diode array spectrophotometer (Agilent, model 8453, Palo Alto, USA). In addition, UV–vis spectra of the β-cryptoxanthin acidified system were recorded at room temperature during 120 min.

2.3. Carotenoid analysis in the simulated juice

The carotenoids were exhaustively extracted from the simulated juice with ethyl acetate, by vortexing (Phoenix, model AP 56, Araraquara, Brazil) during 1 min. The organic phases were transferred to a separatory funnel, washed with water until neutral pH was obtained, and dried under a N_2 stream. Prior to HPLC-PDA-MS/MS analysis, the carotenoid extract was completely solubilised in methanol/methyl *tert*-butyl ether (MeOH/MTBE) (70:30) and filtered through Millipore membranes (0.22 μm).

The carotenoids were analysed in a Shimadzu HPLC (Kyoto, Japan), connected in series to a PDA detector (Shimadzu, model SPD-M20A) and a mass spectrometer with an ion-trap analyser and atmospheric pressure chemical ionisation (APCI) source from Bruker Daltonics, model Esquire 4000 (Bremem, Germany). The UV–visible spectra were obtained between 250 and 600 nm, and the chromatograms were processed at 286, 347, and 450 nm. The MS parameters were set as previously reported (De Rosso & Mercadante, 2007a). For all samples, carotenoid separation was carried out on a C_{30} YMC column (3 μm, 250 × 4.6 mm id.) (Waters, Wilmington, USA) using as mobile phase, a linear gradient of MeOH/MTBE from 95:5 to 70:30 for 30 min, to 50:50 for 20 min. To verify the presence of degradation compounds with low molecular weight which may elute in the dead volume using the mobile phase described above, another linear gradient of MeOH/MTBE/H_2O from 85:5:10 to 95:5:0 for 30 min, to 89:11:0 for 25 min, to 75:25:0 for 40 min, to 50:50:0 for 60 min was used. Independent of the mobile phase, the flow rate was set at 0.9 ml/min and column temperature was kept at 22 °C.

The carotenoids were identified considering the combined information obtained from the following parameters: elution order on the C_{30} HPLC column, co-chromatography with authentic standards, UV–visible spectrum characteristics (λ_{max}, spectral fine structure (% III/II), and *cis* peak intensity (% A_B/II)), and mass spectrum (molecular ion and fragments) compared to standards and data available in the literature (Britton, Liaaen-Jensen, & Pfander, 2004; De Rosso & Mercadante, 2007a, 2007b; Enzell & Back, 1995; Mercadante, Steck, & Pfander, 1999).

The carotenoids were quantified using external calibration curves for all-*trans*-lutein, all-*trans*-zeaxanthin, all-*trans*-β-cryptoxanthin, all-*trans*-β-carotene, and all-*trans*-α-carotene with a minimum of five concentration levels. Neoxanthin, neochrome, antheraxanthin, mutatoxanthin, *cis*-violaxanthin, luteoxanthin, and auroxanthin were quantified using the curve of lutein; the β-cryptoxanthin epoxides, 12′-apo-β-carotenal, using the curve of all-*trans*-β-cryptoxanthin; α-cryptoxanthin and zeinoxanthin by the curve of all-*trans*-α-carotene; β-carotene epoxides by the curve of all-*trans*-β-carotene; and the *cis*-isomers of lutein, β-cryptoxanthin and β-carotene using the curve of the corresponding all-*trans*-isomers. Total carotenoid content was calculated considering all identified peak areas.

3. Results and discussion

3.1. Identification of carotenoids

The carotenoids separated in all systems were identified based on the combined information obtained from chromatographic elution, co-chromatography with standards, UV/visible and mass spectra characteristics (Table 1). Since a detailed description of carotenoid identification using the above information was already reported by De Rosso and Mercadante (2007a, 2007b), only considerations regarding the carotenoids not identified in the previous reports were discussed below.

Table 1
Chromatographic, UV–Vis and mass spectra characteristics of carotenoids detected in the β-cryptoxanthin system and in the simulated cashew apple juice.

Peak[a]	Carotenoids	t_R range (min)[b]	λ_{max} (nm)[c]	III/II (%)	A_B/II (%)	$[M + H]^+$ (m/z)	MS/MS (m/z)
1	12′-Apo-β-caroten-12′-al	7.8–7.8	421–423	0	0	351	333 [M + H − 18], 295, 227 [M + H − 18 − 106], 209, 177, 119
2	*cis*-Neoxanthin	8.4–8.4	326, 415, 437, 468	72	0	601	583 [M + H − 18], 565 [M + H − 18 − 18], 547 [M + H − 18 − 18 − 18], 509 [M + H − 92], 221
3	Neochrome	9.6–10.0	399, 421, 447	80	0	601	583 [M + H − 18], 565 [M + H − 18 − 18], 547 [M + H − 18 − 18 − 18], 221
4	Auroxanthin	11.1–11.7	380, 401, 425	99	0	601	583 [M + H − 18], 565 [M + H − 18 − 18], 547 [M + H − 18 − 18 − 18], 509 [M + H − 92], 491 [M + H − 18 − 92], 221
5	*cis*-Violaxanthin	12.2–12.3	326, 411, 434, 463	64	24	601	583 [M + H − 18], 565 [M + H − 18 − 18], 509 [M + H − 92], 491 [M + H − 18 − 92], 221
6	Luteoxanthin – type carotenoid	12.1–12.8	396–400, 417–423, 442–443	30–60	0	601	583 [M + H − 18], 509 [M + H − 92], 491 [M + H − 18 − 92], 221
7	Antheraxanthin	13.1–13.9	419–426, 443–445, 469–472	25	0	585	567 [M + H − 18], 549 [M + H − 18 − 18], 493 [M + H − 92], 221
8	Mutatoxanthin	14.1–15.2	405, 426, 451	40	0	585	567 [M + H − 18], 549 [M + H − 18 − 18], 493 [M + H − 92], 221
9	All-*trans*-lutein	14.6–15.4	420, 444, 472	60	0	569	551 [M + H − 18], 533 [M + H − 18 − 18]
10	*cis*-Lutein	14.9–15.5	330–332, 415–417, 435–439, 467–469	n.c[d].	0	569	551 [M + H − 18], 533 [M + H − 18 − 18]
11	All-*trans*-zeaxanthin	15.6–16.7	424, 449, 472	20	0	569	551 [M + H − 18], 533 [M + H − 18 − 18]
12	5,6-Epoxy-β-cryptoxanthin	16.9–17.9	420, 444, 470	n.c.	0	569	551 [M + H − 18], 533 [M + H − 18 − 18], 463 [M + H − 106], 221
13	15-*cis*-β-Cryptoxanthin[f]	16.5–18.0	337, 420, 447, 477	12	55	553	535 [M + H − 18], 497 [M + H − 56], 461 [M + H − 92]
14	13-*cis*-β-Cryptoxanthin[g]	18.1–19.2	337, 418, 442, 468	13	46	553.	535 [M + H − 18], 497 [M + H − 56], 461 [M + H − 92], 339
15	13′-*cis*-β-Cryptoxanthin[g]	18.5–19.6	337, 418, 443, 470	13	48	553	535 [M + H − 18], 497 [M + H − 56], 461 [M + H − 92], 339
16a	Phytofluene	19.6–20.7	331, 347, 367	94	n.d[e].	543	461, 406 [M + H − 137], 338 [M + H − 205]
16b	Zeinoxanthin	19.6–20.7	418, 444, 472	60	n.d.	553	535 [M + H − 18]
17	All-*trans*-α-cryptoxanthin	22.0–22.8	420, 442, 470	60	0	553	535 [M + H − 18]
18	All-*trans*-β-cryptoxanthin	23.4–24.8	421, 451, 477	20	0	553	535 [M + H − 18], 495, 461 [M + H − 92]
19a	Phytofluene	25.2–25.8	332, 347, 367	55	0	543	461, 406 [M + H − 137], 338 [M + H − 205]
19b	15-*cis*-β-Carotene	25.2–25.8	337, 420, 449, 470	10	60	537	444 [M − 92]
20	5,8-Epoxy-β-carotene	25.8–26.5	403, 429, 451	n.c.	0	553	535 [M + H − 18], 461 [M + H − 92], 205
21	13-*cis*-β-Carotene	26.1–27.5	337, 420, 444, 470	17	50	537	481 [M + H − 56], 444 [M − 92]
22	9-*cis*-β-Cryptoxanthin[h]	27.1–28.1	339, 420, 445, 471	20	15	553	535 [M + H − 18], 460 [M − 92]
23	9′-*cis*-β-Cryptoxanthin[h]	27.7–28.6	339, 420, 445, 471	28	12	553	535 [M + H − 18], 460 [M − 92]
24	All-*trans*-α-carotene	28.2–29.6	420, 445, 473	55	0	537	481 [M + H − 56], 444 [M − 92]
25	All-*trans*-β-carotene	33.0–34.5	421, 451, 478	25	0	537	444 [M − 92]
26	9-*cis*-β-Carotene	35.6–37.2	338, 427, 447, 472	25	21	537	444 [M − 92]

[a] Numbered according to Figs. 1 and 3.
[b] Retention time on the C_{30} column.
[c] Linear gradient of MeOH/MTBE.
[d] Not calculated.
[e] Not detected.
[f] Tentatively identified as 15-*cis*-β-cryptoxanthin or 15′-*cis*-β-cryptoxanthin.
[g] Tentatively identified as 13-*cis*-β-cryptoxanthin or 13′-*cis*-β-cryptoxanthin.
[h] Tentatively identified as 9-*cis*-β-cryptoxanthin or 9′-*cis*-β-cryptoxanthin.

Peak 1 was identified as 12′-apo-β-caroten-12′-al, considering the lack of spectral fine structure and λ_{max} at 423 nm, early elution order (7.8 min), a protonated molecule at m/z 351, and co-elution with an authentic standard. The MS/MS spectrum showed fragments at m/z 333, due to the loss of water from the protonated molecule, and at m/z 227 resulting from the combined elimination of water and xylene. The 12′-apo-β-caroten-12′-al was detected in both simulated cashew apple juice and all-*trans*-β-cryptoxanthin systems only after 1 h of heating at 90 °C, suggesting that this apocarotenoid was derived from the thermal degradation of all-*trans*-β-cryptoxanthin (peak 18).

Peak 4 was identified as auroxanthin by its characteristic λ_{max}, high spectral fine structure and a protonated molecule at m/z 601. The second order tandem MS spectrum showed fragments due to consecutive losses of one (583 u), two (565 u), and three (547 u) water molecules, elimination of toluene (509 u), and toluene plus water (491 u). The presence of an epoxide and/or furanoid group in the 3-hydroxy-β-ring was confirmed by the presence of a fragment at m/z 221.

Antheraxanthin (peak 7) was identified considering the UV–visible and the highest intensity of the protonated molecule ion (585 u) as compared to that from the loss of water (567), as observed in the MS of zeaxanthin. As cited above for auroxanthin, the presence of an epoxide and/or furanoid group in the 3-hydroxy-β-ring was confirmed by the fragment at m/z 221. Peak 8 showed the same MS features as peak 7, but an hypsochromic shift of 20 nm, being identified as mutatoxanthin.

3.2. Effect of heating and acid addition on β-cryptoxanthin degradation in an aqueous-based system

Fig. 1 shows the chromatograms obtained for non-heated and heated synthetic all-*trans*-β-cryptoxanthin systems. The initial systems were found to contain 94.7% of all-*trans*-β-cryptoxanthin,

along with five isomers in small amounts (5.3%), as shown in Table 2.

In the non-acidified system, the total amounts of *cis* isomers increased from 5.3 to 14.7 μg/ml and a further increase to 17.4 μg/ml, respectively, after 1 and 2 h of heating at 90 °C. As a result of citric acid addition to the system, similar amounts of the *cis* isomers of β-cryptoxanthin were detected, namely 14.9 μg/ml after 1 h and 16.1 μg/ml after 2 h of heating. Di-*cis* isomers of β-cryptoxanthin were not detected in these systems.

Two oxidation compounds (peaks 1 and 12, Fig. 1), not previously detected in the unheated system, were formed in small amounts after heating all-*trans*-β-cryptoxanthin in both non- and acidified systems at 90 °C (Table 2). Although the levels of 5,6-epoxy-β-cryptoxanthin increased with heating time, especially in the acidified system, the amount of citric acid added was not strong enough to induce the epoxy to furanoid rearrangement. In fact, the detection of 5,6-epoxy-β-cryptoxanthin in this system after 2 h of heating is surprising, considering that 5,6-epoxides of carotenoids are easily rearranged to their corresponding 5,8-furanoids during thermal processing of foods (Dhuique-Mayer et al., 2007; Meléndez-Martínez, Vicario, & Heredia, 2007b) and that small amounts of 5,8-epoxy-β-cryptoxanthin were detected in some cashew apple processed products (Assunção & Mercadante, 2003a). Di-epoxides of β-cryptoxanthin were not detected. The apocarotenoid, 12′-apo-β-carotenal, was only formed after heating at 90 °C for 1 h; however, it was not detected anymore after 2 h of heating. Other apocarotenoids with lower molecular weight or higher polarity containing the 3′-hydroxy β-end-group of β-cryptoxanthin were not detected, although another mobile phase with water was used (chromatogram not shown).

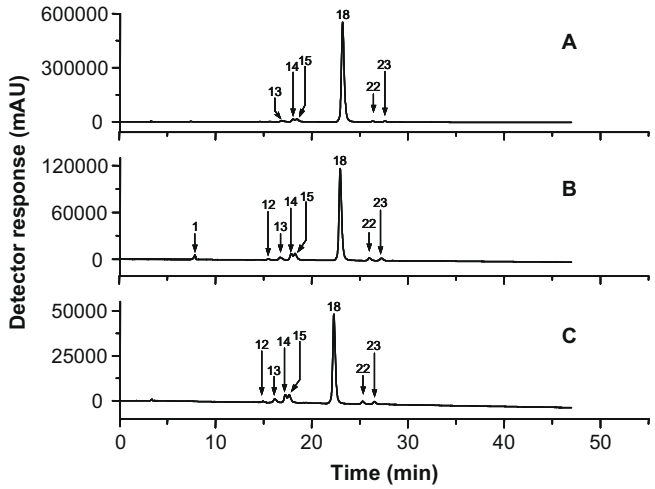

Fig. 1. Chromatogram processed at 450 nm, obtained by HPLC-PDA-MS/MS, of the synthetic β-cryptoxanthin in an aqueous-based acidified system, unheated (A), after 1 h heated at 90 °C (B), and after 2 h heated at 90 °C (C). See text for chromatographic conditions, peak characterisation is given in Table 1.

In addition, in the present study, a high stability of all-*trans*-β-cryptoxanthin was observed in a system at room temperature in the presence of citric acid. The absorptions in the red (700 nm) and near red infrared (950 nm) regions, corresponding respectively to mono and diprotonated carotenoids (Mortensen & Skibsted, 2000), were not observed (Fig. S2 from Supplementary data). This fact was not a surprise, since only strong and moderately strong acids, such as nitric and trifluoroacetic acids, were able to form protonated carotenoids (Mortensen & Skibsted, 2000). When all-*trans*-β-cryptoxanthin was heated in a non-acidified system at 90 °C, a decrease was also observed in the visible region at 440–520 nm with concomitant formation of soft coloured products in the near visible (~400 nm) and non-coloured compounds in the UV region (<360 nm) (inset of Fig. S3 from Supplementary data). These facts clearly indicated that heat was responsible for the major route of all-*trans*-β-cryptoxanthin degradation in an aqueous-based system.

Since similar qualitative and quantitative carotenoid profiles were found in both systems, with the absence and presence of citric acid in a similar concentration as that found in commercial tropical fruit juices, addition of acid did not affect the type of compounds formed during thermal degradation of all-*trans*-β-cryptoxanthin. In addition, the results from Table 2 showed that heating all-*trans*-β-cryptoxanthin for 60 min yielded the same *trans*/*cis* ratio values, 79:21, in non-acidified and acidified systems. Further heating for 120 min changed this isomeric proportion to 60:40 in non-acidified and to 63:37 in acidified systems.

Taking into account the structures of the thermal degradation products formed in both β-cryptoxanthin heated aqueous systems, isomerisation from *trans* to *cis* configurations, epoxidation and cleavage were the main degradation reactions observed in the present study (Fig. 2). Moreover, degradation to non-coloured compounds was also observed since the amounts of β-cryptoxanthin degraded was not compensated by the amounts of products formed (Table 2). In fact, Kanasawud and Crouzet (1990) proposed a reaction mechanism with mono- and di-epoxides of β-carotene as intermediates for the formation of carotenoid-derived volatiles from β-carotene in a heated aqueous medium.

The predominant *cis* isomers formed were 13-*cis*- + 13′-*cis*-β-cryptoxanthin, 7.0 and 7.3%, followed by smaller amounts of 9-

Table 2
Carotenoid changes (μg/ml) in β-cryptoxanthin systems heated at 90 °C.

Carotenoids	Unheated system[a]	Non-acidified heated system[a]		Acidified heated system[a]	
		1 h	2 h	1 h	2 h
12′-Apo-β-caroten-12′-al	n.d.[b]	1.5 ± 0.4	n.d.	1.6 ± 0.5	n.d.
5,6-Epoxy-β-cryptoxanthin	n.d.	1.3 ± 0.7	1.6 ± 0.6	1.4 ± 0.5	2.3 ± 0.3
15-*cis*-β-Cryptoxanthin[c]	0.8 ± 0.3	2.3 ± 0.3	2.8 ± 0.5	2.7 ± 0.4	2.5 ± 0.2
13-*cis*-β-Cryptoxanthin[d]	1.3 ± 0.3	3.1 ± 0.4	3.5 ± 0.3	3.3 ± 0.3	3.5 ± 0.3
13′-*cis*-β-Cryptoxanthin[d]	1.4 ± 0.3	3.2 ± 0.5	3.8 ± 0.2	3.1 ± 0.5	3.6 ± 0.4
All-*trans*-β-cryptoxanthin	94.8 ± 2.0	54.2 ± 1.4	26.2 ± 1.3	57.2 ± 1.8	27.6 ± 2.1
9-*cis*-β-Cryptoxanthin[e]	0.9 ± 0.5	3.0 ± 0.3	3.8 ± 0.4	2.8 ± 0.4	3.4 ± 0.3
9′-*cis*-β-Cryptoxanthin[e]	0.9 ± 0.5	3.1 ± 0.2	3.5 ± 0.2	3.0 ± 0.5	3.1 ± 0.4
Total	100.1 ± 0.9	71.7 ± 2.0	45.2 ± 0.6	75.1 ± 0.6	46.0 ± 2.5

[a] Mean and standard deviation of two independent experiments.
[b] n.d. not detected.
[c] Tentatively identified as 15-*cis*-β-cryptoxanthin or 15′-*cis*-β-cryptoxanthin.
[d] Tentatively identified as 13-*cis*-β-cryptoxanthin or 13′-*cis*-β-cryptoxanthin.
[e] Tentatively identified as 9-*cis*-β-cryptoxanthin or 9′-*cis*-β-cryptoxanthin.

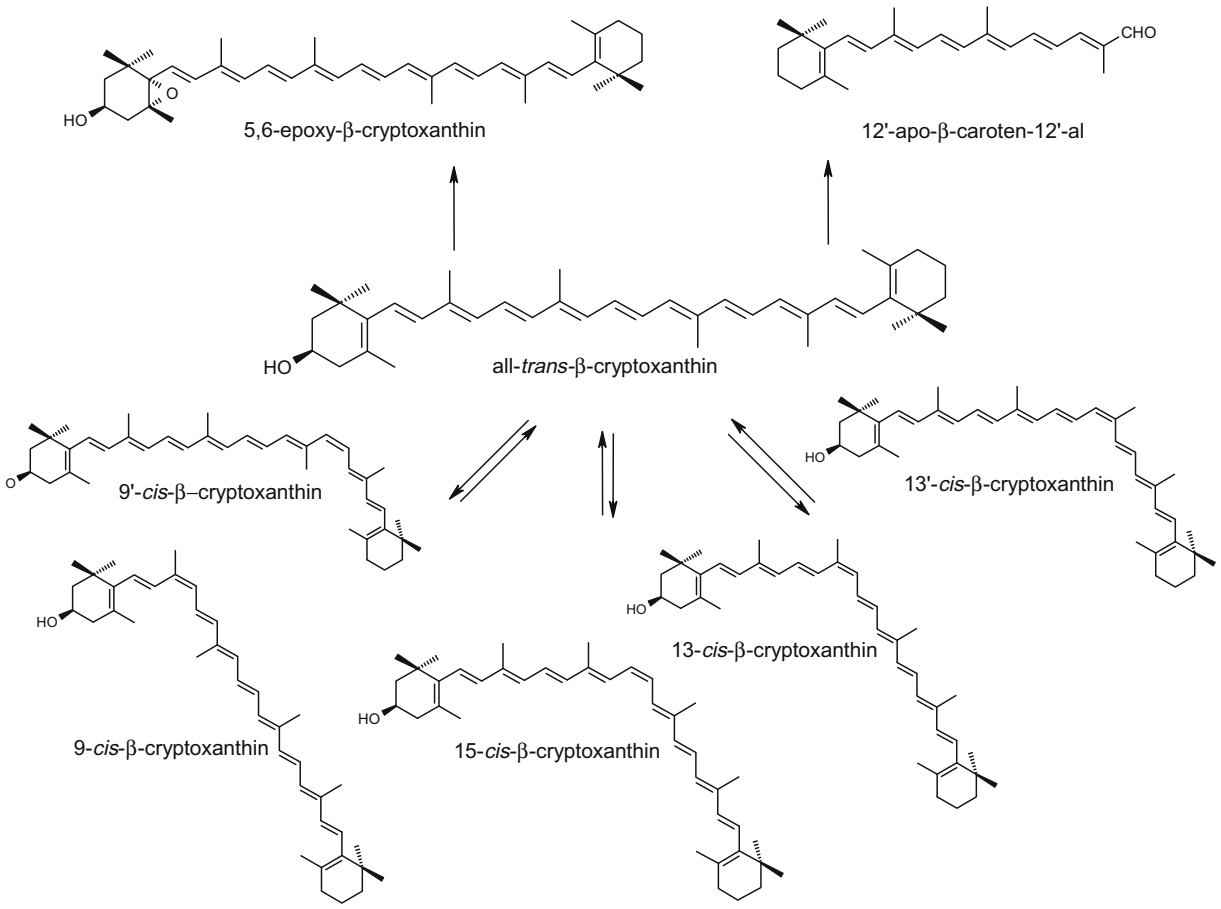

Fig. 2. Proposed mechanism for thermal degradation of all-*trans*-β-cryptoxanthin to coloured compounds in aqueous-based systems.

cis- + 9′-*cis*- isomers (5.3 and 5.9%) and 15-*cis*- or 15′-*cis*-β-cryptoxanthin (2.9 and 3.1%), respectively in non- and acidified aqueous systems after heating for 1 h at 90 °C (Table 2). The predominance of β-cryptoxanthin isomers with *cis* double bonds closer to the molecule centre is explained by the lower activation energy for *trans* to *cis* isomerisation about the central double bond as compared to those about other double bonds, as previously reported for β-carotene (Zechmeister, 1944).

3.3. Influence of heating on carotenoid degradation in a simulated cashew apple juice

The impact of thermal treatment on the composition of carotenoids in a simulated cashew apple juice can be seen in Fig. 3 and Table 3. In general, as expected, the levels of all-*trans* carotenoids decreased with a concomitant increase in the amounts of *cis* isomers and oxidation products, as time and heating temperature increased. Moreover, breakdown non-coloured compounds were also formed since the increased levels of *cis* isomers and oxidation compounds did not compensate for the losses of total carotenoid contents.

A total of 19 different carotenoids were separated in the simulated cashew apple juice not submitted to heat (Fig. 3A). All-*trans*-β-cryptoxanthin was the major carotenoid (57.7%), followed by all-*trans*-β-carotene (22.1%). As a consequence of the heat treatment at both 60 and 90 °C, *cis*-neoxanthin, neochrome, *cis*-violaxanthin, lutein and all-*trans*-α-cryptoxanthin disappeared, whereas two *cis* isomers (*cis*-lutein and 15-*cis*-β-carotene) and four epoxide-derivatives (auroxanthin, mutatoxanthin, 5,6-epoxy-β-cryptoxanthin and 5,8-epoxy-β-carotene) were formed (Table 3). Additional luteoxanthin and one short-chain product 12′-apo-β-carotenal were only formed at 90 °C (Table 3, Fig. 3B).

These facts indicated that the geometric isomerisation and formation of oxidation compounds already observed in the β-cryptoxanthin systems also occurred for the major carotenoids in the simulated cashew apple juice. However, epoxy-furanoid rearrangement was confirmed to have occurred in the simulated cashew ap-

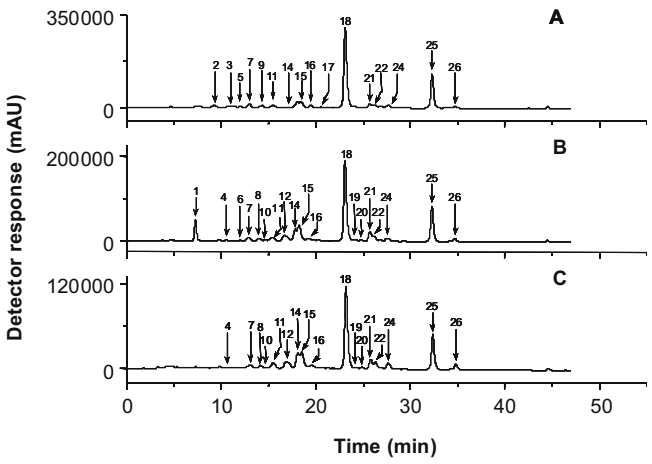

Fig. 3. Chromatogram processed at 450 nm, obtained by HPLC-PDA-MS/MS, of carotenoids from simulated cashew apple juice, unheated (A), after 1 h heated at 90 °C (B), and after 4 h heated at 90 °C (C). See text for chromatographic conditions, peak characterisation is given in Table 1.

Table 3
Carotenoid changes (μg/ml) in simulated cashew apple juice during heating.

Carotenoid	Unheated [a]	60 °C [a]		90 °C [a]	
		1 h	4 h	1 h	4 h
12′-Apo-β-caroten-12′-al	n.d.[b]	n.d.	n.d.	3.0 ± 0.3	n.d.
cis-Neoxanthin	2.1 ± 1.8	n.d.	n.d.	n.d.	n.d.
Neochrome	1.2 ± 0.4	n.d.	n.d.	n.d.	n.d.
Auroxanthin	n.d.	0.3 ± 0.4	0.4 ± 0.3	1.0 ± 0.4	0.9 ± 0.3
cis-Violaxanthin	3.4 ± 0.5	n.d.	n.d.	n.d.	n.d.
Luteoxanthin-type carotenoid	n.d.	n.d.	n.d.	0.9 ± 0.2	n.d.
Antheraxanthin	2.8 ± 0.7	3.0 ± 0.4	2.5 ± 0.5	2.9 ± 0.6	3.0 ± 0.5
Mutatoxanthin	n.d.	n.d.	0.8 ± 0.4	1.0 ± 0.2	0.6 ± 0.4
All-*trans*-lutein	2.0 ± 0.5	1.6 ± 0.4	n.d.	n.d.	n.d.
cis-Lutein	n.d.	n.d.	0.6 ± 0.5	0.7 ± 0.2	0.8 ± 0.3
Zeaxanthin	1.7 ± 0.5	1.5 ± 0.7	1.3 ± 0.2	1.3 ± 0.2	1.1 ± 0.3
5,6-Epoxy-β-cryptoxanthin	n.d.	2.3 ± 0.3	2.4 ± 0.8	2.0 ± 0.3	4.5 ± 0.8
13-*cis*-β-Cryptoxanthin[c]	1.9 ± 0.4	2.7 ± 0.6	3.0 ± 0.6	4.1 ± 0.9	4.6 ± 0.5
13′-*cis*-β-Cryptoxanthin[c]	1.6 ± 0.7	2.4 ± 0.8	3.8 ± 0.8	3.4 ± 1.0	4.8 ± 0.4
Zeinoxanthin	0.7 ± 0.4	0.6 ± 0.3	0.5 ± 0.6	0.9 ± 0.4	0.9 ± 0.4
All-*trans*-α-cryptoxanthin	0.4 ± 0.2	n.d.	n.d.	n.d.	n.d.
All-*trans*-β-cryptoxanthin	61.4 ± 2.5	50.0 ± 2.0	36.3 ± 1.8	40.5 ± 2.0	25.5 ± 1.8
15-*cis*-β-Carotene	n.d	0.5 ± 0.3	0.6 ± 0.7	0.6 ± 0.3	0.9 ± 0.3
5,8-Epoxy-β-carotene	n.d	0.4 ± 0.6	0.6 ± 0.5	0.6 ± 0.3	1.1 ± 0.3
13-*cis*-β-carotene	1.3 ± 0.4	2.5 ± 0.5	2.8 ± 0.5	2.1 ± 0.5	2.5 ± 0.5
9-*cis*-β-Cryptoxanthin[d]	1.0 ± 0.4	2.3 ± 0.3	2.1 ± 0.6	2.4 ± 0.3	2.6 ± 0.6
9′-*cis*-β-Cryptoxanthin[d]	n.d.	n.d.	1.0 ± 0.8	n.d.	n.d.
α-Carotene	0.6 ± 0.3	0.4 ± 0.3	0.5 ± 0.5	0.4 ± 0.3	0.5 ± 0.5
All-*trans*-β-carotene	23.5 ± 1.6	20.3 ± 0.6	14.1 ± 1.4	17.0 ± 0.6	12.0 ± 1.4
9-*cis*-β-Carotene	0.8 ± 1.7	0.9 ± 0.3	1.1 ± 0.3	1.7 ± 0.3	2.1 ± 0.3
Total	106.4 ± 3.8	91.7 ± 1.98	74.4 ± 3.2	86.5 ± 4.8	68.4 ± 5.6

[a] Mean and standard deviation of two independent experiments.
[b] n.d. not detected.
[c] Tentatively identified as 13-*cis*-β-cryptoxanthin or 13′-*cis*-β-cryptoxanthin.
[d] Tentatively identified as 9-*cis*-β-cryptoxanthin or 9′-*cis*-β-cryptoxanthin.

ple juice at both heating temperatures. Despite the high number of stereoisomers of β-carotene and β-cryptoxanthin that can be formed by heating, our experiments confirmed that the 13-*cis*- isomers of both carotenoids were preferentially formed, as previously reported (Dhuique-Mayer et al., 2007; Doering et al., 1995; Kuki et al., 1991; Mercadante, 2008a).

Similar *trans*/*cis* ratio values were found for β-cryptoxanthin and β-carotene in all the simulated cashew apple juice systems, changing respectively from 93:7 to 80:20 and from 92:8 to 79:21 after 1 h of heating at 90 °C. Further heating for 4 h changed the ratio values to 68:32 for β-cryptoxanthin and 69:31 for β-carotene.

Heating at both temperatures also caused loss of lutein, along with formation of *cis*-lutein. Violaxanthin thermal degradation followed the epoxy-furanoid rearrangement, through two sequential transformations of 5,6-epoxy to 5,8-furanoid end-groups, giving rise respectively to luteoxanthin and auroxanthin. It is known that violaxanthin easily undergoes this type of rearrangement, based on the example of the reported losses of 46.4% of violaxanthin and 19.7% of *cis*-violaxanthin after pasteurisation of Valencia orange juice (Lee & Coates, 2003). In addition, violaxanthin was found in fresh cashew apples; however, in commercially processed cashew juice, violaxanthin was not detected while auroxanthin, not present in the fruits, appeared at a detectable level, due to the conversion of the 5,6-epoxide groups of violaxanthin to the 5,8-furanoid groups of auroxanthin (Assunção & Mercadante, 2003a, 2003b).

Since neochrome was the only 5,8-epoxide found in unheated systems, the epoxy-furanoid rearrangement of *cis*-neoxanthin had probably already occurred during extraction or system preparation. It is interesting to note that in twenty-two of the twenty-five genotypes of orange, whenever neoxanthin was found, neochrome was also detected (Fanciullino et al., 2006).

In summary, the overall results show losses of non-detected low molecular weight degradation compounds, isomerisation of *trans* to *cis*-configuration, accompanied by epoxide to furanoid rearrangement and formation of oxidation compounds during carotenoid heating in aqueous-based systems. All reactions were induced mainly by heat in both synthetic β-cryptoxanthin and simulated cashew juice systems. It is important to consider that in the fruit, carotenoids are part of a much more complex system, and are in close proximity to other components such as protein and lipids, frequently in organised and ordered structures such as membranes and vesicles (Britton, 1995). However, heat treatment can facilitate the interaction between acids and carotenoids in the real juice.

Acknowledgements

The authors thank the Brazilian Funding Agency FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) for its financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2009.03.071.

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Food Chemistry 117 (2009) 35–41

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Antioxidant and antidiabetic activity of *Dangyuja* (*Citrus grandis* Osbeck) extract treated with *Aspergillus saitoi*

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ARTICLE INFO

Article history:
Received 14 October 2008
Received in revised form 16 February 2009
Accepted 17 March 2009

Keywords:
Dangyuja extract
Antioxidant activity
ORAC
Antidiabetic activity
Rat intestinal α-glucosidase inhibition

ABSTRACT

Dangyuja is a citrus fruit of Cheju Island in Korea, which is known to have a high content of flavanone glycosides, such as naringin and neohesperidin. Flavanone glycosides of *Dangyuja* (*Citrus grandis* Osbeck) extract were converted into their aglycones by naringinase and hesperidinase, and into their hydroxylated forms by *Aspergillus saitoi*. *Dangyuja* extract treated with *A. saitoi* had significantly higher antioxidant and antidiabetic activity than both its glycosides and aglycones in oxygen radical absorption capacity (ORAC) and supercoiled DNA strand scission assay, rat intestinal α-glucosidase and porcine pancreatic α-amylase inhibition, suggesting that the fermentation of flavanone aglycones of *Dangyuja* extract with *A. saitoi* can increase antioxidant and antidiabetic activity, compared to their glycosides and aglycones. This result suggests that *Dangyuja* extract prepared with enzymatic and microbial treatments could be used for the development of pharmaceutical foods to control the blood glucose level of diabetic patients by inhibiting α-amylase and α-glucosidase in the intestinal tract.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a common, progressive and complex disorder that is difficult to treat effectively in the long term. Recent studies have demonstrated that absorption inhibitors have a role in the prevention of T2DM in high-risk populations (Cheng & Josse, 2004). Inhibitors of intestinal α-glucosidase enzymes retard the rate of carbohydrate digestion, thereby providing an alternative means to reduce postprandial hyperglycaemia (Krentz & Bailey, 2005). Several α-glucosidase inhibitors, such as acarbose (Schmidt et al., 1977), trestatin (Watanabe, Furumai, Sudoh, Tokose, & Maruyama, 1984), amylostatin (Murao, Ohyyama, & Ogura, 1977) and valiolamine (Horii et al., 1987) have been isolated from microorganisms. Acarbose isolated from *Actinoplanes sp.* is commonly used in the management of Type 2 diabetes. The main drawback of using drugs such as acarbose is side effects such as abdominal distention, flatulence, meteorism and possibly diarrhoea (Hollander, 1992). It has been suggested that such adverse effects might be caused by the excessive inhibition of pancreatic α-amylase, resulting in abnormal bacterial fermentation of undigested carbohydrates in the colon (Horii et al., 1987). Natural α-amylase and α-glucosidase inhibitors from food-grade plant sources offer an attractive strategy to control post-prandial hyperglycaemia. Natural inhibitors from plants, which have been shown to have a low inhibitory effect against α-amylase activity and a strong inhibition activity against α-glucosidase, can be used as an effective therapy for postprandial hyperglycaemia with minimal side effects (Horii et al., 1987).

Recently, extensive studies of citrus flavanones have reported that naringenin (NE; 4',5,7-trihydroxyflavanone) reduced glucose uptake to inhibit intestinal and renal Na^+-glucose co-transporter (SGLT1) (Li, Chen, Lau, Leung, & Cheng, 2006), and naringin (NI; naringenin-7-rhamnoglycoside) and hesperidin (HI; hesperetin-7-rutinoside) both significantly increased the glucokinase mRNA level, whilst NI also lowered the mRNA expression of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase in the liver (Jung, Lee, Park, Kang, & Choi, 2006). However, there have been few studies on α-glucosidase inhibition activity. Our previous analyses have demonstrated that aglycone forms (NE and hesperetin; HE; 3,5,7-trihydroxy-4-methoxyflavanone) of citrus flavanones showed significantly higher inhibitory activity than glycoside forms (NI and HI) on baker's yeast α-glucosidase enzyme assay. In addition, both glycoside and aglycone forms of citrus flavanones exhibited moderate inhibitory activity on rat intestinal α-glucosidase enzyme. It has been reported that most baker's yeast α-glucosidase inhibitors did not show any activity against mammalian α-glucosidase, due to the difference of molecular recognition in the target binding site of this enzyme (Gao & Kawabata, 2005), and that 5,6,7-trihydroxyflavanone structure was crucial for exerting high activity (Gao & Kawabata, 2004). Miyake et al. (2003) reported that hydroxylated flavanones were produced with *Aspergillus saitoi* from hesperidin or naringin, which are flavanone glycosides in citrus fruit, and the hydroxylated flavanones produced from naringin were identified as carthamidin (6-hydroxynaringenin) and isocarthamidin (8-hydroxynaringenin), and that from hesperidin was identified as

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doi:10.1016/j.foodchem.2009.03.072

8-hydroxyhesperetin (8-HHE). These products demonstrated more antioxidant capacity than both glycoside and aglycone forms using the DPPH radical-scavenging assay (Miyake et al., 2003).

The flavonoids of citrus fruit have three types: flavanones, flavones and flavonols; there exist about 60 species (Rhyu, Kim, Bae, & Park, 2002). The citrus flavanones have two forms, glycone and aglycone, present in the metabolic process. The glycoside forms include NI, narirutin (NR; naringenin-7-rutinoside), HE and neohesperidin (NHI; hesperetin-7-neohesperidoside), and aglycone forms include NE and HE. In past, citrus flavanones such as NI and NHI had been recognised as bitter components, which affect consumer acceptability. However, lately these citrus flavanones have become components of special interest, through the development of a debittering method, which converts NI into its aglycone (Puri, Banerjee, & Banerjee, 2005), as well as the antioxidant activity (Di Majo et al., 2005), hypotensive effect (Son, Kim, Kwon, & Ju, 1992), and anti-inflammatory and anti-tumuorigenic efficacies of HI (Sakata, Hirose, Qiao, Tanaka, & Mori, 2003), the anti-cholesterol effect of HE through inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and acyl coenzyme A (ACAT) (Lee et al., 1999), and hypolipidaemic and hypoglycaemic effects of NI and HI (Jung et al., 2006).

Unshiu orange, citrus Unshiu is a major citrus fruit of Cheju Island in Korea. Recently, Unshiu orange fell in price due to overproduction. Therefore, the development of an alternative fruit is considered to be an important subject. *Dangyuja*, *Citrus grandis* Osbeck is a citrus fruit of Cheju in Korea has potential as a substitute for Unshiu orange. HPLC analysis has indicated that *Dangyuja* has a higher content of total flavanones than Unshiu orange (data not shown). The major flavanones of *Dangyuja* are neohesperidin and naringin, which are high in the seedcase of unripe citrus fruits (Chung, Kim, Choi, Song, & Kim, 2000) and its extract showed antioxidant activity through free radical-scavenging *in vitro* and to reduce reactive oxygen species in H_2O_2-treated HepG2 cells (Lim, Yoo, Moon, Jeon, & Cho, 2006).

In this study, we carried out the conversion of flavanone glycosides of *Dangyuja* extract into their hydroxylated flavanones (carthamidin, isocathamidin and 8-HHE) by the sequential treatments with naringinase and hesperidinase, and *A. saitoi*. Antidiabetic and antioxidant activity was investigated using rat α-glucosidase and porcine α-amylase inhibition assay, and ORAC and DNA strand scission assay, respectively.

2. Materials and methods

2.1. Materials

Dangyuja powder was kindly supplied by Kunpoong Bio Co. Ltd. (Cheju, Korea). *A. saitoi* IAM 2210 was purchased from the Institute of Applied Microbiology at Tokyo University (Tokyo, Japan). Hesperidin, naringin, neohesperidin, hesperetin, naringenin, yeast α-glucosidase enzyme powder, rat-intestinal acetone powder, *p*-nitrophenyl-α-glucopyranoside (pNPG), porcine pancreatic α-amylase enzyme powder, naringinase, hesperidinase, dimethylsulfoxide (DMSO), fluorescein, potato dextrose agar (PDA) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO). Narirutin was purchased from Indofine Chemical Company, Inc. (Hillsborough, IL). 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Preparation of Dangyuja extract

Four and a half grams of *Dangyuja* powder were suspended in 300 ml of distiled water, and the suspension was heated in autoclave at 121 °C for 15 min. After filtration with filter paper (Whatman No. 1), the filtrate was lyophilised using a vacuum freeze dryer (Biotron Co. Ltd., Puchon, Korea). The extract was kept at −20 °C until analysed.

2.3. Conversion of glycosides in Dangyuja extract by enzymatic and microbial treatment

Preparation of converted samples refers to the slightly modified method of Miyake et al. (2003). Half a gram of *Dangyuja* extract was dissolved in 1 ml of DMSO, and 99 ml of 10% potato dextrose agar solution was added. The mixture was incubated at 37 °C for 1 day with naringinase and hesperidinase powder (each at a concentration of 2.5 mg/ml). After the incubated mixture has been adjusted to pH 5.0 with 0.1 N HCl, it was sterilised at 121 °C for 15 min. After the sterilised solution was inoculated with 1 ml of *A. saitoi*; the inoculated solution was shake-cultured with the shaking incubator (Vision Scientific Co. Ltd., Seoul, Korea) at a speed of 100 rpm/min and 30 °C. Five millilitre aliquots of solution were periodically taken out during the incubation and culture period and blended by tissue tearer (Biospec Products, Inc., Bartlesville, OK). All blended samples were measured for antidiabetic and antioxidant activity.

2.4. HPLC analysis

HPLC analysis was performed on a Tosoh (Tosoh Corporation, Tokyo, Japan) 8010 series equipped with a diode-array UV–Vis detector (UV 8010), a degasser (SD-8023) and a column oven (CO-8010). The instrument was also equipped with a Futecs gradient controller (NS-1000A) and gradient pump (Acuflow Series IV; Futecs Co., Daejeon, Korea); integration and data elaboration were performed by MultiChro software (Yullin Technology, Seoul, Korea). The column used was RP 5 μm C_{18}, (15 cm × 4.5 cm); (Daiso Co. Ltd., Osaka, Japan). HPLC analysis of sample used the method of Kanaze, Kokkalou, Georgarakis, and Niopas (2004), with slight modifications. The mobile phase consisting of methanol/water/acetic acid (25:73:2, v/v/v) was filtered through a 0.45 um pore size cellulose acetate filter (Sartorius, Goettingen, Germany) and degassed by ultrasonic treatment before use. The HPLC system was isocratically operated at a flow rate of 1 ml/min at 45 °C and the detector was set at 288 nm. Before the measurement, DMSO solution was added to all samples at the same amount.

2.5. In vitro antioxidant activity assay

2.5.1. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was carried out on a Tecan GENios multi-functional plate reader (Salzburg, Austria) with fluorescent filters (excitation wavelength: 485 nm and emission wavelength: 535 nm). In the final assay mixture, fluorescein (40 nM) was used as a target of free radical attack with either AAPH (20 mM) as a peroxyl radical generator in peroxyl radical-scavenging capacity ($ORAC_{ROO\cdot}$) assay (Kurihara et al., 2004) or H_2O_2–$CuSO_4$ (H_2O_2, 0.75%; $CuSO_4$, 5 μM) as mainly a hydroxyl radical generator in hydroxyl radical-scavenging capacity ($ORAC_{OH\cdot}$) assay (Cao, Sofic, & Prior, 1997). Trolox (1 μM) was used as a control standard and prepared fresh daily. The analyser was programmed to record the fluorescence every 2 min after AAPH or H_2O_2–$CuSO_4$ was added. All fluorescence measurements were expressed relative to the initial reading. Final results were calculated based on the difference in the area under the fluorescence decay curve between the blank and each sample. $ORAC_{ROO\cdot}$ and $ORAC_{OH\cdot}$ were expressed as micromoles of Trolox equivalents (TE). One ORAC unit is equivalent to the net protection area provided by 1 μM Trolox.

2.5.2. pBR322 DNA strand scission assay

Plasmid supercoiled strand DNA (pBR322 DNA) was dissolved in 10 mM PBS (pH 7.4). DNA (2 μg/ml) was mixed with sample

solution in PBS before the oxidation initiators were added. For generating the peroxyl radical, AAPH in PBS, pH 7.4, was added to a final concentration of 5 mM, and reaction mixtures were incubated at 37 °C for 2 h (Lim, Hu, & Kitts, 2001). Gel electrophoresis was performed in a Tris acetic acid EDTA buffer (40 mM Tris, 2 mM EDTA, pH 8.5), using a horizontal submarine gel electrophoresis apparatus (Amersham Pharmacia Biotech, Uppsala, Sweden) at 3 V/cm for 1.4 h. DNA strands were stained with ethidium bromide (0.5 μg/ml in deionised water), visualised under ultra-violet light and photographed. Photographic negatives were scanned and analysed by imaging densitometer with molecular analysis software to quantitate DNA breakage in term of percentage of supercoiled DNA which was nicked by peroxyl radicals. The protective effects of samples were compared by the retention percentage (% retention), calculated by the following equation:

$$\text{Retention}(\%) = (\text{concentration}_{\text{sample}}/\text{concentration}_{\text{control}}) \times 100,$$

where $\text{concentration}_{\text{sample}}$ and $\text{concentration}_{\text{control}}$ represent the concentrations of supercoiled DNA with oxidative radical and without oxidative radical, respectively.

2.6. Antidiabetic activity assay

2.6.1. Rat intestinal -glucosidase inhibition assay

Rat intestinal α-glucosidase assay referred to the method of Kwon, Vattem, and Shetty (2006) with slight modification. One gram of rat-intestinal acetone powder was suspended in 3 ml of 0.9% saline, and the suspension was sonicated twelve times for 30 s at 4 °C. After centrifugation (10000*g*, 30 min, 4 °C), the resulting supernatant was used for the assay (Ohta, Sasaki, Oohori, Yoshikawa, & Kurihara, 2002). Fifty microlitres of sample solution were pre-incubated with 100 μl of rat-intestinal α-glucosidase solution at 37 °C for 10 min. After pre-incubation, 50 μl of 5 mM *p*-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) were added. The reaction mixtures were incubated at 37 °C for 15 min. Absorbance readings were recorded at 405 nm by ELISA absorbance reader (Tecan Trading AG, Salzburg, Austria) and compared to a control which had 50 μl of solvent in place of the sample solution. The rat α-glucosidase inhibitory activity was expressed as inhibition (%) and was calculated as follows:

$$\text{Inhibition}(\%) = [(A_0 - A_1)/A_0] \times 100$$

where A_0 was the absorbance of the control, and A_1 was the absorbance of the sample.

2.6.2. Porcine pancreatic -amylase inhibition assay

Porcine pancreatic α-amylase inhibition referred to the method of Kwon et al. (2006). Two hundred microlitres of sample solution and 500 μl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α-amylase solution (0.5 mg/ml) were incubated at 25 °C for 10 min. After pre-incubation, 500 μl of a 1% starch solution in 0.02 M sodium phosphate buffer were added. The reaction mixture was then incubated at 25 °C for 10 min. The reaction was stopped with 1.0 ml of dinitrosalicylic (DNS) acid, colour reagent. The reaction mixture was then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 10 ml of water, and absorbance was measured at 540 nm with ELISA microplate reader (Tecan Trading AG).

2.7. Statistical analysis

All data are presented as means ± SD. Statistical analyses were done using SPSS (Statistical Package for Social Science, SPSS Inc., Chicago, IL) program, and significance of each group was verified with one-way ANOVA followed by Duncan's test ($p < 0.05$).

3. Results and discussion

3.1. The flavanone content of Dangyuja extract

The flavanones of *Dangyuja* powder were extracted using autoclaving (121 °C, 15 min) in water and analysed using HPLC. The total content of flavanone glycosides tended to change with the harvesting time. Their percentage increased in order: October (4.02%) < September (6.66%) < August (10.48%) < July (20.66%). The result was consistent with those reported by previous researchers (Chung et al., 2000; Rhyu et al., 2002). Rhyu et al. (2002) reported that the content of flavonoid decreased with maturing of citrus fruit and Chung et al. (2000) reported that naringin and hesperidin rapidly decreased during maturation. Referring to these results, we extracted *Dangyuja* harvested in July. The yield of extraction was 58.66%, and the content of NHI was the highest (16.52%) amongst flavanone glycosides, compared to NR (0.12%), NI (11.12%) and HI (0.80%). This well agrees with the report that *Dangyuja* has a higher content of NI and NHI than other flavanones (Rhyu et al., 2002).

3.2. Conversion of flavanone glycosides of Dangyuja extract into their hydroxylated flavanones by enzymatic and microbial treatment

Flavanone glycosides of *Dangyuja* were subsequently converted into their aglycones and hydroxylated flavanones by naringinase and hesperidinase, and *A. saitoi*, respectively. The conversion of flavanone glycosides was confirmed using HPLC. The retention time of NR, NI, HI and NHI, flavanone glycosides, were 6.18 min, 7.05 min, 8.15 min and 9.32 min, respectively. Those of NE and HE, flavanone aglycones, were 14.48 min and 19.95 min, respectively. Deglycosylation of flavanone glycosides was done by treatment with naringinase and hesperidinase for one day. The result of deglycosylation (Table 1) indicates that all flavanone glycosides were converted into their aglycones. NE (6.04%) was made from NR (0.12%) and NI (11.12%), and HE (12.28%) was derived from HI (0.8%) and NHI (16.52%).

According to Table 1, when the flavanone aglycones of Dangyuja extract were treated with *A. saitoi* for 6 days, the concentration of NE decreased from 6.04% to almost 0%, suggesting that all NE may be converted into its hydroxylated forms by *A. saitoi*. On the other hand, the content of HE decreased from 12.28% to 5.40% after treatment for 2 days and then increased from 5.40% to 7.68% between 2 and 6 days. Miyake et al. (2003) has reported that hydroxylated flavanones were produced with *A. saitoi* from hesperidin or naringin, flavanone glycosides in citrus fruit, and the hydroxylated flavanones produced from NE and HE were identified as carthamidin (6-hydroxynaringenin) and isocarthamidin (8-hydroxynaringenin), and 8-hydroxyhesperetin (8-HHE), respectively (Fig. 1). This result indicates that most of flavanone aglycones such as NE and HE could be converted, by hydroxylation with *A. saitoi,* into carthamidin and isocarthamidin, and 8-hydroxyhesperetin, respectively. The increase of HE concentration between 2 and 6 days treatment with *A. saitoi* may be due to the hydroxylated forms, such as carthamidin (6-hydroxynaringenin) and isocarthamidin (8-hydroxynaringenin), and 8-hydroxyhesperetin (8-HHE) derived from NE and HE, which were eluted at a retention time close to HE under our conditions of HPLC analysis. Even though hydroxylated forms of NE and HE were not identified in the HPLC profile, it was assumed that the flavanone aglycones decreased because they were converted into carthamidin, isocarthamidin and 8-HHE. The antioxidant activities of these hydroxylated flavanones have been reported as being higher than those of flavanone glycosides (Miyake et al., 2003). The structure of carthamidin is similar to that of baicalein which is crucial for exerting high activity on rat intestinal α-glucosidase inhibition (Gao & Kawabata, 2004).

Table 1
The flavanone content of *Dangyuja* extract harvested in July (De), treated with naringinase and hesperidinase (E), and fermented with *A. saitoi* for 2–6 days (F2, F4, and F6).

Sample	Content (%)					
	Narirutin	Naringin	Hesperidin	Neohesperidin	Naringenin	Hesperetin
De	0.12 ± 0.01	11.12 ± 0.04	0.8 ± 0.02	16.52 ± 0.08	ND	ND
E	ND[a]	ND	ND	ND	6.04 ± 0.04	12.28 ± 0.06
F2	ND	ND	ND	ND	2.96 ± 0.04	5.40 ± 0.05
F4	ND	ND	ND	ND	0.32 ± 0.02	6.48 ± 0.08
F6	ND	ND	ND	ND	ND	7.68 ± 0.05

Data are presented as mean values ± standard deviation. Values are presented as g/100 g of extract (dry weight basis).
[a] Not detectable.

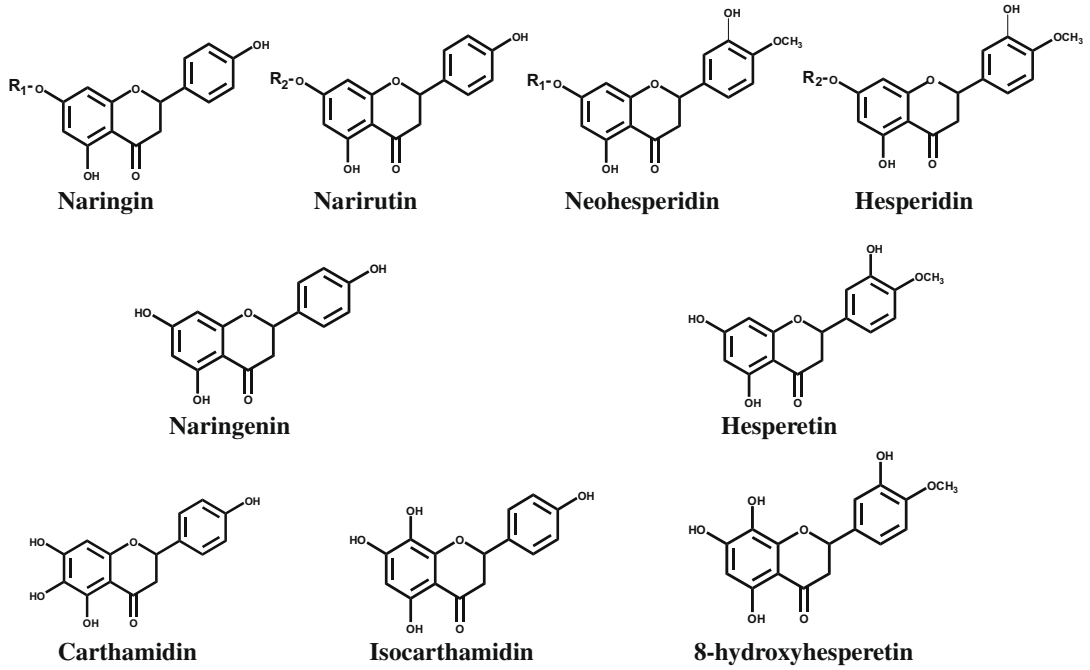

Fig. 1. Chemical structure of citrus flavanones and their derivatives. R1, Neohesperidose; R2, Rutinose.

3.3. Antioxidant activity of converted Dangyuja extract

The antioxidative activity of *Dangyuja* extract containing flavanone glycosides and aglycones, and hydroxylated compounds formed due to treatment with enzyme and *A.* saitoi, was measured by using the ORAC assay, which has been used successfully to analyse the scavenging capacity for peroxyl and hydroxyl radicals, the most harmful and reactive oxygen species (Aruoma, 1994). ORAC assay was carried out with AAPH as a peroxyl radical generator, and $CuSO_4$ and H_2O_2 as a hydroxyl radical generator. All antioxidant capacities were indicated in terms of Trolox equivalant (TE). The capacity to scavenge peroxyl radical increased in order of F6 (18.31 TE) < De (21.86 TE) < E (22.98 TE) < F4 (23.65 TE) < F2 (24.59 TE) at 2 μg/ml of sample concentration (Fig. 2), and the hydroxyl radical-scavenging activity increased in order of De (33.07 TE) < F6 (33.25 TE) < E (37.68 TE) < F4 (40.96 TE) < F2 (41.21 TE) at 10 μg/ml of sample concentration (Fig. 3). The treatment with naringinase and hesperidinase converted flavanone glycosides of *Dangyuja* extract into their aglycones, which contributed to an increase in the antioxidant activity of *Dangyuja* extract, which is in good agreement with published results (Kim, Jung, & Jang, 2008). Furthermore, after the treatment with naringinase and hesperidinase, the fermentation of *Dangyuja* extract with *A. saitoi* for 2 or 4 days caused a significant increase in its ability to scavenge peroxyl and hydroxyl radicals, indicating that hydroxylated forms from flavanone aglycones of *Dangyuja* extract may significantly demonstrate higher antioxidant activity in scavenging peroxyl and hydroxyl radical than their glycosides or aglycones. Therefore,

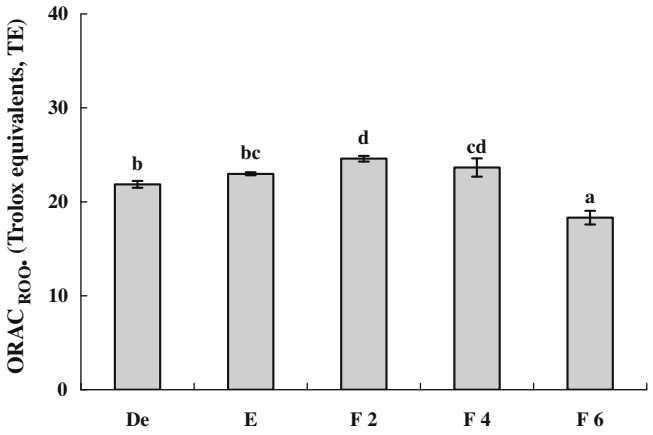

Fig. 2. Effect of enzymatic and microbial treatment on the peroxyl radical-scavenging activity of *Dangyuja* extract. De = *Dangyuja* extract; E = *Dangyuja* extract treated with naringinase and hesperidinase; F2 = *Dangyuja* extract fermented with *A. saitoi* for 2 days; F4 = *Dangyuja* extract fermented with *A. saitoi* for 4 days; F6 = *Dangyuja* extract fermented with *A. saitoi* for 6 days. All values are mean ± S.D. Means with the same letter are not significantly different ($p < 0.05$).

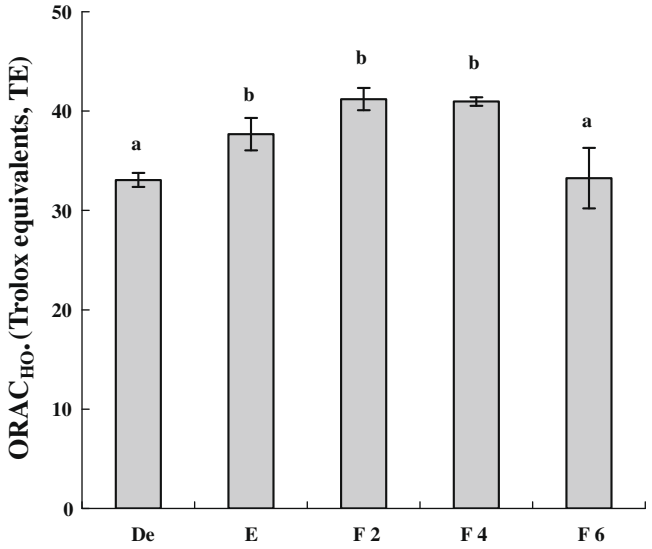

Fig. 3. Effect of enzymatic and microbial treatment on the hydroxyl radical-scavenging activity of *Dangyuja* extract. De = *Dangyuja* extract; E = *Dangyuja* extract treated with naringinase and hesperidinase; F2 = *Dangyuja* extract fermented with *A. saitoi* for 2 days; F4 = *Dangyuja* extract fermented with *A. saitoi* for 4 days; F6 = *Dangyuja* extract fermented with *A. saitoi* for 6 days. All values are mean ± S.D. Means with the same letter are not significantly different ($p < 0.05$).

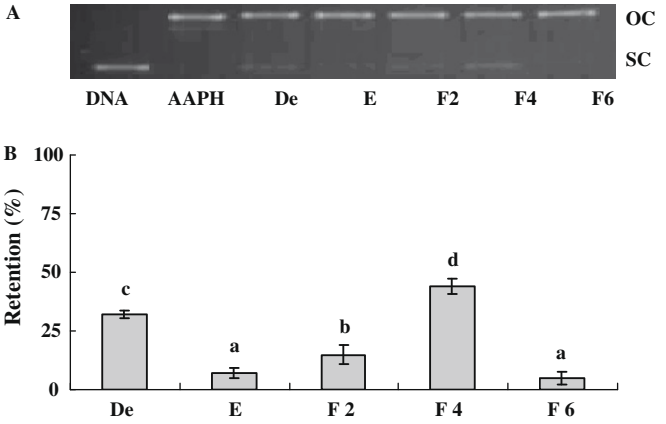

Fig. 4. (A) Electrophoretic diagram of pBR322 plasmid DNA and (B) effect of enzymatic and microbial treatment on the peroxyl radical-scavenging activity of *Dangyuja* extract. DNA = pBR322 plasmid; AAPH = pBR322 plasmid DNA treated with AAPH; De = pBR322 plasmid DNA with AAPH and *Dangyuja* extract; E = pBR322 plasmid DNA with AAPH and *Dangyuja* extract treated with naringinase and hesperidinase; F2 = pBR322 plasmid DNA with AAPH and *Dangyuja* extract fermented with *A. saitoi* for 2 days; F4 = pBR322 plasmid DNA with AAPH and *Dangyuja* extract fermented with *A. saitoi* for 4 days; F6 = pBR322 plasmid DNA with APH and *Dangyuja* extract fermented with *A. saitoi* for 6 days. All values are mean ± S.D. Means with the same letter are not significantly different ($p < 0.05$).

the conversion of flavanone glycosides of *Dangyuja* extract into hydroxylated forms by their sequential treatment with naringinase and hesperidiase, and *A. saitoi* could increase the ability of the extract to scavenge peroxyl and hydroxyl radical.

Most oxidative damage occurring in biological systems is due to peroxyl radicals that have comparatively long half-lives and thus greater affinity to diffuse into biological fluids in cells and tissues (Halliwell, Aeschbach, Lőliger, & Aruoma, 1995). While the reactions of peroxyl radicals are slower than those of hydroxyl radicals, they are more specific. This suggests that peroxyl radicals may be more efficient than hydroxyl radicals in damaging important cellular macromolecules, such as DNA, proteins, and carbohydrates (Lee, Kwon, Shetty, & Jang, 2004).

The protection effect of *Dangyuja* extract prepared with enzymatic and microbial treatment on the oxidative damage of pBR322 plasmid DNA caused by peroxyl radical was shown in Fig. 4. In each lane, the lower band was due to supercoiled DNA (SC) and the upper to open-circular DNA (OC). Only a single strand scission caused the conversion of SC into OC. Second strand scission converts OC into linear DNA that is located between SC and OC on agarose gel electrophoresis (Yang & Schaich, 1996). Therefore, the measurement of SC in plasmid DNA treated with peroxyl radicals could indicate the effectiveness of protective activity against oxidative stress of plasmid DNA. The percentage of retention increased in order of F6 (5.04%) < E (7.04%) < F2 (14.89%) < De (32.09%) < F4 (43.77%) at 10 μg/ml of sample concentration (Fig. 3). *Dangyuja* extract treated with *A. saitoi* for 4 days, containing hydroxylated forms from NE and HE, showed the highest protective effect against the oxidative DNA damage induced by peroxyl radical amongst samples tested. However, *Dangyuja* extract having flavanone aglycones, treated with naringinase and hesperidinase, demonstrated lower protective activity against oxidative stress of plasmid DNA than extract containing flavanone glycosides; the reason why was unclear.

3.4. Antidiabetic effect of converted Dangyuja extract

The α-glucosidase inhibitors, which interfere with enzymatic action in the brush-border of the small intestine, could slow the liberation of D-glucose from oligosaccharides and disaccharides, resulting in delaying glucose absorption and decreasing postprandial plasma glucose levels (Gao & Kawabata, 2005). Our previous experiment using yeast α-glucosidase assay indicated that aglycones such as NE and HE had significantly higher inhibitory activity than glycosides, such as NI and NE. However, both glycosides and their aglycones exhibited low inhibitory activity, and there was no difference between glycosides and aglycones on rat α-glucosidase inhibition activity (data not shown). It has been reported that most yeast α-glucosidase inhibitors did not show any activity against mammalian α-glucosidase, due to the difference of molecular recognition in the target binding site of enzymes (Gao & Kawabata, 2005), and that a 5,6,7-trihydroxyflavanone structure was crucial for exerting high activity (Gao & Kawabata, 2004), Therefore, we prepared *Dangyuja* extract having hydroxylated forms of flavanone by the conversion using the fermentation method with *A. saitoi* and investigated the inhibitory activity on rat intestinal α-glucosidase.

With rat α-glucosidase inhibition assay, the inhibitory activity of hydroxylated flavanone was compared with those of their glycoside and aglycone forms. The inhibition activity increased in order of De (16.66%) < F6 (27.43%) < E (30.86%) < fermentation < F2 (45.65%) < F4 (73.15%) (Fig. 5). *Dangyuja* extract prepared with *A. saitoi* for 4 days after the enzymatic treatment showed the highest inhibitory activity amongst all samples tested. On the other hand, the fermentation of *Dangyuja* extract with *A. saitoi* for 6 days decreased the inhibition activity on rat intestinal α-glucosidase. The inhibitory activity on porcine pancreatic α-amylase assay was conducted using the same samples and concentration in rat α-glucosidase inhibition assay. The inhibition activity increased in order of De (−2.63%) < E (3.28%) < F2 (11.58%) < F6 (16.17%) < F4 (48.15%) (Fig. 6). This result indicates that the tendency of *Dangyuja* extract to inhibit porcine α-amylase is similar to its tendency to inhibit rat intestinal α-glucosidase.

According to the result of Gao and Kawabata (2004), carthamidin was shown to exert substantial activity on rat intestinal α-glucosidase inhibition amongst hydroxylated flavanones, such as carthamidin, isocarthamidin and 8-HHE produced from NE and HE with *A. saitoi*. As shown above, the present finding of *Dangyuja* extract prepared by fermenting with *A. saitoi* for 4 days to inhibit

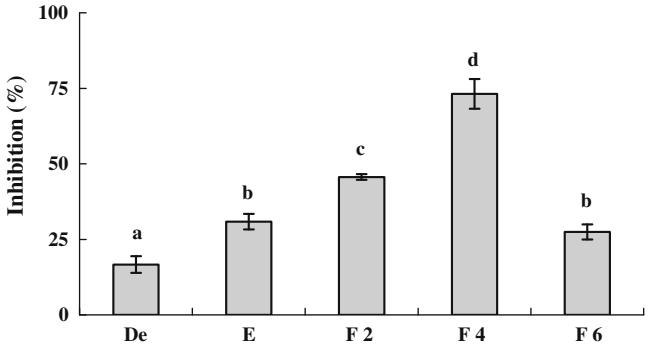

Fig. 5. Effect of enzymatic and microbial treatment on rat intestinal α-glucosidase inhibitory activity of *Dangyuja* extract. De = *Dangyuja* extract; E = *Dangyuja* extract treated with naringinase and hesperidinase; F2 = *Dangyuja* extract fermented with *A. saitoi* for 2 days; F4 = *Dangyuja* extract fermented with *A. saitoi* for 4 days; F6 = *Dangyuja* extract fermented with *A. saitoi* for 6 days. All values are mean ± S.D. Means with the same letter are not significantly different ($p < 0.05$).

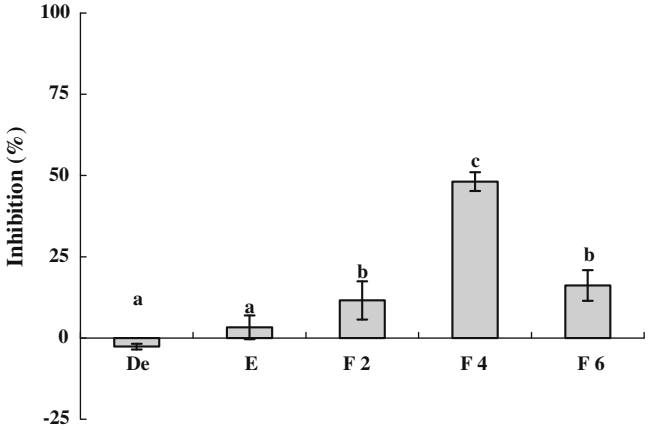

Fig. 6. Effect of enzymatic and microbial treatment on porcine pancreatic α-amylase inhibitory activity of *Dangyuja* extract. De = *Dangyuja* extract; E = *Dangyuja* extract treated with naringinase and hesperidinase; F2 = *Dangyuja* extract fermented with *A. saitoi* for 2 days; F4 = *Dangyuja* extract fermented with *A. saitoi* for 4 days; F6 = *Dangyuja* extract fermented with *A. saitoi* for 6 days. All values are mean ± S.D. Means with the same letter are not significantly different ($p < 0.05$).

significantly both rat intestinal α-glucosidase and porcine pancreatic α-amylase suggests that it may contain hydroxylated flavanone as carthamidin or other bioactive inhibiting compounds. Recent studies have demonstrated that absorption inhibitors have a role in the prevention of Type 2 diabetes in high-risk populations (Cheng & Josse, 2004; Krentz & Bailey, 2005). Therefore, it is suggested that hydroxylated product from *Dangyuja* extract with enzymatic and microbial treatment may be used for the development of pharmaceutical foods to control the blood glucose level of diabetic patients by inhibiting α-glucosidase and α-amylase in the intestinal tract.

4. Conclusions

Flavanone glycosides of *Dangyuja* extract were efficiently converted into their aglycones by naringinase and hesperidinase and their hydroxylated flavanones by *A. saitoi.* Antioxidant capacity was analysed using oxygen radical absorbance capacity (ORAC) and supercoiled DNA strand scission assay. Antidiabetic activity of flavanone products of *Dangyuja* was measured using rat intestinal α-glucosidase and porcine pancreatic α-amylase. Hydroxylated flavanones of *Dangyuja* extract prepared with enzymatic and microbial treatment showed significantly higher antioxidant and antidiabetic activity than both their glycosides and aglycones in ORAC and supercoiled DNA strand scission assay, and rat intestinal α-glucosidase and porcine pancreatic α-amylase inhibition, suggesting that the fermentation of flavanone aglycones of *Dangyuja* extract with *A. saitoi* can increase antioxidant and antidiabetic activities, compared to their glycosides and aglycones. These results indicate that the hydroxylated product from *Dangyuja* extract with enzymatic and microbial treatment could be used for the development of pharmaceutical foods to control the blood glucose level of diabetic patients by inhibiting α-amylase and α-glucosidase in the intestinal tract.

Acknowledgement

This study was supported by a research grant from Hannam University, Daejeon, Korea in 2009.

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Food Chemistry 117 (2009) 42–47

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Protective effect of selenium-polysaccharides from the mycelia of *Coprinus comatus* on alloxan-induced oxidative stress in mice

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ARTICLE INFO

Article history:
Received 12 December 2008
Received in revised form 18 February 2009
Accepted 19 March 2009

Keywords:
Coprinus comatus
Polysaccharide
Selenium
Alloxan-induced diabetic mice
Oxidative stress
Antioxidant

ABSTRACT

We investigated the effect of selenium-polysaccharide (SPS) isolated from selenium-enriched mycelia of *Coprinus comatus* on hypoglycaemic, hypolipidemic and antioxidant activities in diabetic mice. Compared with untreated diabetic mice, the administration of SPS for 20 days caused a significant decrease ($p < 0.05$) in blood glucose levels. Simultaneously, the alteration in lipid metabolism was partially attenuated as evidenced by decreased serum total cholesterol (TC), triglyceride (TG) and low-density lipoprotein cholesterol (LDL) levels and by increased high-density lipoprotein cholesterol (HDL) concentration in diabetic mice ($p < 0.05$). In addition, the SPS caused a significant decrease ($p < 0.05$) in the level of malondialdehyde (MDA) and a significant increase ($p < 0.05$) in the activities of enzymic antioxidants and the levels of non-enzymic antioxidants in liver and kidney of diabetic mice. Furthermore, the effects of SPS was more potent than that of polysaccharide (PS) from mycelia of *C. comatus* at the same dose.

1. Introduction

The essential trace element selenium, element number 34 with atomic weight of 78.84, forms a key constituent of selenoproteins such as glutathione peroxidases (Beckett & Arthur, 2005), and acts as a critically important antioxidant in many different cell types (Mukherjee, Anbazhagan, Roy, Ghosh, & Chatterjee, 1998). Selenium has also been found to have insulin-mimetic activities in vitro and in vivo (Stapleton, 2000), and has been shown to stimulate glucose uptake in adipocytes in vitro (Ezaki, 1990) and to regulate vital metabolic processes such as glycolysis and gluconeogenesis (Becker et al., 1996). Since it is generally believed that organic selenium compounds are better and safer than inorganic selenium as a dietary supplement, a variety of selenium-enriched biological products including garlic, yeast and lactic acid bacteria have been commercialised. The selenium supplementation using microorganisms has received much attention in the past decade (Chasteen & Bentley, 2003). In our previous study, it was reported that *Coprinus comatus*, a rare edible fungus, is able to absorb and accumulate the trace element selenium (Yu, Cui, Cui, & Wang, 2008).

The basidiomycete, *C. comatus*, is a delicious and highly nutritious edible fungus, and is also considered as a source of valuable medicinal compounds. It possesses various bioactive functions such as immunomodulation, hypoglycaemic, hypolipidemic, antitumor, and antibacterial effects (Fan et al., 2006). However, there is little information regarding the biological activity of these polysaccharides isolated from the fermentation mycelia of *C. comatus* and selenium-enriched fermentation mycelia of *C. comatus*. Moreover, no study has even compared the effects of these polysaccharides on hypoglycaemic, hypolipidemic and antioxidant activities in diabetic mice. In addition, several studies have shown that tissue (particularly in liver and kidney) antioxidant status may be an important factor in the aetiology of diabetes and that antioxidant treatment reduces diabetic complications (Kakkar, Mantha, Radhi, Prasad, & Kalra, 1998; Mohamed, Bierhaus, & Schiekofer, 1999). Therefore, this study evaluated the effect of these polysaccharides on blood glucose, blood lipid metabolism and oxidant stress in alloxan-induced diabetic mice.

2. Materials and methods

2.1. Chemicals

Alloxan and 3, 3′-diaminobenzidine (DAB) were purchased from Sigma Chemical Company (St. Louis, Mo). Sodium selenite was obtained from Tianjin Kemi Chemical Research Center (Tianjin, China). Blood glucose test strips were from Tyson Bioresearch Inc. (Taiwan, China). Reagent kits for the determination of TC, HDL, LDL and TG were from Beijing BHKT Clinical Reagent Co., Ltd (Beijing, China). Reagent kits for the determination of MDA, Vc, V_E, GSH, SOD, CAT and GSH-Px were from Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals

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doi:10.1016/j.foodchem.2009.03.073

were of the highest commercial grade available on the domestic market. The freshly prepared redistiled water was used in the present study.

2.2. Selenium-enriched fermentation mycelia of C.comatus

The seed of *C. comatus* was purchased from Shanxi Agricultural University of China.

First, the seed was grown at 28 °C for 5 days on PDA comprehensive medium slants (1000 ml 20% potato extract liquid + 20.0 g dextrose + 2.0 g peptone + 2.0 g KH_2PO_4 + 1.0 g $MgSO_4 \cdot 7H_2O$ + 0.01 g VB_1 + 20.0 g agar) and then maintained at 4 °C in a refrigerator. Three to four pieces of the mycelia of *C. comatus* were transferred from a slant into a 250 ml Erlenmeyer flask containing 100 ml liquid medium (1000 ml 30.0 g maize power + 30.0 g dextrose + 4.0 g peptone + 2.0 g KH_2PO_4 + 1.0 g $MgSO_4 \cdot 7H_2O$ + 0.01 g VB_1). The culture was incubated at 25 °C on a shake platform at 100 r/min for 4 days.

The 4-day-old liquid culture was homogenised using a sterilized blender and inoculated to a 250 ml Erlenmeyer flask containing 70 ml fermentation medium (1000 ml 40.0 g maize power + 20.0 g glucose + 4.0 g peanut meal + 2.0 g KH_2PO_4 + 1.0 g $MgSO_4 \cdot 7H_2O$ + 10.0×10^{-3} g VB_1 + 4.4×10^{-3} g Na_2SeO_3). The volume of inoculums was 3.5 ml, which was then cultivated under the same condition. After 5 days, selenium-enriched fermentation mycelia of *C. comatus* were harvested, washed with distiled water and stored at −20 °C.

2.3. Fermentation mycelia of C. comatus

The fermentation mycelia of *C. comatus* was produced using the same method to produce selenium-enriched fermentation mycelia of *C. comatus* except that there was no Na_2SeO_3 in the fermentation culture medium.

2.4. Preparation of polysaccharides

The fresh selenium-enriched fermentation mycelia of *C. comatus* was agitated into a homogenate with a blender, and then was treated with a microwave for 3 min. The sample was extracted with 2-fold volumes of distiled water for 4 h at 70 °C. After centrifuging to remove debris fragments (7000 r/min, 10 min), the solution was concentrated 3-fold in a rotary evaporator. Protein was removed with Sevag method (Vilkas & Radjabi-Nassab, 1986). The solution was dialysed in a DEAE cellulose bag against distiled water for 2 days to remove low-molecular-weight materials. Then, the solution was precipitated with 3-fold volumes of absolute ethanol for 48 h at 4 °C. The precipitates were collected by centrifugation (7000 r/min, 10 min), and then vacuum-dried at 50 °C, giving a brown selenium-polysaccharide powder (SPS).

The polysaccharide (PS) from the fermentation mycelia of *C. comatus* was produced using the same method to produce selenium-polysaccharide.

2.5. Analysis of polysaccharides

The content of polysaccharide was determined by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and the selenium content was determined by the method of Cheng (1956). In addition, protein in the polysaccharide was quantified according to Bradford's method (1976) using bovine serum albumin (BSA) as the standard.

The infrared spectra of samples (KBr disc) were recorded, as were the ultraviolet spectra of samples.

2.6. Animal and experimental design

Fifty male Kun-Ming mice weighing 28 ± 3 g were obtained from Guangdong Research Animal Center at Guangzhou, China. The mice were housed in a room maintained at 24–26 °C with a normal day/night cycle. Mice were provided with a basal diet (purchased from Guangdong Research Animal Center, Guangzhou, China) and free access to drinking water. The ingredient and nutrient compositions of the basal diet are shown in Table 1. The approval of this experiment was obtained from the Institutional Animal Ethics Committee of Shantou University (Shantou, China).

Mice were adapted to diet for 1 week before the experiment began. After a 12-h fasting, 40 mice were induced with a single injection of alloxan prepared freshly at a dose of 260 mg/kg body weight. Seventy-two hours later after alloxan injection, whole blood samples were obtained from the tail vein of the overnight fasted mice and their glucose levels were tested by blood glucose test strips (Tyson Bioresearch Inc., Taiwan, China). The mice having blood glucose levels greater than 11.1 mmol/l were considered diabetic and were used for the study.

Mice were randomly divided into four experimental groups with six mice each: Group I (NM): normal mice, received intraperitoneal injection with physiological saline; Group II (DM): alloxan-induced, untreated diabetic mice received intraperitoneal injection with physiological saline; Group III (DM + PS): diabetic mice, received intraperitoneal injection with 150 mg/kg body weight of the PS (without selenium); Group IV (DM + SPS): diabetic mice, received intraperitoneal injection with 150 mg/kg body weight of the SPS (organic selenium content: 15.21 μg/g). The treatments lasted for 20 days. At the end of the experiment, the body weight and blood glucose level were measured when the mice were fasted overnight. Blood samples were collected and immediately centrifuged for 5 min at 7000 r/min at 4 °C to obtain serum. Organs (liver and kidney) were excised from the animal and stored at −70 °C.

2.7. Biochemical analysis

Blood samples were obtained from the tail vein of the overnight fasted mice and their glucose levels were tested by blood glucose test strips (Tyson Bioresearch Inc., Taiwan, China).

The serum concentration of total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL) and triglyceride (TG) were determined using commercially available kits (BHKT Clinical Reagent Co., Ltd, Beijing, China) and according to the kits' manuals. Lipid peroxidation (as malondialdehyde, MDA) levels in liver and kidney were estimated by the

Table 1
The composition of basal diet.

Ingredients	Content (%)
Corn starch	59.00
Casein	18.00
Sucrose	8.50
Soybean oil	4.00
α-cellulose	5.00
L-cystine	0.50
Premix of minerals and trace elements (without selenium)[a]	3.50
Premix of vitamins[b]	1.00
Choline chloride	0.50

[a] Minerals and trace elements added per kg diet: Ca, 5000 mg; P, 1761 mg; K, 3400 mg; Na, 1091 mg; Cl, 1852 mg; S, 300 mg; Mg, 753 mg; Fe, 100 mg; Mn, 75 mg; Cu, 10 mg; Zn, 30 mg; I, 0.5 mg; F, 0.8 mg; Mo, 0.2 mg; Co, 0.25 mg.

[b] Vitamins added per kg diet: vitamin A, 13,000 IU; vitamin D, 1000 IU; vitamin E, 40 IU; vitamin K, 3 mg; vitamin B_1, 8 mg; vitamin B_2, 10 mg; vitamin B_6, 6 mg; vitamin B_{12}, 0.02 mg; niacin, 45 mg; pantothenic acid, 17 mg; Biotin, 0.2 mg; vitamin C, 150 mg.

method of Jain, Levine, Duett, and Hollier (1990), based on thiobarbituric-acid reactivity.

The superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) activities in liver and kidney samples were measured using commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China) and according to the kits' manuals. The levels of V_C and V_E and glutathione (GSH) were also estimated using commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China) and according to the kits' manuals.

2.8. Statistical analyses

All results are expressed as mean ± SD for six mice in each group. To determine the effect of treatment, data were analyzed using one-way ANOVA repeated measures. *p*-values of less than 0.05 were regarded as significant. Significant values were assessed with Duncan's multiple range test. Data were analyzed using the statistical package "SPSS 12.0 for Windows".

3. Results

3.1. Result of analysis of samples

Content of the polysaccharide in PS and SPS were both more than 99%, and organic selenium were 0 and 15.21 μg/g, respectively. The data on UV and IR analysis of samples are shown in Figs. 1 and 2. It had a negative response to the Bradford test and no absorption at 280 or 260 nm in the UV spectrum, indicating the absence of proteins and nucleic acids. The IR spectrum of the PS showed a strong band at 3421.2 cm^{-1} attributed to the hydroxyl stretching vibration of the polysaccharide. The bands in the region of 2927.5 cm^{-1} are due to C–H stretching vibration, and the bands in the region of 1654.7 cm^{-1} are due to associated water. The band at 920.5 cm^{-1} was ascribed to α-D-Glucopyranose. The absorptions at 1027.9, 1078.0 and 1151.3 cm^{-1} also indicated the α-pyranose form of the glucosyl residue. Moreover, the SPS revealed similar spectral characteristic to the PS. Therefore, we can only surmise that the combination of selenium may not affect the main structure of the polysaccharide.

3.2. Changes of body weight and blood glucose in diabetic mice after PS and SPS administration

The body weight and blood glucose level of different experimental groups are shown in Table 2. The alloxan-induced diabetic mice exhibited a significant increase ($p < 0.05$) in fasting blood glucose and a significant loss of body weight ($p < 0.05$) when compared with NM group. The administration of the PS (150 mg/kg body weight, without selenium) and SPS (150 mg/kg body weight, organic selenium content: 15.21 μg/g) for 20 days in diabetic mice caused a significant decrease in blood glucose levels ($p < 0.05$) when compared with DM group. Simultaneously, the body weight of diabetic mice was also observed after administration of the PS and SPS, but there was no significant increase ($p > 0.05$) when compared with DM group. The administration of SPS showed more effective than that of PS.

3.3. Effect of PS and SPS administration on concentration of serum TC, TG, HDL and LDL in diabetic mice

Diabetes mellitus usually is complicated with hyperlipoproteinemia. The concentration of serum TC, TG, HDL and LDL in different experimental groups are shown in Table 3. The present results showed that the TC, TG and LDL concentrations in the serum were significantly increased ($p < 0.05$), whereas serum HDL level was significantly decreased ($p < 0.05$) in the alloxan-induced diabetic mice when compared with the NM group. The administration of the PS (150 mg/kg body weight, without selenium) for 20 days, the alteration in lipid metabolism was partially attenuated as evidenced by decreased serum TC and TG levels and by increased HDL concentration in diabetic mice ($p < 0.05$), and the administration of the SPS (150 mg/kg body weight, organic selenium content: 15.21 μg/g) for 20 days, the alteration in lipid metabolism was partially attenuated as evidenced by decreased serum TC, TG and LDL levels and by increased HDL concentration in diabetic mice ($p < 0.05$) when compared with the DM group. The effect of SPS was more potent than that of PS.

3.4. Effect of PS and SPS administration on MDA level and the activities of antioxidant enzymes and concentration of non-enzymic antioxidants in liver and kidney in diabetic mice

The MDA level and the activities of antioxidant enzymes like SOD, GSH-Px and CAT and the concentration of non-enzymic antioxidants like GSH, vitamin C and vitamin E in liver and kidney of mice in different experimental groups are shown in Table 4. There appears to be a significant increase ($p < 0.05$) in the level of MDA associated with the diminution of the activities of SOD, GSH-Px and CAT and the levels of GSH, vitamin C and vitamin E in liver and kidney of mice in the DM group in comparison with those of the NM group. The administration of the PS (150 mg/kg body weight, without selenium) and SPS (150 mg/kg body weight, organic selenium content: 15.21 μg/g) for 20 days caused a significant decrease in the level of MDA ($p < 0.05$) and a significant increase in the activities of enzymic antioxidants ($p < 0.05$) and

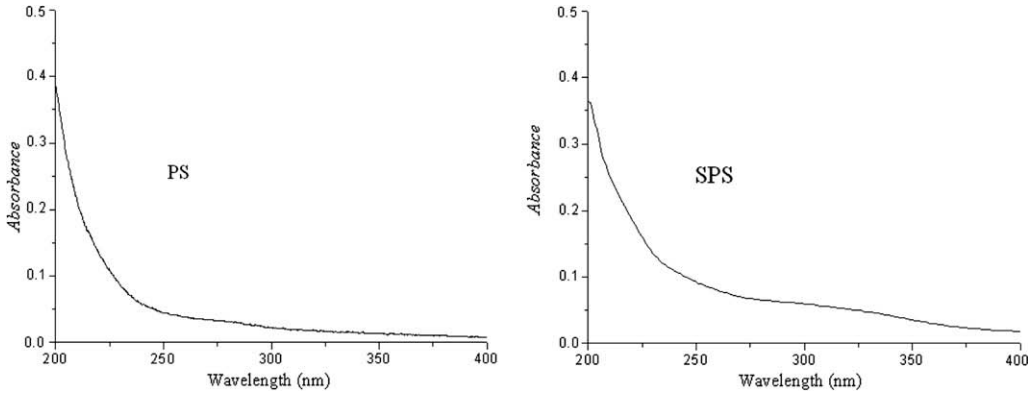

Fig. 1. UV spectra of the polysaccharide (PS) and selenium-polysaccharide (SPS) from mycelia of *C. comatus*.

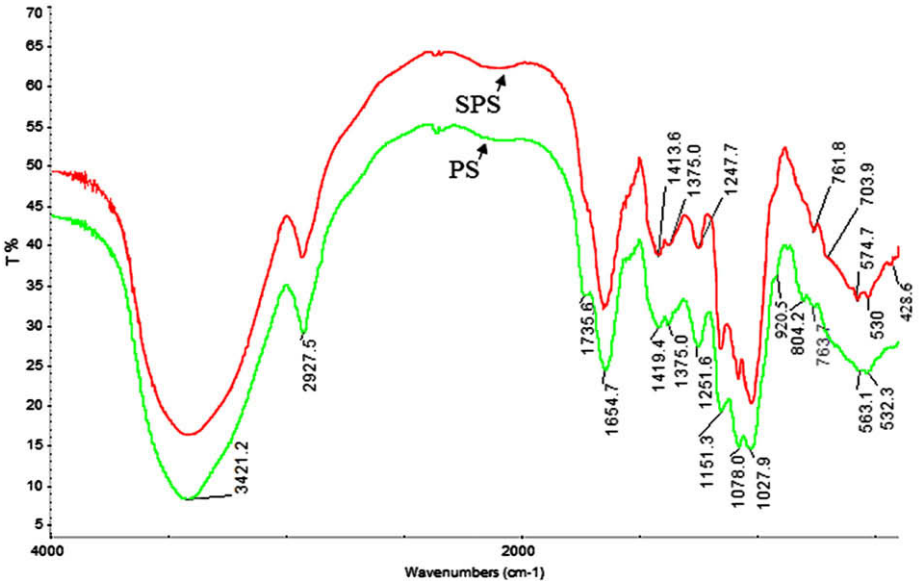

Fig. 2. IR spectra of the polysaccharide (PS) and selenium-polysaccharide (SPS) from mycelia of *C. comatus*.

Table 2
Effect of the polysaccharide (PS) and selenium-polysaccharide (SPS) from mycelia of *C. comatus* on body weight and blood glucose in diabetic mice.

Groups	Body weight (g)		Blood glucose (mmol/l)	
	Pre-experiment	Post-experiment	Pre-experiment	Post-experiment
NM	28.31 ± 3.06[b]	31.13 ± 3.49[b]	6.38 ± 0.42[b]	6.53 ± 0.46[b]
DM	25.87 ± 4.69[a]	24.60 ± 4.93[a]	18.70 ± 3.78[a]	19.70 ± 3.38[a]
DM + PS	25.50 ± 5.19[a]	25.96 ± 3.89[a]	18.94 ± 3.14[a]	11.50 ± 2.81[ab]
DM + SPS	25.02 ± 4.37[a]	25.90 ± 2.79[a]	18.68 ± 3.46[a]	9.66 ± 1.08[ab]

Values are expressed as mean ± SD for six mice in each group. One-way ANOVA repeated measures with Duncan's multiple rang test was used to calculate statistical significance.
[a] Indicates statistical significance of $p < 0.05$ compared to the NM group.
[b] $p < 0.05$ compared to the DM group.

the levels of non-enzymic antioxidants ($p < 0.05$) when compared with DM group. Moreover, the effect of SPS was more prominent compared with that of PS.

4. Discussion

Alloxan is widely used to induce experimental diabetes in animals. The mechanism of action in beta-cells of the pancreas is mediated by reactive oxygen species. The action of reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of beta-cells (Szkudelski, 2001). In the present experiment, the alloxan-induced diabetic mice exhibited a significant increase ($p < 0.05$) in fasting blood glucose and a significant loss of body weight ($p < 0.05$) when compared with normal mice. The administration of the PS (150 mg/kg body weight, without selenium) and SPS (150 mg/kg body weight, organic selenium content: 15.21 μg/g) for 20 days caused a significant decrease in blood glucose levels ($p < 0.05$) when compared with untreated diabetic mice. However, it did not normalise the body weight completely as it remained lower than normal mice. The decrease in body weight observed in diabetic mice might be the result of protein wasting due to unavailability of carbohydrate for utilisation as an energy source (Chen & Ianuzzo, 1982). Diabetes mellitus is one of the most common human metabolic diseases, and derangements in lipid metabolism in diabetes are often important determinants of the course and status of the diseases (Fumelli, Romagnoli, Carlino, Fumelii, & Boemi, 1996). When compared with normal mice, the present results also showed that the TC, TG and LDL concentrations in the serum were significantly increased ($p < 0.05$), whereas serum HDL level was significantly decreased ($p < 0.05$) in the alloxan-induced diabetic mice. After administration of the PS (150 mg/kg body weight, without selenium) and SPS (150 mg/kg body weight, organic selenium content: 15.21 μg/g) for 20 days, the alteration in lipid metabolism was partially attenuated when compared with untreated diabetic mice. Our study revealed that the hypoglycaemic and hypolipidemic effects of SPS was more prominent compared with that of PS.

Diabetes mellitus is associated with generation of reactive oxygen species leading to oxidative damage particularly in liver and

Table 3
Effect of the polysaccharide (PS) and selenium-polysaccharide (SPS) from mycelia of *C. comatus* on concentration of serum TC, TG, HDL and LDL in diabetic mice.

Groups	TC (mmol/l)	TG (mmol/l)	HDL (mmol/l)	LDL (mmol/l)
NM	2.24 ± 0.26[b]	1.11 ± 0.38[b]	0.53 ± 0.06[b]	0.58 ± 0.09[b]
DM	3.56 ± 0.18[a]	2.04 ± 0.37[a]	0.18 ± 0.05[a]	0.74 ± 0.07[a]
DM + PS	3.43 ± 0.31[a]	1.27 ± 0.26[b]	0.35 ± 0.09[ab]	0.75 ± 0.06[a]
DM + SPS	2.61 ± 0.15[ab]	1.19 ± 0.13[b]	0.41 ± 0.06[ab]	0.38 ± 0.02[ab]

Values are expressed as mean ± SD for six mice in each group. One-way ANOVA repeated measures with Duncan's multiple rang test was used to calculate statistical significance.
[a] Indicates statistical significance of $p < 0.05$ compared to the NM group.
[b] $p < 0.05$ compared to the DM group.

Table 4
Effect of the polysaccharide (PS) and selenium-polysaccharide (SPS) from mycelia of *C. comatus* on MDA level and the activities of antioxidant enzymes and concentration of non-enzymic antioxidants in liver and kidney in diabetic mice.

Groups	NM	DM	DM + PS	DM + SPS
Liver				
MDA (nmol/mg protein)	2.42 ± 0.33[b]	5.48 ± 0.43[a]	2.56 ± 0.25[b]	2.43 ± 0.29[b]
SOD (U/mg protein)	173.98 ± 10.50[b]	155.83 ± 11.81[a]	170.41 ± 8.37[b]	185.52 ± 10.56[ab]
GSH-Px (U/mg protein)	657.63 ± 43.87[b]	562.80 ± 52.79[a]	708.56 ± 60.62[ab]	773.86 ± 16.13[ab]
CAT (U/mg protein)	98.39 ± 9.62[b]	55.41 ± 5.51[a]	76.71 ± 5.69[a]	73.61 ± 4.03[a]
GSH (μg/mg protein)	1.73 ± 0.07[b]	1.17 ± 0.28[a]	1.58 ± 0.34[ab]	2.25 ± 0.41[ab]
V_C (μg/mg protein)	7.17 ± 0.26[b]	2.76 ± 0.32[a]	5.64 ± 0.44[ab]	5.39 ± 0.46[ab]
V_E (μg/g tissue)	20.18 ± 3.19[b]	14.18 ± 3.46[a]	17.82 ± 1.75[ab]	20.00 ± 3.92[b]
Kidney				
MDA (nmol/mg protein)	2.16 ± 0.28[b]	4.18 ± 0.29[a]	2.47 ± 0.13[ab]	2.11 ± 0.20[b]
SOD (U/mg protein)	152.82 ± 21.59[b]	109.46 ± 12.63[a]	151.11 ± 6.52[b]	171.43 ± 33.81[ab]
GSH-Px (U/mg protein)	532.31 ± 33.76[b]	331.50 ± 44.46[a]	766.99 ± 40.05[ab]	852.19 ± 27.19[ab]
CAT (U/mg protein)	43.83 ± 2.54[b]	33.44 ± 1.53[a]	39.38 ± 2.09[ab]	40.19 ± 2.39[b]
GSH (μg/mg protein)	1.90 ± 0.18[b]	1.21 ± 0.17[a]	1.43 ± 0.21[ab]	2.13 ± 0.28[ab]
V_C (μg/mg protein)	2.61 ± 0.39[b]	1.06 ± 0.25[a]	1.33 ± 0.10[ab]	1.24 ± 0.43[ab]
V_E (μg/g tissue)	45.27 ± 6.14[b]	18.36 ± 0.63[a]	28.36 ± 7.58[ab]	45.09 ± 5.06[b]

Values are expressed as mean ± SD for six mice in each group. One-way ANOVA repeated measures with Duncan's multiple rang test was used to calculate statistical significance.
[a] Indicates statistical significance of $p < 0.05$ compared to the NM group.
[b] $p < 0.05$ compared to the DM group.

kidney (Kakkar et al., 1998; Mohamed et al., 1999). Oxidative stress in diabetes coexists with a decrease in the antioxidant status (Picton, Flatt, & Mcclenghan, 2001), which can increase the deleterious effects of free radicals. Antioxidants play a major role in protecting biological systems against reactive oxygen species and reflect the antioxidant capacity of the system (Irshad & Chaudhuri, 2002). The components of the defence system that have evolved to reduce the injury from free radical attack include several enzymes and a few free radical scavenger molecules (Vendemiale, Grattagliano, & Altomare, 1999). In the present experiment, we have also observed a significant increase ($p < 0.05$) in the level of MDA associated with the diminution of the activities of SOD, GSH-Px and CAT and the levels of GSH, vitamin C and vitamin E in liver and kidney of diabetic mice in comparison with normal mice. These results further confirm that there is a strong correlation between oxidative stress and diabetes occurrence. The administration of the PS (150 mg/kg body weight, without selenium) and SPS (150 mg/kg body weight, organic selenium content: 15.21 μg/g) for 20 days, caused a significant decrease in the level of MDA ($p < 0.05$) and a significant increase in the activities of enzymic antioxidants ($p < 0.05$) and the levels of non-enzymic antioxidants ($p < 0.05$) in liver and kidney of diabetic mice. Our study also revealed that the antioxidant effect of SPS was more potent than that of PS.

Vitamin C is a potent antioxidant, which widely acts on oxygen free radicals as well as by interaction with vitamin E (Garg & Bansal, 2000). Both the vitamin C and vitamin E significantly decreased in the liver and kidney of diabetic mice. The administration of the PS (150 mg/kg body weight, without selenium) and SPS (150 mg/kg body weight, organic selenium content: 15.21 μg/g) for 20 days increased the vitamin C and vitamin E levels. This indicates that vitamin E is used in combating free radicals and if vitamin C is present, vitamin E levels are preserved. Also vitamin C regenerates vitamin E from its oxidised form. GSH has a multifactorial role in antioxidant defence. It is a direct scavenger of free radicals as well as a co-substrate for peroxide detoxification by GSH-Px. Loven, Schedl, Wilson, and Diekus (1986) suggested that the decrease in tissue GSH could be the result of decreased synthesis or increased degradation of GSH by oxidative stress in diabetes. We observed a decrease in GSH in the liver and kidney of diabetic mice. The administration of the PS (150 mg/kg body weight, without selenium) and SPS (150 mg/kg body weight, organic selenium content: 15.21 μg/g) for 20 days increased the content of GSH in the liver and kidney of diabetic mice. This indicates that the PS and SPS can either increase the biosynthesis of GSH or reduce the oxidative stress leading to less degradation of GSH, or have both effects.

SOD has been postulated as one of the most important enzymes in the enzymatic antioxidant defence system which catalyses the dismutation of superoxide radicals to produce H_2O_2 and molecular oxygen (Mc-Crod, Keele, & Fridovich, 1976), hence diminishing the toxic effects caused by their radical. The observed decrease in SOD activity could result from inactivation by H_2O_2 or by glycation of enzymes (Sozmen, Sozmen, Delen, & Onat, 2001). The superoxide anion has been known to inactivate CAT, which involved in the detoxification of hydrogen peroxide (Chance, Green Stein, & Roughton, 1952). Thus, the increase in SOD activity may indirectly play an important role in the activity of catalase. CAT is a hemeprotein which catalyses the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals (Searle & Wilson, 1980). This decrease in CAT activity could result from inactivation by glycation of the enzyme (Yan & Harding, 1997). Reduced activities of SOD and CAT in the liver and kidney have been observed in diabetic mice. The administration of the PS (150 mg/kg body weight, without selenium) and SPS (150 mg/kg body weight, organic selenium content: 15.21 μg/g) for 20 days increased the activities of SOD and CAT in the liver and kidney of diabetic mice. GSH-Px plays a primary role in minimising oxidative damage. Glutathione peroxidase (GSH-Px), an enzyme with selenium, and glutathione-s-transferase (GST) works together with glutathione in the decomposition of H_2O_2 or other organic hydroperoxides to non-toxic products at the expense of reduced glutathione (Bruce, Freeman, & James, 1982). Reduced activity of GSH-Px may result from radical-induced inactivation and glycation of the enzyme (Hodgson & Fridovich, 1975). Reduced activities of GSH-Px in the liver and kidney has been also observed in diabetic mice. The administration of the PS (150 mg/kg body weight, without selenium) and SPS (150 mg/kg body weight, organic selenium content: 15.21 μg/g) for 20 days increased the activity of GSH-Px in the liver and kidney of diabetic mice. This means that the PS and SPS can reduce the potential glycation of enzymes or they may reduce reactive oxygen free radicals and improve the activities of antioxidant enzymes.

In conclusion, the present investigation showed that the PS and SPS possess potent antioxidant activity, which may be directly or

indirectly responsible for its hypoglycaemic and hypolipidemic properties. Furthermore, the effect of SPS was more potent than that of PS which may due to the co-effect of polysaccharide and selenium. Therefore, the SPS should be considered as a candidate for future studies on diabetes.

Acknowledgment

This work was supported by Department of Biology and Marine Biology Institute, Shantou University.

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Food Chemistry 117 (2009) 48–54

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Characteristics and antioxidant activity of ultrafiltrated Maillard reaction products from a casein–glucose model system

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ARTICLE INFO

Article history:
Received 5 December 2008
Received in revised form 17 March 2009
Accepted 19 March 2009

Keywords:
Maillard reaction products (MRPs)
Casein–glucose
Browning
Free amino group
Antioxidant activity

ABSTRACT

Maillard reaction products (MRPs) were prepared from casein–glucose by refluxing for 130 min at 102 °C and initial pH 12.0 without pH control to investigate the characteristics of casein–glucose Maillard reaction and the antioxidant activity difference among different fractions of MRPs. Browning and intermediate products increased, however, the pH of the system decreased with increase in the heating time. Free amino group content decreased 78% during first 10 min and did not change nearly thereafter. Amino acid analysis indicated that lysine and arginine decreased significantly, and casein was partially hydrolysed to peptides or free amino acid. High molecular weight compounds were dominant in the MRPs, determined by high performance gel-filtration chromatography. After ultrafiltration, antioxidant activity of each MRPs fraction was investigated by DPPH radical-scavenging activity, reducing power, Fe^{2+} chelating activity and lecithin oxidation assay. MRPs of different molecular weight exhibited distinctly different antioxidant activities.

1. Introduction

Maillard reaction (MR) is a very complex reaction between carbonyls and amines. It occurs spontaneously during food processing and storage, mainly by the reaction between reducing sugars and the amino groups of proteins (Liu, Yang, Jin, Hsu, & Chen, 2008). MR usually produces a wide range of products with odors and colours, antioxidant, antiallergenic, antimicrobial and cytotoxic properties (Liu et al., 2008; Plavsic, Cosovic, & Lee, 2006; Rufian-Henares & Morales, 2006). MR also leads to the reduction of protein nutrition, digestive incompatibility and potential toxicity (Ajandouz, Desseaux, Tazi, & Puigserver, 2008).

Maillard reaction products (MRPs) of amino acid–sugar have been found to exhibit strong antioxidant activity. Various mechanisms are involved in the antioxidant activity of MRPs, like radical chain-breaking activity (Eichner, 1981; Morales & Jimenez-Perez, 2001), scavenging of reactive oxygen species (Bersuder, Hole, & Smith, 2001), decomposing hydrogen peroxide and metal chelation (Wijewickreme, Kitts, & Durance, 1997).

The high molecular weight melanoidins prepared from xylose and glycine has been found to possess a higher browning, reducing power and antioxidant activity (Yamaguchi, Koyama, & Fujimaki, 1981). Yen and Tsai (1993) investigated the antioxidant activity of partially fractionated MRPs prepared by refluxing glucose (0.5 M) and tryptophan (0.5 M) at pH 11.0 and 100 °C for 10 h, the results showed that the high molecular weight fraction achieved higher reducing power and antioxidant activity. Rufian-Henares and Morales (2007) investigated the effect of in vitro enzymatic digestion on antioxidant activity of coffee melanoidins and fractions. The results acquired two plausible options: modifying/releasing the ionically bound compounds and/or genesis of new more active structures from the melanoidin skeleton after enzymatic treatment.

Most studies have been done to investigate the mechanism of MR from proteins and sugars. More than half a century ago, Lea and Hannan (1949) published some valuable kinetic data on the influence of temperature, pH and water activity on the Maillard reaction in a glucose–casein model system. In subsequent kinetic studies, model systems such as those based on soy proteins, egg albumin (Kato, Matsuda, Kato, & Nakamura, 1989) and milk were used (Brands & Van Boekel, 2003).

MRPs of protein–sugar have been investigated as suitable encapsulant to protect sensitive core material against oxidation (Augustin, Sanguansri, & Bode, 2006; Drusch et al., 2009; Kosaraju, Weerakkody, & Augustin, 2009). Glycation of casein with glucose or lactose resulted in an enhancement of antioxidant activity when compared with native casein (McGookin & Augustin, 1991), but the antioxidant mechanism and the active components are not understood clearly. The objectives of this study were (1) to describe the characteristics of casein–glucose MR at high pH, the possible changes in chemicals during heating and (2) to investigate the

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doi:10.1016/j.foodchem.2009.03.074

possible antioxidant activity difference among different fractions of MRPs.

2. Materials and methods

2.1. Chemicals

Ferrozine and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Casein, an isoelectric precipitate containing 14.5% nitrogen, was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Lecithin was purchased from East China Norm University Chemical Reagent Co., Ltd. (Shanghai, China). The other solvents/chemicals used were of analytical grade and obtained from Shanghai Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Preparation of Maillard reaction products

Casein–glucose model systems were used as reported by Drusch et al. (2009) with some modifications. The model consisted of casein (5.0 g) and glucose (10.0 g), dissolved in distilled de-ionised water (dd H_2O) and adjusted to pH 12.0 with 5.0 M NaOH and 1.0 M HCl solution, the substrate concentration was adjusted to 20% (w/v) with ddH_2O. The solution was transferred to a 250 ml 3-neck round-bottom flask and heated at 102 °C for 130 min under refluxing in an oil bath with magnetic stirring at 40 rpm. The heated casein–glucose mixtures were termed as Maillard reaction products (MRPs). Five milliliters sample of MRPs was removed with a pipet at different intervals and placed in an ice bath to cool down. The samples from the heating for 130 min were lyophilised with Stoppering Tray Dryer (LABCONOCO, USA) for further chemical and biological experiments. The lyophilisation was proceeded under 0.12 mbar vacuum with four different segments in the order of (1) 1.0 h at −30 °C, (2) 20 h at −10 °C, (3) 20 h at −4 °C, (4) finally 0.5 h at 15 °C. All determinations were carried out at least two times and mean values were calculated.

2.3. Ultrafiltration of the Maillard reaction products

Water-soluble MRPs were isolated by ultrafiltration according to the method as described by Yen and Tsai (1993). Briefly, after ice bath cooling, MRPs obtained from 130 min heating were adjusted to pH 7.0 with 1 M NaOH and then centrifuged at 3000 rpm for 15 min to obtain the water-soluble MRPs. Two hundred milliliters water-soluble MRPs diluted solution was subjected to ultrafiltration, using an Amicon ultrafiltration cell model 8400 (Amicon, Beverly, MA, USA) equipped with 50 30, 10, 5, and 1 kDa nominal molecular mass cut-off membrane. The 20 ml retentate was topped up to 200 ml with water and washed again. Washing step was repeated at least three times. Each fraction was collected and lyophilised, then stored in desiccators at 4 °C until analysis.

2.4. Measurement of pH

A pH meter (Model SP-71, METTLER TOLEDO, Inc., Shanghai, China) was used for the determination of system's pH values.

2.5. Measurement of the absorbance

The absorbance of the heated solutions was measured using a spectrophotometer (UNICO UV-2100, Shanghai, China) at 294 and 420 nm, as markers at the intermediate and final stages of the reactions, respectively (Ajandouz, Tchiakpe, Ore, Benajiba, & Puigserver, 2001). Samples were diluted 100 and 50-fold with 0.1% (w/v) sodium dodecyl sulfate (SDS) to obtain absorbance value at 294 and 420 nm, respectively.

2.6. Measurement of free amino group content

The quantity of available amino groups was determined by the modified *o*-phthaldialdehyde (OPA) method (Guan, Qiu, Liu, Hua, & Ma, 2006). The OPA reagent was prepared daily. Forty milligram of OPA was dissolved in 1.0 ml of methanol and 3.0 ml ddH_2O and named as CA. Twenty five milliliter 100 mM sodium tetraborate, 2.5 ml of 20% sodium dodecyl sulfate (SDS) and 100 μl of β-mercaptoethanol were mixed, and then diluted to a final volume of 50 ml with water, this solution was termed as CB. Two hundred microliter sample solution was added to the mixture of 0.3 ml CA and 3.7 ml CB. The solution was mixed briefly and incubated for 2 min at ambient temperature, and the absorbance was read at 340 nm using a UNICO UV-2100 spectrophotometer. The blank was determined in the same manner, except that ddH_2O was used instead of sample. A calibration curve was obtained by using 0.25 ∼ 2 mM L-lysine as a standard. Amino group loss was calculated from the ratio of the volume of OPA used after heating to the volume of OPA used for original sample. Casein was heated without glucose under identical conditions as the preparation of MRPs for 130 min, and termed as casein heated. Total nitrogen content was determined using the Kjeldahl method and confirmed the protein content of each solution. Results were the average of three measurements and expressed as free amino group loss (%).

2.7. Amino acid analysis

The samples were hydrolysed with 6 M hydrochloric acid at 110 °C for 24 h under vacuum. The hydrolysate was submitted to automated online derivatisation with *o*-phthalaldehyde and reversed phase high performance liquid chromatography (RP-HPLC) analysis in Agilent 1100 (Agilent Technology, Palo Alto, CA, UAS) assembly system using a Zorbax 80A C_{18} column (4.6 i.d. × 180 mm), running at 0.5 ml/min. The results acquired were analysed with the aid of ChemStation for LC 3D software (Agilent Technology, Palo Alto, CA, USA). Results were expressed as percentage (%) of amino acid of the measurement.

2.8. Estimation of molecular weight (MW) distribution

The high performance gel-filtration chromatography (HPGFC) was performed with HPLC system (Waters 600, Waters Inc., USA) equipped with a column (Ultrahydrogel™Linear 300 mm × 7.8 mmid × 2) and Empower ChemStation. A refractive index detector was used to detect samples eluting from the column. The effluent was 0.1 M $NaNO_3$ at 0.9 ml/min. The standard compounds for molecular weight estimation were: (1) MW 2000 kDa (2) MW 133.8 kDa (3) MW 7.1 kDa (4) MW 2.5 kDa and (5) MW 0.18 kDa. The sample was injected after being dissolved in effluent and filtrated with 0.45 um membrane. The molecule weight was calculated as follows;

$$\text{Log MW} = 12.6 - 0.479\,T \quad (1)$$

where MW means molecular weight and *T* means elution time.

2.9. Determination of reducing power

The reducing power of MRP samples was determined according to the method of (Oyaizu, 1986) with some modifications. One milliliter of MRPs sample (100-fold dilution) was mixed with 1.0 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 1.0 ml of 1% potassium ferricyanide ($K_3Fe(CN)_6$). The reaction mixtures were incubated in a temperature-controlled water bath at 50 °C for 20 min,

followed by addition of 1.0 ml of 10% trichloroacetic acid after cooling to room temperature. The mixtures were then centrifuged at 750 g using a centrifuge (TG16-WS, Xiangyi, Changsha, China) for 10 min at 25 °C. The supernatant obtained (1.0 ml) was treated with 1.0 ml of distilled water and 200 μl of 0.1% $FeCl_3$. The absorbance of the reaction mixture was measured at 700 nm with UNICO UV-2100 spectrophotometer. Reducing power was expressed as absorbance at 700 nm, the lowest value of the tests was used as the initial reference value, and an increase in absorbance was used as the measure of the increased reducing power. For the comparison, the assay was conducted in the same manner but butylhydroxytoluene (BHT) was added instead of sample solution. The results were expressed as absorbance units (AU).

2.10. Determination of DPPH radical-scavenging activity

DPPH radical-scavenging activity was determined according to the method of Benjakul, Visessanguan, Phongkanpai, and Tanaka (2005) with some modifications. An aliquot of MRPs sample (1.0 ml) was added to 2.0 ml of 0.1 mM DPPH in ethanol. The solution was then mixed vigorously and allowed to stand at room temperature in the dark for 20 min. The mixture was centrifuged for 10 min at 750g. The absorbance of supernatant was measured at 517 nm using a UNICO UV-2100 spectrophotometer. The blank was prepared in the same manner, except that distilled water was used instead of MRPs samples. For the control, the assay was conducted in the same manner but ethanol was added instead of DPPH solution. For the comparison, the assay was conducted in the same manner but BHT was added instead of sample solution. The percentage of DPPH radical-scavenging activity was calculated as follows;

$$\text{Radical-scavenging activity}(\%) = \left[1 - (A517\ \text{nm}_{\text{sample}} - A517\ \text{nm}_{\text{control}})/A517\ \text{nm}_{\text{blank}}\right] \times 100 \quad (2)$$

where A517 nm_{sample} is the absorbance of sample, A517 $nm_{control}$ is the absorbance of the control and A517 nm_{blank} is the absorbance of the blank.

2.11. Determination of chelating activity on Fe^{2+}

Chelating activity of MRPs was determined by the method of Dinis, Madeira and Almeida (1994) with modifications. One milliliter sample solution was mixed with 1.85 ml ddH_2O and 0.05 ml 2.0 mM $FeCl_2$, the mixture was allowed to rest at room temperature (28 °C) for 30 s. The reaction mixture thus obtained was later added with 0.1 ml 5 mM ferrozine (Sigma, St. Louis, MO, USA) and mixed, absorbance at 562 nm was determined with UNICO UV-2100 spectrophotometer after 10 min resting time at room temperature and 5 min centrifugation at 3000 rpm. For the blank, the assay was conducted in the same manner but ddH_2O was added instead of sample solution. The percentage of chelating activity was calculated as follows;

$$\text{Chelating activity}(\%) = \left(1 - A562\ \text{nm}_{\text{sample}}/A562\ \text{nm}_{\text{blank}}\right) \times 100 \quad (3)$$

where A562 nm_{sample} is the absorbance of sample and A562 nm_{blank} is the absorbance of the blank.

2.12. Inhibition of lipid peroxidation induced by iron

Lecithin was suspended in phosphate buffered saline (0.01 M, pH 7.4) at a concentration of 10.0 mg/ml with magnetic stirring (Yi, Meyer, & Frankel, 1997). The solution was labeled as LLS. Fifteen gram trichloroacetic acid (TCA), 0.37 g thiobarbituric acid (TBA) and 2 ml concentrated hydrochloric acid (HCl) were added to ddH_2O, and the volume was adjusted to 100 ml with ddH_2O. The solution was labeled as TCA–TBA–HCl. One milliliter LLS, 1.0 ml 400 uM $FeCl_3$, 1.0 ml 400 uM ascorbic acid and 1.0 ml sample solution were mixed to the flasks in order and mixed well. The solution was placed in water bath at 37 °C in dark for 60 min, and then 2.0 ml TCA–TBA–HCl was added. The system was cooled down with ice water after heating in water bath at 90 ~ 100 °C for 15 min. The pink solution was centrifuged at 750g for 10 min, and the absorbance of the supernatant was read at 532 nm, and called as As. The blank was made with 1.0 ml ddH_2O substitution for 1.0 ml sample, named as Ac. The inhibition percentage (%) was calculated as formula;

$$\text{Inhibition percentage}\ (\%) = (\text{Ac} - \text{As})/\text{Ac} \times 100. \quad (4)$$

2.13. Statistical analysis

Collected data were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was performed and means comparisons were carried out by Student–Newman–Keuls' tests. A value of $p < 0.05$ was considered significant. Data were analysed by using a statistical software package (SPSS for Windows, 13.0, 2005, SPSS Inc. USA).

3. Results and discussion

3.1. Changes of pH

The pH of casein-glucose system decreased as the heating time is extended up to 130 min (Table 1). The pH decreased at a higher rate during the first 30 min, thereafter, it slightly decreased up to 130 min, and the pH of casein–glucose MRPs decreased from approximately 12.0 to 5.5 ~ 6.5. The phenomena may be due to the production of formic and acetic acid from glucose, which is partially degraded to these compounds when it is heated at high pH with protein or not (Rufian-Henares, Delgado-Andrade, & Morales, 2006). Other acids such as lactic, levulinic, propionic, butyric, pyruvic, citric and saccharinic acids might have been formed (Brands & Van Boekel, 2003).

At the intermediate stage of the MR, because of the generation of some acidic compounds with buffering capacities including formic acid, acetic acid, methylglyoxal, glyoxal, etc. and the inhibition of acidity to MR, decrease in pH was leveled off with prolonged heating time. Therefore, it can be concluded that the consumption of amino group, together with formation of acids, could be the mechanism of pH decreasing tendency in the MR system (Liu et al., 2008).

3.2. Changes in absorbance

The color progress in MR was investigated by measuring absorbance at 420 nm. As can be seen from Table 1, a sharp increase

Table 1
Changes in pH and absorbance (mean ± SD) at different wavelengths of the mixture during heating to 130 min.

Heating time (min)	pH	Absorbance at 420 nm	Absorbance at 294 nm
0	$12.0^{a} \pm 0.21$	$0.007^{a} \pm 0.002$	$0.138^{a} \pm 0.023$
10	$9.41^{b} \pm 0.71$	$0.511^{b} \pm 0.051$	$2.311^{b} \pm 0.035$
30	$7.63^{c} \pm 0.37$	$0.697^{c} \pm 0.072$	$2.533^{c} \pm 0.097$
60	$6.86^{c,d} \pm 0.42$	$0.757^{c,d} \pm 0.108$	$2.602^{c} \pm 0.069$
90	$6.51^{d} \pm 0.35$	$0.803^{c,d} \pm 0.054$	$2.623^{c} \pm 0.036$
130	$6.10^{d} \pm 0.55$	$0.853^{d} \pm 0.076$	$2.658^{c} \pm 0.012$

[a–d]Different letter superscripts denote significant difference ($p < 0.05$). SD: standard deviation from triplicate determinations.

browning was observed during the first 30 min. The similar results were obtained by Rufian-Henares, Garcia-Villanova, and Guerra-Hernandez (2004), although the colour intensity in the paper was higher. This difference could be related to the different pH used. The initial pH value of the reaction system was considered to affect Maillard reaction significantly. In alkaline condition, Schiff-base formed easily and promoted the MR further. Consequently, the brown components of MR system were produced quickly. During the development of brown color caused by the MR, caramelisation can occur simultaneously. Caramelisation reactions contribute to overall non-enzymatic browning, especially in the alkaline pH ranges (Ajandouz et al., 2001; Benjakul et al., 2005).

The slight increases in browning intensities of MRPs samples were observed after 30 min of heating (Table 1).The results might be due to the consumption of the amino group and production of acid by MR, shifting systems into more acidic condition and lowered the browning reaction.

Absorbance at 294 nm was used to investigate the formation of the intermediate compounds of the MR (Ajandouz et al., 2001). A significant increase ($p < 0.05$) in A294 nm of MRPs samples was observed during 30 min of heating (Table 1). However, no significant changes in A294 nm of MRPs were found with prolonged heating time up to 130 min. The results suggested that intermediate products were produced to a great extent within the first 30 min. During extended heating, some intermediate products might undergo polymerisation to form the melanoproteins whereas only a small amount of intermediate products was generated (Lertittikul, Benjakul, & Tanak, 2007).

3.3. Changes in free amino group content

Table 2 shows the results of amino group changes determined with the OPA method. Since no amino groups were modified on native casein, all results were reported relative to 100% of the amino groups of native casein. When casein was heated without glucose at pH 12, thirteen percent amino groups could not been detected (Table 2). This may be due to the S–S covalent bond between *k*-casein and steric obstruction. Amino group losses have been previously reported in proteins heated alone (Smith & Friedman, 1984).

As can be seen in Table 2, a significant ($p < 0.05$) decrease in free amino group content of MRPs was observed when heated for the first 10 min. About 78% free amino groups were lost due to the attachment to the reducing compounds. Then the amino group loss was not changed significantly with the extended heating time to 130 min. This indicates that heating quickly induces alkali catalysed interaction of free amino groups, such as ε-NH_2 groups of lysine, with sugar via glycation process within 10 min. High pH quickly induced the condensation reaction between free amino groups of casein and the carbonyl groups of glucose and its degradation products (Brands & Van Boekel, 2003).

From the above results, the decrease in free amino group was in accordance with the increase in browning and absorbance at 294 nm (Table 1). The reaction rate of glycation between casein and sugar depended on the percentage of the acyclic form and the electrophilicity of the carbonyl groups (Naranjo, Malec, & Vigo, 1998). As the pH of the casein–sugar systems dropped during the extended heating time and in addition, due to protonation of the lysine residues, the reactivity of the protein decreased, resulting in the insignificant changes in the free amino group loss after 10 min heating.

Table 2
Free amino group loss percentage (mean ± SD) in casein–glucose MRPs heated for different time and in heated casein in absence of glucose.

Samples	Free amino loss (%)
0 min	$0^{a} \pm 0.73$
10 min	$78.07^{b} \pm 2.63$
30 min	$79.83^{b} \pm 0.88$
60 min	$80.71^{b} \pm 0.72$
90 min	$80.92^{b} \pm 1.35$
130 min	$78.07^{b} \pm 2.47$
Casein heated	$13.16^{c} \pm 0.65$

[a–c]Different letter superscripts denote significant difference ($p < 0.05$); SD: standard deviation from triplicate determinations.

3.4. Amino acid analysis

The amino acids in the MRPs of MW higher than 50 kDa decreased almost 21.73% compared with native casein (Table 3). Amino acid percentage composition of all three types of MRPs showed that MR caused a significant loss in lysine and arginine compared to native casein. Moreover, arginine and lysine of unfractioned MRPs decreased to 1.43% and 7.77%, respectively (Table 3) compared to the original values in the native casein. This indicated that glucose and glucose degraded products attached to casein or casein peptides. The lost amino groups were probably originated largely from the side chains of lysine residues, but guanidino groups of arginine might also have been involved. Losses in cystine and lysine with the application of heat have also been attributed to the formation of cystine-derived crosslink. Heat results in the formation of a dehydroalanyl residue from cystine, which is capable of binding with the ε-amino group of lysine to form lysinoalanine. This indicates that lysinoalanine can be formed only at basic pH (Ajandouz et al., 2008).

Amino acids can be seen in the MRPs of MW from 5 to 10 kDa, though the quantity is small (Table 3). It showed that casein was degraded to peptides or free amino acid when casein was heated at high pH and high temperature. These compounds can react with glucose and its degraded products.

3.5. Molecular weight distribution of MRPs

Three peaks are found as shown in Fig. 1. Area of peak 1 was the largest, which means that the high molecular weight MRPs dominate in the mixture. According to the formula (1), the molecular weight distributions were: peak1, 4.404–2.400 kDa; peak2, 0.765–4.404 kDa; peak3, 0.018–0.765 kDa.

MRPs from casein–glucose comprise a wide range of compounds. Casein was covalently attached to glucose and glucose degradation products (GDPs), which results in high MW MRPs (melanoproteins). Caramelisation of glucose heated at high pH induces the formation of low and medium MW compounds, while casein hydrolysates reacted with glucose and GDPs also contribute to this kind of MRPs (Drusch et al., 2009).

3.6. Reducing power

During the reducing power assay, the presence of reductants in the tested samples result in reducing Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}). The Fe^{2+} can therefore be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Yoshimura, Iijima, Watanabe, & Nakazawa, 1997).

Reducing power of MRPs increased with the increased concentration of MRPs (Table 4), it reached to 0.668 of absorbance at 700 nm with concentration of 2 mg/ml, but increased slightly afterwards. This result revealed that MRPs could function as electron donors, but its reducing power was less than that of BHT (Table 4).

Reducing power of each fraction of MRPs and the unfractionated MRPs are shown in Table 5, reducing power was found in this order, MW higher than 50 kDa = MW 10–30 kDa > MW 1–5 kDa = unfractionated MRPs > MW 5–10 kDa = MW lower than 1 kDa =

Table 3
Amino acid percentage composition (%) of native casein and different types of Maillard reaction products (MRPs).

Amino acids	Casein	MW[a] > 50 kDa[b] MRPs	Unfractioned MRPs	MW 5–10 kDa MRPs
Aspartic acid	6.44	5.28	2.18	0.30
Glutamic acid	20.63	17.24	6.84	0.41
Serine	3.64	3.58	1.41	0.10
Histidine	2.17	1.64	0.66	0.04
Glycine	1.62	1.27	0.53	0.15
Threonine	3.37	2.77	1.10	0.07
Arginine	2.80	0.16	0.04	0.008
Alanine	2.73	2.39	0.96	0.05
Tyrosine	4.43	3.24	0.95	0.02
Cystine-s	0.08	0.04	0.02	0.005
Valine	5.79	4.66	1.86	0.11
Methionine	2.21	1.68	0.58	0.02
Phenylalanine	4.47	3.75	1.50	0.11
Ileucine	4.80	3.78	1.51	0.11
Leucine	8.21	6.72	2.69	0.12
Lysine	6.69	1.53	0.52	0.02
Proline	11.29	9.91	3.55	0.13
Total percentage (%)	91.37	69.64	26.9	1.77

MW > 50 kDa MRPs: the MRPs of molecular weight higher than 50,000 Da; unfractioned MRPs: MRPs without ultrafiltration; casein: native casein; MW 5–10 kDa MRPs: MRPs of molecular weight from 5000 to 10,000 Da.
[a] MW means molecular weight.
[b] kDa means kilo Dalton.

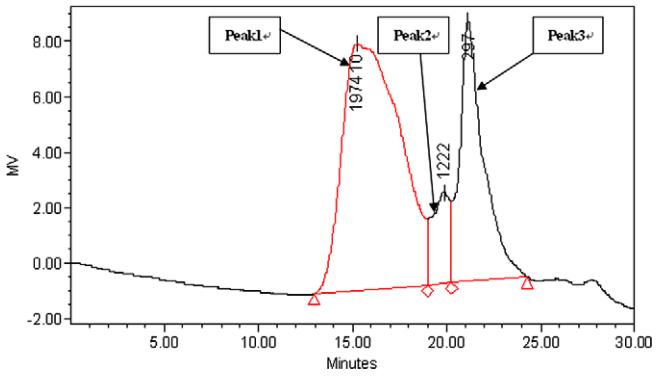

Fig. 1. Distribution of molecular weight of casein–glucose MRPs by high performance gel-filtration chromatography.

MW 30–50 kDa. The mixture of the heated casein–glucose was a wide range of compounds, consisting of glucose degradation products, protein, protein hydrolysates, caramelisation products, glycated protein, glycated peptides, etc. Each kind of product might exhibit distinctly different reducing power. The high molecular weight (MW higher than 50 kDa) MRPs exhibited the highest reducing power. Hydroxyl groups of advanced MRPs may play a role in reducing activity. Additionally, the intermediate compounds of MRPs were reported to break the radical chain by donation of a hydrogen atom (Eichner, 1981). These results are also in agreement with the previous reports of Delgado-Andrade, Rufian-Henares, and Morales (2005) and Yamaguchi et al. (1981), who described that a high molecular weight of melanoidin prepared from xylose and glycine achieved a higher browning and reducing power.

3.7. DPPH radical-scavenging activity

DPPH radical-scavenging activity increased as the concentration of MRPs increased. This activity increased drastically ($p < 0.05$) at the low concentration of MRPs, while nearly reached a plateau after that concentration (Table 4). DPPH radical was scavenged by MRPs by donation of hydrogen to form a stable DPPH-H molecule. The color change from purple to yellow by acceptance of hydrogen radical from MRPs and it become a stable diamagnetic molecule (Benjakul et al., 2005).

Either intermediates or the final brown polymer can function as hydrogen donors, sugar caramelisation can also contribute to the antiradical activity measured by DPPH test (Benjakul et al., 2005) As can be seen from Table 4, the DPPH radical-scavenging activity of MRPs was less stronger than that of BHT. Based on the reducing power and radical-scavenging activity of MRPs from the casein–glucose, however, it can be used as antioxidant for food products.

As Table 5 indicates, the radical-scavenging activity of low MW MRPs was greater than that of high MW MRPs. This phenomenon was not consistent with the results of reducing power. This may be due to the complexity of MRPs, which quench hydrophilic radicals more efficiently than hydrophobic (Jing & Kitts, 2002). The higher ability to quench hydrophilic radicals is due to the higher water solubility of the melanoproteins. More investigation needs to be conducted to elucidate this phenomenon.

3.8. Chelating activity on Fe^{2+}

Ferrous ion chelating activity of MRPs from casein–glucose is shown in Table 4. The chelating activity increased as the concentration of MRPs increased. Chelating activity increased from 31.24% to 93.87% as the MRPs concentration increased from 0.50 to 2.0 mg/ml. MRPs have been found to be effective as metal chelating compounds (Wijewickreme et al., 1997)

High MW fractions exhibited higher metal-chelating power, whereas low MW fractions did not show this kind of activity (Table 5). The high molecular weight MRP was reported to possess greater metal chelating potential than low molecular weight MRPs. The chelating activity can possibly be attributed to hydroxyl groups originating from MRPs (Yoshimura et al., 1997).

3.9. Inhibition of lipid peroxidation

As far as lipid peroxidation is concerned, free radicals abstract hydrogen from a fatty acid double bond to produce fatty acid free radicals, which further react with oxygen to produce fatty acid hydroperoxide. The hydroperoxide is unstable and decomposes readily to shorter chain hydrocarbons such as aldehydes, etc. The intermediate products can be determined as thiobarbituric acid reactive substances (TBARS) assay. Table 4 shows the inhibition

Table 4
Reducing power, radical-scavenging activity, chelating activity and lipid peroxidation inhibition percentage of MRPs or BHT (mean ± SD).

Concentration of MRPs[a] or BHT[b] (mg/ml)	Reducing power[d]	Radical-scavenging (%)	Chelating activity of MRPs (%)	Inhibition percentage of MRPs (%)
0.5 (0.1)	$0.572^{a} \pm 0.033$ ($0.553^{a} \pm 0.041$)	$28.52^{a} \pm 2.37$ ($59.34^{a} \pm 3.76$)	$31.24^{a} \pm 2.16$	$9.10^{a} \pm 1.12$
1.0 (0.2)	$0.609^{a,b} \pm 0.056$ ($0.807^{b} \pm 0.025$)	$33.33^{a,b} \pm 2.76$ ($63.08^{a,b} \pm 4.31$)	$62.26^{b} \pm 1.73$	$20.96^{b} \pm 1.31$
2.0 (0.3)	$0.668^{b,c} \pm 0.037$ ($1.038^{c} \pm 0.072$)	$34.78^{b} \pm 1.74$ ($70.25^{b} \pm 5.02$)	$93.87^{c} \pm 3.27$	$31.73^{c} \pm 2.09$
4.0 (0.4)	$0.720^{c} \pm 0.043$ ($1.342^{d} \pm 0.042$)	$36.41^{b} \pm 3.12$ ($78.48^{c} \pm 4.18$)	—[c]	$38.32^{d} \pm 2.15$
8.0 (0.5)	$0.758^{c} \pm 0.038$ ($1.418^{d} \pm 0.059$)	$38.52^{b} \pm 3.26$ ($80.47^{c} \pm 3.17$)	—	$41.21^{d} \pm 2.83$

[a–d]Different letter superscripts denote significant difference ($p < 0.05$). SD: standard deviation from triplicate determinations. The data in parentheses are antioxidation results of BHT.
[a] MRPs: Maillard reaction products were prepared as the Section 2.2 report, the heating time was 130 min.
[b] BHT means butylhydroxytoluene and the concentration is indicated in the parenthesis.
[c] — : not determined.
[d] Absorbance of the reaction mixture was measured at 700 nm.

Table 5
Reducing power, radical-scavenging activity, chelating activity and lipid peroxidation inhibition percentage of different MRPs fractions of different molecular weights (mean ± SD).

Samples	Reducing power[c]	Radical-scavenging activity (%)	Chelating activity (%)	Inhibition percentage (%)
MW[a] < 1 kDa[b]	$0.4845^{a} \pm 0.019$	$40.39^{c,d} \pm 1.68$	0	$33.82^{c} \pm 1.03$
MW 1–5 kDa	$0.5235^{b} \pm 0.024$	$42.96^{d} \pm 3.10$	0	$57.11^{d} \pm 2.45$
MW 5–10 kDa	$0.501^{a} \pm 0.004$	$22.86^{b} \pm 0.28$	0	$61.72^{e} \pm 3.51$
MW 10–30 kDa	$0.5395^{c} \pm 0.018$	$36.06^{c} \pm 4.29$	0	$57.83^{d} \pm 1.12$
MW 30–50 kDa	$0.471^{a} \pm 0.027$	$19.81^{b} \pm 1.19$	$59.43^{a} \pm 1.61$	$62.24^{e} \pm 3.6$
MW > 50 kDa	$0.5685^{c} \pm 0.015$	$5.52^{a} \pm 0.32$	$88.27^{b} \pm 2.20$	$6.87^{a} \pm 0.88$
Unfractionated MRPs	$0.520^{b} \pm 0.021$	$24.04^{b} \pm 3.24$	$85.42^{b} \pm 1.93$	$24.75^{b} \pm 4.21$

[a–e]Different letter superscripts denote significant difference ($p < 0.05$).
[a] MW means molecular weight.
[b] kDa means kilo Dalton; unfractioned MRPs: MRPs without ultrafiltration; SD: standard deviation from triplicate determinations.
[c] Absorbance of the reaction mixture was measured at 700 nm.

percentage of MRPs to lecithin oxidation, inhibition percentage increased evidently with increased concentration of MRPs.

As can be seen from Table 5, MW 30–50 and 5–10 kDa fractions of MRPs had greater lecithin antioxidant inhibition percentage than others, and close to the 1–5 and 10–30 kDa fractions. The results are partially not consistent with the other three antioxidant tests. In the presence of MRPs, the propagation step might be inhibited and lead to lower oxidation. Owing to chelating property of the ferrous ions, MRPs might retard the lipid oxidation by eliminating their pro-oxidant activity. It was likely that higher concentration of MRPs prevented oxidation in lecithin system via greater iron chelating potential (Benjakul et al., 2005). In general, MRPs of casein–glucose are very complicated, and the mechanism of lipid oxidation assay is very complex to elucidate.

4. Conclusion

Casein–glucose model system was chosen to prepare MRPs at high pH. The decrease in free amino groups and pH were consistent with the increase in browning and absorbance at 294 nm of the MR system. Amino acid analysis indicated that casein and glucose both degraded partially, all the compounds of the mixture took part in a complicated reaction. High molecular weight MRPs are the dominant compounds in the mixture of heated casein–glucose by HPGFC analysis. MRPs showed increased antioxidant activity with increasing concentration, different fractions of ultrafiltrated MRPs exhibited different antioxidant activity; this may be due to the complexity of MRPs, which could act simultaneously with different mechanisms of action. In this respect, research on the mechanisms of formation and the identification of the structure for the active compounds in Maillard reaction are also needed in the future study.

Acknowledgements

This study was funded by the National Key Technology R&D Program of China (2006BAD27B04) and Key Technology R&D Program of Jiangsu (BE2008374).

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Food Chemistry 117 (2009) 55–63

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Impact of edible coatings and packaging on quality of white asparagus (*Asparagus officinalis*, L.) during cold storage

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ARTICLE INFO

Article history:
Received 18 December 2008
Received in revised form 14 February 2009
Accepted 19 March 2009

Keywords:
White asparagus spears quality
Edible coatings
Texture
Anthocyanins
Lignification

ABSTRACT

The effects of edible coatings and plastic packaging on quality aspects of refrigerated white asparagus spears were studied using two different experimental protocols. The first included four coating formulations based on carboxymethyl-cellulose and sucrose fatty acid esters, whey protein isolate alone and in combination with stearic acid, and pullulan and sucrose fatty acid esters, and an uncoated sample serving as a control. The second set consisted of four treatments; uncoated asparagus spears (control), coated with a carboxymethyl-cellulose formulation, packaged in plastic packaging and combination of coated and packaged asparagus spears. All products were stored at 4 °C and the quality parameters such as weight loss, texture, visual appearance, lignin and anthocyanins concentration, and colour were evaluated during their storage. Edible coatings exhibited a beneficial impact on the quality of asparagus by retarding moisture loss, reducing hardening in their basal part and slowing down the purple colour development. The plastic packaging had a remarkable influence in reducing weight loss and retarding hardening but its impact to the rest of the quality parameters was similar to that of the edible coatings. The combination of packaging and edible coating did not seem to offer any additional advantage on asparagus spears apart from the fact that the product had a brighter appearance at the middle part of the stem compared to the packaged spears alone.

1. Introduction

Asparagus (*Asparagus officinalis*, L.) has very short shelf life due to its high respiration rate, which continues after harvesting. White asparagus undergoes a sequence of considerable physiological and biochemical changes which quickly influence its chemical composition and lead to quality loss, mainly due to an increase of hardness, water loss and the synthesis of anthocyanins (Chang, 1987). Therefore, ensuring an extended shelf-life by adequate post-harvest conservation appears to be very challenging for commercialization of this highly valued vegetable (Villanueva, Tenorio, Sagardoy, Redondo, & Saco, 2005). Postharvest treatments to increase storage and shelf life included storage in modified atmosphere packaging (MAP) (Siomos, Sfakiotakis, & Dogras, 2000) and prestorage hot water treatments (Siomos, Gerasopoulos, & Tsouvaltzis, 2005).

The role of packaging on vegetable conservation, distribution and marketing is also quite popular and is frequently used in combination with other conservation methods, an approach called hurdle technology (Hoover, 1997). However, the final disposal of the packaging materials leads to ecological problems and additional recycling costs (Viña et al., 2007). On the other hand, the application of edible coatings appears to be one of the most innovative approaches to extend the commercial shelf life of fruits and vegetables by, among other mechanisms, acting as a barrier against gas transport and showing similar effects to storage under controlled atmospheres (Park, 1999). Indeed, over the last two decades the development and use of bio-based packaging materials to prolong the shelf-life and improve the quality of fresh products has been receiving increased attention. The reasons for such an interest are mainly related to environmental issues due to disposal of conventional synthetic food-packaging materials. However, in order such edible films and coatings to be used at a commercial level in food products they must fulfill some basic requirements: acceptable sensorial characteristics, appropriate barrier properties, good mechanical strength, reasonable microbial, biochemical and physicochemical stability, safety, low cost and simple technology for their production (Diab, Biliaderis, Gerasopoulos, & Sfakiotakis, 2001). The effectiveness of edible coatings for protection of fruits and vegetables also depends on controlling the wettability of the coating solutions, which affects the coating thickness (Park, 1999). Thus, edible coating formulations must wet and spread uniformly on the vegetable's surface and, after drying, a coating that has adequate adhesion, cohesion and durability to function properly must be formed (Ribeiro, Vicente, Teixeira, & Miranda, 2007).

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doi:10.1016/j.foodchem.2009.03.076

Edible films and coatings are generally based on biological materials such as proteins, lipids and polysaccharides. The main polysaccharides that can be included in edible coating formulations are starch and starch derivatives, cellulose derivatives, chitosan, pectin, alginate and other gums. Carboxymethyl-cellulose is a cellulose derivative that has received considerable attention with several examples of applications in many fruits and vegetables. A commercial edible coating formulation based on carboxymethyl-cellulose and sucrose fatty acid esters, named Semperfresh™, has been applied to pears (Zhou et al., 2008), cherries (Yaman & Bayoindirli, 2002) and many other fruits. Pullulan, an extracellular polysaccharide produced by *Aureobasidium pullullans*, also is capable of forming edible films but has not been largely exploited as a coating material in fruits and vegetables, presumably because of its high water solubility. One example of pullulan used as a coating hydrocolloid was for strawberries and kiwifruit (Diab et al., 2001). Proteins that can also be used in formulations of edible coatings for fruits and vegetables include those derived from animal sources, such as casein and whey proteins, or obtained from plant sources, like corn zein, wheat gluten and soy protein (Vargas, Pastor, Chiralt, McClements, & Gonzalez-Martinez, 2008). Whey protein based coatings have been extensively used to extend the shelf life of fruits and vegetables (Cisneros-Zevallos & Krochta, 2003; Lerdthanangkul & Krochta, 1996).

To the best of our knowledge there are no available data regarding the effect of edible coatings on postharvest quality aspects of white asparagus spears. Therefore, the aims of the present work were to evaluate the effect of different edible coating formulations on quality parameters of white asparagus spears during refrigerated storage, to compare the impact of one of these edible coatings with that of a plastic packaging on the extension of asparagus postharvest life, and to explore if there is any additional beneficial effect of a combined treatment using edible coating and packaging with the synthetic film.

2. Materials and methods

2.1. Plant material

White asparagus (*Asparagus officinalis*, L.) spears were harvested from commercial farms in the regions of Imathia and Pella, Greece. The spears were hydrocooled and transported to the laboratory within 3 h after harvest, under refrigerated conditions. Straight, undamaged samples, around 18–22 mm in diameter, with closed bracts were carefully selected and cut at 18 cm from the tip.

2.2. Coating solutions preparation and application

Sodium carboxymethyl-cellulose (CMC 2500F Tic Gums, USA), whey protein isolate (WPI) (Bi-Pro®, Davisco Foods International, USA) and pullulan (Hyashibara Biochem Laboratory Inc., Okayama, Japan) were used as biopolymer matrices in the coating formulations. Other substances used were sucrose fatty acid ester F-50 with an HLB value of six (Dai Ichi Kogyo Seyaku Co., Ltd., Tokyo, Japan), polyethylenoglycol PEG 400 (Merck, Darmstadt, Germany), sorbitol and stearic acid (Sigma–Aldrich GmbH, Steinheim, Germany). Ethyl alcohol was reagent grade and water used in all experiments was distilled.

The carboxymethyl-cellulose based coating (CMC) was prepared by firstly dissolving the CMC (0.2% w/w) in a water–ethanol mixture (4:1 v/v) under magnetic stirring at 60 °C and then adding the plasticizer PEG (0.1% w/w) and the sucrose fatty acid ester F-50 (0.8% w/w), followed by stirring for 1 h. Two different coatings using the whey protein isolate were prepared; WPI 1 and WPI 2, which included 4% w/w WPI and 1% w/w sorbitol as a plasticizer, both dissolved in distilled water. The solutions were denatured for 30 min in a 90 °C water bath under continuous shaking and then were rapidly cooled in an ice bath, in order to stop further protein denaturation, and finally equilibrated to room temperature. WPI 2 also included 1% w/w stearic acid as a lipid constituent, which was incorporated to the coating solution just after the denaturation step by homogenization at 19,000 rpm for 4 min using an UltraTurrax T25 homogenizer (IKA Labortechnik, Staufen, Germany). The pullulan based coating solution (P) was obtained by dissolving pullulan (5% w/w) in distilled water under magnetic stirring at 60 °C with subsequent addition of sorbitol (1% w/w) and a sucrose fatty acid ester F-50 (1% w/w), followed by stirring for 1 h. All the coating solutions were left overnight at 4 °C in order to eliminate air bubbles. The composition of the coatings used in this work was selected among a large number of other coatings examined in preliminary trials.

Coating was carried out at room temperature by dipping the asparagus spears for 30 s in the formulated suspensions and then slow drying at ambient conditions, by turning them from time to time, for about 2 h. After the coating treatment the samples were placed in plastic trays and subjected to cold storage.

2.3. Coating characterization

The surface tension of each suspension was measured by the Ring Method using a Kruss tensiometer at 20 °C. Rheological characterization of the coating formulations was conducted by a rotational Physica MCR 300 rheometer (Physica Massterchnic GmbH, Stuttgart, Germany) using a double gap cylindrical geometry; temperature was regulated by a Paar Physica circulating bath and a controlled peltier system (TEZ 150P/MCR) with an accuracy of ±1 °C. The data of the rheological measurements were analyzed with the supporting software US200 V2.21. Flow curves were performed by measuring steady shear viscosity (η) over a range of shear rates between 0.1 and 1200 s^{-1} at 25 °C. The contact angles at the asparagus surface were measured following the Choi and Han procedure (Choi & Han, 2002), using a digital microscope (Intel QX3, Mattel Inc., El Segundo, CA). All the liquid drops used for the measurements were axecimetric.

The estimation of the critical surface tension (γ_C) of the white asparagus surface was obtained by extrapolation from the Zisman plot (Zisman, 1964), which was developed using water (HPLC grade), glycerol, ethylene glycol and dimethyl sulfoxide (DMSO) (Sigma–Aldrich GmbH, Steiheim, Germany), as reference liquids. Their surface tensions are 72.8, 63.4, 48.0 and 44.0 mN/m^2, respectively. To avoid changes on the asparagus surface, the measurements took place in less than 30 s. Ten replicates of contact angle measurements were obtained at 20 (±1) °C.

2.4. Sample treatments

Two different experimental sets were adopted. The first (set A) included application of four different coatings, CMC, WPI 1, WPI 2 and P on white asparagus spears and uncoated spears served as control (C). The samples were placed in a cold room at 4 °C and 95% RH for 11 days and the quality parameters were evaluated on days 6 and 11 after harvest. In the second experimental set (set B), four different treatments were employed: (a) uncoated samples (control – C), (b) CMC coated spears, (c) packaged asparagus in plastic trays wrapped with a 16 μm stretch film (Fabri Arti Grafiche S.R.L.-Vignola, Modena, Italy). The synthetic film had O_2 and CO_2 transmission rates of 583 and 1750 $ml\ m^{-2}\ h^{-1}\ atm^{-1}$, respectively, and a moisture vapor transmission rate of 14.6 $g\ m^{-2}\ h^{-1}\ atm^{-1}$ at 39 °C and 90% RH (film permeabilities were measured by the manufacturer) (Pack). The fourth treatment (d) involved a combination of CMC coating and the above packaging (Pack + CMC), in order to investigate if there is any additional

effect when combining edible coating and synthetic film packaging. All trays were placed in cold storage at 4 °C and 95% RH for 17 days. The determination of asparagus quality was held at the day of harvest (day 0) and at days 11 and 17. Each sample, containing five lots (6–7 spears/lot), was included for each experimental set, storage condition and sampling time.

2.5. Weight loss

Weight loss was measured periodically by weighting five trays containing 6–7 spears, for each treatment. The results were expressed as the percentage loss of initial weight.

2.6. Texture determination

The asparagus spears, 18 cm in length, were marked at 6-cm intervals from the tip and then sectioned at the ink markings into three cylindrical portions; apical, middle and basal, since it is known that there is evidence for significant differences in texture over the different parts of white asparagus (Rodríguez et al., 2004). The texture was measured at the middle of each of the three sections of each spear by applying the cutting test using a Texture Analyzer (TA-XT2i, Stable Microsystems). Values were expressed as maximum force (kg), using a Warner–Blatzler cell with a blade (0.3 cm width) that cut the spears at a speed of 5 mm/s (average of 20 replicates).

2.7. Visual evaluation of appearance

The general appearance of the products was assessed by at least 10 trained panelists. Samples were evaluated using the following hedonic scale: 1 = bad, 2 = fairly good, 3 = good, 4 = very good and 5 = excellent. A value of three was considered as the commercial acceptability threshold.

2.8. Colour measurements

Colour readings of the spears were performed with a chromatometer (Minolta CR-400/410, Minolta, Osaka, Japan), equipped with an 8-mm measuring head. The meter was calibrated using the manufacturer's standard white plate. Colour changes were quantified in the L^*, a^*, b^* colour space. Chroma value ($\text{Chroma} = \sqrt{a^{*2} + b^{*2}}$) was also calculated in order to compare changes among treatments. On each spear two readings in two different areas were taken; the first at 2 cm from the tip, in order to study if there is a violet colour development, and the second at 10 cm, so as to explore if there is any 'greying' appearance on the spear surface.

The colour was also assessed visually by a 10-member trained panel using the following hedonic scale: 5 = total white, 4 = violet colour development at 2 cm from the tip, 3 = violet colour at 3 cm, 2 = violet colour at 4 cm, 1 = violet colour at 5 cm and 0 = violet colour development over longer distance.

2.9. Lignin determination

The lignin content of the different parts of the asparagus spears (apical, middle, and basal) was determined with the thioacidoglycolysis method, as described by Bruce and West (1989). About 150 g of tissue was homogenised with 95% ethanol for 5 min. The mixture was vacuum filtered, the residue was washed with 100 ml of ethanol and then dried at 50 °C for 24 h. About 0.25 g of the above dry residue was mixed with 7.5 ml of 2 N HCl and 0.5 ml of thioglycolic acid which was then boiled with occasional shaking for 4 h and centrifuged at 7500*g* for 15 min. The residue (lignin thioglycolate) was washed with 10 ml of water, suspended again in 10 ml of 0.5 N NaOH with occasional shaking for 18 h at room temperature and centrifuged; 2 ml of concentrated HCl was added to the supernatant liquid. The lignin thioglycolic acid complex was precipitated at 4 °C for 4 h, centrifuged (7500*g*, 15 min) and the residue was dissolved in 10 ml of 0.5 N NaOH. After the appropriate dilutions, the absorbance was read at 280 nm, using a Metertech UV/vis SP8001 (Taipei, Taiwan) spectrophotometer. Quantification was carried out using a standard lignin curve. The lignin standard was purchased from Sigma–Aldrich GmbH (Steinheim, Germany).

2.10. Anthocyanin determination

The anthocyanin content was determined on the peel of asparagus, which was obtained by a sharp razor from 1 cm from the tip until 6 cm from the tip; this region is where most of the colour development occurs. The method of Fuleki and Francis (1968) was employed for the determination of anthocyanins with a few changes which were proposed by Flores, Oosterhaven, Martínez-Madrid, and Romojaro (2005). The plant tissue was chopped in a mortar, and 2 g were extracted with 5 ml of extractant solution; the latter was 100% ethanol/0.5 N HCl (85:15 v/v), and was kept in agitation with the tissue for 1 min. The resulting homogenate was incubated for 4 h in darkness. Every step was carried out in an ice bath in order to keep the temperature at 4 °C. The homogenate was then centrifuged at 7500*g* for 15 min and the supernatant was used to perform the spectrophotometric measurement at 533 nm, using the extraction medium as blank. The extinction coefficient of anthocyanins, 984 $(\text{g}/100\ \text{ml})^{-1}\ \text{cm}^{-1}$, was used for calculation of the anthocyanin content in the peel of white asparagus (Fuleki & Francis, 1968). The results were expressed as mg anthocyanins per g FW of peel.

2.11. Statistical analysis

All results are means ± s.e., and the data were statistically evaluated by ANOVA with mean differentiation by the Duncan's multiple range test ($\alpha = 0.05$). The statistical software used was the SPSS, version 15.0.

3. Results and discussion

3.1. Coating characterization

For coating solutions, Cisneros-Zevallos and Krochta (2003) have found that the average liquid film thickness on coated apples is a function of viscosity, draining time, density of the biopolymer solutions, surface tension of the fruit, surface tension of the liquid, and the surface roughness. Since the film thickness can greatly affect the gas and water barrier properties of the coatings, it was considered important to study the asparagus surface properties and the surface tension and rheological properties of the coating solutions.

The critical surface tension of the white asparagus peel, derived from the Zisman plot, was 26.7 mN/m. This indicates that the asparagus peel is a solid surface with low surface energy. Several authors have reported the values of critical surface tension obtained from a Zisman plot for various fruits and vegetables like garlic peel with a value of 18.3 mN/m (Hershko & Nussinovitch, 1998), strawberry with a value of 18.8 mN/m (Ribeiro et al., 2007), and tomato and carrot with values of 17.4 and 24.1 mN/m, respectively (Casariego et al., 2008). The peel or surface of many fruits and vegetables has low surface tension for protection purposes; however, this natural advantage is a shortcoming for application of aqueous coatings on plant tissues (Viña et al., 2007).

Table 1
Characterization of the coating formulations.

Coating formulation	Surface tension (mN/m)	Apparent viscosity at 100 s^{-1}, η_{ap} (mPa s)
CMC	37.1 ± 1.0	52.9
WPI 1	50.0 ± 1.0	2.8
WPI 2	49.2 ± 1.0	3.5
P	38.3 ± 0.6	19.6

The surface tension of a coating suspension is an essential factor for determining coating success. Table 1 shows the surface tensions of the four different coatings applied on white asparagus spears. The coatings CMC and P had a surface tension of 37.0 and 38.3 mN/m, respectively. These values were significantly lower than those of coatings based on whey protein isolate; the WPI 1 and WPI 2 had values of 50.0 and 49.0 mN/m, respectively, typical for protein solutions. The first two coatings, CMC and P, had a low surface tension due to the sucrose fatty acid ester which was included in their formulation as a surface active ingredient. Another reason for the low surface tension of the CMC coating may be the fact that the diluent was a water–ethanol mixture 4:1. These results show that the CMC and P coatings were more prone to spreading on white asparagus peel since their surface tension values were closer to the critical surface tension of the asparagus peel obtained from the Zisman's plot, compared to the respective values of WPI 1 and WPI 2.

The flow properties of a film forming liquid greatly affect its coating quality in the solid state. Moreover, the smoothness of the surface to be coated has a strong influence on the coating appearance. Levelling of the coating surface takes place after application of the liquid and during drying, due to solvent evaporation and is a crucial part of the coating process. The levelling of irregularities in liquid coating depends on the rheological properties and surface tension of the liquid, the effects of gravity and the characteristics of the surface to coat. A positive characteristic of coatings in the liquid state is the presence of a yield stress or a viscosity (η_0) high enough to prevent gravity effects (sagging and dripping), but sufficiently small to allow capillarity-driven levelling (Peressini, Bravin, Lapasin, Rizzotti, & Sensidoni, 2003).

In Table 1, the apparent viscosities at 100 s^{-1} of the four different coatings applied on white asparagus are given. The CMC coating exhibited a pseudoplastic behavior (flow curves not shown) as the viscosity decreased with the increase of shear rate and it showed the highest apparent viscosity at 100 s^{-1} (52.9 mPa s), compared to the rest of the coating solutions. The pullulan-based (P) coating showed Newtonian behavior with an apparent viscosity of 19.6 mPa s. The whey protein isolate coating solutions, WPI 1 and WPI 2, also showed Newtonian-like behavior and their apparent viscosities were lower than the others, with values of 2.8 and 3.5 mPa s, respectively. The addition of stearic acid in the whey protein solution did not seem to alter the apparent viscosity to a great extent. In a previous study, starch-based coatings exhibited a pseudoplastic behavior and the most viscous formulation (medium amylose content starch-based with 20 g l^{-1} of glycerol) showed an apparent viscosity of 22.6 mPa s at 512 s^{-1} (Garcìa, Martino, & Zaritzky, 1998).

3.2. Weight loss

This quality parameter is quite crucial, since every loss in weight is translated into an economic loss. Additionally, the weight loss has a strong impact on the spear appearance, due to shrinkage, and an 8% weight loss makes asparagus spears unsaleable (Siomos, 2003). Fig. 1 shows the weight loss of the asparagus spears for both experimental sets A and B. The weight loss increased progressively

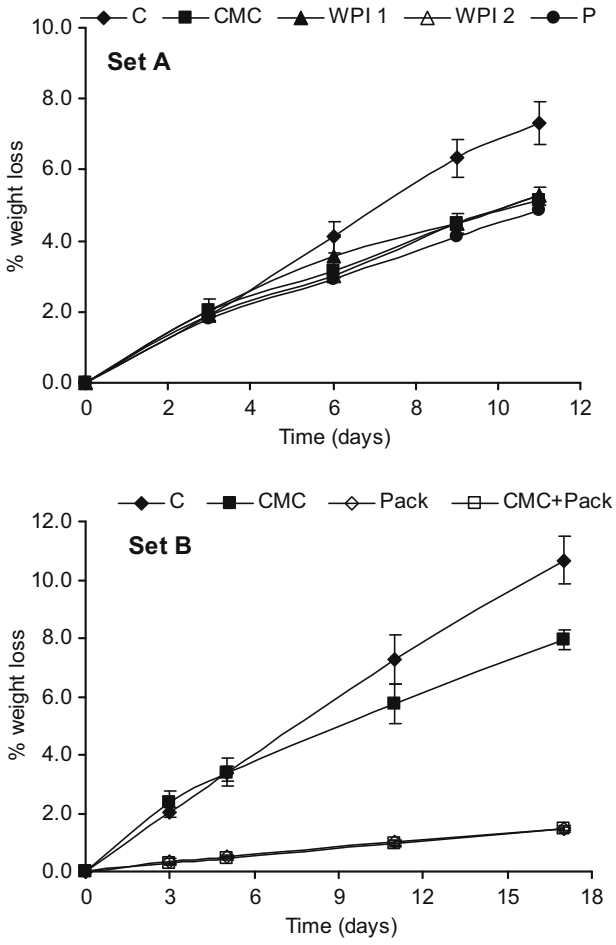

Fig. 1. Effect of various edible coating formulations and packaging on weight loss (%) of white asparagus spears during storage at 4 °C for 11 days (set A) and for 17 days (set B); the vertical bars represent the standard deviation ($n = 5$).

upon storage, and is mainly attributed to the water loss by transpiration due to differences in the water vapour pressure of water between the atmosphere and the asparagus surface (Park, Chinnan, & Shewfelt, 1994). After 11 days of storage (set A), the weight losses of the control and the coated samples were 7.3% and ~5.0%, respectively. The edible coatings applied on white asparagus spears significantly reduced the weight loss, probably due to the hydrophobic ingredients they contained, such as the sucrose fatty acid ester, and stearic acid, which may had decreased the water vapour permeability of the surface. However, the WPI 1 coating, without including any of these substances, also acted as a water vapour barrier, presumably due to denaturation of its protein components. Among all the coatings employed, there were no major differences, in spite of the variation in the solids content, biopolymer type and other substances used.

Several studies had dealt with the effect of various coatings based on polysaccharides in controlling the weight loss of several fruits and vegetables. Many of them referred to the application of commercial formulations that contained carboxymethyl cellulose and sucrose fatty acid esters, e.g., Semperfresh™ and TAL Pro-long, exerting a better weight loss control of many products such as cherries (Yaman & Bayoindirli, 2002). Pullulan coatings applied on strawberries and kiwifruit also exhibited a positive impact by extending their shelf life (Diab et al., 2001). Furthermore, the properties of protein based films have been extensively studied in applications of fruits and vegetables. More specifically, whey protein edible films attracted much of attention as it was found that

they can afford moderate potential as moisture barriers for food systems. The reduction in water vapour permeability of WPI based films could be further enhanced through the addition of lipids (McHugh & Krochta, 1994). Placing the asparagus spears in plastic packaging significantly reduced their weight loss, as shown in Fig. 1 for set B, whereas the combination of coating with packaging offered no additional protection to weight loss, compared to the packaging alone. The prevention of weight loss due to the maintenance of a high relative humidity environment is a major advantage of vegetable packaging; the beneficial effect of this approach in asparagus spears, as evidenced by the present findings is in agreement with the findings of Siomos et al. (2000). In another work, the effects of starch-based coatings and combination treatments of plastic film packaging with the coatings on weight loss of Brussels sprouts were studied (Viña et al., 2007); the coatings did not seem to reduce the weight loss during storage, whereas the plastic packaging substantially reduced the weight loss below the maximum admissible level in coated or uncoated sprouts.

3.3. Visual evaluation of appearance

After six days of refrigerated storage (set A) the appearance of the coated samples was rated as "excellent" or "very good", in contrast to the uncoated control C which fell into the "good" category. The uncoated asparagus spears exhibited a more dehydrated surface than the coated spears. The differences among the four different coating treatments were significant, with the WPI 1 and P giving the highest rating values in the appearance, probably because of the glossiness that these coating formulations imparted to the surface of the plant tissue. Samples coated with WPI 2 had the lowest score, possibly due to the incorporation of stearic acid which can lead to an undesirable opaque appearance after drying. After 11 days of storage the uncoated samples showed a strongly dehydrated surface, especially at the basal part of the spears, thus leading to quality ratings well below the acceptability threshold. Instead, all the asparagus spears treated with the CMC, WPI 1, WPI 2 and P coatings maintained their quality above the acceptance threshold level (see Fig. 2).

With respect to experimental set B, after 11 days of refrigerated storage, the uncoated product exhibited significantly lower quality levels compared to the other treatments and far below the acceptance level. In contrast, the treatments with CMC, Pack and the combination Pack + CMC did not differ significantly in their quality ratings. At day 17, the asparagus spears that were not packaged showed significantly lower quality ratings, compared to the treatments of Pack and Pack + CMC which were ranked under the "very good" category. No differences were observed among the last two treatments (Pack, Pack + CMC), probably because the packaging in a plastic container has a stronger impact on quality preservation than the edible coating alone.

3.4. Texture

The texture of asparagus has been related to fibrousness and the process of hardening that occurs after harvesting; the latter is accompanied by the lignification of the pericyclic (schlerenchyma) fibres (Rodríguez et al., 2004). Additionally, changes in texture may also reflect losses of tissue water and increases in other phenolic compounds apart from lignin. Several methods have been developed to determine textural changes along with the fibre content and the degree of lignification. Currently, textural measurements involve shearing through the asparagus spears by using the Warner–Blatzler geometry (Rodríguez et al., 2004).

Fig. 3 represents the texture of the three distinct asparagus parts for the different treatments of set B samples, measured as maximum cutting force F_{max}. Regarding the apical part of asparagus

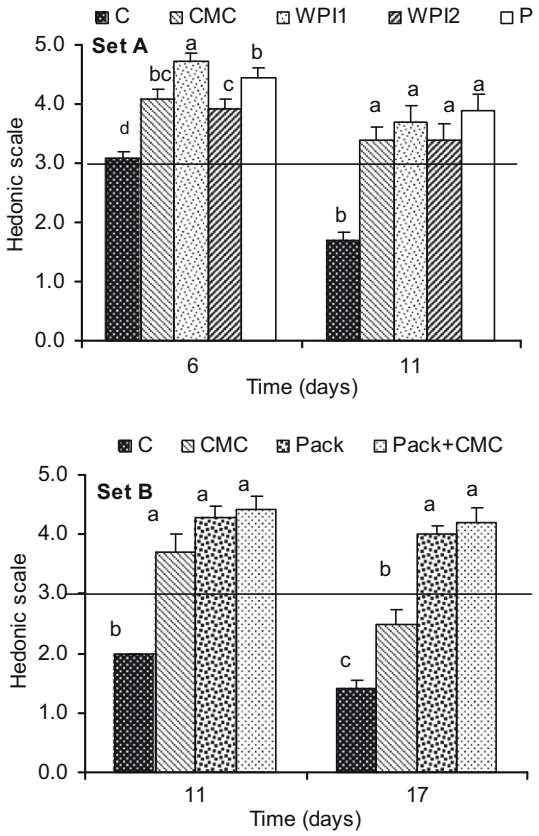

Fig. 2. Effect of different edible coatings and packaging on the visual evaluation of appearance of white asparagus spears during storage at 4 °C for 11 days (set A) and for 17 days (set B). The line corresponds to the commercial acceptability threshold (value three in the hedonic scale). Means with different superscript letters are significantly different ($p < 0.05$) for each sampling time; the vertical bars represent the s.e.

spears all the samples (C, CMC, Pack and Pack + CMC) exhibited significantly increased firmness values during storage, compared to the day of harvest (day 0). On the other hand, the firmness values in the middle part of samples Pack and Pack + CMC were maintained to similar levels with the fresh asparagus (day 0), while the uncoated (C) and the coated with CMC spears showed significantly increased values compared to the day of harvest (day 0). Similarly, the basal parts of the packaged samples (Pack and Pack + CMC) retained their texture values during storage at levels similar to the fresh asparagus. The basal part of CMC coated asparagus seemed to maintain its texture to initial firmness levels after 11 days of storage, but after 17 days the firmness value significantly increased. The CMC coating used is expected to modify the internal gas composition of white asparagus, especially by reducing oxygen and elevating carbon dioxide concentration, thus retarding the biochemical reactions that lead to hardening; the latter might explain the slower textural changes observed for the basal part of the coated spears. Additionally, the packaging helped maintain the texture of asparagus spears. In a previous work it was found that the use of Modified Atmosphere Packaging in green asparagus exhibited a beneficial effect in retarding the hardening process, especially of the basal part of the stalks (Villanueva et al., 2005). Finally, the uncoated spears exhibited significantly higher firmness value in the basal part during storage compared to the day of harvest.

3.5. Lignin content

Lignin is the cell wall component frequently associated with tissue hardening. The lignification in asparagus is controlled by

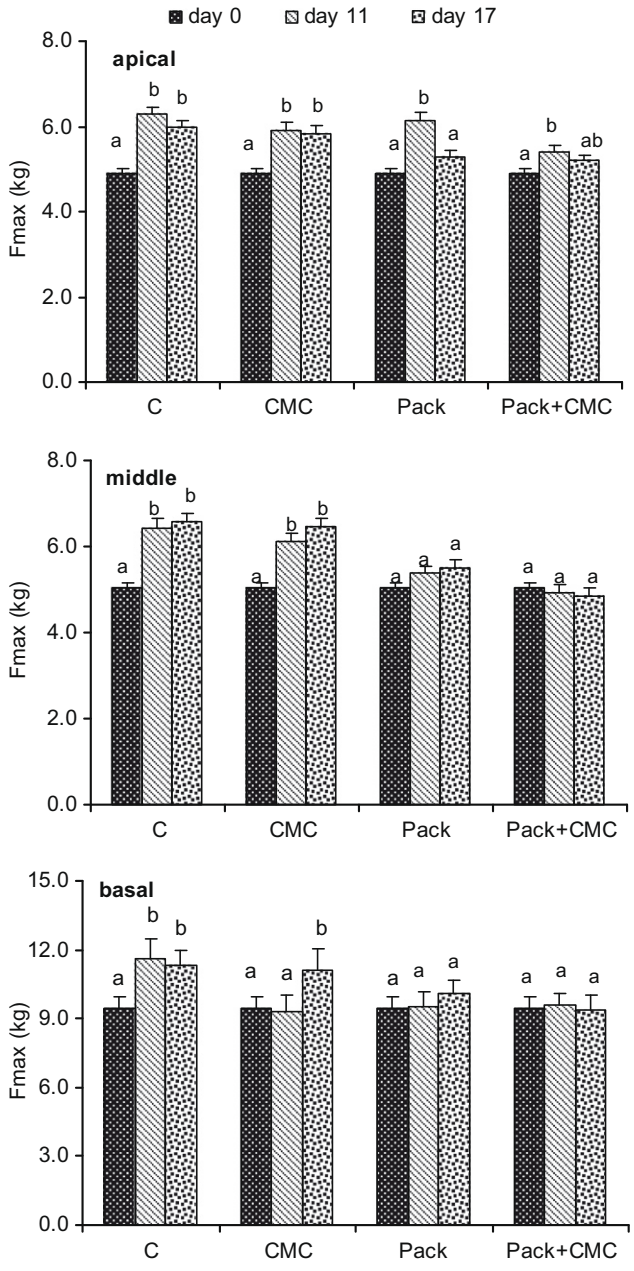

Fig. 3. Texture of asparagus expressed in maximum cutting force F_{max} (kg) for the apical, middle and basal parts during storage for 17 days (set B). Means with different superscript letters are significantly different ($p < 0.05$) for each treatment; the vertical bars represent the s.e.

different enzymes, among them phenylalanine ammonia-lyase (PAL) which is a key enzyme in the biosynthetic pathway of flavonoids such as anthocyanins and lignin; its activity is stimulated by low temperature and light (Flores et al., 2005). Therefore, the inhibition or regulation of this enzyme activity could help to maintain some of the white asparagus quality attributes.

Fig. 4 represents the lignin content of the three distinct asparagus parts for the different treatments of set B samples, expressed in mg of lignin/kg fresh weight (FW). All three parts of asparagus spears, for all the samples (C, CMC, Pack and Pack + CMC) exhibited significantly increased lignin content during storage, compared to the day of harvest, showing that none of the treatments applied was able to maintain the lignin levels close to those of the fresh product.

According to Rodrìguez, Jiménez, Guillén, Heredia, and Fernández-Bolaños (1999), lignin is concentrated mainly in the middle and basal sections of fresh white asparagus upon refrigerated storage, i.e., the lignin content of the apical section remains unchanged, while those of the middle and basal parts increase. They also suggested that the tissue toughening process is not simply related to the concentration of lignin, as has been previously proposed (Lipton, 1990), but it may be connected to the macromolecular structure of lignin and to its cross-linking with other cell wall components, either polysaccharides or proteins or both. Both monomeric and dimeric (cross-links) phenolic acids have been isolated and characterized from several plant sources, and have been suggested as important texture determinants of some fruits and vegetables (Rodrìguez et al., 1999). Indeed, Jaramillo et al. (2007) have recently attributed the process

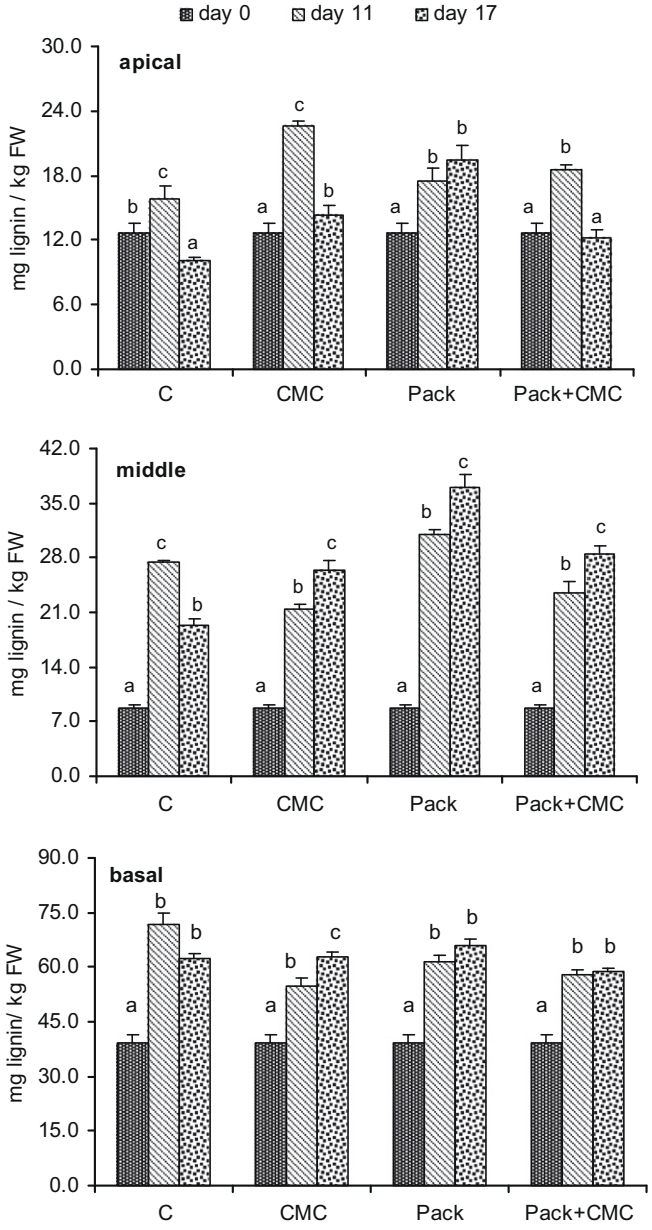

Fig. 4. Lignin concentration in asparagus spears, expressed in mg of lignin/ kg of fresh weight (FW), for the apical, middle and basal parts during storage for 17 days (set B). Means with different superscript letters are significantly different ($p < 0.05$) for each treatment; the vertical bars represent the s.e.

of toughening of white asparagus spears to the accumulation of diferulates.

3.6. Anthocyanins and colour assessment

The degree of tip colouration is a basic quality characteristic for shorting white asparagus into commercial grades (Commission of the European Communities, 1999); spears with a purple colour are judged to be of an inferior quality. The presence of anthocyanins in white asparagus spears is the reason for the purple colour developing mostly in their apical part. Evaluation of colour development is based on three different methods: determination of anthocyanins concentration in the peel of asparagus, visual assessment and colourimetric assessment.

Biosynthesis of anthocyanins in the epidermis of white asparagus spears either requires or is enhanced by light and the key enzyme regulating the accumulation of these pigments, i.e., phenylalanine ammonia-lyase (PAL) (Flores et al., 2005). Although very little is known about the regulation of anthocyanin synthesis in harvested white spears, undesirable colouration could be reduced by the use of modified atmosphere packaging (MAP) with semi-permeable plastic films (Siomos et al., 2000). These authors also found that, after an initial stimulation by light, anthocyanin synthesis occurs in harvested white asparagus spears, irrespective of temperature and light conditions during storage in air (Siomos et al., 2000).

The degree of purple colouration in the surface of asparagus was visually assessed, for both experimental sets A and B (Fig. 5). In experimental set A, the coated samples retained their white colour in higher levels compared to the control, as shown for day 11. This may be due to the fact that edible coatings can function as gas barriers, thus modifying the internal atmosphere in the asparagus spears (high levels of CO_2 and low levels of O_2) and this in turn may retard the biochemical reactions leading to anthocyanins synthesis. In experimental set B, for both days of assessment, the control C showed the lowest score, about 2.5; instead, the treatments CMC and Pack + CMC seemed to retain their original white colour for the storage period of 11 and 17 days, with scores around 4.0. Regarding the uncoated packaged samples (Pack), it was noticed that on day 11 they had developed purple colouration at the same levels as the control, but on day 17 the scores obtained from colour assessment did not differ significantly between the control and the coated treatments CMC and Pack + CMC.

Fig. 6 represents the concentration of anthocyanins in the peel of asparagus spears during storage for the different treatments of experimental set B. After 11 days of storage, the uncoated control C and the uncoated packaged samples (Pack) showed a significant increase in anthocyanins content; instead, the coated treatments CMC and Pack + CMC exhibited lower levels of anthocyanins. Similar results were obtained for day 17, where the control exhibited the highest value of anthocyanins concentration compared to the rest of the treatments. From the above findings, it is obvious that the CMC coating can slow down significantly the anthocyanin synthesis as previously discussed for experimental set A.

In Table 2, the data obtained from the colourimeter readings at 2 and 10 cm from the tip are given (i.e., values L^*, a^* and Chroma); the 2 cm colour values represent the purple colour formation in that part of the asparagus spears. The coated samples, CMC and Pack + CMC at day 17 had higher, but no significantly different L^* values compared to the control C. After 17 days of storage the uncoated control C exhibited a significantly higher ($p < 0.05$) a^* value, compared to the coated samples, CMC and Pack + CMC; a high a^* value and above zero indicates purple colour formation. The middle part (10 cm from the tip) of the packaged spears (treatment Pack) became grayish, as indicated by a decline in the L^* values (Table 2). In contrast, the control, the CMC coated and the combination Pack + CMC did not seem to darken, since their L^* values showed no significant changes over the harvest values, and were significantly higher than those of the packaged spears without the coating after 17 days of storage. These findings may indicate that the packaging can have a negative effect on the asparagus surface appearance; however, the combination treatment with edible coating CMC can minimize this impact.

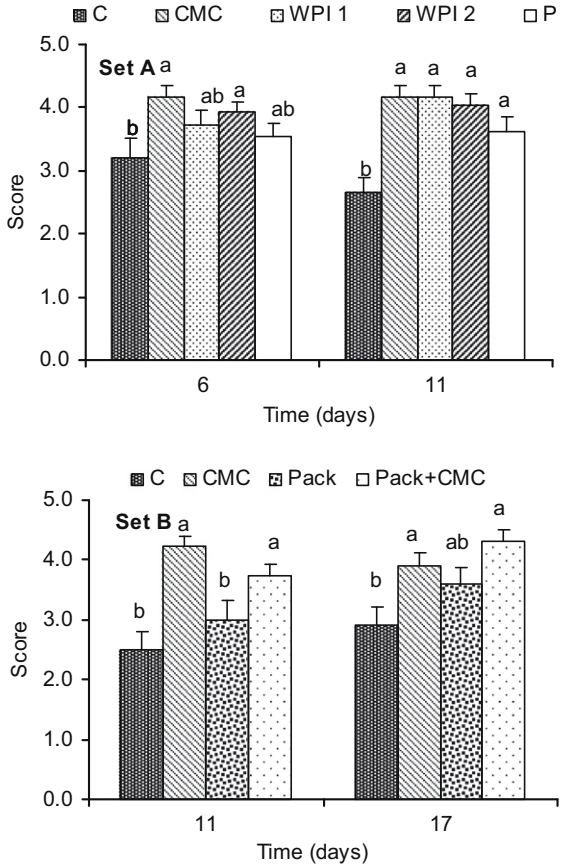

Fig. 5. Visual assessment of colour in asparagus spears for the two different experimental sets, A and B. Means with different superscript letters are significantly different ($p < 0.05$) for each sampling time; the vertical bars represent the s.e.

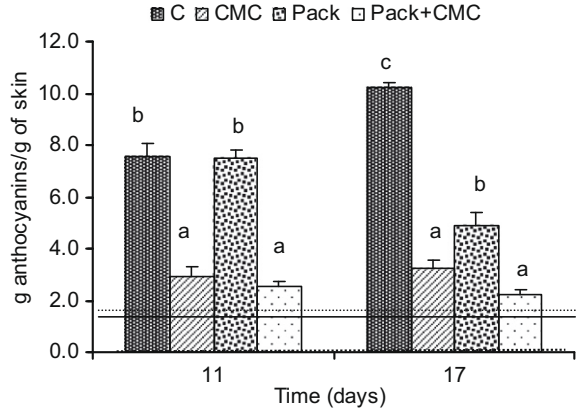

Fig. 6. Anthocyanins concentration in the peel of asparagus spears (set B). Means with different superscript letters are significantly different ($p < 0.05$) for each sampling time; the vertical bars represent the s.e. The horizontal solid line represents the anthocyanins concentration at the day of harvest (day 0) and the dotted line the +s.e.

Table 2
Colour parameters on the surface of asparagus spears (set B). Means with different superscript letters are significantly different ($p < 0.05$) for each sampling time.

Spear part	Day	Treatment	Colour parameters		
			L^*	a^*	Chroma
2 cm from the tip	0		76.19	−1.85	18.12
	11	C	72.99^{b}	2.90^{ab}	17.06^{a}
		CMC	74.26^{b}	1.05^{ab}	16.44^{a}
		Pack	69.41^{a}	3.34^{b}	17.81^{a}
		Pack + CMC	74.41^{b}	0.61^{a}	18.13^{a}
	17	C	70.79^{ab}	3.39^{c}	17.11^{a}
		CMC	74.72^{b}	0.10^{ab}	19.81^{ab}
		Pack	69.85^{a}	1.95^{bc}	17.79^{ab}
		Pack + CMC	72.34^{ab}	−0.88^{a}	19.90^{b}
10 cm from the tip	0		80.20	−1.35	8.41
	11	C	80.59^{a}	−1.51^{a}	10.94^{a}
		CMC	80.44^{a}	−1.43^{a}	10.91^{a}
		Pack	78.94^{a}	−1.23^{a}	10.58^{a}
		Pack + CMC	80.84^{a}	−1.17^{a}	10.44^{a}
	17	C	78.94^{ab}	−1.39^{a}	14.11^{c}
		CMC	80.18^{ab}	−1.46^{a}	12.00^{ab}
		Pack	78.72^{a}	−1.02^{b}	13.84^{bc}
		Pack + CMC	80.35^{b}	−1.43^{a}	11.87^{a}

4. Conclusions

The edible coatings CMC, WPI 1, WPI 2 and P seemed to have a beneficial impact on white asparagus spears quality retention during refrigerated storage by retarding their weight loss, the purple colour formation on the surface of the tips, and by maintaining the spears in high quality levels; however, no significant differences were observed among these coatings. In a second experimental set, the carboxymethyl-cellulose based coating (CMC) appeared to reduce hardening in the basal part of the stored products and anthocyanin synthesis. These results most likely reflect the ability of the coating to act as a protective gas barrier, modifying the internal atmosphere by increasing the levels in carbon dioxide and decreasing oxygen levels. Such changes affect the metabolic processes involved in lignification and anthocyanins synthesis. The plastic packaging seemed to be the most effective approach for white asparagus quality preservation since it showed the largest reductions in weight loss, remarkably retained the quality of the spears and it seemed to retard the processes of hardening. Combination treatment of CMC coating and plastic packaging did not impart any further quality improvement of the asparagus spears quality attributes, compared to the packaging alone, apart from the fact that it reduced the 'grayish' appearance of the packaged spears; the latter was attributed to the glossiness offered by the coating solution on the spear sample surface. Among all treatments examined, the combined action of carboxymethyl-cellulose based coating with plastic packaging was the most effective in preserving the quality attributes of the white asparagus spears.

Acknowledgements

The work was supported by PAVET 05 189, General Secretariat of Research and Technology, Greek Ministry of Development. The author M. Tzoumaki would also like to thank the State Scholarship Foundation (IKY) for awarding her a graduate fellowship.

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Food Chemistry 117 (2009) 64–68

Contents lists available at ScienceDirect

Food Chemistry

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In vitro starch hydrolysis and estimated glycaemic index of bread substituted with different percentage of chempedak (*Artocarpus integer*) seed flour

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ARTICLE INFO

Article history:
Received 24 November 2008
Received in revised form 23 February 2009
Accepted 19 March 2009

Keywords:
Glycaemic index
Hydrolysis index
In vitro starch hydrolysis
Resistant starch
Chempedak seed flour
Bread

ABSTRACT

Chempedak (*Artocarpus integer*) seed flour (CSF) was substituted for wheat flour at different levels (0%, 10%, 20%, 30% w/w) in bread. Assessment on the *in vitro* starch hydrolysis was carried out to evaluate the hydrolysis index (*HI*) and estimated glycaemic index (*EGI*) of bread substituted with different levels of CSF. Kinetics of *in vitro* starch hydrolysis in all bread samples (with the exception for white bread) indicated a gradual increase with respect to time intervals. Bread of 30% CSF exhibited significantly lower ($p < 0.05$) *in vitro* starch hydrolysis, as compared with the other samples. Results showed that *HI* value decreased significantly ($p < 0.05$) as the levels of CSF substitution increased. Resistant starch (RS) content in bread samples was inversely related with *HI* value as CSF substitution levels increased, thus lowering the *EGI* value.

1. Introduction

Chempedak (*Artocarpus integer* Merr.), which belongs to the Moraceae family, is found abundantly in Peninsular Thailand, Borneo and Peninsular Malaysia, particularly in Perak and Kedah. Chempedak flesh is usually eaten fresh or cooked, while the seeds are considered as waste. Jansen (1991) reported that chempedak seeds are made up of 10–25% of the fresh fruit weight, comprised mainly of carbohydrate, protein and dietary fibre.

Carbohydrates constitute a major proportion of food consumed and represent the primary energy source, contributing to nearly 55–70% of the total energy consumed (Osorio-Díaz et al., 2002). Nutritionally, carbohydrate is classified as available (starch and sugar) and unavailable carbohydrates (non-digestible food components, i.e., dietary fibre) (Ahmad, 1995).

Bread is the best source of nutritional needs among cereal and cereal products. Bread is a valuable source of vitamins, e.g., B-complex vitamins and minerals, e.g., calcium and iron (Dhingra & Jood, 2001). Since starch is the main component of bread (Hug-Iten, Handschin, Conde-Petit, & Escher 1999), the glycaemic index (*GI*) for bread is high, due to rapid carbohydrate breakdown in the digestive system which raises blood glucose (Cavallero, Empilli, Brighenti, & Stanca 2002).

GI is a way of ranking food products, particularly carbohydrate-based, on the postprandial blood glucose response, compared to a reference food (Jenkins et al., 1981). The main factor that affects *GI* is the rate or absorption of carbohydrate in the presence of food products. Regular consumption of foods with high *GI* values, particularly refined or processed carbohydrate, is associated with the development of certain chronic diseases, such as obesity, Type II diabetes, increased risk of cardiovascular diseases and certain forms of cancer (Marques et al., 2007; Schnell, de Delahaye, & Mezones, 2005).

Foster-Powell, Holt, and Brand-Miller (2002) reported that addition of fibre to food products reduced their *GI* value. Resistant starch (RS) is another functional material that has a similar effect to some dietary fibres. In the small and large intestines, RS increased the indigestible carbohydrate ingested, thus lowering the *GI* value of food products (Yamada et al., 2005). This is attributed to the starch digestion rate, which subsequently releases glucose into the bloodstream at a slower rate, resulting in reduced glycaemic and insulinemic postprandial responses for food products containing high amounts of dietary fibre (Jenkins et al., 1982; Tovar, Sáyago-Ayerdi, Peñalver, Paredes-López, & Bello-Pérez, 2003).

The addition of non-wheat flour to conventional wheat bread to improve the nutritive value in terms of dietary fibre, essential mineral and lysine are of predominant interest among food researchers. Wheat flour was substituted with barley, cowpea and jackfruit seed at different levels in bread formulation and physicochemical properties of the bread were assessed (Cavallero et al., 2002; Dhingra & Jood, 2001; Tulyathan, Tanuwong, Songjinda, & Jaiboon 2002).

The objectives of this study were to assess the utilisation of chempedak seeds as a substitute to wheat flour at different percentage levels in bread formulation and to evaluate the effect of

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0308-8146/$ - see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2009.03.077

the substitution on the *in vitro* starch hydrolysis and RS content in the bread samples.

2. Materials and methods

Chempedak seeds were obtained from a local farm in Baling, Kedah, Malaysia. All the ingredients for breadmaking were purchased from Sim Co., Penang, Malaysia.

2.1. Processing of chempedak seed flour

Chempedak seed flour (CSF) was prepared according to the modified method of Tulyathan et al. (2002). Chempedak seeds were cleaned and the seed coats were peeled off. The seeds were boiled for 30 min, chopped into small pieces, air-dried for 24 h at 60 °C (AFOS Mini Kiln; AFOS Ltd., Hessle, UK) and ground to 0.25 μm diameter (Universal Bench Top Grinder, Germany). The flour was then stored in an airtight plastic container at 5 °C prior to use.

2.2. Preparation of bread samples

Bread samples were prepared by slight modification of the Sponge and Dough Method procedure in AACC (2000). CSF was substituted with wheat flour at four different levels (0%, 10%, 20%, 30% w/w), with the addition of 100 ppm α-amylase (M500FL) (Millbo, S.p.A., Trecate, Italy) and maltodextrin (DE 15) (Behn Meyer FoodTech, Subang Jaya, Malaysia). All ingredients were mixed and baked at 165 °C for 25 min. Bread samples were allowed to cool at ambient temperature for an hour prior to analyses.

2.3. Proximate composition

Chempedak seed, CSF and bread samples were analysed for moisture (Method 44–15A), crude protein ($N \times 6.25$) (Method 46–13), crude fibre (Method 32–10), crude fat (Method 30–25) and ash (Method 08–01) content according to AACC (2000).

Carbohydrate was determined by difference:

$$[100 - (\text{moisture} + \text{crude protein} + \text{crude fibre} + \text{crude fat} + \text{ash})].$$

All analyses were carried out in triplicate.

2.4. Starch digestibility

2.4.1. Resistant starch content

Resistant starch (RS) content was determined according to the *in vitro* method of Goñi, García-Diz, Mañas, and Saura-Calixto (1996). Briefly, defatted sample (100 mg) was pre-incubated with pepsin solution (Sigma–Aldrich, St Louis, MO) (40 °C, 60 min, pH 1.5) and then α-amylase solution (Sigma–Aldrich) was added for starch hydrolysis with constant agitation (37 °C, 16 h, pH 6.9).

After amylolysis, the sample was centrifuged (3000*g*, 15 min) and the residue (isolated RS) was washed twice with distilled water. The final residue was dispersed with KOH (30 min, room temperature with constant shaking) and further incubated with 80 μl amyloglucosidase (23,000 units/g solids; Sigma–Aldrich) at 60 °C (45 min, pH 4.75). Glucose was determined in the aliquots by using glucose solution in the GOD-PAP kit (Randox Laboratories Ltd., Crumlin, UK). RS concentration of the sample was calculated as mg of glucose × 0.9. RS content was analysed in triplicate.

2.4.2. In vitro kinetics of starch digestion

In vitro kinetics of starch digestion was determined according to the method of Goñi, García-Diz, and Saura-Calixto (1997), and Goñi and Valentín-Gamazo (2003). Bread sample (50 mg) was incubated with pepsin solution at 40 °C (60 min, pH 1.5) for protein hydrolysis. The sample solution was added with Tris-maleate buffer and pH was adjusted to 6.9.

Starch hydrolysis was initiated by addition of 5 ml of Tris-maleate containing 2.6 IU porcine pancreatic α-amylase solution (Sigma–Aldrich). The sample solution was then incubated at 37 °C with moderate agitation. At 30 min intervals, over 0 to 3 h, 1 ml of sample was taken out and placed in a different tube. The α-amylase was inactivated immediately by incubating at 100 °C for 5 min with vigorous shaking. The digested starch was completely hydrolysed into glucose by adding 60 μl amyloglucosidase (23,000 units/g solids). Glucose was measured as mg of glucose × 0.9. The rate of starch digestion was expressed as the percentage of total starch (TS) hydrolysed at different times. Each sample was analysed in triplicate.

A non-linear model established by Goñi et al. (1997) was applied to describe the kinetics of starch hydrolysis. The area under the hydrolysis curve (*AUC*) was calculated using the first order equation:

$$AUC = C_\infty(t_f - t_0) - (C_\infty/k)[1 - \exp - k(t_f - t_0)].$$

C_∞ corresponds to the concentration at equilibrium (t_{180}), t_f is the final time (180 min), t_0 is the initial time (0 min) and k is the kinetic constant. The hydrolysis index (*HI*) was obtained by dividing the area under the hydrolysis curve of each sample by the corresponding area of a reference sample (white bread). The estimated glycaemic index (*EGI*) was calculated using the following equation:

$$EGI = 39.71 + (0.549HI).$$

3. Results and discussion

3.1. Proximate composition

The proximate composition of chempedak seed and chempedak seed flour (CSF) is presented in Table 1. Processing of chempedak seeds into flour significantly reduced ($p < 0.05$) the protein and ash content. Mubarak (2005) reported that a similar result was obtained in raw mung bean when subjected to boiling. However, the crude fibre in CSF was found to be higher than jackfruit seed flour as reported by Tulyathan et al. (2002).

Ultimate level of CSF substitution in bread samples showed a significant increase ($p < 0.05$) in the moisture, crude fibre and ash contents while the carbohydrate and crude fat content decreased significantly ($p < 0.05$) (Table 2).

The amount of water added to the bread formulation increased with increase in CSF substitution level. Non-wheat flour induces a higher water absorption capacity than wheat flour (Tulyathan et al., 2002; Wang, Rosell, & Benedito de Barber 2002). The higher

Table 1
Proximate composition and resistant starch content of chempedak seed and chempedak seed flour (g/100 g dry weight).

Sample	Chempedak seed	Chempedak seed flour
Moisture	56.57[A] ± 0.12[a]	5.93 ± 0.01[b]
Protein	12.88 ± 0.47[a]	8.78 ± 0.46[b]
Crude fat	0.99 ± 0.05[a]	0.96 ± 0.07[a]
Crude fibre	2.44 ± 0.71[a]	2.35 ± 0.43[a]
Ash	2.57 ± 0.14[a]	2.21 ± 0.09[b]
Carbohydrate[B]	24.55 ± 0.69[b]	79.74 ± 0.52[a]
Resistant starch	29.72 ± 0.45[a]	14.77 ± 0.14[b]

[A] Calculated by difference.
[B] Values are mean of triplicates. Mean values followed by different letters in the same row are significantly different ($p < 0.05$).

Table 2
Proximate composition of bread (g/100 g dry weight) substituted with different levels of chempedak seed flour (CSF).

Sample	Bread I (Control)	Bread II (10% CSF)	Bread III (20% CSF)	Bread IV (30% CSF)
Moisture	34.64[B] ± 0.09[d]	37.15 ± 0.10[c]	39.75 ± 0.20[b]	43.53 ± 0.14[a]
Protein	13.29 ± 0.16[a]	12.91 ± 0.75[a]	12.88 ± 0.05[a]	12.69 ± 0.04[a]
Crude fat	1.50 ± 0.05[a]	1.10 ± 0.03[b]	0.84 ± 0.03[c]	0.75 ± 0.05[d]
Crude fibre	1.75 ± 0.11[d]	2.39 ± 0.08[c]	3.00 ± 0.05[b]	3.64 ± 0.02[a]
Ash	1.43 ± 0.01[d]	1.57 ± 0.02[c]	1.74 ± 0.02[b]	1.79 ± 0.00[a]
Carbohydrate[A]	47.40 ± 0.19[a]	44.87 ± 0.88[b]	41.79 ± 0.17[c]	37.61 ± 0.12[d]

[A] Calculated by difference.
[B] Values are means of triplicates. Mean values followed by different letters in the same row are significantly different ($p < 0.05$).

amount of fibre in non-wheat flour binds a relatively large amount of water through the hydroxyl groups existing in the fibre structure (Wang et al., 2002). Water content played a significant role in the final quality of the bread as it affects the gelatinisation of starch in bread (Thorvaldsson & Skjöldebrand, 1998).

3.2. Resistant starch content

Resistant starch (RS) content in CSF was significantly lower ($p < 0.05$) than in raw chempedak seed (Table 1). Processing of starchy foods promotes disintegration and/or microstructural damage of the seeds and affects the water absorption capability (Niba, 2003).

At a higher substitution level of CSF, the RS in bread samples increased significantly ($p < 0.05$); (Table 3). RS content increased by 16.3% in 10% CSF bread, compared with control bread. Meanwhile, RS content in 20% and 30% CSF bread samples increased by almost twofold, compared with the control bread.

According to Niba (2003), heat treatment followed by cooling in breadmaking resulted in formation of retrograded starch (RS3), due to an increase in gelatinised starch. Thus, repeated heating and cooling process applied during breadmaking resulted in higher RS content in bread samples (Niba, 2003).

RS formation is associated with retrogradation of starch, particularly amylose molecules, whereby enzyme-resistant amylose-amylose linkages are formed (Onyango et al., 2005). Åkerberg, Liljeberg, and Björck (1998) stated that apart from amylose content, amylopectin plays a significant role in RS formation in bread. They suggested that linear molecules of amylopectin might participate in starch retrogradation, consequently increasing RS content in bread samples.

Although amylose/amylopectin concentration is closely related to starch retrogradation and RS formation, other factors such as presence of other food components and type of processing applied contributes to RS content of food products (Rosin, Lajolo, & Menezes 2002). High molecular weight (HMW) molecules of maltodextrin reassociate to form double helices among themselves or with starch molecules. Therefore, at low maltodextrin concentration, the presence of starch in bread may help the reassociation of HMW molecules of maltodextrin and further increase the RS content (Wang & Jane, 1994). Furthermore, the extent of RS formation was also affected by the amount of dietary fibre and moisture content, which promotes the degree of starch gelatinisation (Tas & El, 2000).

3.3. In vitro starch hydrolysis

An *in vitro* starch hydrolysis method was carried out in this study, to simulate the *in vivo* situation of carbohydrate digestion characteristics and to estimate the metabolic glycaemic response to a food (Goñi et al., 1997).

The kinetics of *in vitro* starch hydrolysis for white bread (reference) and bread substituted with different levels of CSF with the addition of maltodextrin and α-amylase are summarised in Table 4. According to Cavallero et al. (2002), bread exhibits a sponge structure which is highly accessible to α-amylase and tends to elicit high glycaemic responses.

At a higher CSF substitution level, there was significantly lower ($p < 0.05$) *in vitro* starch hydrolysis. In the first 90 min the degrees of *in vitro* starch hydrolysis in 10% and 20% CSF breads were not significantly different ($p > 0.05$). Meanwhile, the 30% CSF bread underwent significantly lower ($p < 0.05$) *in vitro* starch hydrolysis as compared to the other bread samples at all time intervals. *In vitro* starch hydrolysis for 10%, 20% and 30% CSF breads was significantly reduced ($p < 0.05$) by 9.5%, 11.0% and 22.6%, respectively, as compared with control bread.

The profile of *in vitro* starch hydrolysis (Fig. 1) exhibited a gradual increment in all tested bread samples as time increased. White bread underwent a higher starch hydrolysis during 30–120 min, when compared with the other bread samples.

Table 3 showed the resistant starch (RS), hydrolysis index (*HI*) and estimated glycaemic index (*EGI*) for bread substituted with different levels of CSF with the addition of maltodextrin and α-amylase. At higher CSF substitution levels, *HI* decreased significantly ($p < 0.05$). *HI* values of 10%, 20% and 30% CSF bread samples decreased by 6.8%, 9.9% and 16.2%, respectively, when compared to control bread. Significant decreases of *HI* value in bread samples corresponded with significantly decreased ($p < 0.05$) *EGI*.

El (1999) reported that bread with the addition of a mixture of 10% rye flour and 15% wheat bran exerted a hypoglycaemic effect. Soluble dietary fibre reduced the dietary carbohydrate absorption rate by forming a viscous gel in the small intestine, hence reducing

Table 3
Model parameters, resistant starch (RS), hydrolysis index (*HI*) and estimated glycaemic index (*EGI*) of bread substituted with different levels of chempedak seed flour (CSF) added with maltodextrin and α-amylase.

Sample	RS (% db)	C_∞	k	Calculated *HI*	*EGI*
Bread I (Control)	16.86[A] ± 0.88[a]	15.48 ± 1.17[a]	0.09 ± 0.03[a]	43.23 ± 2.28[a]	63.44 ± 1.25[a]
Bread II (10% CSF)	19.60 ± 0.05[b]	14.81 ± 0.92[a]	0.06 ± 0.01[a]	40.28 ± 1.81[ab]	61. 83 ± 0.99[ab]
Bread III (20% CSF)	29.63 ± 0.20[c]	14.19 ± 0.32[ab]	0.07 ± 0.01[a]	38.96 ± 0.67[bc]	61.10 ± 0.37[bc]
Bread IV (30% CSF)	30.69 ± 0.75[c]	12.90 ± 0.65[b]	0.11 ± 0.07[a]	36.24 ± 2.77[c]	59.61 ± 1.52[bc]

C_∞: equilibrium constant; k: kinetic constant.
[A] Values are mean of triplicates. Mean values followed by different letters in the same column are significantly different ($p < 0.05$).

Table 4
In vitro kinetics of starch hydrolysis (% total starch hydrolysed at different time intervals) of white bread and bread substituted with different levels of chempedak seed flour (CSF) added with maltodextrin and α-amylase.

Time	White bread (reference)	Bread I (Control)	Bread II (10% CSF)	Bread III (20% CSF)	Bread IV (30% CSF)
0	0	0	0	0	0
30	20.70[A] ± 0.95[a]	14.07 ± 0.11[b]	12.74 ± 0.52[c]	12.52 ± 0.39[c]	10.89 ± 0.22[d]
60	25.85 ± 0.32[a]	14.14 ± 0.16[b]	13.24 ± 0.03[c]	13.00 ± 0.03[c]	12.04 ± 0.75[d]
90	35.90 ± 1.59[a]	14.61 ± 0.61[b]	13.64 ± 0.20[c]	13.42 ± 0.39[c]	12.25 ± 0.64[d]
120	45.30 ± 0.64[a]	14.71 ± 0.71[b]	13.93 ± 0.25[b]	13.87 ± 0.34[b]	12.50 ± 0.62[c]
150	45.65 ± 0.64[a]	14.89 ± 0.74[b]	14.42 ± 0.64[b]	13.96 ± 0.43[b]	12.66 ± 0.50[c]
180	45.65 ± 0.64[a]	15.48 ± 1.17[b]	14.81 ± 0.93[b]	14.19 ± 0.32[bc]	12.90 ± 0.65[c]

[A] Values are means of triplicates. Mean values followed by different letters in the same row are significantly different ($p < 0.05$).

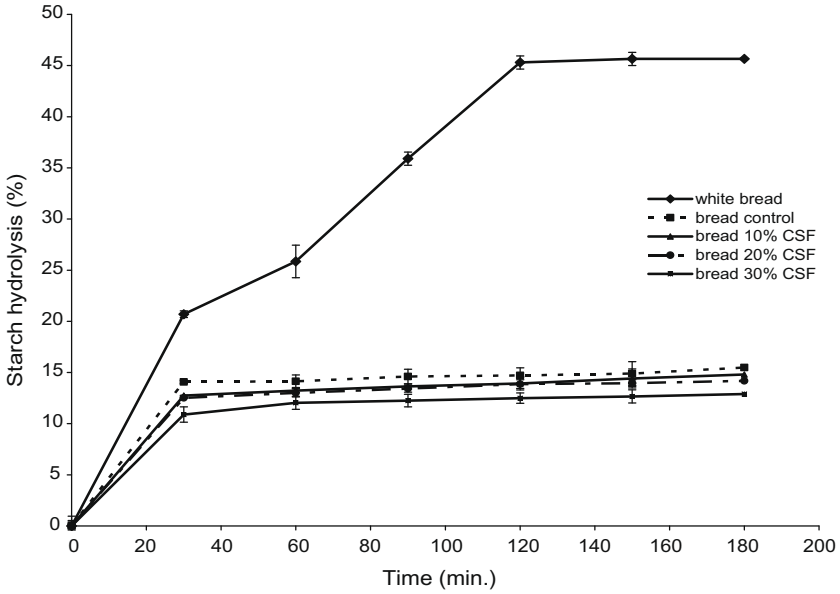

Fig. 1. Profile of *in vitro* starch hydrolysis (%) in white bread (reference) and bread substituted with different levels of chempedak seed flour (CSF) added with maltodextrin and α-amylase.

the postprandial blood glucose response. However, Wolever (1990) strongly suggested that encapsulation of nutrients within plant cell walls in insoluble dietary fibre inhibits starch digestion, which is more important in the further decrease of the glycaemic index.

The glycaemic effects of food depend on the food texture and particle size (Tovar et al., 2003), type of starch (amylose:amylopectin ratio) (Behall & Schofield, 2005), physical entrapment of starch molecules within the food, food processing and other ingredients, such as sugars, fat (Marques et al., 2007), protein, dietary fibre and antinutrient content (Cavallero et al., 2002; El, 1999). Products with high content of RS were reported to have lower glycaemic and insulinaemic responses in healthy human subjects (Åkerberg, Liljeberg, & Björck, 1998; Behall & Schofield, 2005).

From the results obtained, RS content in bread samples with higher CSF substitution levels is inversely related with *HI* value ($y = -2.1747 + 110.48$); ($R^2 = 0.8164$) (Fig. 2). The result is in agreement with previous studies that higher RS content leads to a reduction of *in vitro* starch hydrolysis rate, hence reducing the *GI* value of a food product (Åkerberg et al., 1998; Behall & Schofield, 2005; Goñi & Valentín-Gamazo, 2003; Rosin et al. 2002; Yamada et al., 2005). RS is known to exert a similar effect to some dietary fibres, by increasing the indigestible carbohydrate portion in small and large intestines (Yamada et al., 2005), thus reducing the rate of dietary carbohydrate absorption (El, 1999).

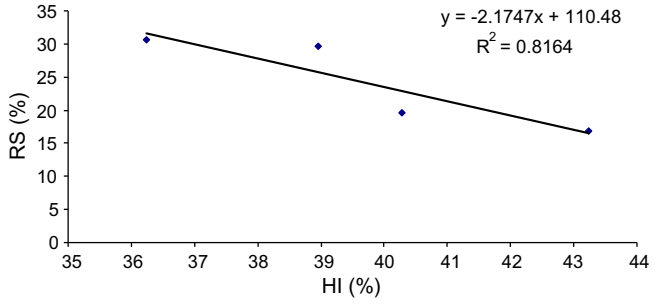

Fig. 2. Correlation between resistant starch (RS) content and hydrolysis index (*HI*) value in bread substituted with different levels of chempedak seed flour (CSF) added with maltodextrin and α-amylase.

4. Conclusion

From the study conducted, *in vitro* starch hydrolysis of bread samples was significantly affected ($p < 0.05$) by the amount of chempedak seed flour added in the bread formulation. Resistant starch content increased significantly ($p < 0.05$) by twofold in 20% and 30% CSF bread samples, compared with the control. Hence, *in vitro* starch hydrolysis is closely related to the amount of RS present in bread samples. RS content is inversely related with hydrolysis index value, which resulted in lower estimated glycaemic index values at higher added CSF levels.

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Food Chemistry 117 (2009) 69–74

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Functional properties of the Maillard reaction products of rice protein with sugar

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ARTICLE INFO

Article history:
Received 5 December 2008
Received in revised form 23 February 2009
Accepted 19 March 2009

Keywords:
Rice protein
Maillard reaction
Emulsifying properties
Protein solubility

ABSTRACT

The glycation of rice proteins with reducing sugars was investigated in an attempt to improve their solubility and functionality. Rice proteins isolated from a Chinese milled medium-grain rice were glycated with glucose, lactose, maltodextrin, or dextran in 2% aqueous dispersions. The sugar that provided the most improvement of the solubility, emulsification activity (*EA*) and emulsification stability (*ES*) of the Maillard reaction products was glucose. The optimum reaction conditions were at pH 11, 100 °C, and reaction time 15 min, which increased the solubility, *EA* and *ES* of rice protein from 20%, 0.46 and 11.1 to 92%, 0.64 and 18.2, respectively. Extending the reaction time beyond 15 min continued the development of latter-stage Maillard browning products without improvements in the functional properties of the Maillard reaction products. SEC–HPLC analysis with light scattering detection showed a decrease of the weight-averaged molecular weight from 500 to 100 K during the initial 15 min.

1. Introduction

Rice protein has been considered to have a high biological value and low hypoallergenic characteristics. However, the use of rice protein as an ingredient in foods has been limited, due to its poor functional properties, such as solubility. Whole rice grains contain different types of proteins which have been isolated and characterised. Over 80% of rice proteins are glutelins, which have low solubility at neutral pH. With low solubility the presence of other functional properties, such as foaming, gelling and emulsifying, are also limited (Agboola, Ng, & Mills, 2005).

Numerous attempts have been made to improve the functional properties of proteins through physical, chemical, enzymatic and genetic modifications (Matheis & Whitaker, 1984; Matsudomi, Sasaki, Kato, & Kibayashi, 1985). A chemical method to improve the functional properties of proteins has been the reaction of proteins with reducing sugars via the Maillard reaction (Wooster & Augustin, 2006). The modified proteins have often showed better solubility and improved emulsifying properties (Oliver, Melton, & Stanley, 2006). This reaction is natural and spontaneous and does not require the addition of extraneous chemicals. Thus it has been considered a promising approach to the improvement of the functional properties of proteins for food uses.

Different proteins, such as ovalbumin, lysozyme and soy proteins, have been conjugated with various sugars, in order to improve their solubility, emulsifying, and heat stability properties (Chevalier, Chobert, Popineau, Nicolas, & Haetle, 2001; Miralles, Martinez-Rodriguez, Santiago, van de Laemaat, & Heras, 2007). The improvement of the functionality of rice proteins by the Maillard reaction has not been reported. However, rice proteins contain a moderately high ratio of lysine, which has a free amino group that is liable to undergo Maillard reaction.

There are two approaches, which have been used to carry out the Maillard reaction (Guan, Qiu, Liu, Hua, & Ma, 2006). The first method involves the heating of a dry dispersion of a protein and saccharide under controlled temperature and relative humidity conditions. A disadvantage of this method is that some of the products are insoluble aggregates with poor surface wetting properties; a second method, which can overcome some of these problems, is to carry out the reaction in an aqueous solution or dispersion (Jing & Kitts, 2002; Lertittikul, Benjakul, & Tanaka, 2007). The latter method has the advantage of better control and shorter reactions times. Its disadvantages include the removal of the water and buffer when recovering the products.

In our initial studies, rice protein was reacted with sugars by dry heating. Initial results showed that the modified rice proteins exhibited slightly improved emulsifying activity, but there was no significant improvement in solubility. The goal of this study was to improve the solubility and emulsifying properties of rice proteins by glycation under wet reaction conditions. The influence of pH, type of sugar, the weight ratio of protein:sugar, reaction temperature and reaction time were studied. The degree of substitution and browning were also studied. The use of high pHs was chosen to solubilise the rice proteins and to enhance the rate of

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doi:10.1016/j.foodchem.2009.03.078

Maillard reaction, with the hypothesis that improvements in solubility and emulsifying properties would be made.

2. Materials and methods

2.1. Preparation of rice protein concentrate

The common method for the separation of rice protein from the starch of milled rice grains is by alkali solubilisation of the proteins followed by acid precipitation (Connor, Saunders, & Kohler, 1977; Yamamoto, Sumie, & Toshio, 1973). In this study, milled medium-grain rice (Nan Jing 39 from Jiangsu Baobao Group Co., Rudong, China) was washed with deionised water, and then soaked in three times its weight of deionised water for 15 h. The rice and water were then placed in a food blender and blended into a slurry. The protein was extracted from the slurry with 0.1 M NaOH at 40 °C (the ratio of slurry to NaOH solution was 1:3 w:w). The slurry was continuously stirred for 2 h and then centrifuged (6000*g* for 10 min). The supernatant was removed, and its pH was adjusted to 5.5 using 0.1 M HCl. It was left undisturbed for a cold precipitation overnight (4 °C). The supernatant was carefully siphoned off from the precipitate. The precipitate was washed 4 times with deionised water. The final slurry was neutralised to a pH 7.0 with 0.1 M NaOH and lyophilised. The protein content of the lyophilised substrate was determined by the Kjeldahl method (The value of 5.95 was used as a protein conversion factor), and the obtained rice protein content was 92% (dry weight).

2.2. Preparation of Maillard reaction products (MRPs) from rice protein–sugar model systems

Rice protein (2.4 g) was dispersed in 200 ml deionised water and the pH was adjusted to 12 with 10 M NaOH. The protein was thoroughly dispersed by vigorous and continuous agitation for 1 h. Glucose (2.4 g) was added to the dispersion. The pH of dispersion was readjusted to pH 11 with 0.1 M HCl. Aliquots of 15 ml were transferred to each of twelve 50 ml screw-capped glass tubes; they were capped to prevent evaporation during heating. The samples were heated in a water bath at 100 °C. Two sample tubes were removed during heating at the following times: 0, 5, 10, 15, 20, and 30 min. When removed the tubes were immediately placed in an ice-bath and then stored at 4 °C prior to analysis. Two additional samples were removed after 15 min of heating and freeze–dried and used for further experiments. Samples with other pH values, reaction temperatures, and ratios of protein to reducing sugar were prepared by the same procedure.

2.3. Solubility measurement of MRPs

Ten millilitres of an MRP sample was diluted with 10 ml of various buffers (0.05 M sodium phosphate buffer, pH 5, 6.5 and 8; 0.05 M sodium hydrogen carbonate buffer, pH 9.5 and 11; 0.05 M citrate buffer, pH 3.5 and 0.05 M KCl–acetic acid buffer, pH 2). The pH of the solution was readjusted to the buffer pH with 0.1 M HCl or NaOH. Samples were centrifuged for 20 min at 10,000*g*. Protein content in the supernatant was determined by the Kjeldahl method. Solubility was expressed as a percentage of protein in the supernatant to the total protein content.

2.4. Measurement of hydrophobicity

The hydrophobicity of the MRPs was determined according to the ANS method. A freeze–dried MRP sample was dissolved in 0.01 M phosphate buffer (pH 8) at different concentrations. The sample was centrifuged at 7000 rpm for 20 min. The concentration of the protein in the supernatant was measured by the Folin method. An aliquot of 2 ml was mixed with 40 μl of 1-anilinonaphthalene-8-sulfonic Acid (ANS) (8 mM) at 25 °C. The fluorescence intensity was measured with a fluorimeter. The excitation and emission wavelengths were set at 394 and 473 nm, respectively. The fluorescence intensity was measured as a function of protein concentration. The slope of the function was expressed as the hydrophobicity index.

2.5. Emulsifying activity and stability measurement of MRPs

The emulsifying properties of the samples were determined by the method of Pearce and Kinsella (1978) with some modifications. An MRP sample was diluted with buffer (0.05 M sodium phosphate buffer, pH 8) to a final protein concentration of 0.4% for the analysis of emulsifying properties. To form an emulsion, 15 ml of a 0.4% sample solution and 5 ml of soy oil were mixed together, with continuous agitation. The crude emulsion was homogenised using an Ultra-Turrax T25 homogeniser (IKA Werke GmbH & Co. KG, Staufen, Germany) at 10,000 rpm for 1 min. A sample of the emulsion (50 μl) was taken from the bottom of the test tube at 0, 3, 5, and 10 min and immediately diluted with 5 ml of 0.1% sodium dodecyl sulphate solution. The absorbance of the diluted emulsion was then determined at 500 nm. The emulsifying activity (*EA*) was determined from the absorbance measured immediately after the emulsion had formed (0 min). The emulsion stability (*ES*) was determined as the half-life of the initial turbidity of the emulsion (Pearce & Kinsella, 1978; Tang, Hettiarachchy, Horax, & Eswaranandam, 2003).

2.6. Determination of the degree of substitution of the MRPs

The degree of substitution (*DS*) of the rice proteins was determined from the analysis of the free amino groups in the rice proteins and MRPs. The level of free amino groups in the rice protein–sugar MRPs was determined with the TNBS method (Adler-Nissen, 1979). A protein sample (1.2% w/v) was dispersed in 0.1% SDS to obtain a solution with 0.2% protein concentration. It was then mixed with 2.0 ml of 0.21 M phosphate buffer, pH 8.2 and 1.0 ml of 0.1% 2,4,6-trinitrobenzenesulfonic acid (TNBS) solution. The solution was thoroughly mixed and placed in a temperature-controlled water bath at 50 °C for 1 h in the dark. The reaction was terminated with the addition of 2.0 ml of 0.1 M sodium sulphite. The mixture was cooled at room temperature for 30 min. The absorbance was measured at 340 nm.The degree of substitution was determined as:

$$DS\,(\%) = ((A_o - A_t)/A_o) \times 100\%,$$

where A_o was the absorbance of the sample before heating, and A_t was the absorbance of the sample after heating for t min.

2.7. Colour measurement of MRPs

For measurement of the extent of browning, MRPs were diluted in 0.1% SDS to a concentration of 0.2% of protein. The extent of browning was recorded as a measure of the absorbance at 420 nm.

2.8. Fluorescence analysis of MRPs

MRPs were dissolved in 0.2 M borate buffer (pH 8.5), then centrifuged at 7000 rpm for 20 min. The fluorescence intensity of the supernatant was measured with a fluorescence spectrometer (Hitachi 650-60). The excitation wavelength was 347 nm, and a spectrum was recorded from 380 to 480 nm with a slit width of 5 nm.

2.9. Amino acid analysis of MRPs

Measurement of the amino acid content of the MRPs was carried out by HPLC analysis on an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA) with on-line pre-column derivation by o-phthaldialdehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC-Cl, for proline analysis). Analysis was performed on a Hypersil ODS C_{18} column (4.6 mm × 150 mm, 5 μm film thickness). The UV detector was set at a wavelength of 338 nm. The column temperature was 30 °C. Gradient elution was used. The gradients were formed by 20 mM sodium acetate (**A**) and 20 mM sodium acetate:methanol:acetonitrile (1:2:2 V/V/V; solvent **B**). The elution system was:–10 min, 50% **B**; 10–20 min, 50–100% **B**; 20–25 min, 100–50% **B**. The flow rate was 1.0 ml/min.

2.10. Size exclusion chromatography – multi-angle laser light scattering analysis of MRPs

The high performance size-exclusion chromatography/multi-angle laser light scattering (HPSEC/MALLS) system consisted of an HP1050 series pump, an HP1050 autoinjector (Hewlett Packard, Valley Forge, PA), a MALLS detector (Dawn DSP-F, Wyatt Technologies, Santa Barbara, CA), a differential refractometer detector (ERC-7512, ERMA Incorporated, Tokyo, Japan) and a UV detector (Shimadzu, Kyoto, Japan). For the SEC analysis, two PL aquagel-OH linear columns (Polymer Laboratories, Amherst, MA) were connected in series, and an aqueous solution of 0.1 N $NaNO_3$ was used as the mobile phase at a flow rate of 0.6 ml/min. The column temperature was maintained at 50 °C with a column heater (Bio-Rad, Hercules, CA).

3. Results and discussion

Many cereal proteins lack functionality, largely due to their insolubility. The covalent bonding of polysaccharides or smaller reducing sugars with proteins *via* the Maillard reaction can be a useful way of increasing their solubility and functionality. For other proteins this has been shown to be the case (Oliver et al., 2006). However, the establishment of optimum reaction conditions for preparing useful MRPs is needed for the synthesis of more functional rice proteins. An important functional property of MRPs is their emulsifying properties. The emulsifying properties of MRPs depend on the degree of the glycation of the protein. The emulsifying properties have been reported as being enhanced during the early stages of conjugation, and at longer times, they gradually decreased. To produce an effective emulsifying agent there should be a good balance between the degrees of hydrophobic and hydrophilic properties. In addition, the loss of emulsifying power is presumably caused by the degradation of initial MRPs, which has been suggested by the appearance of low molecular weight products found by electrophoresis gel analysis at longer reaction times (Miralles et al., 2007).

3.1. Influence of varieties of sugars

The effect of the type of saccharide (glucose, lactose, maltodextrin, or dextran) on the functionality of MRPs formed by glycation of rice proteins (weight ratio of protein:sugar of 1:1) at pH 11 was investigated at a reaction temperature of 100 °C (Table 1). The *DS* and solubility of the MRPs increased with a decrease in size of the reactant saccharide. Several investigations have shown that the reaction mechanisms of monosaccharides and disaccharides differ and that reaction products obtained from monosaccharides are different from those obtained from disaccharides (Kato, Matsuda, Kato, & Nakamura, 1988). There has been reported a linear correlation between the *DS* and the saccharide size (Nacka, Chobert, Burova, Leonil, & Haertle, 1998).

There were effects on other properties of the MRPs as well. There was little difference observed between MRPs formed with glucose or lactose as compared to larger differences between the MRPs formed with maltodextrins or dextrans. Lactose showed a greater effect than glucose on the emulsion activity (*EA*), and glucose had a greater affect on the emulsion stability (*ES*). Lactose had a greater extent of browning, and lowering of pH, but both saccharides had greater effects on these properties than the maltodextrin and dextran. The lower reactivity of the maltodextrin and dextran have been reported to be related to steric hindrance effects (Kato, 2002).

The effect of saccharide type on *DS*, pH, and extent of browning was measured at intervals over a 30 min heating period. The *DS* increased during the first 15 min of heating and remained rather constant upon continued heating (Fig. 1). The rice protein–glucose MRPs exhibited the highest *DS*. Similar trends for the solubility, *EA*, and *ES* were also observed (not shown). The extent of browning and acidity also increased during the first 15 min of heating (Huiying, Tao, Dongxiang, & Fang, 2007), and continued to increase during the latter 15 min of the 30 min period (Fig. 2). The reduction of pH occurring during the Maillard reaction could be partially due to the degradation of MRPs into acids, such as formic and acetic acids (Brands & Van Boekel, 2002). On the other hand, the decrease in pH could also be attributed to the reaction of amines to form compounds of lower basicity (Van Boekel & Brands, 1998).

The extent of browning of the protein–lactose MRPs was greater than the MRPs formed with other sugars, and the pH of the protein–lactose MRPs decreased more than other MRPs. Brands and Van Boekel (2001) reported that compared to monosaccharide systems, about four times more formic acid was formed with disaccharide-casein MRPs. The pH of all MRPs decreased markedly from their initial values within the first 15 min. Thereafter, the reduction of pH values was less during the last 15 min. It has been reported that pH has an effect on both the reaction rate and the reaction mechanism (Brands & Van Boekel, 2002).

From these results, it appeared that useful functional properties for the MRPs were optimum when formed with glucose at 15 min, and they did not appear to increase with longer reaction times. The best solubility of the MRPs (90%) was obtained with glucose. How-

Table 1
Effect of saccharide type on Maillard browning reaction products (MRPs) formed with rice protein after 15 min at 100 °C*.

Saccharide	Degree of substitution (%)	Solubility (%)	Emulsifying activity	Emulsifying stability	Browning absorbance (420 nm)	pH
No saccharide	0^{a}	20.0^{a}	0.496^{a}	10.3^{a}	0.07^{a}	11^{e}
Glucose	28.0^{b}	90.0^{e}	$0.620^{b,c}$	18.0^{d}	0.247^{d}	8.7^{b}
Lactose	26.8^{b}	83.2^{d}	$0.618^{c,d}$	16.5^{c}	0.268^{e}	8.3^{a}
Maltodextrin	17.7^{c}	73.0^{c}	0.589^{b}	13.6^{b}	0.223^{c}	9.3^{c}
Dextrin	12.2^{d}	60.1^{b}	0.585^{b}	13.6^{b}	0.128^{b}	10.6^{d}

* Concentrations of initial reactants were 1% saccharide and 1% rice protein. Values are means of three replicate values and different superscript letters within the same column represent a significant difference ($p < 0.05$).

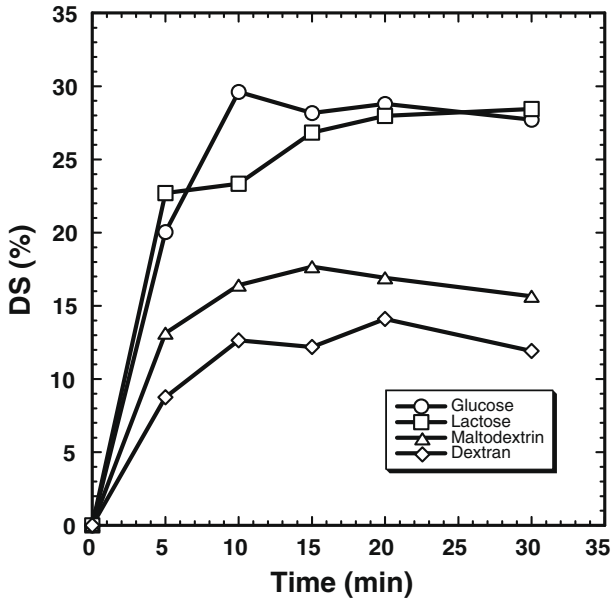

Fig. 1. The effect of saccharide on the degree of substitution (*DS*) of the free amino groups on rice proteins was measured over 30 min at 90 °C and an initial pH of 11.

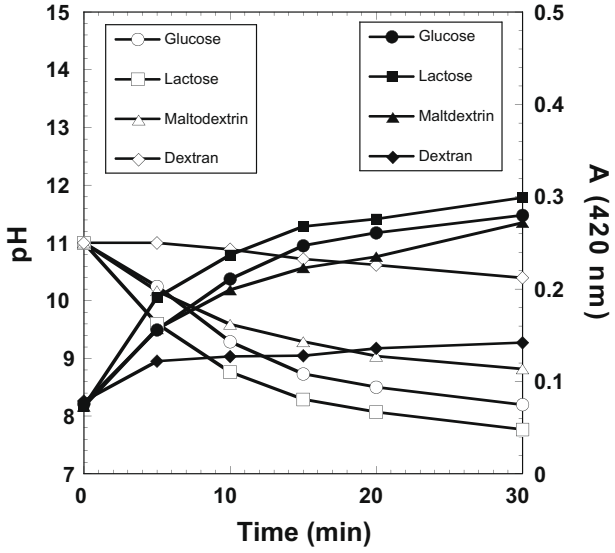

Fig. 2. The effect of saccharide on the pH and extent of browning of the reaction of saccharide with rice proteins was measured over 30 min. The open symbols represent the pH values, and the closed symbols represent absorption (*A*) at 420 nm.

ever, an increase in the extent of browning and reduction of pH continued throughout the 30 min reaction period.

3.2. Study of the effect of reaction conditions on the formation of MRPs

For rice proteins, pH may have several roles in the formation of MRPs. High pH enhances rice protein solubility and increases the rate of glycation. Rice proteins were glycated with glucose (weight ratio 1:1) at pH values 10 and 11 at 100 °C. The *DS* for the MRPs at pH values of 10 and 11 increased rapidly during the first 10 min and reached values of 10% and 34%, respectively. With continued heating after 10 min, the *DS* values increased more slowly, to reach values of 12% and 37%, respectively, after 30 min.

When the rice proteins were reacted with glucose at 100 °C with different weight ratios (*WR*) of protein:glucose (2:1, 1:1 and 1:2) with the same total weight (2%) for 30 min, differences among the functional properties of the MRPs were observed. Even though the *DS* values for the *WR* 1:2 and 2:1 showed the same overall trend as *WR* 1:1 over the reaction period (Fig. 1), the *DS* values at 15 min were 26, 28, and 21 for the *WR*s of 1:2, 1:1, and 2:1, respectively. The ranking of the final *DS* values at 30 min was the same as the 15 min ranking order. The trends of the pHs of the reaction mixtures and development of brown colour (420 nm) of the *WR*s 1:2 and 2:1 were also similar to the *WR* 1:1 (Fig. 2). The pH values at 15 min were 8.0, 8.7 and 9.1 for the *WR* of 1:2, 1:1, and 2:1, respectively. Likewise, the absorbances at 420 nm were 0.33, 0.25, and 0.22 for *WR* 1:2, 1:1, and 2:1, respectively. Thus for a fixed total concentration (2% w/w) of glucose and protein, the *WR* of 1:1 gave the highest *DS*. The reduction of pH and development of brown colour increased with an increase in *WR* of glucose under these conditions.

The effect of three reaction temperatures (80, 90, and 100 °C) on the solubility of the MRPs formed with *WR* 1:1 with the above reaction conditions was measured. After 15 min, MRPs were isolated from the reaction mixtures with *WR* of 1:1 and their solubility was measured. The solubilities of the MRPs were 15%, 86% and 90% at 80, 90, and 100 °C, respectively.

With a *WR* of 1:1 (2% w/w), and initial pH of 11, the effect of reaction times between 0 and 30 min was evaluated at 100 °C (Table 2). Both *DS* and degree of solubility of the MRPs showed rapid increases during the first 10 min and became constant after 15 min. The degree of hydrophobicity decreased with the increase in *DS* and solubility, as would be expected. The *EA* and *ES* values increased during the first 15 min and then showed a slight decrease in their values during the latter 15 min. A maximum in the *EA* and *ES* values at 15 min may suggest that the best balance between the hydrophobic and hydrophilic balance had been obtained at that time. As compared to native rice proteins, the amounts of the amino acids S-Cys, Lys, Pro and Arg decreased by 61.1%, 48.3%, 28.7% and 10.9% in the MRPs, which were formed after 15 min, respectively. Lys is commonly believed to be the most reactive amino acid in terms of glycation with reducing sugars. Arg (Yen, Tsai, & Lii, 1992), S-Cys (Billaud, Maraschin, & Nicolas, 2004) and Pro (Davidek, Clety, Devaud, Robert, & Blank, 2003) were also found to be lost in the Maillard reaction with proteins. The decrease of S-Cys content also reduces the chance of protein aggregation through –S–S bonds, which could help to increase the solubility of the MRPs in water.

The objective of this work was to improve the functional properties of rice proteins by the formation of MRPs. The results suggest that the reaction conditions which favoured the formation of useful MRPs with limited amounts of latter-stage products were WR 1:1, pH 11, 100 °C for 15 min. These MRPs had optimum emulsifying activity and solubility. In order to confirm that reaction times of longer than 15 min did produce latter-stage MRPs, several experiments were made to detect evidence for their production.

3.3. Analysis of latter-stage MRPs

The Maillard reaction is associated with the development of fluorescent compounds formed prior to the generation of brown pigments (Morales, Romero, & Jimenez-Perez, 1996; Morales & Van Boekel, 1997), which may be precursors of brown pigments. The presence of fluorescent compounds was analysed during the course of the reaction, in order to find possible markers of the development of latter-stage MRPs. The development of fluorescent compounds was found to increase with longer heating times of the rice protein and glucose (Fig. 3). It appeared that after 10 min of heating at 100 °C there was a large increase in the concentration of fluorescent compounds. Even as these compounds may have been formed prior to the generation of brown pigments, their con-

Table 2
Effect of reaction time on properties of MRPs formed with rice proteins (1%) and glucose (1%) at 100 °C.

	Reaction time (min)	Degree of substitution (%)	Solubility (%)	Hydrophobicity	Emulsifying activity	Emulsifying stability	Browning absorbance (420 nm)	pH
MRPs	0	–	10.9[a]	2491[f]	0.461[a]	11.1[a]	0.08[a]	11.0[e]
	5	20.0[a]	18.6[b]	1747[e]	0.539[b]	11.4[a]	0.16[b]	10.2[d]
	10	29.6[b]	85.6[c]	1564[d]	0.631[c,d]	14.0[b]	0.21[c]	9.3[c]
	15	28.2[b]	90.3[d]	1416[c]	0.642[d]	18.2[d]	0.25[d]	8.7[b]
	20	28.8[b]	90.0[d]	1288[b]	0.599[c]	15.4[c]	0.26[d,e]	8.5[b]
	30	27.7[b]	90.0[d]	1143[a]	0.563[b,c]	14.4[b,c]	0.28[e]	8.2[a]

Values are means of three replicate values and different superscript letters within the same column represent a significant different ($p < 0.05$).

centrations continued to increase during the latter 15 min, as the *EA* and *ES* values were decreasing and the solubility of the MRPs showed no significant change. This suggested that at longer heating times the rate of production of latter-stage MRPs did increase, which was also supported by the increase in brown colour (Table 2).

In order to further study the latter-stage MRPs, HPSEC-MALLS was used with UV detection at 280 nm and multi-angle laser light scattering. With HPSEC-MALLS the MRPs were separated by molecular weight. With a laser light scattering detector the weight-averaged molecular weights (*Mw*) of the larger region were measured. Chromatographs of the products from each reaction time (0, 5, 10, 15, 20 and 30 min) were recorded; each was divided into two fractions, the first fraction had a low *Mw* cutoff of 10,000 Daltons and the second was the fraction, which had *Mw*s of less than 10,000 (Fig. 4). For each fraction, the integration of absorbencies at 280 nm was calculated. For the high *Mw* fraction, the recovered mass increased during the first 15 min and remained relatively constant during the latter 15 min of heating. This trend was similar to the increase in solubility of the MRPs (Table 2). The *Mw* of the larger molecular weight fraction decreased from 500 to 80 kDa during the first 15 min and then remained relatively constant during the latter 15 min. This suggested that initially the rice proteins may have been aggregated. During the initial 15 min of the reaction, the degree of aggregation decreased with the formation of MRPs. The final *Mw*s of these fractions for the heating times after 10 min of heating were between 100 and 80 kDa. For the lower

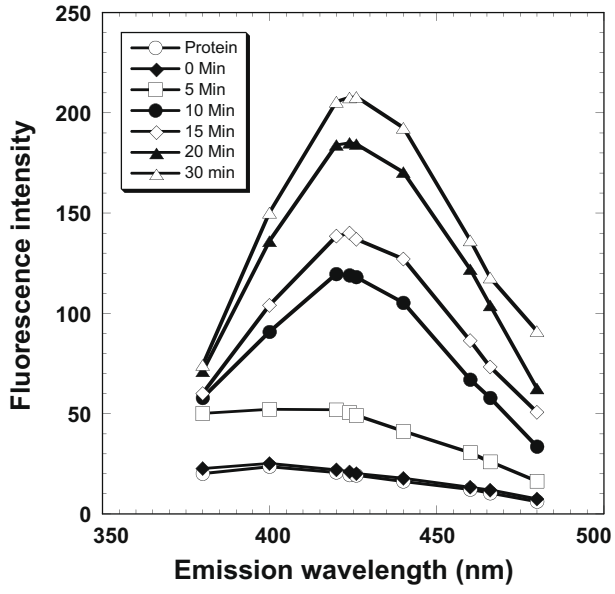

Fig. 3. The effect of reaction time on the fluorescence spectra of glucose MRPs measured with an excitation frequency of 347 nm. The MRPs were formed at 100 °C.

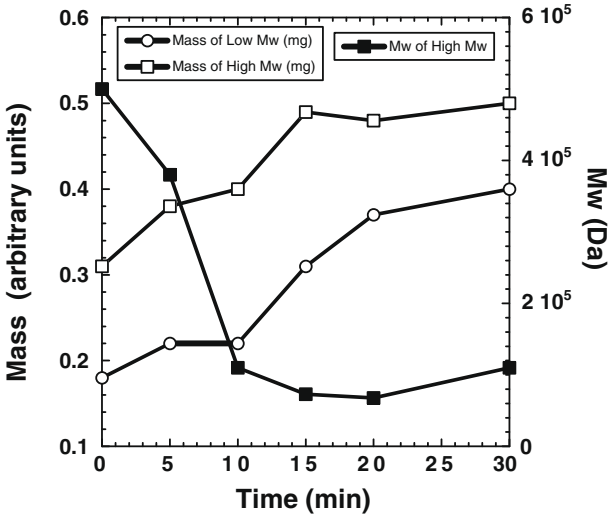

Fig. 4. Glucose MRP products analysed by HPLC size-exclusion analysis. The collection samples were divided into two fractions. The high molecular fraction was in the range of 1000–10 kDa. The low *Mw* range is that below 10 kDa. The recovered mass of each fraction is in arbitrary units. *Mw* is the weight average of the molecular weight of each fraction.

Mw fraction, there was little change in the recovered mass during the first 10 min and its Mw was also constant at 40 kDa. At later times the concentrations of the low *Mw* species absorbing at 280 nm increased continuously and the final *Mw* was 20 kDa. These products may have been latter-stage MRPs, which were compounds that were dissociated from glycated proteins, and contained chromophores absorbing at 280 nm.

4. Conclusion

The Maillard reaction can be successfully used as a coupling method for rice protein and glucose to form MRPs with improved functional properties. Using methods to detect latter-stage MRPs, optimum reaction conditions were found which provided improved functional properties of rice proteins with reduced levels of latter-stage MRPs. At a reaction time of 15 min the MRP had the best emulsifying capacity and solubility. The conditions for obtaining these MRPs were a 2% (w/w) protein:glucose (*WR* 1:1) aqueous dispersion with an initial pH of 11 which was heated for 15 min at 100 °C. At these conditions, the functional properties (the emulsifying activity, emulsifying stability and solubility of the MRPs) increased by factors of 1.29, 1.77 and 8.30 times, respectively. However, there was a loss of 48% of the original lysine content of the rice proteins. Longer reaction times did not improve the yield or functional properties and there was evidence that at longer times other products were formed.

Acknowledgements

This work was financially supported by National 863 Program 2006AA10Z327, Nature Science Foundation of Jiangsu Province, China (BK 2007502), NSFC 30871744, 111 project-B07029 and PCSIRT0627.

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Food Chemistry 117 (2009) 75–82

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Mechanisms by which flavonol aglycones inhibit lipid oxidation better than glycosylated flavonols in comminuted muscle tissue

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ARTICLE INFO

Article history:
Received 19 August 2008
Received in revised form 30 January 2009
Accepted 19 March 2009

Keywords:
Quercetin
Quercetin-β-D-glucoside
Flavonols
Mechanically separated turkey
Chicken muscle
Lipid oxidation
Membranes
Partitioning

ABSTRACT

The effect of glycosylation on the ability of flavonoids to inhibit lipid oxidation in comminuted muscle tissue has not been previously examined. This work examined the ability of quercetin and quercetin-β-D-glucoside to inhibit lipid oxidation in mechanically separated turkey (MST). Quercetin inhibited formation of lipid peroxides and thiobarbituric acid reactive substances (TBARS) more effectively compared to quercetin-β-D-glucoside during frozen storage ($p < 0.05$). The possible mechanisms that cause glycosylation to decrease inhibition of lipid oxidation were also examined. Hydroxyl radical scavenging activity was similar when comparing quercetin and quercetin-β-D-glucoside, which indicated that the free hydroxyl group in 3 position of C ring in quercetin did not enhance its hydroxyl radical scavenging ability. Since muscle membrane lipids are susceptible to lipid oxidation, the ability of quercetin and quercetin-β-D-glucoside to incorporate into cellular membranes was studied. After adding quercetin and quercetin-β-D-glucoside to minced chicken muscle, flavonol content in the membrane fraction was determined. Around 32% of added quercetin partitioned into the membranes whereas quercetin-β-D-glucoside was not detected in the membranes. Similar trends were observed when each flavonol was added to isolated membranes. These studies suggest that glycosylation of flavonols weakens their ability to inhibit lipid oxidation in muscle tissue partly by decreasing the amount of flavonols in the membrane phase. In order to understand whether metal chelation by flavonols is a likely mechanism involved in the inhibition of lipid oxidation in MST, the role of endogenous metals in promoting lipid oxidation was examined. Addition of the metal chelators ethylenediamine tetraacetic acid (EDTA) and tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) to MST did not inhibit lipid oxidation, which suggests that endogenous metals present in MST were not promoters of lipid oxidation. Hence it seems unlikely that the mechanism of inhibition by flavonols involved metal chelation in the comminuted muscle.

1. Introduction

Muscle foods are susceptible to lipid oxidation which negatively affects the quality and economic value of the muscle products. Incorporation of antioxidants into foods can effectively retard lipid oxidation (Labuza, 1971). In our previous work, we found that a fraction isolated from an amphiphilic extract prepared from cranberry juice powder was an effective inhibitor of lipid oxidation in mechanically separated turkey (MST). The compound responsible for the inhibitory activity was then identified as quercetin. It was evident from these studies that glycosylated quercetin in the extract did not contribute to the efficacy of the isolated fraction in MST (Kathirvel, Gong, & Richards, 2009).

For a better understanding of how glycosylation of flavonols affects the antioxidant activity in comminuted muscle tissue systems, a comparative study between quercetin and quercetin-β-D-glucoside was conducted. Quercetin and quercetin-β-D-glucoside belong to the class of flavonoids known as flavonols. Quercetin and quercetin-β-D-glucoside are structurally similar flavonols except for the presence of a glucose moiety at 3-hydroxy position in quercetin-β-D-glucoside (Fig. 1). Flavonols are widely distributed in plant foods (Bahorun, Luximon-Ramma, Crozier, & Aruoma, 2004; Justesen, Knuthsen, & Leth, 1998; Vvedenskaya & Vorsa, 2004) and have received considerable attention for their antioxidant properties (Crozier et al., 2000; Mira et al., 2002; Plumb, Price, & Williamson, 1999). The structure–activity relationship of flavonols depends on the test system used to determine lipid oxidation. The antioxidant activity of flavonols has been extensively studied in methyl lineolate, liposome systems and fish lipids (Hopia & Heinonen, 1998; Liao & Yin, 2000; Pazos, Gallardo, Torres, & Medina, 2005). Free radical scavenging and metal chelation have been described to be the key factors responsible for the antioxidative

Abbreviations: MST, mechanically separated turkey; TBARS, thiobarbituric acid reactive substances; HPLC, high performance liquid chromatograph; EDTA, ethylenediamine tetraacetic acid; TPEN, tetrakis(2-pyridylmethyl) ethylenediamine.

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doi:10.1016/j.foodchem.2009.03.079

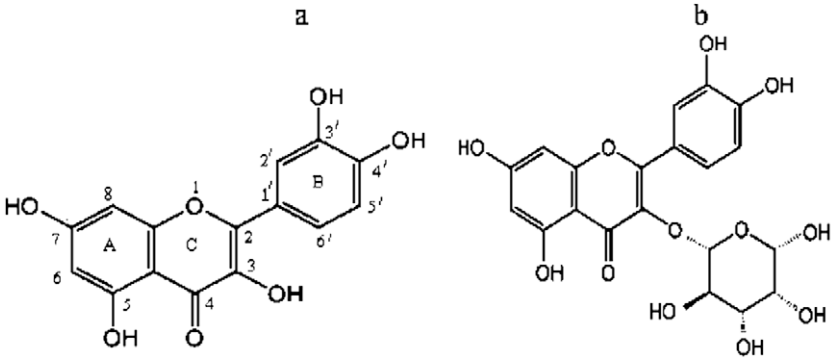

Fig. 1. Structure of (a) quercetin and (b) quercetin-β-D-glucoside.

activities of flavonols (Bors, Heller, Michel, & Saran, 1990; Mira et al., 2002).

Partitioning of flavonols in octanol and water phases has been reported (Rothwell, Day, & Morgan, 2005) and has been described as a useful way to determine the behaviour of flavonols towards the membrane phase. In muscle foods, membrane phospholipids are believed to be the most susceptible lipid fraction to lipid oxidation compared to neutral lipids (e.g. triacylglycerols), mainly due to the higher degree of fatty acid unsaturation and increased surface area of the membranes (Pikul, Leszczynski, & Kummerow, 1984). Partitioning of antioxidants to sites where the lipids are most susceptible to oxidation might be important in reducing the extent of lipid oxidation in muscle foods. Studies that probe the importance of flavonoid partitioning in muscle membranes are still lacking. The purpose of the work here was to compare the ability of two commonly occurring flavonols in plant foods, quercetin and quercetin-β-D-glucoside to inhibit lipid oxidation in mechanically separated turkey (MST) as well as to assess the possible mechanisms by which these flavonols exhibit their antioxidative properties in muscle tissue. MST was used as a substrate for oxidation study mainly due to its high susceptibility to lipid oxidation and use as a raw material in formulation of processed meats such as bologna and wieners. This study helps in the better understanding of how structural changes influence the antioxidant activity of flavonols in a muscle tissue matrix.

2. Materials and methods

2.1. Materials

Mechanically separated turkey (MST) was obtained from Kraft-Oscar Mayer (Newberry, SC) and was vacuum packaged and stored at −80 °C until use. Broiler chicks (Cornish Rock) obtained from Sunnyside Farms, WI were raised in the Poultry Research Lab (PRL), UW-Madison. Chicken breast muscle used for partitioning studies was harvested from broiler chickens raised in the PRL. Sealable, polyethylene bags (10 cm × 15 cm) and vacuum pouches (3 ml standard barrier) were purchased from Koch Supplies (Chicago, IL). Ethanol (absolute, 200 proof) was obtained from Aaper Alcohol and Chemical Co. (Shelbyville, Kentucky). 2-Thiobarbituric acid, sodium chloride, potassium hydroxide, sodium hydroxide, disodium ethylenediamine-tetraacetic acid, propyl gallate, potassium dihydrogen phosphate, deoxyribose, ferric chloride, ferrous sulphate, hydrogen peroxide, sodium ascorbate, Proteinase, HEPES buffer (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffer hemisodium salt), ethylene diamine tetraacetic acid (EDTA), tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), quercetin dihydrate and quercetin-β-D-glucoside were procured from Sigma A/S (St. Louis, MO). Ammonium thiocyanate, barium chloride, hydrochloric acid, sulphuric acid, trichloroacetic acid, sodium carbonate, methanol, 1-butanol and chloroform were purchased from Fisher Scientific (Chicago, IL). The solvents used were of HPLC grade.

2.2. High Performance Liquid Chromatograph (HPLC) apparatus and chromatographic conditions

HPLC analysis of flavonols were performed on a Agilent 1100 HPLC system equipped with an Agilent 1100 series binary pump and photodiode array detector (Agilent, Wilmington, DE). A C18 reversed phase column (Discovery 5 μm, 25 cm × 0.46 cm i.d.) (Supelco, Bellefonte, PA) was used. The binary solvent system used was: Solvent A, 0.1% trifluoroacetic acid in water and solvent B, 0.1% trifluoroacetic acid in methanol. To determine the flavonols that partitioned into the membranes, membrane lipids were extracted using chloroform:methanol (1:1 v/v) and NaCl solution and both the chloroform and methanol/water phases were analyzed. To analyze the chloroform phase, it was first evaporated and lipids were redissolved in methanol:butanol (1:1v/v) before injecting into the HPLC system. A linear gradient of 0–100% B from 0 to 40 min was carried out at a flow rate of 1 ml/min. Multiwavelength detection was monitored at 280, 320, 360 and 520 nm. Quercetin and quercetin-β-D-glucoside were used as standards.

2.3. Addition of flavonols to MST

MST was vacuum packaged in 500 g portions and stored at −80 °C until use. The frozen packs were then thawed for 2 h at room temperature (without breaking the vacuum seal) before using them for treatments. Quercetin and quercetin-β-D-glucoside were added at 100 μmol/kg MST and thoroughly mixed with a spatula to ensure uniform distribution. All samples were stored in zip-lock polyethylene bags at −4 °C during the study period. The carrier solvent was 100% ethanol and was used at 1% of the final sample weight. A control sample containing 1% ethanol without the flavonols was also prepared for the respective trials.

2.4. Isolation of crude membranes from MST

The method described by Raghavan and Hultin (2005) was used for the isolation of crude membranes from MST. Ten grams of muscle was mixed with four volumes of cold 0.1 M HEPES buffer containing 0.2% (w/v) sodium ascorbate (pH 7.5) and homogenized at speed 6 for 40 s using a Kinematica Polytron PT 10–35 homogenizer (Brinkmann Instruments, Westburg, NY). The pH of the homogenate was adjusted to 7.5 and was centrifuged at 10,000*g* for 20 min at a sample temperature of 7–9 °C using a Beckman

L5–65 ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). The supernatant obtained was centrifuged at 130,000*g* for 30 min to obtain crude membrane as sediment.

2.5. Addition of quercetin and quercetin-β-D-glucoside to crude membranes isolated from MST

A 2 ml methanol:water solution containing approximately 367 μg quercetin or 464 μg quercetin-β-D-glucoside were added to membranes prepared from 10 g MST and hand homogenized using a Potter-Elvehjem tissue grinder for 2 min. The level of addition of flavonols to the membranes corresponds to the level used for the oxidation study in MST (100 μmol/kg MST). This suspension was then centrifuged at 130,000*g* for 30 min. The flavonol content in the supernatant was quantified by HPLC. The resulting membrane pellet was then resuspended in 3 ml of 0.1 M HEPES buffer and was used for lipid, phospholipid and flavonol determinations.

2.6. Adddition of quercetin and quercetin-β-D-glucoside to chicken breast muscle

Broiler chickens (8-weeks old) raised at UW-Madison were sacrificed by asphyxiation using carbon dioxide. The chickens were bled before collecting the breast muscles. Breast muscles were removed and stored at 0–4 °C until used (10–16 h). The muscles were ground twice through 5 mm perforations in a KitchenAid model KSM90 grinder (KitchenAid Inc., St. Joseph, MI). Sodium ascorbate and proteinase (a proteolytic enzyme preparation) were added to ground muscle at 0.2% and 0.005% (w/w) respectively and minced for 20 s in the food processor. Proteinase was added to break down structural proteins and to aid the release of membrane material. Sodium ascorbate provided protection to the flavonols added to the muscle. The minced muscle was then incubated for 1 h at refrigerated conditions (0–4 °C). After incubation, quercetin and quercetin-β-D-glucoside were added to the muscle at 100 μmol/kg using 1% ethanol as a carrier solvent and minced for 30 s in the food processor. An incubation period of around 20 min was implemented prior to isolating the membranes. Membranes were then isolated from 10 g of muscle as described above and suspended in 3 ml of 0.1 M HEPES buffer which was then used for lipid, phospholipid and flavonol determinations. All the procedures were done in iced conditions unless otherwise noted.

2.7. Total lipid, phospholipid and flavonol determination

Total lipid content of membranes were determined by extracting 3 ml of membrane suspension with 30 ml of 1:1 (v/v) chloroform/methanol mixture in a separatory funnel using 12 ml of 0.5% (w/v) NaCl solution for phase separation (Sigfusson & Hultin, 2002). The funnel was stored at refrigerated temperatures during phase separation. For extraction of muscle lipids, 10 g of muscle sample was homogenized using a Kinematica Polytron PT 10–35 homogenizer (Brinkmann Instruments, Westburg, NY) at speed 6 for 60 s with 100 ml chloroform/methanol mixture. The homogenate was filtered through Whatman #4 filter paper into a separatory funnel, followed by the addition of 40 ml of 0.5% (w/v) NaCl solution for phase separation. The chloroform layer was separated and aliquots of chloroform were used for total lipid, phospholipid and flavonol determination. Total lipid content was determined by driving off the chloroform layer using nitrogen gas. For determining quercetin and quercetin-β-D-glucoside in membrane lipids, lipid obtained by driving off an aliquot of chloroform layer was redissolved in 1 ml of methanol:butanol (1:1v/v) and filtered through 0.45 μm pore size PTFE disk filters prior to injection to the HPLC. The methanol:water phase was also analyzed for the presence of flavonols.

The phosphorous content of the membrane lipid extract was determined spectrophotometrically (Anderson & Davis, 1982). In brief, aliquots of the chloroform layer obtained after lipid extraction were transferred to test tubes and dried under nitrogen gas. Concentrated sulphuric acid (98%) (0.1 ml) was added to the samples, vortexed and digested at 155 °C for 10 min. Tubes were then cooled and samples were oxidized with hydrogen peroxide at 155 °C for 40 min. After cooling the tubes, 2 ml of distilled water was added and mixed properly. This was followed by the addition of 0.8 ml of ammonium molybdate-ascorbate solution (1:1 v/v) and the mixture was heated for 7 min in a boiling water bath. The absorbance was then read at 797 nm after cooling of the samples. Sodium phosphate (dibasic) was used as a standard. A factor of 25 was used for converting lipid phosphorous to phospholipids, based on an average molecular weight of phosphatidylcholine divided by the atomic weight of phosphorous.

2.8. Hydroxyl radical scavenging assay

The scavenging activity of quercetin and quercetin-β-D-glucoside on the hydroxyl radical ($OH^{\cdot}$) was measured by the deoxyribose method (Aruoma, 1994). The only exception was that Adenosine Triphosphate (ATP) was used as the iron chelator instead of EDTA. This assay uses the Fenton reaction to generate $OH^{\cdot}$ in the presence of ascorbic acid which then attacks the sugar deoxyribose to form products that on heating with thiobarbituric acid yields a pink chromogen detected at 532 nm. The reaction mixture contained the following reagents at final concentrations stated: deoxyribose (2.8 mM), $FeCl_3$ (25 μM), ATP (100 μM) [ATP and $FeCl_3$ are mixed prior to the addition of deoxyribose], H_2O_2 (2.8 mM), KH_2PO_4/KOH buffer at pH 7.4 (10 mM), quercetin and quercetin-β-D-glucoside at two concentrations of 18 μg and 36 μg. In order to avoid the interference caused by solvents and to make the flavonols soluble in aqueous media, stock solutions of flavonols were prepared by dissolving 2.5 mg of flavonols in 100 μl of 2 N NaOH and then solution made up to 25 ml with distilled water. The pH of the flavonol solution was adjusted to 7.4. The reaction was started by adding ascorbate (100 μM). The final reaction volume was 1.2 ml. The reaction mixture was incubated in a water bath for 1 h at 37 °C. After incubation, 1 ml of 1% (w/v) thiobarbituric acid and 1 ml of 2.8% (w/v) trichloroacetic acid were added to the mixture and heated in a boiling water bath at 100 °C for up to 20 min. The sample was then cooled and the absorbance was measured at 532 nm. The reaction mixture not containing flavonols was used as control. The hydroxyl radical scavenging activity (HRSA) was expressed as

$$\mathrm{HRSA}(\%) = \left[1 - \frac{\text{Absorbance at 532 nm in the presence of flavonols}}{\text{Absorbance at 532 nm in the absence of flavonols}}\right] \times 100$$

2.9. Ability of metal chelators to inhibit lipid oxidation in MST

In order to indirectly understand whether metal chelation by flavonols were one of the mechanisms by which they inhibit lipid oxidation in MST, metal chelators were added to MST to see whether the chelators bind endogenous metals present in MST and prevent lipid oxidation. Ethylene diamine tetraacetic acid (EDTA) and tetrakis (2-pyridylmethyl) ethylene diamine tetraacetic acid (TPEN) were used as metal chelators. EDTA is an aqueous metal chelator where as TPEN is a membrane permeable metal chelator. EDTA and TPEN were added at 2 mM/kg MST and were thoroughly mixed with a spatula. The carrier solvent was 100% ethanol and was used at 1% of the final sample weight. Since EDTA was

not soluble in ethanol, it was first dissolved in distilled water followed by ethanol before adding to MST. A control sample containing 1% ethanol without any metal chelators was also prepared for the respective trials. All samples were stored in zip-lock polyethylene bags at −4 °C during the study period.

2.10. Determination of thiobarbituric acid reactive substances (TBARS)

The progress of lipid oxidation in MST was followed by periodically removing samples and quantifying TBARS formed by the method described by (Lemon, 1975). Approximately, 1 g of the sample was mixed with 6 ml of trichloroacetic acid (TCA) solution (7.5% TCA, 0.1% disodium ethylenediamine tetraacetic acid [EDTA], 0.1% propyl gallate) and homogenized with a Polytron Type (PT) 10/35 (Brinkmann Instruments, Westbury, NY) for 30 s. The homogenate was filtered through Whatman no. 1 filter paper. One millilitre of the filtrate was then mixed with 1 ml of thiobarbituric acid (TBA) (0.02 M) and incubated at 100 °C for 40 min. After cooling the reaction mixture in cold water for 5 min and centrifuging (2000*g* for 5 min) in a Beckman J-6B centrifuge (Beckman Instruments, Inc., Palo Alto, CA), the absorbance was measured at 532 nm. TBARS value was expressed as μmol TBARS/kg of tissue. A standard curve was constructed using tetraethoxypropane as a precursor of malonaldehyde.

2.11. Determination of lipid peroxides

Lipid peroxides were determined according to the method described by Richards and Dettman (Richards & Dettmann, 2003). Approximately 0.3 g of the tissue was homogenized using a Kinematica Polytron PT 10–35 homogenizer (Brinkmann Instruments, Westburg, NY) at speed 6 for 30 s in 10 ml of chloroform:methanol (1:1 v/v). A volume of 3.08 ml of 0.5% NaCl was then added to separate the mixture into two phases. The mixture was then centrifuged at 2000*g* for 5 min and 2.0 ml of chloroform layer was removed using a glass syringe. A volume of 1.33 ml of chloroform: methanol (1:1 v/v) was added to the removed aliquot followed by addition of 25 μl of ammonium thiocyanate and 25 μl of fresh ferrous chloride solution (made by mixing equal volumes of barium chloride (8 g/l) in 0.4 N HCl and ferrous sulfate (20 g/l)). After incubation at room temperature for 20 min, absorbance was read at 500 nm. The standard used was cumene hydroperoxide.

2.12. Statistical analysis

The MIXED procedure of the SAS system was used to analyze the data obtained from lipid oxidation and partitioning studies. This procedure implements random effects in the statistical model and permits modeling the covariance structure of the data (Littell, Henry, & Ammerman, 1998). For lipid oxidation studies, two separate reaction mixtures for each treatment were prepared and used as the source of variation for the statistical analysis. Two indicators of lipid oxidation were measured for each treatment during the storage study. Means were separated by least significance difference test. Samples were drawn from each experimental unit at various time points and hence repeated measures were obtained for each treatment. In this model, the variation between the experimental units within a treatment is specified by the 'random' statement where as the covariation within the experimental units is specified by the 'repeated' statement. Since the repeated measures were not taken at equal intervals of time, 'sp(pow)(d)' was fitted into the model to account for this variation. Partitioning studies in isolated MST membranes were done in duplicates. For determining the partitioning of quercetin and quercetin-β-D-glucoside in chicken breast muscle membranes, two separate studies were done. In each study, the treatments were conducted in duplicates. For the partitioning and scavenging studies, Student's t-test was performed. Significance was defined at $p \leqslant 0.05$.

3. Results

3.1. Ability of quercetin and quercetin-β-D-glucoside to inhibit lipid oxidation in MST

A direct comparison between quercetin and quercetin-β-D-glucoside to inhibit lipid oxidation was carried out in MST at −4 °C. The level of addition was 100 μmol/kg MST. The antioxidative activity was measured by the ability of the flavonols to inhibit the formation of TBARS and lipid peroxides in MST during the storage study (Fig. 2). With regard to TBARS formation, the lag phase was defined as the time it took for TBARS values to exceed 20 μmol/kg of MST during the storage period. TBARS reached 20 μmol/kg within a 14 day period in the control, whereas the samples containing quercetin-β-D-glucoside and quercetin took an additional 3 days and 13 days respectively to reach the 20 μmol/kg threshold. Significant inhibition ($p < 0.05$) in TBARS formation was observed in samples with quercetin compared to samples with quercetin-β-D-glucoside. There was no significant difference ($p > 0.05$) in terms of inhibiting lipid oxidation when comparing control and samples containing quercetin-β-D-glucoside. Similarly significant inhibition ($p < 0.05$) in lipid peroxide formation was observed in quercetin treated samples when compared to samples containing quercetin-β-D-glucoside.

3.2. Partitioning of quercetin and quercetin- β-D-glucoside in crude membrane isolates

To study the partitioning of querectin and quercetin- β-D-glucoside, a model system consisting of membranes isolated from MST and aqueous methanolic solutions of flavonols were used. Quantification of flavonols was done by HPLC. Quercetin dissolved in methanol:water (1:1 v/v) was added to membrane at 367 μg, homogenized and centrifuged. The concentration of quercetin in

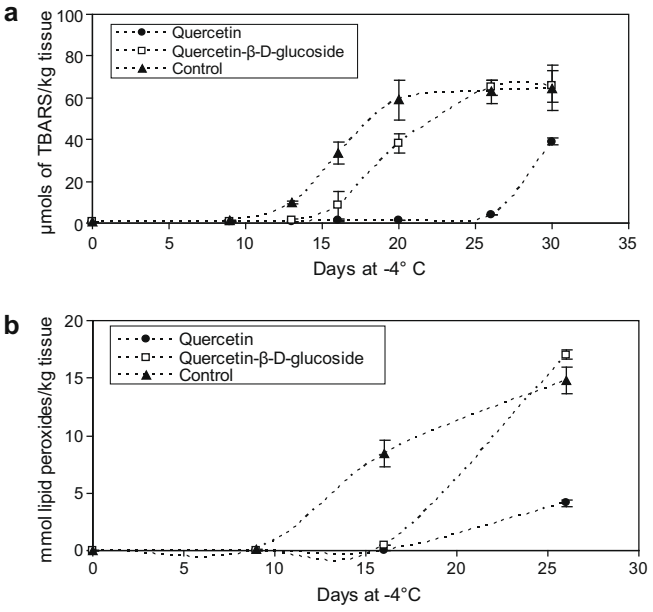

Fig. 2. Effect of quercetin and quercetin-β-D-glucoside on the formation of (a) TBARS and (b) lipid peroxides in MST. Both quercetin and quercetin-β-D-glucoside were added at 100 μmol/kg MST. Ethanol was used as the carrier solvent at 1% of the final sample weight. Results are expressed as average ± standard deviation from duplicate samples.

Table 1
Partitioning of quercetin and quercetin-β-D-glucoside in crude membrane suspensions[a].

Sample	Flavonol added to membranes[b] (μg)	Flavonol recovery (μg)		
		Supernatant phase[c]	From membranes	
			Methanol:water phase[d]	Chloroform phase[d]
Quercetin	367	267.5 ± 0.002	43 ± 0.01	2.41 ± 0.75
Quercetin-β-D-glucoside	464	368.0 ± 0.70	35 ± 5.65	ND

ND – not detected. Results are expressed as average ± standard deviation from duplicate samples.
[a] $n = 2$, where n = number of replicates for each experiment.
[b] Amount of quercetin and quercetin-β-D-glucoside (μg) added to membranes isolated from 10 g of MST.
[c] The amount of quercetin and quercetin-β-D-glucoside determined in the solution after sedimentation of the membranes by ultracentrifugation.
[d] The amount of quercetin and quercetin-β-D-glucoside that partitioned into the membranes and recovered from methanol:water and chloroform phases used for extracting lipids and flavonols from membranes.

the supernatant was 267.5 μg. The quercetin that partitioned from the aqueous suspension into the membrane pellet was then extracted using chloroform:methanol and the two phases were separated using sodium chloride solution. Quercetin from the membranes was found to partition between methanol-water (43 μg) and chloroform phases (2.41 μg). When quercetin-β-D-glucoside was added to membranes at 464 μg, the supernatant contained 368 μg of glucoside whereas the recovery of the glucoside in the methanol–water phase was 35 μg. No glucoside could be detected in the chloroform layer (Table 1). When expressed on a percentage basis, around 12.4% of added quercetin partitioned into the membranes whereas only 7.5% of added quercetin-β-D-glucoside partitioned into the membranes ($p = 0.09$).

3.3. Partitioning of quercetin and quercetin-β-D-glucoside in chicken breast muscle

There was a low membrane total lipid ratio (around 0.38) in membranes isolated form MST (Table 2). This indicated a substantial amount of contamination of neutral lipids with the membrane phospholipids. This is problematic when estimating the amount of flavonols that partitions into the membrane phase when flavonols are first added to muscle. Membranes isolated from minced, chicken breast muscle had a membrane phospholipid/total lipid ratio ranging from 0.63–0.72 which indicated a relatively pure membrane preparation. The chemical characteristics of membranes obtained from MST and chicken breast muscle are shown in Table 2. The higher neutral lipid contamination in membranes isolated from MST is likely due to the higher amount of neutral lipid present and greater amount of shearing and tissue disruption when preparing MST compared to minced chicken breast.

Quercetin and quercetin-β-D-glucoside dissolved in ethanol (1% muscle weight) were added to minced chicken breast muscle at 100 μmol/kg tissue and minced for 30 s. The cellular membranes were then physically isolated from the muscle. The isolated membranes were then dissolved in a mixture of chloroform and methanol followed by addition of sodium chloride solution in an effort to capture all of the flavonols since these compounds could have some solubility in each of the different solvent phases. Quercetin that separated with the isolated membranes partitioned into both the chloroform phase and methanol-water phase resulting in recoveries of 20.26 μg and 88.42 μg, respectively (Table 3). This constituted a 32.1% recovery based on the added level (338.26 μg quercetin/10 g muscle). When quercetin-β-D-glucoside was added to the breast muscle, the compound was not detected in either the chloroform or the methanol-water phase (Table 3). Recovery of flavonols was calculated based on the assumption that the concentration of flavonols in the membrane fraction obtained by centrifugation was the same as that of the unrecovered fraction of membranes in the muscle tissue.

Table 2
Comparison of chemical characteristics of mechanically separated turkey (MST) and chicken breast muscle (CBM).

Sample	Membrane TL content (mg)	Membrane PL content (mg)	PL(membrane)/TL(membrane)	PL(mem)/PL(muscle)
MST	16.45 ± 0.07	6.12 ± 0.73	0.38 ± 0.03	0.22 ± 0.02
CBM	24.20 ± 1.41	16.24 ± 1.06	0.67 ± 0.08	0.35 ± 0.02

The abbreviations TL and PL represent total lipid and phospholipid contents respectively. Membrane TL content indicates the total lipid extracted from membranes obtained from 10 g of MST or minced chicken breast muscle. Membrane PL content indicates the phospholipid content of membranes obtained from 10 g of MST or minced chicken breast muscle. PL (muscle) indicates the phospholipid content of 10 g of MST or chicken breast muscle. Results are expressed as average ± standard deviation from duplicate measurements.

Table 3
Partitioning of quercetin and quercetin-β-D-glucoside in membranes after each flavonol was added to minced chicken breast muscle[a].

Sample	μg added flavonol/10 g muscle[b]	Flavonol recovery (μg)		% Flavonol recovery[d]
		Methanol:water phase[c]	Chloroform phase[c]	
Quercetin	338.26	88.42 ± 4.79	20.26 ± 2.39	32.13%
Quercetin-β-D-glucoside	464.38	ND	ND	–

ND- not detected. Results are expressed as average ± standard deviation.
[a] n = 4, where n = number of replicates for each experiment.
[b] Amount of quercetin and quercetin-β-D-glucoside (μg)/10 g of minced chicken breast muscle.
[c] The amount of quercetin and quercetin-β-D-glucoside that partitioned into the membranes isolated from 10 g of muscle and recovered from methanol:water and chloroform phases used for extracting lipids and flavonols from membranes.
[d] Recovery of flavonols was calculated based on the assumption that the concentration of flavonols in the membrane fraction obtained by centrifugation was the same as that of the unrecovered fraction of membranes in the muscle tissue.

Table 4
Hydroxyl radical scavenging activities of quercetin and quercetin-β-D-glucoside. Results are expressed as average ± standard deviation from duplicate samples.

Flavonol (μg/1.2 ml reaction mixture)	HRSA (%)
Quercetin – 18 μg	22.81 ± 0.20
Quercetin – 36 μg	35.05 ± 0.13
Quercetin-β-D-glucoside – 18 μg	24.95 ± 2.01
Quercetin-β-D-glucoside – 36 μg	37.24 ± 1.14

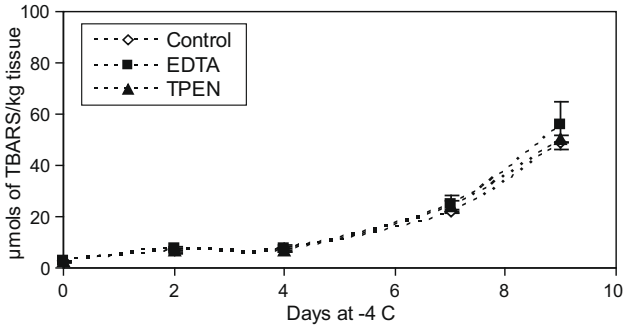

Fig. 3. Effect of metal chelators, EDTA and TPEN on the formation of TBARS in MST. Both the metal chelators were added at 2 mM/kg MST. Ethanol was used as the carrier solvent at 1% of the final sample weight. Results are expressed as average ± standard deviation from duplicate samples.

3.4. Hydroxyl radical scavenging activity

It was thought that part of the mechanism by which quercetin more effectively inhibited lipid oxidation in MST compared to quercetin-β-D-glucoside could be due to differences in free radical scavenging abilities. The deoxyribose assay was used to assess the ability of each flavonol to scavenge hydroxyl radicals. The scavenging ability of quercetin and quercetin-β-D-glucoside at concentrations of 18 and 36 μg per 1.2 ml of reaction mixture are shown in Table 4. Both quercetin and its glucoside at varying concentrations exhibited similar scavenging activities.

3.5. Ability of metal chelators to inhibit lipid oxidation in MST

Another mechanism by which flavonols act as antioxidants involves the chelation of metals ions like iron and copper. Neither EDTA nor TPEN inhibited lipid oxidation in MST during the storage study (Fig. 3). Thus, endogenous metals present in MST did not seem to play a role as lipid oxidation catalysts in MST which makes it unlikely that the mechanism of inhibition by flavonols involved metal chelation in the muscle matrix.

4. Discussion

The growing interest in the substitution of synthetic food antioxidants by naturally occurring phenolic compounds in fruits and vegetables has led to the screening of compounds in food systems for their antioxidative efficacy. Quercetin and quercetin-β-D-glucoside (Fig. 1) are two flavonols commonly found in berries, citrus fruits, onions, leafy vegetables, etc. (Crozier et al., 2000). Even though the antioxidant efficacy of flavonols have been studied in liposomes, fish lipids and mitochondria (Liao & Yin, 2000; Pazos et al., 2005; Ratty & Das, 1988), little attention has been given towards the structure–activity relationship of flavonols in comminuted muscle food systems. The reason for choosing quercetin and quercetin-β-D-glucoside for comparative study was due to the fact that quercetin identified in a fraction isolated from cranberry juice powder extract was a potent inhibitor of lipid oxidation in MST whereas its glycosylated counterpart did not contribute to the efficacy of the fraction in MST (Kathirvel et al., 2009).

When quercetin and quercetin-β-D-glucoside were added separately to MST, quercetin effectively inhibited lipid oxidation in MST compared to quercetin-β-D-glucoside (Fig. 2). In muscle foods, membrane lipids are the substrates most susceptible to oxidation compared to neutral lipids. Location of flavonols at sites where lipids are prone to oxidation might be an important prerequisite for inhibiting lipid oxidation in MST. In our partitioning study, we did find that quercetin partitioned into the membranes more effectively than quercetin-β-D-glucoside (Tables 1 and 3). The results indicate that the presence of sugar in quercetin-β-D-glucoside was affecting its inhibitory properties in muscle tissue probably by partitioning it away from the membranes. When quercetin-β-D-glucoside was added to minced chicken muscle, no glucoside could be determined in the membranes, which indicates that the polarity conferred by the sugar moiety affected the membrane incorporation of quercetin-β-D-glucoside in the muscle matrix. On the contrary, the non-polar nature of quercetin compared to its glucoside might have enabled quercetin to interact with the polar head groups of phospholipids at the lipid-water interface through the formation of hydrogen bonds. Such an association with the membranes is necessary to scavenge the free radicals at the lipid-water interface and inhibit the propagation of lipid oxidation in the muscle tissue. Studies in membranes composed of dipalmitoylphosphatidylcholine indicated that flavonoids rich in hydroxyl groups strongly interact with the polar portion of the phospholipid molecule at the lipid-water interface (Ollila, Halling, Vuorela, Vuorela, & Slotte, 2002). Quercetin, a planar flavonol has been shown to intercalate into the organized structures of the phospholipids within the vesicle membranes when compared to naringenin, a non-planar flavonone (van Dijk, Driessen, & Recourt, 2000).

The partitioning coefficient (log P) of quercetin in octanol-water was significantly higher than quercetin-3-glucoside, which demonstrates higher lipophilicity of quercetin than its glucoside (Rothwell et al., 2005). In this study, log P of quercetin was 1.82 compared to 0.76 for quercetin-3-glucoside. Log P best represents how a compound behaves in a membrane environment. In the partitioning study carried out in chicken muscle, quercetin-3-glucoside did not associate with the muscle membranes (Table 3). Even though the experimentally determined log P value for quercetin-3-glucoside shows that it partitions into the octanol phase comparatively lesser than quercetin, it seems that the actual partitioning of glycosylated quercetin in biological membranes is quite different. One possible explanation for this difference in partitioning could be due to the complex characteristics of the muscle system compared to the octanol–water system. The different partitioning data obtained while using isolated membranes from MST and chicken muscle system could also be due to the differences in the test system studied. When the glucoside is added directly to the isolated membrane system, the chances of glucoside interacting with the membranes are higher compared to when it is added directly to muscle. It seems that when the glucoside is added directly to muscle, it comes in contact with the myofibrillar proteins and the aqueous phase of the muscle and remains there without becoming incorporated into the muscle membranes because of its hydrophilic nature compared to quercetin.

A potential mechanism of inhibition involves the ability of flavonols to scavenge hydroxyl radicals. Hydroxyl radicals form in muscle tissue mostly due to the ability of low molecular weight metals like iron and copper to react with hydrogen peroxide (Koppenol, 1993; Rowley & Halliwell, 1983). Our results indicated similar hydroxyl radical scavenging abilities of quercetin compared to quercetin-β-D-glucoside (Table 4). The 3′, 4′ hydroxyl groups in the

B ring (catechol group), the 2, 3 double bond in conjugation with the 4-oxo group in the C ring and the presence of hydroxyl groups in position 3 and 5 are believed to be responsible for the free radical scavenging activity of quercetin (Hudson & Lewis, 1983; Pietta, 2000)(Fig. 1). Our data suggests that the presence of the hydroxyl group in position 3 of C ring in quercetin did not enhance its hydroxyl radical scavenging ability compared to quercetin-β-D-glucoside in which the 3 position is occupied by linkage to the sugar moiety. Since both the flavonols showed similar aqueous radical scavenging abilities, it seems that scavenging chain-propagating lipid radicals at the lipid-water interface or the hydrophobic core of the membrane by quercetin is necessary to inhibit lipid oxidation in MST rather than the scavenging of radicals in the aqueous phase of the muscle tissue. Quercetin showed a greater protective effect than rutin in inhibiting autoxidation of rat cerebral membranes (Saija et al., 1995) where as quercetin showed lesser antioxidant activity than rutin in inhibiting Fe^{2+}induced lipid oxidation in both liposome and human erythrocyte membrane ghost systems (Liao & Yin, 2000). In the first case, the protective effect could be attributed to quercetin interacting with the membranes and scavenging the free radicals produced near the membranes thereby inhibiting the propagation of lipid oxidation. In the second case, rutin being hydrophilic was able to interact with water soluble oxidants like $Fe^{2+}$and prevent lipid oxidation where as quercetin associated more with the membranes and was not available to scavenge the aqueous radicals. These studies support the fact that the antioxidant activity of flavonols not only depends on the ability of flavonols to associate with the biomembranes, but also the type of oxidants involved in the test system being studied. Trace amounts of lipid peroxides have been reported to be present in muscle tissue (Nakamura, Tanaka, Higo, Taira, & Takeda, 1998) and has been shown to promote methemoglobin induced lipid oxidation in fish muscle (Richards & Hultin, 2000) and Cu^{2+}stimulated oxidation of low density lipoproteins (Thomas, Kalyanaraman, & Girotti, 1994). Lipid radicals such as alkoxyl and peroxyl radicals are constantly generated in the membranes by the breakdown of preexisting lipid peroxides by trace metals and heme proteins (Kanner, German, & Kinsella, 1987; Patel, Svistunenko, Wilson, & Darley-Usmar, 1997). These lipid radicals generated can abstract a hydrogen atom from neighboring polyunsaturated fatty acids and propagate further lipid peroxidation. Hence the presence of flavonols at or near the membranes might be conducive to scavenge the free radicals and inhibit lipid oxidation processes. The results from partitioning as well as oxidation studies demonstrates how differences in the ability of quercetin and quercetin-β-D glucoside to partition into the muscle membranes influenced the inhibitory properties in muscle tissue.

Another antioxidant mechanism of flavonols involves chelation of metal ions like iron and copper which can be promoters of lipid oxidation in muscle tissue. The metal chelates once formed are only slightly active in the promotion of free-radical reactions. Quercetin has been shown to reduce $Fe^{3+}$to $Fe^{2+}$and chelate Fe^{2+} (Mira et al., 2002). This study described pH dependant changes in metal complexation sites of flavonols. At pH 5.5, the complexation site was probably between the C5 hydroxyl group and 4-oxo group whereas the chelating ability of the catechol group increased at alkaline pH (7.4) due to ionization of phenolic groups at high pH. This suggests that the better ability of quercetin to inhibit lipid oxidation in MST compared to its glucoside was not due to metal chelating differences between C5 hydroxyl group and 4-oxo group as these groups are available in both compounds. Another study with rutin, a flavonol glycoside, and quercetin showed similar abilities of both the compounds to prevent Cu^{2+}induced peroxidation of LDL which suggests that the presence of a sugar moiety at position 3 of the C ring did not affect the Cu^{2+}chelating ability of rutin (Brown, Khodr, Hider, & Rice-Evans, 1998). In other words, this study suggests that the 3′, 4′ hydroxyl groups in the B ring (catechol group) of flavonols is responsible for metal chelation rather than the 4-oxo group in the C ring with either of its adjacent hydroxyl groups.

Even though sufficient information regarding the metal chelating abilities of flavonols is available from literature, we wanted to test if the metals present in MST were promoters of lipid oxidation in muscle tissue. This would help us understand whether metal chelation by flavonols is a significant contributor towards the antioxidant activity of flavonols in the muscle matrix. Two metal chelators, EDTA and TPEN were added to MST and their ability to inhibit the formation of TBARS was determined. EDTA is a known water soluble metal chelator where as TPEN has been shown to permeate the membranes and chelate metals (Karck et al., 1992). MST has some calcium content because its preparation process involves the passage of turkey frames and necks through a deboner which can incorporate fine bone fragments in the tissue. The content of calcium in MST has been estimated to be 145 mg per 100 g tissue where as iron and copper contents have been reported to be 1.61 mg and 0.093 mg per 100 g tissue respectively (USDA nutrient database). Since metal ions like iron and copper are considered to be promoters of lipid oxidation in muscle tissue, the metal chelators were added in excess at 2 mM/kg MST to allow for all of the iron and copper to be chelated along with any of the calcium. Neither of the metal chelators inhibited lipid oxidation (Fig. 3), which suggests that endogenous metals present in MST were not promoters of lipid oxidation in MST. Based on this study, it seems that metal chelation did not contribute towards the ability of flavonols to inhibit lipid oxidation in MST. Studies on the inhibitory effects of flavonoids on LDL oxidation induced by heme protein (metmyoglobin) suggests that metal chelation is less important compared to peroxyl radical scavenging activity (Rice-Evans, Miller, & Paganga, 1996).

One of the reasons for the high propensity of MST to lipid oxidation is due to its compositional nature. MST is rich in hemoglobin, lipids and low levels of tocopherol (Dawson & Gartner, 1983). Studies show that flavonoids can interact with hemoglobin and cause conformational changes to the heme protein (Xi & Guo, 2007). Moreover their study showed that the binding capacity of rutin, a flavonol glycoside, to bovine hemoglobin is lower compared to quercetin. One plausible explanation for this difference is due to the steric hindrance caused by the bulky sugar moiety in rutin which affects its penetration into the hydrophobic pocket of hemoglobin. It is not known whether structural differences between quercetin and quercetin-β-D-glucoside could cause any change in the mode of binding to hemoglobin in MST and whether the binding characteristics could influence hemoglobin-mediated lipid oxidation in MST. If such a mechanism occurred in MST, based on the results from the oxidation study (Fig. 2) and aforementioned study, it seems that querectin may be able to bind hemoglobin better than its glucoside and inhibit heme protein-mediated lipid oxidation in MST.

Flavonoids have been reported to induce changes in membrane fluidity which is a determinant in the rate of membrane oxidation. Increase in membrane fluidity causes an increased motion of fatty acids which was described to lower the probability of lipid radical interactions with other fatty acids thereby reducing the propagation of lipid oxidation (Oteiza et al., 2005). The antioxidant activity of flavonoids in liposomes composed of dipalmitoylphospatidylcholine has been related to its capacity to induce a decrease in the membrane transition temperature (T_m), which is indicative of a less ordered membrane (Saija et al., 1995). On the other hand, an increase in T_m has been described for the flavonoids in 1-stearoyl-2-linoleoylphosphatidylcholine liposome membranes (Arora, Byrem, Nair, & Strasburg, 2000). These opposite findings have been attributed to the different composition of the membranes studied. In the first study, the liposomes composed of dipalmitoyl-

phospatidylcholine had an ordered arrangement that was interrupted by the incorporation of the flavonoids between the acyl chains of the phospholipids whereas in the second study the liposomes composed of 1-stearoyl-2-linoleoyl phosphatidylcholine had a less ordered phase first that was modified to a highly ordered phase and thus an increased T_m due to the flavonoid incorporation in the bilayer core. Apart from membrane fluidity changes, the localization of flavonoids in the membrane layer can sterically hinder diffusion of free radicals and thereby decrease the kinetics of free radical reactions (Arora et al., 2000). The ability of quercetin to effectively inhibit lipid oxidation in MST might be due to its ability to cause changes in the membrane fluidity as well as to form a barrier against the access of oxidants into the membranes layer. Further research is required to understand how different flavonoids modulate the fluidity of biological membranes and how these changes influence lipid oxidation processes.

In conclusion, our studies indicate that quercetin is a potent inhibitor of lipid oxidation in MST compared to its glycoside. The presence of the sugar moiety decreased the inhibitory properties of quercetin-3-D-glucoside in MST, probably by partitioning it away from the membranes. The activity of quercetin in MST seems to be due to its higher membrane affinity which enables it to be an efficient free radical scavenger. Factors such as changes in membrane fluidity and flavonol–hemoglobin association also may have contributed to increased oxidative stability of MST. The physical location and orientation of flavonols at sites where the lipids are prone to oxidation is likely to be a determinant in reducing lipid oxidation in muscle tissues. Future research should focus on investigating strategies to drive the flavonols into membrane lipids which are the target sites of lipid oxidation in muscle tissue.

Acknowledgements

This work was supported in part by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, HATCH project WIS04904, the Wisconsin Cranberry Board, Inc. and the University of Wisconsin – Madison Industrial and Economic Development Research (I & EDR) Program.

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Food Chemistry 117 (2009) 83–87

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Composition and characteristics of oil extracted from flaxseed-added corn tortilla

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ARTICLE INFO

Article history:
Received 25 September 2008
Received in revised form 4 February 2009
Accepted 19 March 2009

Keywords:
Tortilla
Flaxseed
Chemical composition
Unsaturated fatty acids

ABSTRACT

Flaxseed has recently gained attention as a functional food, and the effect of adding flaxseed (10%, 15%, and 20%) to tortillas was evaluated. The physicochemical characteristics and the free fatty-acid (FFA) content of the oil extracted from flaxseed-added corn tortilla were determined. The results showed that the lipid (4.27%) and protein content (9.10%) of the control sample was statistically lower ($p \leqslant 0.01$) than the tortillas added with flaxseed. In general, the total amylose content did not change with the flaxseed-added content; however, the amylose apparently decreased with the amount of flaxseed, indicating the presence of starch–lipid complexes. The saponification value was 95.37 (mg KOH/g oil) in the tortilla added with 10% flaxseed and increased to 100 (mg KOH/g oil) for the 20% flaxseed treatment. The peroxide value was observed to increase ($p < 0.05$) when the flaxseed flour was added at 10%, 15%, and 20%. In this study, the tortillas exhibited a high amount of total unsaturated fatty acids, 26.32–30.08% (oleic acid). Thus, the flaxseed-added corn tortilla could represent a valuable staple in improving the nutritional value of the original food product.

1. Introduction

In Mexico and some Central American countries, tortillas are the main source of protein (50%) and calories (70%) in the diet of the people with low economic resources (Bressani, 1990; Gomez, Rooney, & Waniska, 1987). Masa for tortillas is traditionally made by cooking corn in a calcium carbonate solution, steeping, and milling to produce nixtamal (Martínez-Flores, Martínez-Bustos, Figueroa, & González-Hernández, 2002; Ortega, Villegas, & Vasal, 1986; Vivas, Waniska, & Rooney, 1987); however, amaranth also was nixtamalised for tortilla production (Valdez-Niebla, Paredes-López, Vargas-López, & Hernández-López, 1993). Recently, in order to improve the nutritional quality of tortilla, transgenic maize (genetically modified maize with the cDNA of amarantin) was nixtamalised and some technological and nutritional characteristics were evaluated (Ayala-Rodriguez et al., 2009). On the other hand, dry instant corn-masa flours have been developed for industrial production and introduced into the marketplace (Almeida-Dominguez, Cepeda, & Rooney, 1996). Dry instant corn-masa has a much longer shelf-life than the traditional wet corn-masa, and contains other added ingredients, such as amaranth flour, vitamins and minerals, hydrocolloids (Rendón-Villalobos, Agama-Acevedo, Islas-Hernández, Sánchez-Muñoz, & Bello-Pérez, 2006), etc., to increase the functionality of these flours. As a result, the demand for instant corn-masa is increasing in Mexico and the United States.

The whole nixtamalised corn kernel includes the germ (embryo), which contains most of the oil of the grain. The germs are ground in the process of making instant corn-masa, liberating oil and coating the endosperm particles with oil (Vidal-Quintanar, Love, Love, White, & Johnson, 2003). Nixtamalised corn may also be a good source of essential fatty acids contained in the germ oil. It is known that corn oil is a good source of unsaturated fatty acids, such as linoleic acid, which are essential fatty acids for human beings. Essential fatty acids cannot be produced by humans and need to be ingested from food. Generally, corn oil contains 61.9% of linoleic acid (Weber, 1999). According to Ulusoy, Karabulut, and Turan (2004), oils are not stable products, and the factors that are responsible for their low stability include light, oxygen, temperature, unsaturated fatty acids content, and the presence or absence of antioxidants. Oxidation of unsaturated fatty acids can lead to the production of free radicals that create hydroperoxides that may represent a potential danger to human health. However, highly polyunsaturated corn oil is a reasonably stable oil, because it contains high levels of natural antioxidants and <1% linolenic acid (Martínez-Flores, Garnica-Romo, Romero, & Yahuaca, 2006). Linolenic acid is considered as being unstable because it is easily oxidised and is responsible for the development of off-flavour and off-odour in the oil. At present, nutritionists consider that the ideal edible oils should contain about 3.0% linolenic acid (Yoshida, Kanrei, Tomiyama, & Mizushina, 2006).

Flaxseed is a natural source of flavonoids, lignans, and phenolic compounds. It is making its mark on the world's food supply as a functional food because of its nutrients. Flaxseed is rich in

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.03.080

polyunsaturated fatty acids (PUFAs), lignan, dietary fibre, and protein. Furthermore, flaxseed does not contain gluten (Wu, Huff, & Hsiehi, 2007).

The health benefits derived from flaxseed include prevention of cardiovascular disease, stroke, cancer, osteoporosis, diabetes, and improvement of immune function, blood pressure, and kidney function (Morris, 2004). Several flaxseed food products are now available in the market. For example, whole or ground flaxseed is added to rolls, muffins, bagels, pasta, cereal, energy bars, and dry mixes for pancakes (Ahmed, 1999; Manthey, Lee & Hall, 2002; Morris, 2004; Muir & Westcott, 2000; Nesbitt & Thompson, 1997). Furthermore, consumers can also purchase whole and ground flaxseed and flaxseed oil in the supermarkets or health food stores.

Phospholipids (PLs) composed of PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have attracted attention as important minor nutrients (Siswoyo, Mukoyama, & Morita, 2000). Recently, PLs have been extensively used throughout the world as an emulsifier, to improve the functional properties in food processing: improvement in bread texture and volume, dough tolerance, baking properties, and to retard the bread-becoming state (Kweon et al., 1994; Lin & Czuchajowska, 1998). The PUFA-PLs can also improve the quality of bread and retard the retrogradation of starch, owing to the formation of complexes between starch and lipids. Supplementation of bread with the PUFA-PLs is another way to obtain these nutrients for people who dislike eating fish (Siswoyo et al., 2000).

Research has been conducted on the characterisation of flaxseed, but less attention has been paid to the effect of incorporating whole flaxseed meal into foods.

In recent years, considerable interest has been generated in the development and consumption of foods enriched with various healthy ingredients. However, there is no information available about the chemical changes that occur in the lipid fraction of tortilla with added flaxseeds. Therefore, the objective of this study was to supplement nixtamalized corn flour (NCF) with flaxseed to evaluate the physicochemical characteristics and free fatty-acid content of oil extracted from flaxseed-added corn tortilla.

2. Materials and methods

2.1. General

Corn tortillas were produced in the Quality Control Laboratory, CEPROBI-IPN. The NCF (MASECA®, León, Mexico) and flaxseed flour (Nutrisa®, SA de CV, Distrito Federal, México) were obtained from selected supermarket in Cuernavaca, Mor. Mexico.

2.2. Tortilla preparation

Corn tortillas were produced using the method described by Islas-Hernández, Rendón-Villalobos, Agama-Acevedo, Tovar, and Bello-Pérez (2007). Tortillas were processed from 100% NCF (control) and from a mixture of 10%, 15%, and 20% flaxseed flour with NCF, respectively, followed by the addition of water to form masa. The masa was pressure-molded and extruded into thin circles to make tortillas of 1 mm thickness. Tortillas were baked in a gas-fired domestic oven for 1 min per side at ≈250 °C, cooled to 30 °C, and then freeze-dried in liquid nitrogen.

2.3. Proximate analyses

Moisture content of the sample was determined by heating a sub-sample of 3 g in a vacuum oven at 50°C for 1 h (44–15, AACC International, 2000). Ash and protein were analysed according to the Approved Methods 08–01 and micro-Kjeldahl method 46–1 (AACC International, 2000). The total dietary fibre (TDF) was measured according to the AOAC method 985.29 (AOAC, 2000). Samples were gelatinized with a heat-stable -amylase (pH 6, 100 °C, 30 min), and then enzymatically digested sequentially with protease (pH 7.5, 60 °C, 30 min) and amyloglucosidase (pH 6, 0 °C, 30 min) to remove protein and starch. The TDF was precipitated with ethanol, and after washing and drying, the residue was weighed. The results were corrected for protein and ash. Lipid content was assessed with the 30–25 AACC method (AACC International, 2000). The amylose content of the isolated starches was expressed as the apparent amylose and total amylose content using the method of Hoover and Ratnayake (2002).

2.4. Oil extraction

Free lipids (oil) were extracted from each sample with ethyl ether as a solvent, for 5 h using a Soxhlet extractor, following the AACC Method 30-25 (AACC International, 2000).

2.5. Determination of saponification and peroxide values, and free fatty-acid (FFA) contents

The saponification value (SV) was determined by AOAC (2000) method 920.160. In duplicate, about 2 g of the oil sample was added to a flask with 30 ml of ethanolic KOH, and then attached to a condenser for 30 min to ensure that the sample was fully dissolved. After the sample was cooled, 1 ml of phenolphthalein was added and titrated with 0.5 M HCl until a pink endpoint was reached. Tortilla oil oxidation was determined using the official AOCS Peroxide Value (PV) method Cd 8–53 (AOCS, 1997, revised 2004). This iodometric titration method measures the peroxides and hydroperoxides that are the initial products of lipid oxidation (Pike, 1994). The results were expressed as milliEquivalents (mEq) of active oxygen per kg of oil. The FFA content in the oil was determined by using a modified FFA method (AOCS, 1997 revised 2004). Heated (60 °C) isopropyl alcohol (0.75 ml) was added to 75 mg of oil in a 25-ml Erlenmeyer flask. Then, phenolphthalein (0.5% in 95% ethanol) was added to the solution. The fatty-acid content was determined by titrating with 0.001 N NaOH.

2.6. Statistical analysis

All the tests were carried out in triplicate and the values were expressed as mean ± SE. Differences among the treatments were evaluated by one-way ANOVA with *post hoc* mean ranking test using Tukey. Mean values with a statistical difference of $p \leqslant 0.05$ were considered as significant. The data figures were plotted using Sigmaplot 2.0 (Systat Software, Inc. San Jose, CA, USA).

3. Results and discussion

3.1. Proximate analyses

Moisture value in the flaxseed-added tortilla decreased; however, the flaxseed level did not show any statistical difference ($p < 0.05$) (Table 1). This pattern might be related with the hydrophobic character of the flaxseed oil. An increase in the protein content was observed with respect to the level of flaxseed added to the tortilla. Flaxseed flour has 21% higher protein level (Ramcharitar, Badrie, Mattfeldt-Bemam, Matsuo, & Ridley, 2005) than that in the NCF (9.33%) (Agama-Acevedo et al., 2004), which influenced this parameter. Figueroa et al. (2001) observed 3% higher protein content when the tortilla was fortified with 4% of defatted soy. This increase in the protein content in flaxseed-added tortilla might be important to increase the essential amino acid level that is deficient in corn.

Table 1
Average chemical composition (%) of tortilla containing different flaxseed level.

Sample	Protein[A,B]	Ash[A]	TDF	Moisture
Control	9.10 ± 0.12[a]	1.31 ± 0.01[a]	16.45 ± 0.23[a]	47.60 ± 0.34[a]
Tortilla + 10% flaxseed	10.87 ± 0.03[b]	1.54 ± 0.01[a]	19.75 ± 0.04[b]	43.80 ± 0.58[b]
Tortilla + 15% flaxseed	11.60 ± 0.32[b,c]	1.44 ± 0.01[a]	21.84 ± 0.06[c]	44.10 ± 0.32[b]
Tortilla + 20% flaxseed	12.93 ± 0.03[d]	1.64 ± 0.03[a]	23.50 ± 0.17[d]	44.13 ± 0.32[b]

Results are means of three replicates ± SEM. Means with the same superscript letters within a column are not significantly different at $p < 0.05$ level by Tukey's multiple range test.
SEM: standard error of the means ($n = 9$).
TDF = total dietary fiber.
[A] Composition expressed on a dry weight basis.
[B] $N \times 5.85$.

As judged from the equivalent ash concentration recorded in both types of tortillas, the substitution level employed was not enough for altering the mineral content of the baked products in a significant way ($p > 0.05$). Similar result was obtained for tortillas added with amaranth flour (1.4% for tortilla from 100% maize, and 1.5% for corn/amaranth tortilla) (Islas-Hernández et al., 2007).

Furthermore, a significant increase in the TDF content was also obtained with the flaxseed-flour addition (approximately between 20% and 50%), demonstrating that flaxseed is a good TDF source (28%) (Ramcharitar et al., 2005). Today, flaxseed is used as a good source of soluble and insoluble fibre to reduce blood cholesterol and promote laxation (Payne, 2000). Soluble fibre is approximately a quarter of the total fibre in the flaxseed. Most of the soluble fibre is made up of mucilaginous gum, and up to 7–10% flaxseed is mucilage (Carter, 1993). Mucilage is mostly composed of pentosan and arabinoxylan (Erskine & Jones, 1957; Garden-Robinsosn, 1994; Muralikrishna, Salimath, & Tharanathan, 1987), which has water-binding properties similar to guar gum (Fedeniuk & Biliaderis, 1994). Owing to this increase in the TDF, flaxseed-added tortilla can be considered as a nutraceutical food, because dietary fibre content is one of the interesting health-related features of food (Sambucetti & Zuleta, 1996).Therefore, tortilla could represent a valuable staple in improving the nutritional value of the original food product, providing additional health benefits These types of products are commonly known as functional foods, defined as "food similar in appearance to conventional foods that are consumed as part of the normal diet and have demonstrated physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions" (Health Canada, 1998) .

The amount of lipids in the flaxseed-added tortilla increased approximately between 100% and 200% (Table 2). This increase is important in two senses: from (1) the nutritional point of view, because flaxseed contains 40–60% lipid, in which about 50% is α-linolenic acid (ALA) (Dorrel, 1970). The ALA, a short-chain omega-3 fatty acid, is the precursor fatty acid for the synthesis of EPA and DHA, both of which have been linked to controlling cardiovascular diseases (Bibus, Stitt, & Holman, 1998; Goodnight, 1993; Simopoulos, 1999), and (2) the technological point of view, where the lipids decrease the starch retrogradation of the starchy products and increase the shelf-life, owing to the formation of amylose–lipid complexes (Biliaderis, 1991), as was demonstrated in bread.

Certain starches contain lipids, and additional lipids or surfactants are added to modify the texture. For example, emulsifiers are added to reduce the stickiness in pasta, mashed potato, and candy, and to prolong the shelf-life of bread (Biliaderis, 1991; Eliasson & Wahlgren, 2004). In general, the total amylose content was not different ($p < 0.05$) in the diverse tortilla analysed (Table 2). This pattern is due to the fact that flaxseed has a low level of starch (Carter, 1993) that does not influence the amylose content of flaxseed-added tortilla. When the apparent amylose level was determined in the tortilla, this parameter decreased when the flaxseed level increased in the blend (Table 2). This pattern demonstrates that amylose complexed with lipids when they are eliminated, and no difference was observed in the total amylose quantified among the different tortillas analysed. However, the apparent amylose amount is in agreement with the oil content determined in the flaxseed-added tortilla. When amylose is quantified using the iodine staining method, the results depend on whether the starch has been defatted or not, to separate the lipid of the amylose and determine the polysaccharide. However, both amylose and amylopectin can form inclusion complexes with lipid-like molecules. The inclusion complex in itself is probably very similar between the two starch polymers; however, the organisation and supramolecular structures differ between them (Eliasson & Wahlgren, 2004). On the other hand, the enzymatic hydrolysis of amylose in the amylose–lipid complexes was reduced, and a delay in the amylolysis of the amylose in the amylose–lipid complexes was observed (Cui & Oates, 1999; Tufvesson, Skrabanja, Björck, Liljeberg, & Eliasson, 2001). The presence of starch polymer–lipid complexes in the flaxseed-added tortilla, determined by the apparent amylose content, is in agreement with the higher resistant starch content and lower hydrolysis percentage when the flaxseed level in the tortilla increased (Rendón-Villalobos, Bello-Pérez, and Tovar, 2009).

Table 2
Oil and amylose content (%) in tortilla containing different flaxseed level.

Sample	Oil	Total amylose	Apparent amylose
Control	4.27 ± 0.03[a]	26.29 ± 0.16[a]	24.53 ± 0.39[a]
Tortilla + 10% flaxseed	8.23 ± 0.01[b]	25.27 ± 0.00[a]	22.38 ± 0.16[b]
Tortilla + 15% flaxseed	9.98 ± 0.08[c]	24.92 ± 0.32[a]	21.02 ± 0.32[b]
Tortilla + 20% flaxseed	12.00 ± 0.16[d]	24.34 ± 0.16[a]	18.67 ± 0.32[c]

Results are means of three replicates ± SEM. Means with the same superscript letters within a column are not significantly different at $p < 0.05$ level by Tukey's multiple range test.
SEM: standard error of the means ($n = 9$).

3.2. Saponification value (SV)

The results of this study revealed that the SV increased when the flaxseed level rose in the tortilla (Fig. 1). The increase in SV value is owing to the formation of higher amount of short fatty acids and glycerides (Sankaram, 1966), because when higher amount of lipids are present, a determined level of them are complexed with amylose and the rest are free, because during the cooking of the tortilla, the free lipids are degraded to short fatty acids.

However, the SV determined in the flaxseed-added tortilla is considered low, when compared with other usual vegetable oils, such as castor (176–184), soybean (188–198), and rapeseed (170–180) (Codex Alimentairus, 1993), suggesting better stability (Bisht, Sivasankaran, & Bhatia, 1993; Sivasankaran et al., 1988) because flaxseed-added tortilla presented low probability of rancidity problem.

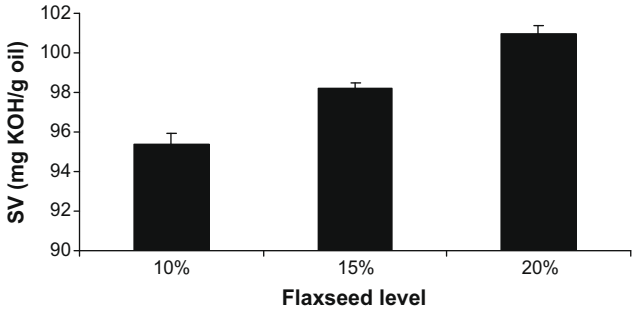

Fig. 1. Saponification value (SV) of oil extracted from tortillas added with different flaxseed level. Error bars represent standard error of the means ($n = 9$).

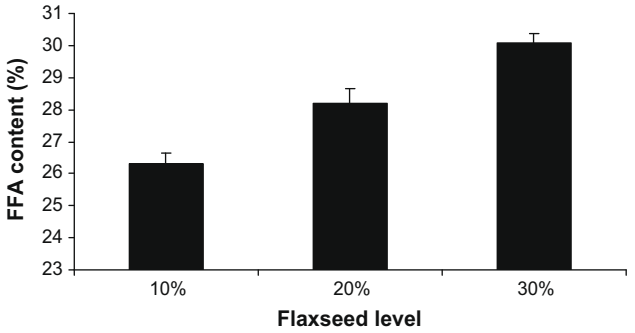

Fig. 3. Free Fatty Acid (FFA) content of oil extracted from tortillas added with different flaxseed level. Error bars represent standard error of the means ($n = 9$).

The high degree of unsaturation of $n-3$ fatty acids indicates that they can become highly susceptible to rancidity, owing to factors, such as heat, light, and oxygen during processing and storage (Goh, Ye, & Dale, 2006).

3.3. Peroxide values (PV)

The results demonstrated the occurrence of a gradual increase ($p < 0.001$) in the PV when the flaxseed flour was added (Fig. 2). This pattern was similar to that of the SV. The PV value is related to the oil oxidation and consequently to the rancidity. These values are below 10, observed in the majority of conventional oils (Codex Alimentarius, 1993).

It was reported that flaxseed oil had a PV value of 0.24 (Jaswir, Kitts, Che Man, & Hassan, 2005), which was lower than those determined in the commercial oils, such as olive (3.37), castor (0.46), and soybean oil (0.5) (Miyasawa, Fujimoto, Kinoshita, & Usuki, 1994; Moh & Stang, 1999; Vekiari, Papadopoulou, & Kiritsakis, 2007), which reflects the low PV value in the flaxseed-added tortilla. The low PV value is an additional characteristic of the flaxseed-added tortilla, because it can be stored for a longer time with no rancidity problems and with soft texture.

3.4. Free fatty-acid (FFA)

The FFA content of the tortilla increased with the level of flaxseed flour added, ranging between 26.32% and 30.08%, with statistical difference ($p > 0.05$) (Fig. 3). The quantification of FFA is related to the SV and PV levels, because the FFA are more susceptible to oxidation and therefore must be taken into consideration with respect to the stability and shelf-life of the food products (Pérez de, Alfaro, & Padilla, 1999). The FFA content is a problem in the food stability, because they are the substrates for lipoxygenase that oxidises unsaturated FFA, resulting in rancidity. The FFA content in durum flour has been reported to range from 9% to 9.9% (Lin, Youngs & D'Appolonia, 1974; Skarsaune, Youngs, & Gilles, 1970).

The monounsaturated fatty acids have great importance owing to their nutritional implication and effects on the oxidative stability of the oils (Aguilera, Ramírez-Tortosa, Mesa, & Gil, 2000). Mixtures of linoleic acid and ALA, as FFA, have been reported to prevent elevations of blood cortisol and cholesterol levels that accompany stressful situations, and check the rapid and repeated eye blinking in rats, following chemical induction of dopamine depletion (Mostofsky, Yehuda, Rabinovitz, & Carasso, 2000).

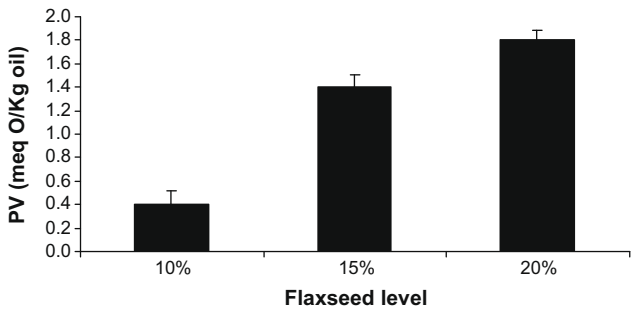

Fig. 2. Peroxide value (SV) of oil extracted from tortillas added with different flaxseed level. Error bars represent standard error of the means ($n = 9$).

4. Conclusions

Flaxseed is a valuable source of various biologically active compounds (essential PUFAs, proteins, phenolic acids, etc.), which serves as the base for a number of effective drugs and prophylactic preparations, as well as important nutritional additives. The formation of starch polysaccharide–lipid complexes was evident owing to the detection of minor apparent amylose content when the flaxseed level increased in the tortilla. Therefore, flaxseed-added corn tortilla could represent a valuable staple in improving the nutritional value of the original food product.

Acknowledgements

The authors wish to acknowledge the economic support from SIP-IPN, COFAA-IPN and EDI-IPN. Technical assistance of Glenda Pacheco-Vargas is also gratefully acknowledged.

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Food Chemistry 117 (2009) 88–93

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Colour, pH stability and antioxidant activity of anthocyanin rutinosides isolated from tamarillo fruit (*Solanum betaceum* Cav.)

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ARTICLE INFO

Article history:
Received 14 November 2008
Received in revised form 20 March 2009
Accepted 23 March 2009

Keywords:
Anthocyanins
Solanum betaceum
Tamarillo fruit
Tree tomato
Colour
Stability
Antioxidant

ABSTRACT

Changes in colour and stability of anthocyanins have been evaluated over pH range 2.0–8.7. The study was made on crude extract (XAD-7 Amberlite-retained fraction) as well as on the following pure pigments isolated from tamarillo fruit (*Solanum betaceum* Cav.): delphinidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranosyl)-3′-*O*-β-glucopyranoside, delphinidin 3-*O*-(6″-*O*-α-rhamnopyranosyl)-β-glucopyranoside, cyanidin 3-*O*-(6″-*O*-α-rhamnopyranosyl)-β-glucopyranoside and pelargonidin 3-*O*-(6″-*O*-α-rhamnopyranosyl)-β-glucopyranoside. The relationships between the colour and the hydroxylation degree of the B ring and the pH have been studied for the first time on rutinosides. The peel extract showed much more colour stability than the jelly extract at all the pH values studied. The replacement of the 3′-OH with a glycosyl group increased the stability of the colour to pH changes, although this substitution yields a less colourful (higher L^* and lower C^*_{ab}) compound (Dp 3-rut-3′-glc), having both hypsochromic and hypochromic shifts relative to the non-glycosylated molecule (Dp 3-rut). Moreover, the influence of the hydroxylation degree of the B ring on the quality and stability of colour, as well as on the antioxidant activity, was determined.

1. Introduction

Most foodstuffs are exposed to some kind of processing before being consumed, which can cause loss of some quality properties, such as colour, aroma or taste; hence producers face the need of replacing these characteristics. Additives (e.g., colourings and flavourings) are used to recover or to emphasize original features, to ensure uniformity, and to guarantee quality.

Pigments are chemical components absorbing radiation in the visible region of the electromagnetic spectrum. The colour is due to a specific molecular group (chromophore) which absorbs energy and, as consequence, the excitation of an electron of external orbitals with major energy occurs; the non-absorbed energy is reflected and refracted and detected by the eyes, where impulses are generated and sent to the brain, and then, interpreted as colour (Delgado-Vargas, Jiménez, & Paredes-Lopez, 2000). Based on the chromophore chemical structure, pigments can be classified as chromophore with conjugated systems (carotenoids, anthocyanins, betalains, caramel and synthetic pigments) or porphyrins with coordinated metals (myoglobin, chlorophyll, and their derivatives).

In the conjugated systems, anthocyanins are specifically important because they are responsible for some red colours in the nature, as monomeric, oligomeric and polymeric anthocyanins. The use of natural extracts of these pigments as food additives has some limitations, due to colour variation caused by pH changes, light exposure and oxygen (Bridle & Timberlake, 1997; Markakis, 1982).

In general, anthocyanins show their highest colour intensity in the flavylium ion form (Harborne & Williams, 1995). It has been demonstrated that anthocyanin stability is influenced by substituents in their structures, sugars and acyl groups (Giusti & Wrolstad, 2003). In recent years, research on anthocyanin chemical structure has increased (Bjorøy, Fossen, & Andersen, 2007; Byamukama, Kiremire, Andersen, & Steigen, 2005; Cabrita, Frøystein, & Andersen, 2000; Fossen & Andersen, 2003; Mateus et al., 2003, 2006; Tian, Giusti, Stoner, & Schwartz, 2006; Wang, Race, & Shrikhande, 2003); however, it is important to study in depth the relationships between colour and chemical composition, which may help to understand the basic principles that influence the anthocyanins' colour.

In Colombia, the tamarillo or tree tomato (*Solanum betaceum* Cav.) is a promising product for export, due to its colour; the red variety has been the most accepted internationally. In this work the stability of tamarillo fruit extracts and isolated individual

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.03.081

anthocyanin rutinosides (Fig. 1) to pH changes has been evaluated by means of colorimetric studies, to obtain more precise information about the change of colour in both crude extracts and individual anthocyanins.

2. Materials and methods

2.1. Chemicals and supplies

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and Amberlite XAD-7 were obtained from Rohm and Haas, Darmstadt, Germany. HPLC-grade acetonitrile, ACS-grade *n*-butanol, methanol, *tert*-butyl methyl ether (TBME), formic acid, hydrochloric acid and potassium persulfate were purchased from Merck, Bogotá, Colombia. CD_3OD, CF_3COOD, and CF_3COOH (TFA) were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Plant material

Tamarillo fruits (5.09 kg) were collected in Puente Nacional (Santander, Colombia). A voucher specimen was coded as Col 343584 at the Instituto de Ciencias Naturales at Universidad Nacional de Colombia.

2.3. Isolation of crude anthocyanins extract

Fresh ripe fruits were washed and peeled; the seeds and the surrounding jelly were manually separated from the flesh. The jelly (250 g), filtered through glass wool, was applied onto an Amberlite XAD-7 column (800 mm long, 40 mm i.d.). The column was washed with 1.25 l of water, and elution of anthocyanins was carried out with 300 ml of a mixture of methanol:acetic acid (19:1, v/v). The eluate was concentrated under reduced pressure at 35 °C and the aqueous solution was lyophilised (crude jelly extract). This procedure was repeated four times to obtain 4.35 g of the crude jelly extract.

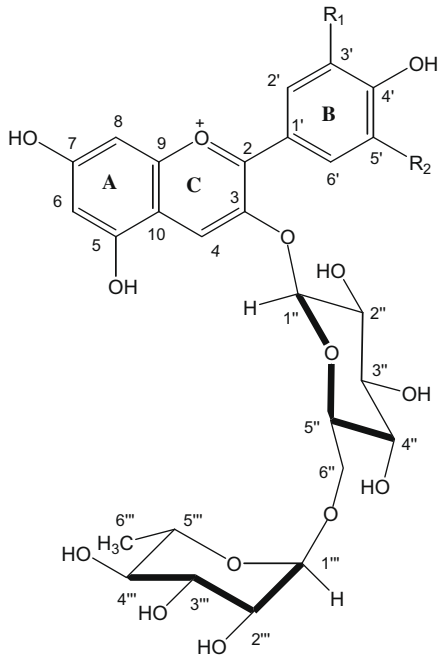

Fig. 1. Basic structure of tamarillo anthocyanins (*Solanum betaceum* Cav). Dp 3-rut-3′-glc = delphinidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranosyl)-3′-*O*-β-glucopyranoside (R_1 = O-glc, R_2 = OH), Dp 3-rut = delphinidin-3-*O*-(6″-*O*-α-rhamnopyranosyl)-β-glucopyranoside (R_1 = R_2 = OH), Cy 3-rut = cyanidin-3-*O*-(6″-*O*-α-rhamnopyranosyl)-β-glucopyranoside (R_1 = OH, R_2 = H), Pe 3-rut = pelargonidin-3-*O*-(6″-*O*-α-rhamnopyranosyl)-β-glucopyranoside (R_1 = R_2 = H). glc = glucopyranoside.

The peelings (2.51 kg) were cut into small pieces (2 cm^2) and extracted with 2 l of methanol:acetic acid (19:1, v/v) for 12 h (maceration). After filtration the organic solvent was evaporated at 35 °C using a rotary evaporator and the remaining aqueous phase was applied onto a XAD-7 column (800 mm long, 40 mm i.d.). The pigments were eluted as indicated before (Degenhard, Knapp, & Winterhalter, 2000), to give an enriched anthocyanins extract of 0.935 g (crude peelings extract). Then, the XAD-7 isolates of jelly and peelings were fractionated by multilayer coil countercurrent chromatography (MLCCC).

2.3.1. Countercurrent chromatography (CCC)

A multilayer coil countercurrent chromatograph (P.C. Inc., Potomac, MD) with tubular column of PTFE (400 ml total volume) was used. Solvent system consisted of *n*-butanol:TBME:acetonitrile:water (2:2:1:5) v/v/v/v, acidified with 0.1% TFA (Degenhard et al., 2000). The organic phase was used as the stationary phase; therefore elution mode was head to tail. Crude anthocyanins extract was dissolved in 5 ml of a mixture of stationary phase and mobile phase (1:1 v/v), and introduced through the injection port. The mobile phase was pumped at 1 ml min^{-1}, while centrifugation was carried out at 800 rpm. Four-millilitre fractions were collected. The sample loads in MLCCC were high (0.6 g), so fractionation of up to several hundred milligrams of sample was achieved in a single MLCCC run. To check the purity, each fraction was analysed by HPLC, and further purification was carried out using preparative HPLC.

2.3.2. High performance liquid chromatography

The analytical HPLC results were obtained with an Agilent 1100 HPLC system (Agilent, Santa Clara, CA) fitted with a photodiode array detector and a Zorbax-SB C_{18} column (4.6 mm × 250 mm; 5 μm film thickness). Two solvents were used for elution: **A** = acetonitrile:formic acid:water (3:10:87, v/v/v) and **B** = acetonitrile:formic acid:water (50:10:40, v/v/v). The elution profile consisted of a gradient from 6% to 20% **B** at 0–10 min, 20% to 40% **B** at 10–20 min, 40 to 50% **B** at 20–30 min, 50% to 6% **B** at 30–35 min. Aliquots of 100 μl (0.1 mg ml^{-1}) were injected and the flow rate was 0.8 ml min^{-1}. Prior to injection, all samples were filtered through a 0.45 μm Millipore membrane filter.

Preparative HPLC was performed using a Luna C_{18} column (10 mm × 250 mm: 5 μm film thickness) and a 6000LP UV detector. An isocratic elution profile was applied (95% **A**, 5% **B**) using acetonitrile, formic acid and water (solvent **A**: 3:10:87, v/v/v; solvent **B**: 50:10:40, v/v/v). The flow rate was 4 ml min^{-1} for 20 min and aliquots of 40 μl (250 mg ml^{-1}) were injected.

2.3.3. Spectroscopy

UV–Vis absorption spectra of anthocyanins were recorded online during HPLC analysis, and the spectral measurements were made over the wavelength range 300–680 nm in steps of 2 nm. ESI–MS analyses were performed on a Shimadzu QP-8000 mass spectrometer (Shimadzu, Japan). The electrospray voltage applied was 4.5 kV, nebuliser gas flow of 4.5 l min^{-1}, probe voltage 4.5 kV, curved desolvation line (CDL) voltage 130 V, CDL temperature of 230 °C, deflector voltage at 45 and 60 V and acquisition from *m/z* 50 to *m/z* 800 in positive ionisation mode. A solution of 1 mg ml^{-1} of each purified pigment was dissolved in a 1:1 mixture of solvent **A** (water:formic acid 9:1) and solvent **B** (acetonitrile:formic acid 9:1). The anthocyanin solutions were injected directly into the system at a flow rate of l00 μl min^{-1}. Low-resolution fast atom bombardment MS of the pigments was performed on an

AutoSpec-Q (Waters Corporation, Milford, MA) in a glycerol-NaI matrix, using positive detection and acquisition from *m/z* 50 to *m/z* 900. Argon was used as collision gas. The complete structures of isolated anthocyanins were established by ^{1}H- and ^{13}C NMR analysis. Full assignments were performed with TOCSY, COSY, HSQC and HMBC experiments. Isolated anthocyanins dissolved in a mixture of CD_3OD:CF_3OOD (19:1, v/v) were measured using a Bruker AMX-500.

Thus, the identities of anthocyanins were determined to be: delphinidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranosyl)-3′-*O*-β-glucopyranoside (Dp 3-rut-3′-glu), delphinidin 3-*O*-(6″-*O*-α-rhamnopyranosyl)-β-glucopyranoside (Dp 3-rut), cyaniding 3-*O*-(6″-*O*-α-rhamnopyranosyl)-β-glucopyranoside (Cy 3-rut) and pelargonidin 3-*O*-(6″-*O*-α-rhamnopyranosyl)-β-glucopyranoside (Pe 3-rut). The chemical structures of these anthocyanins are shown in Fig. 1.

2.4. Quantification of total phenols and determination of anthocyanic indices in the crude extracts

Total phenols (TP) were estimated using the Folin–Ciocalteu method (Singleton & Rossi, 1965). Sample aliquots of 0.5 ml were added to 0.5 ml of water, 5 ml of Folin–Ciocalteau reagent (0.2 N), and 4 ml of a saturated solution of sodium carbonate (75 g/l), and mixed thoroughly. The absorbance was measured at 765 nm with an HP8452 spectrophotometer (Hewlett Packard, Palo Alto, CA) after incubation for 2 h at room temperature. Quantification was made based on a standard curve, generated with 2.6, 5.2, 7.9, 13.1 and 26.2 mg of gallic acid. TP values were expressed in % w/w (100 × mg gallic acid/mg crude extract).

The anthocyanic indices represent approximate measurements of the phenolic constituents and they can be used in comparative evaluations. The anthocyanin extracts include both monomeric anthocyanins and polymeric pigments. When a solution containing anthocyanins is treated with an excess of SO_2 an immediate decolouration of the solution occurs, so the residual colour existing after such treatment is due to polymeric pigment forms.

Aqueous solutions (2 mg ml^{-1}, pH 5.2) of crude extracts were prepared for the chemical indices determination. The index of polymeric pigments (*IPP*) was measured at 520 nm and total anthocyanic colour (*AC*) was measured in 1 M HCl at 520 nm. Polymeric colour (*PC*) was assumed to be equal to 5 × *IPP*/3, and the colour of the monomeric anthocyanins (*MC*) in 1 M HCl was obtained by difference ($MC = AC - (5\ IPP/3)$). The concentration of total monomeric anthocyanins (*AT*) in 1 M HCl was expressed as delphinidin 3-glucoside chloride (molecular mass 500.5) using the molar absorptivity value (ε) of 23,700 l mol^{-1} cm^{-1} (Heredia, Francia-Aricha, Rivas-Gonzalo, Vicario, & Santos-Buelga, 1998) at 520 nm. Hence, *AT* (mg/l) = 21.1 *MC* (Somers & Evans, 1977).

2.5. Determination of the total antioxidant capacity

The total antioxidant capacity was determined by the TEAC method (Re et al., 1999), which is based on the capacity of antioxidants to capture the radical 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$). It was performed using the HP8452 spectrophotometer in kinetic mode. $ABTS^{\cdot+}$ radical cation was produced by reacting 7 mM 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diamonium salt and 2.45 mM potassium persulfate, after incubation at room temperature in the dark for 16 h. The $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.1 at 734 nm. The filtered sample was diluted with ethanol, so as to give 20–80% inhibition of the blank absorbance with 20 μl of sample. $ABTS^{\cdot+}$ solution (1 ml; absorbance of 0.70 ± 0.1) was read at 734 nm and 20 μl of the sample were added and mixed thoroughly. Trolox standards of final concentration 0–15 μM in ethanol were prepared and assayed under the same conditions. Trolox equivalent antioxidant capacity of sample was calculated based on the inhibition exerted by standard Trolox solution at 6 min.

2.6. Colorimetric study

The evaluation of the colour was based on the spectrophotometric measurement of the transmission spectrum in the visible region (380–770 nm) using an HP8452. The colour parameters were obtained through weighted ordinates method ($\Delta\lambda = 2$ nm) from transmission spectra, by using the CromaLab® software (Heredia, Álvarez, González-Miret, & Ramírez, 2004), which takes into consideration the International + Commission on Illumination recommendations (CIE, 2004). D65 standard illuminant, corresponding to the natural daylight, and 10° standard observer were considered in the calculations. Reference blank measurements were made with the cuvette filled with distilled water. CIE 1976 ($L^*a^*b^*$) (CIELAB) uniform colour space was taken into account for the colorimetric analysis. Within the CIELAB uniform space a psychometric index of lightness, L^* (ranging from 0, black, to 100, white), and two colour coordinates, a^* (which takes positive values for reddish colours and negative values for greenish ones) and b^* (positive for yellowish colours and negative for the bluish ones), are defined. From these coordinates, other colour parameters are defined: the hue angle (h_{ab}) is the qualitative attribute of colour, and the chroma (C^*_{ab}) is the quantitative attribute of colour intensity.

2.6.1. The pH effect

The CIELAB parameters (L^*, a^*, b^*, C^*_{ab}, h_{ab}) were determined in 5×10^{-5} M solutions of each anthocyanin at different pH values, ranged from 2.0 to 8.7. Modifications in pH were made by addition of small volumes of NaOH (1 M or 10 M). Crude extracts were diluted in water until 0.8 absorbance units at $\lambda = 520$ nm were obtained in order to study the influence of the pH on the colour; the concentration of the diluted jelly extract was 2.1 mg/ml, and that of the diluted peelings extract was 35 mg/ml.

The colour differences ΔE^*_{ab} were calculated between the initial pH value (pH = 2.0) and after each increase of pH, considering the Euclidean distance between the two colour points: $\Delta E^*_{ab} = ((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)^{1/2}$. Data consisted of the average of two experimental values.

3. Results and discussion

Once both jelly and peelings crude extracts were obtained, different chemical characteristics were determined (anthocyanic indices). The XAD-7 jelly extract showed higher content of total phenols (TP) and total anthocyanins (TA), while the peelings extract showed higher index of polymeric pigments (*IPP*). The higher content of TP and TA in the crude jelly extract is in accordance with the higher antioxidant capacity of this extract (Table 1).

3.1. Influence of the pH on the colour of the anthocyanin crude extracts

The colour variation in the aqueous solutions of anthocyanin crude extracts was studied within the pH range 2.0–6.2, that is to say under and above the most common pH values in foods. As revealed in Table 1 both jelly and peelings extracts showed reddish hues at the lowest pH value (peelings: 32.1°; jelly: 35.0°). As the pH increased to 6.2 the colour of the peelings extract gradually changed towards orange hues (until $h_{ab} = 64.9°$) and the jelly extract towards red-purple hues (until $h_{ab} = 0.4°$). The colour differences ΔE^*_{ab} regarding the pH = 2.0 sample (the most colourful sample) are summarised in Table 2. These differences were higher

Table 1
Total Phenolic (TP), Polymeric Pigment Index (PPI), Total Anthocyanins (TA) and Antioxidant Activity (TEAC) of crude extract and pure anthocyanins isolated from tamarillo fruit (*Solanum betaceum* Cav.).

Sample	λ_{max}	ε	TEAC (pH 5.2)	TP	PPI	TA
Jelly	500	–	1.90 ± 0.125[b]	25.11 ± 0.9	0.20 ± 0.034	20.03 ± 0.85
Peelings	530	–	1.09 ± 0.076[b]	13.69 ± 0.8	1.16 ± 0.107	0.20 ± 0.03
Dp 3-rut-3′-glc	514	15974	2.20 ± 0.026[a]	–	–	–
Dp 3-rut	518	25874	4.91 ± 0.146[a]	–	–	–
Cy 3-rut	512	27268	1.80 ± 0.098[a]	–	–	–
Pe 3-rut	500	36660	1.48 ± 0.045[a]	–	–	–
Ascorbic acid			1.09 ± 0.093[a]	–	–	–

ε: molar absorption (0.1% HCl in ethanol). TEAC: Trolox equivalent antioxidant capacity.
[a] mmol of Trolox/mmol compound.
[b] mmol Trolox/g. Data given are the average of six measurement expressed in terms of mean ± S.D. TP: total phenolics (% p/p), PPI: polymeric pigment index (absorbance units), TA: total anthocyanins (mg delphinidin 3-glucoside/l).

for the jelly extract across the pH range, which is evidence of the lower colour stability (higher sensitivity to pH changes) of this extract. The higher stability of the peelings extract may be due not only to the higher polymeric anthocyanins content, which are components more stable to pH changes (Somers & Evans, 1977), but also to the possible presence of some compounds stabilising the colour in the extract, i.e., phenolic acids, flavones, flavonols, flavanones, flavanols and organic acids (Eiro & Heinonen, 2002; Markovic, Petranovic, & Baranac, 2000; Rein & Heinonen, 2004). The crude extracts were shown to be relatively more stable under low acid conditions (pH 2.0–3.4); however, even in the crude peelings extract (more stable) the colour differences were up to 3 CIELAB units (Table 2) indicating that they can be visually discriminated (Martínez, Melgosa, Pérez, Hita, & Negueruela, 2001).

3.2. UV–vis spectroscopy of individual anthocyanins

For this study, the following anthocyanins isolated from the fruit were studied: Dp 3-rut-3′-glc, Dp 3-rut, Cy 3-rut and Pe 3-rut. The maximum absorption in the visible spectrum of the aqueous solution (5×10^{-5} M; pH 2.0) and the molar absorption (ε) of each anthocyanin are shown in Table 1. Comparing the three rutinosides, of which the structural differences are the number of hydroxyl groups in the B ring, it is observed that Pe 3-rut, having one hydroxyl group, shows a λ_{max} at 500 nm. However, as the number of hydroxyl groups in the *ortho* position increases (Cy 3-rut and Dp 3-rut) the maximum absorption shifts towards higher wavelengths and the absorption intensity decreases. Analysing the spectral characteristics of the delphinidin derivatives, it can be observed that the replacement of the 3′-hydroxyl of the Dp 3-rut with a glucose moiety causes both hypsochromic and hypochromic shifts in the visible spectrum (Table 1).

The spectral measurements were important to study the effect of hydroxylation and glycosylation in the B ring of the aglycone on the visible spectrum; however, with only this data we do not have enough information to explain the chromatic characteristics of the anthocyanins. It is well known that the properties of anthocyanins, including the colour expression, are influenced by the chemical structure and pH (Heredia et al., 1998).

Table 2
CIELAB colour parameters (L^*, a^*, b^*, C^*_{ab}, h_{ab}) and colour differences (ΔE^*) of crude extracts (jelly and peelings) and pure anthocyanins isolated from tamarillo fruit (*Solanum betaceum* Cav.).

	pH 2.0–3.4				pH 3.4–6.2			
	Jelly		Peelings		Jelly		Peelings	
a^*_i	62.1		51.3		55.4		52.4	
a^*_f	55.4		52.4		28.7		24.1	
b^*_i	43.5		32.2		22.5		42.5	
b^*_f	22.5		42.5		0.2		51.6	
L^*_i	45.5		51.2		49.0		46.3	
L^*_f	49.0		46.3		66.1		40.5	
$C^*_{ab,i}$	75.8		60.6		59.8		67.4	
$C^*_{ab,f}$	59.8		67.4		28.7		56.9	
$h_{ab,i}$	35.0		32.1		22.1		39.0	
$h_{ab,f}$	22.1		39.0		0.4		64.9	
ΔE_{ab}	22.3		11.5		38.8		30.3	
	pH 2.0–3.4				pH 3.4 – 6.2			
	Dp 3-rut-3′-glc	Dp 3-rut	Cy 3-rut	Pe 3-rut	Dp 3-rut-3′-glc	Dp 3-rut	Cy 3-rut	Pe 3-rut
a^*_i	28.1	59.1	52.7	44.4	6.5	24.6	28.9	34.3
a^*_f	6.5	24.6	28.9	34.3	1.0	−0.3	5.4	6.0
b^*_i	4.8	15.2	26.8	62.7	4.3	−1.1	5.8	34.8
b^*_f	0.6	−1.1	5.8	34.8	−0.4	0.4	−2.6	2.1
L^*_i	79.7	70.8	74.2	78.3	87.5	87.2	85.0	80.1
L^*_f	87.5	87.2	85.0	80.1	89.0	98.5	85.4	97.6
$C^*_{ab,i}$	32.0	61.0	59.1	76.9	7.8	24.7	29.5	48.9
$C^*_{ab,f}$	7.8	24.7	29.5	48.9	1.0	1.9	29.4	6.3
$h_{ab,i}$	28.6	14.4	26.1	54.7	33.3	−2.5	11.4	45.4
$h_{ab,f}$	33.3	−2.5	11.4	45.4	21.0	−23.9	−22.5	19.0
ΔE_{ab}	23.3	41.5	33.5	29.7	7.4	27.4	24.9	46.7

i = initial (lowest pH value of the range); f = final (highest pH value of the range).

3.3. Influence of pH on the colour of the individual anthocyanins

Fig. 2 represents the chromatic characteristics of the rutinosides solutions on the (a^*b^*)-diagram. This study reveals the impact of anthocyanins structures, such as hydroxylation and glycosidation, on colour and stability at various pH values. The pigments represent variation of the hydroxylation grade of the B ring of many anthocyanins isolated from fruits and vegetables (Brouillard, 1982). Aqueous solutions (5×10^{-5} M) of Dp 3-rut-3′-glc, Dp 3-rut, Cy 3-rut and Pe 3-rut were studied at two pH range: (2.0–3.4) and (3.4–6.2). According to the CIELAB parameters (L^*, a^*, b^*, C^*_{ab}, h_{ab}, ΔE^*_{ab}) their colour stabilities depend highly on the pH and the structure (Fig. 2, Table 2). At the lowest pH value (pH 2.0, where anthocyanins exit basically in flavylium form), Dp 3-rut-3′-glc, Dp 3-rut and Cy 3-rut showed reddish hues (h_{ab} = 28.6°, 14.4° and 26.1°, respectively), while Pe 3-rut showed orange ones (h_{ab} = 54.7°). At this pH, the number of hydroxyl groups of the B ring clearly influenced the colour characteristic; the hue angle (h_{ab}) and lightness (L^*) values were clearly higher for Pe 3-rut (only one OH), followed by Dp 3-rut-3′-glc (two OH), Cy 3-rut (two OH) and Dp 3-rut (three OH). Dp 3-rut-3′-glc underwent small decreases of the hue angle as the pH increased from 2.0 to 6.2, while great decreases in hue were found for Dp 3-rut, Cy 3-rut and Pe 3-rut. It is noticeable that anthocyanins containing aglycones with only two or three hydroxyl groups on the B ring (Cy 3-rut and Dp 3-rut) showed smaller colour differences (ΔE^*_{ab}) in the more alkaline region pH (3.4–6.2), contrary to Pe 3-rut (one OH) which showed greater colour differences (ΔE^*_{ab}) at these pH values (Table 2). On the other hand, at the most acid pH (2.0–3.4) the Dp 3-rut (three OH) was more unstable (the highest ΔE^*_{ab}).

Comparing the ΔE^*_{ab} values at both pH ranges, it was observed that Dp 3-rut, Cy 3-rut and Pe 3-rut were clearly more unstable than Dp 3-rut-3′-glc. The loss of colour occurring due to the base attack on the pigment structure could be related to the solvating grade of each molecule, in such a way that the greater the solvating grade was the higher the stability to pH changes. According to a study on flavonoids (Rezende, Moll, González, Beezer, & Mitchell, 1999), the internal hydrogen bonding (intramolecular interactions) between neighbouring OH reduces the availability of these groups (by both steric and electronic factors) to interact with the solvent. This could explain the lowest colour stability of Dp 3-rut (lowest solvating) at low pH (2.0–3.4). Nevertheless, at pH 3.4–6.2, which induces the formation of quinoidal bases (Brouillard, 1982), different behaviours were observed. Anthocyanins having two or three hydroxyl groups on the B ring showed similar colour differences, ΔE^*_{ab} being 27.4 and 24.9 CIELAB units for Dp 3-rut and Cy 3-rut, respectively. This can be also explained based on intramolecular interactions between neighbouring OH, since it is reasonable to suppose that two neighbouring OH groups allow the formation of hydrogen bonding, which would yield to the most stable system. According to these results, it is evident the existence of relations between the colour and the chemical composition, so that solvating degree significantly influences the final colour characteristics.

With the purpose of observing the effect of replacing the 3′-hydroxyl of Dp 3-rut with a glucosyl group (giving Dp 3-rut-3′-glc) on the colour characteristics and stability to pH changes, the chromatic characteristics of the dilutions of these two pigments are presented on the (a^*b^*)-plane (Fig. 3). The net red hue (h_{ab} values between 10° and 30°) at very acid pH, gradually changed to yellow hues as the pH increased. It is clear that the glycosidic substitution at the 3′- position of the aglycone compared to non-substitution produced relative large decrease of h_{ab}, and less intense colour (C^*_{ab}: 32 and L^*: 79.7 CIELAB units), at pH = 2.0 (Table 2); however, Dp 3-rut-3′-glc is the most resistant to colour changes (lowest ΔE^*_{ab}) as described above. The major colour stability of this anthocyanin compared to the other pigments could be explained by the presence of three sugars in the molecule which protect the flavylium ion from the base attack, due to the "sandwich" configuration of this type of compound (Giusti & Wrolstad, 2003), restricting the hydration possibilities.

On the other hand, the antioxidant capacity of the four pure anthocyanins was determined: Dp 3-rut, Cy 3-rut, Pe 3-rut and Dp 3-rut-3′-glc (all of them glycosylated in the 3-position of the C ring). Dp 3-rut-3′-glc has an additional glycoxyl group in the 3′ position of the B ring. The results showed that the isolated anthocyanins have higher capacity to capture free radicals in aqueous

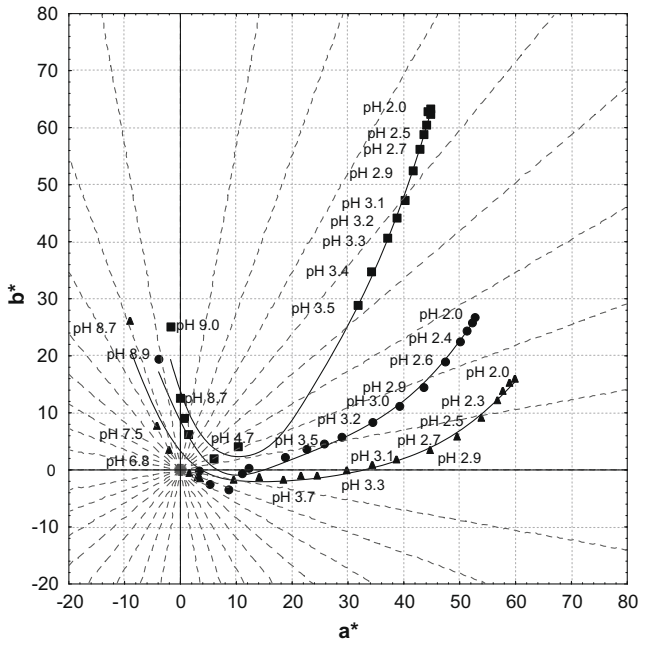

Fig. 2. (a^*b^*)-diagram. Colour changes of the major anthocyanins studied at different pH values (■ Pe 3-rut; ● Cy 3-rut; ▲ Dp 3-rut).

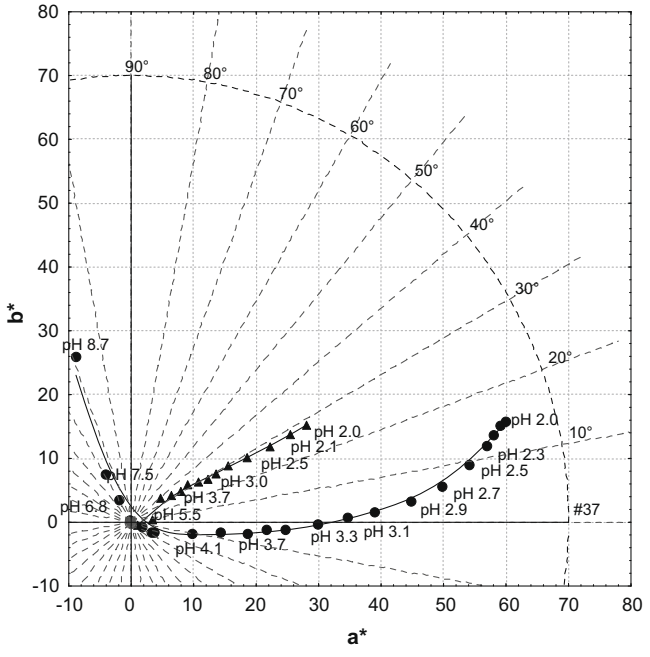

Fig. 3. (a^*b^*)-diagram. Colour changes of Dp 3-rut-3′-glc (▲) and Dp3-rut (●) at different pH values.

solution (pH 5.2) than ascorbic acid (Table 1). It was also observed that the hydroxylation degree of the isolated rutinosides have great influence on the antioxidant capacity.

As observed in Table 1, Dp 3-rut was more efficient at capturing the ABTS radical than Cy 3-rut, and this in turn was more efficient than Pe 3-rut. Thus, the rutinosides having hydroxyl groups in *ortho* position of the B ring, as in the case of Dp 3-rut, Cy 3-rut and Dp 3-rut-3′-glc, have a greater efficiency in capturing free radicals, which can be attributed to the fact that hydroxyl groups in the *ortho* position confer high stability on the formed radical, that is to say they stabilise the formation of the *O*-semiquinone radical (Rice-Evans, Miller, & Paganga, 1996). In comparison, Dp 3-rut-3′-glu, having an additional glycosyl in the 3′-position, was less efficient in capturing radicals in aqueous solution than the similar Dp 3-rut. This indicates that a different glycosylation pattern can considerably modify the antioxidant activity of the anthocyanins, and the extent of this change also depends on the aglycone type.

In summary, the characteristics of colour, stability and antioxidant activity of the crude extracts and isolated rutinosides pigments of Tamarillo fruit have been studied in aqueous solution. Through the application of tristimulus colorimetry, the relations of chemical structure and antioxidant pigments' colour, and their relevance in pH change have been shown.

A relationship between antioxidant activities *in vitro* and phenolic contents has been observed in crude extracts; however, whether this antioxidant potential has an effective role *in vivo* remains to be demonstrated. This study shows the potential value of these extracts as antioxidants and in the improvement of nutritional value of foods and their preservation. Furthermore, the possible use of the peelings (usually waste material) for the production of anthocyanins or natural antioxidant extracts can provide some economic benefits and added value to this material.

Acknowledgements

The authors sincerely acknowledge to Colciencias (Colombia), Universidad de Nariño (Colombia), and IPICS-Uppsala (University Sweden) for the financial support.

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Food Chemistry 117 (2009) 94–98

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Purification of soybean amylase by superparamagnetic particles

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ARTICLE INFO

Article history:
Received 13 October 2008
Received in revised form 20 March 2009
Accepted 23 March 2009

Keywords:
Magnetic carrier technology
Superparamagnetic particles
Amylase
Soybean

ABSTRACT

Many studies of purification technology have focused on the development of supports of substrates for isolating enzymes. In this study, superparamagnetic particles modified by epichlorohydrin and other cross-linking agents to coat with starch were used as a purification support for isolating amylases from soybean proteins after precipitation with ammonium sulphate. The recovery of amylase activity from the crude amylase was 17.50% with a 194-fold purification. The molecular weight of the purified amylase was estimated to be 70 kDa by SDS–PAGE. Both crude and purified amylases showed an optimum pH of 6.0 with optimum temperatures of 70 and 60–70 °C and thermal stability of 20–70 and 20–60 °C, respectively. Since the affinity magnetic carrier could adsorb the target proteins efficiently, the superparamagnetic particles technology can be further combined with other separation technologies in the industries to enhance their purification efficiency.

1. Introduction

Magnetite is used as a magnetic carrier in the magnetic carrier technology that is widely used in the field of medical diagnostics, DNA and RNA purification, enzyme immobilization, immunoassay, environmental analysis, and ferrofluids applications (Liu, Guan, Shen, & Liu, 2005; Sonti & Bose, 1997; Zheng, Shu, & Yan, 2003). The magnetic carrier technology, first developed in 1973, is a novel technique in the separation science in which a non-magnetic target is specifically bound to the customised magnetic particles to form a magnetised complex. When a magnetic field is applied to this complex, the magnet-bound target could be mobilised and separated easily, resulting in the purification of target component (Cui, Gu, Xu, & Shi, 2006; Liu et al., 2005; Sonti & Bose, 1997).

The advantages of this technology are: (1) its operation is rapid; (2) it causes significant reduction in the operation cost; and (3) it facilitates simple separation and recovery of the enzyme (Akgöl, Kacar, Denizli, & Arıca, 2001; Jiang, Long, Huang, Xiao, & Zhou, 2005).

Amylase exists widely in animal, plants, bacteria and fungi (Prigent, Matoub, Rouland, & Cariou, 1998). Starch is the major energy storage of the plant and until now its metabolic pathway is not completely understood. For a long time, it was thought that the α-amylase, β-amylase, debranching enzyme, starch phosphorylase and α-glucosidase were released to decompose plant starch (Lizotte, Henson, & Duke, 1990). β-Amylase is the major starch-hydrolysing enzyme in soybean (Gertler & Birk, 1965), which is an exo-amylase that hydrolyses the α-1,4-glucosidic linkage of a non-reducing polysaccharide. β-Amylase might exist in the cytoplasm, vacuole and chloroplast, and its activity is controlled by several factors such as the stage of the plant growth, carbohydrates, non-biological stress, and plant hormone (Lee, 2007).

The present study was designed to determine the effectiveness of using the starch-coated superparamagnetic iron oxide (starch-SPIO) particles method to further purify β-amylase from the protein precipitated by 40–70% ammonium sulphate. The result demonstrated that the starch-SPIO method was able to recover 17.50% of β-amylase activity with a 194-fold purification of the crude enzymes.

2. Materials and methods

2.1. Materials

Soybean was kindly provided by Chen (University of Illinois, Champaign-Urbana, Illinois, USA). Soluble starch, ammonium sulphate $(NH_4)_2SO_4$, and sodium chloride (NaCl) were purchased from ACROS, Japan. Ferric chloride ($FeCl_3$), ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), epichlorohydrin, and ammonia (NH_4OH) were purchased from Fisher Scientific, USA.

2.2. Preparation of superparamagnetic particles

The chemical co-precipitating method with heating and alkalization was used for preparing superparamagnetic iron oxide

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doi:10.1016/j.foodchem.2009.03.089

(SPIO). Briefly, 3 mol $FeCl_3$ and 2 mol $FeCl_2 \cdot 4H_2O$ were dissolved in 100 ml distilled water, and the pH was adjusted to 11 with 28% ammonia water. The solution was heated to 80 °C for 30 min to cause precipitation. The precipitate was washed with distilled water and ethyl alcohol to remove the impurities such as iron chloride and sulphates, and the particles were dried at 80 °C for 8–10 h in an oven (Koneracka et al., 1999). The dried iron oxides were the superparamagnetic particles. The major component of the magnetic particles was Fe_3O_4, and the water content was about 35%.

2.3. Preparation of affinity superparamagnetic particles

One gram of superparamagnetic particles was mixed with 10 ml of epichlorohydrin. After stirring for 30 min, the precipitate was washed with distilled water, and then 20 ml of 2% soluble starch was added to it and shaken for 2 h. The precipitate was washed with distilled water several times and was dried. The dried particles were used as affinity superparamagnetic particles (starch-SPIO) (Cesar, Olga, Roberto, & Jose, 2000).

2.4. Extraction of crude soybean enzyme

Soybean was soaked in distilled water until germination. The germinated soybean was washed with water and then pureed twice for 5 min to homogenise with Tris buffer (10 mM Tris–HCl, pH 7.0) containing 10 mM NaCl. The homogenate was centrifuged (12,900*g*, 30 min at 4 °C) and the supernatant was called crude soybean enzyme (Mohamed, 2004). The fraction containing crude soybean enzyme was heated at 65 °C in a water bath for 30 min, and was then centrifuged at 12,900*g* for 30 min. The supernatant was precipitated with 40–70% ammonium sulphate and the precipitate was further processed by dialysis, redissolving, and centrifugation. The resulting supernatant was the partially purified soybean enzyme (crude enzyme). The protein-staining method was followed for the determination of protein with bovine serum albumin as the standard (Field, Spielman, & Hand, 1989).

2.5. The absorption and elution of affinity supermagnetic particles

The crude enzymes were mixed and reacted with affinity supermagnetic particles for 2 h. The enzymes adsorbed on the supermagnetic particles were isolated by applying a magnetic field, and the un-adsorbed proteins were washed out with distilled water. The solution of 0.5 M NaCl phosphate buffer having a pH of 7.0 was added to the soybean enzymes adsorbed on the affinity supermagnetic particles. After shaking, the solution was eluted for 1 h. The same procedure was run using a solution of 1.0 M NaCl phosphate buffer having a pH of 7.0. In both cases the solid and liquid were separated by applying a magnetic field. The recovered eluate was the SPIO-purified soybean amylase (Zeng & Cohen, 2000).

2.6. Determination of amylase activities

Soluble starch (2% w/v) was used as a substrate to determine amylase activity. One millilitre of soluble starch solution was mixed with 0.1 ml of the enzyme preparation and was reacted at 37 °C for 10 min. The reaction was stopped by adding 1 ml of 3,5-dinitrosalicylic acid and by heating the reaction mixture in boiling water for another 10 min. After the reaction mixture had been cooled in cold water, the absorbance of 0.1 ml reaction mixture diluted with 0.9 ml distilled water was measured at 546 nm using maltose as the standard. One unit of activity was defined as the amount of enzyme that is able to produce 1 μg of maltose per minute at 37 °C (Zakowski, Gregory, & Bruns, 1984).

2.7. SDS–PAGE electrophoresis

Acrylamide at 12.5% was used as the resolving gel and 3.75% acrylamide was used to prepare the stacking gel. The electrophoresis equipment was Bio-rad, set at a fixed voltage of 120 V and was applied to versatile mini-protein three electrophoresis cells with running buffer having a pH of 8.3 (10 mM tris–glycine). After 2 h, the gel was stained with Coomassie brilliant blue R-250 light for 30 min, and was then decolored for 2–3 h (Wanderley, Torres, Moraes, & Ulhoa, 2004).

2.8. Zymogram method

The enzyme was mixed homogeneously with the running buffer (10 mM tris–glycine, pH 8.3, without β-mercaptoethanol) and the mixture was applied on the polyacrylamide gel, (two gels were simultaneously run in one test). The operation was carried out at a fixed voltage of 120 V for 2 h at 4 °C. One polyacrylamide gel was stained with Coomassie brilliant blue R-250 for 30 min and was then decolored for 2–3 h. The other polyacrylamide gel was reacted with a 2% soluble starch solution (containing 1% (v/v) Triton-X-100) for 2 h at room temperature, and was then washed with distilled water to remove the surface substance on the polyacrylamide gel. The polyacrylamide gel was then stained with iodine until clear bands appeared (Wang et al., 2001).

2.9. Effect of pH on enzyme activity

The amylase activity was determined in the pH range from 2 to 12 using glycine–HCl buffer having pHs 2.0 to 3.0, phosphate–citrate buffer having pHs 4.0 to 7.0, tris–HCl buffer having pHs 8.0 and 9.0, $NaHCO_3$–NaOH buffer having pH 10.0 and NaOH–HCl buffer having pHs 11.0–12.0. The molarity of all the buffered solutions was adjusted to 0.2 M and the reaction was run at 37 °C for 10 min (Prigent et al., 1998).

2.10. Effect of temperature on enzyme activity

The amylase activity was determined in the temperature range from 20 to 100 °C by using 0.1 M phosphate buffer having a pH of 7.0 and the reaction was run for 10 min (Raquel et al., 2006).

2.11. Thermal stability of enzyme activity

The enzyme was first incubated in the temperature range from 20 to 100 °C for 30 min, and the amylase enzyme activity was then determined in 0.1 M phosphate buffer having a pH of 7.0 at 37 °C for 10 min (Muralikrishna & Nirmala, 2005).

3. Results

3.1. Purification of soybean amylase

The crude soybean extract after having been heated in 65 °C water bath for 30 min retained most of the original amylase activity as evidenced from Table 1 showing 96% of the original enzymatic activity with a slight increase in the specific activity (1.18-fold increase). This heated extract was subsequently precipitated with a 40–70% of ammonia sulphate solution. The amylase-specific activity of the precipitated protein was increased by 8.42-fold but only retaining 19.54% of the original activity. The precipitated protein was further purified by the starch-SPIO method, which increased the specific activity of the precipitated protein by 23-fold while retaining 90% of its total amylase

Table 1
Purification of amylase from soybean.

Procedure	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Purification fold	Yield (%)
Crude	318,400	18,790	16.95	1.00	100
Heating 30 min, at 65 °C	306,860	15,966	19.22	1.13	96.38
$(NH_4)_2SO_4$ fraction (40–70% sat'n)	62,225	436	142.69	8.42	19.54
Starch-SPIO	55,734	17	3286.18	193.87	17.50

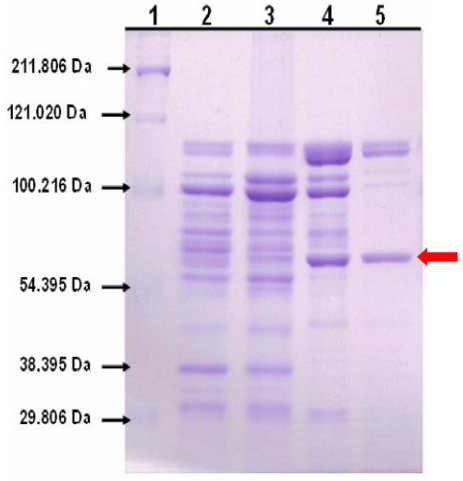
activity. In terms of the original enzyme activity, the starch-SPIO method is highly effective in isolating amylase from proteins precipitated with 40–70% of ammonia sulphate with a 194-fold increase in the specific activity and retaining 17.5% of the original enzymatic activity.

3.2. The molecular weight of soybean amylase

Fig. 1 compares SDS–PAGE gels of various degrees of purification to the marker proteins. There was not much difference between the crude extract (lane 2) and the one after having been heated at 65 °C for 30 min (lane 3). Lane 4, however, showed that the band corresponding to amylase was enhanced after heating the extract in a hot water bath, and followed by precipitation by ammonium sulphate. The final purification by the affinity superparamagnetic particles (starch-SPIO) gave mostly a sharp band of amylase, suggesting that miscellaneous proteins had been removed effectively from the crude soybean extract. The molecular weight of the β-amylase purified by the starch-SPIO method was estimated to be 70 kDa.

3.3. Zymogram method

Fig. 2A and B depict the Zymogram method, which demonstrates Iodine staining and Coomassie brilliant Blue R-250 staining. The molecular weight of amylase from soybean was estimated to be 72 kDa, which is presumably the same as 70 kDa of the amylase purified by the starch-SPIO method.



Lane 1 : Marker ;
Lnae 2 : Crude enzyme;
Lane 3 : Heating 30 mins, at 65 ºC;
Lane 4 : $(NH_4)_2SO_4$ fraction ;
Lane 5 : Starch-SPIO purified enzyme

Fig. 1. Comparison of the SDS–PAGE of purified amylase to crude enzymes from soybean.

3.4. Optimum pH

The crude enzyme was not very sensitive to pH changes from 3 to 9 as depicted in Fig. 3. The amylase activity was around 90% at pH 3, which peaked at pH 6.0 and slightly decreased to about 85% as the pH was increased to 9.0. Even at very alkaline pHs, substantial enzymatic activity remained. As compared to the crude amylase, the purified enzyme was more sensitive to pH changes. The purified amylase activity peaked at pH 6.0 and decreased gradually either by decreasing or by increasing pH from the optimum pH of 6.0.

3.5. Optimum temperature

The optimum temperature for amylase activity of both the crude and purified soybean amylases is illustrated in Fig. 4. The crude amylase was not very sensitive to temperature between 20 and 70 °C as evidenced from <10% change in the amylase activity with an increase in the temperature from 20 to 70 °C. When the temperature was raised from 70 to 100 °C, the crude amylase lost its activity gradually. Thus, the highest activity was observed at a temperature of 70 °C.

In contrast to the crude amylase, the starch-SPIO-purified amylase exhibited temperature sensitivity as normal enzymes do. The amylase activity increased from 35% to 100% as the temperature was raised from 20 to 60 °C and reached a plateau between 60 and 70 °C. The amylase activity decreased sharply to <10% at 80 °C. Thus, the temperature range for the amylase activity of the purified enzyme is narrower than that for the crude enzyme in this study.

3.6. Thermal stability of amylase

As depicted in Fig. 5, the crude amylase from soybean was very stable between 20 and 60 °C, and its activity decreased sharply when the temperature was over 70 °C. The soybean amylase purified by affinity superparamagnetic particles was also stable between 20 and 50 °C, its activity decreased sharply at the temperature of 60 °C and it became inactivated at the temperature of 70 °C and higher.

4. Discussion

The present study using the starch-SPIO method as the final step of the purification of amylase from soybean yielded a protein with an estimated molecular weight of 70 kDa, which is presumably similar to 61.7 kDa molecular weight of β-amylase purified from soybean flour by Gertler and Birk (1965). The molecular weight of β-amylase appears to be different depending on the species: 54 kDa for amylase from wheat seed; 18 kDa for amylase from wheat germ (Sharma, Sharma, & Gupta, 2000), 50 kDa for amylase from sweet potato (Teotia, Khare, & Gupta, 2001), 55–57 kDa for amylase from pea hypocotyls (Lizotte et al., 1990). There are two iso-amylases in the first stage of barley germination with the molecular weight of 43 kDa and 22.5 kDa (Machaiah & Vakil, 1984). Thus, the molecular weight of amylase differs depending on different origins, species, and development stages of the plants.

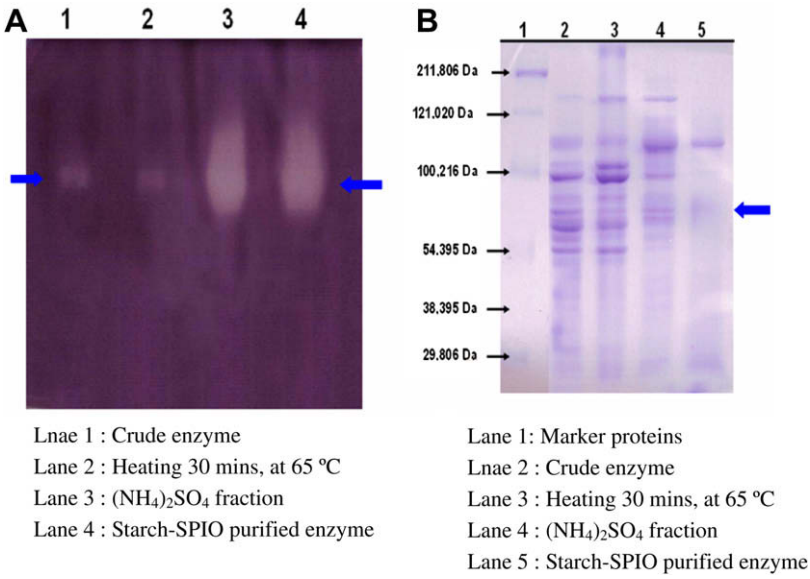


Fig. 2. The zymogram method of amylase from soybean. (A) Iodine staining; (B) Coomassie brilliant blue R-250 staining.

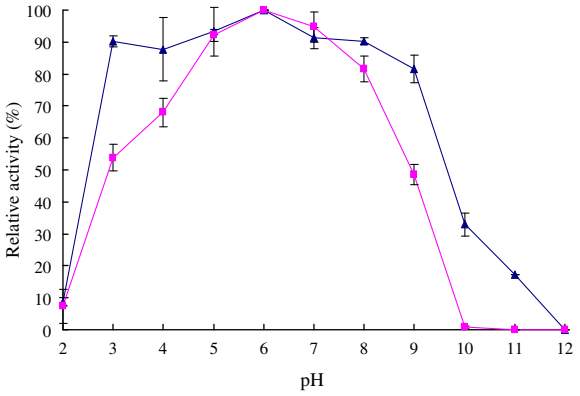


Fig. 3. Effect of pH on the activity of amylase from soybean. (▲) Crude enzyme; (■) Starch-SPIO (starch-coated affinity superparamagnetic particles)-purified enzyme.

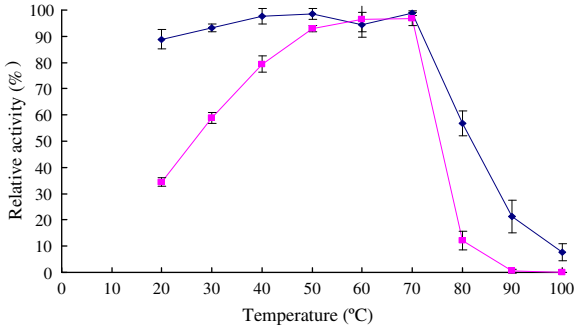


Fig. 4. Effect of temperature on the activity of amylase from soybean. (♦) Crude enzyme; (■) Starch-SPIO (starch-coated affinity superparamagnetic particles)-purified enzyme.

The results of our experiment that examined pH optimum for the purified amylase activity were similar to those of the other studies which showed the optimum pH to be between 5.0 and 6.0: the amylase of *Termitomyces clypeatus* had an optimum pH of 5.5 (Ghosh & Sengupta, 1987); the amylase of *Eleusine clypeatus*, pHs 5.0–5.5 (Nirmala & Muralikrishna, 2003); the β-amylase of soybean, pHs 5–6 (Gertler & Birk, 1965; Kumar, Vishwanath, Singh,

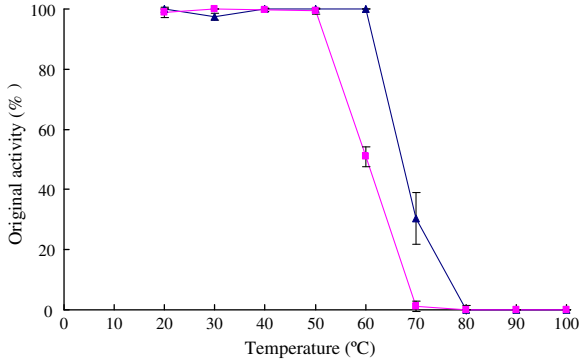


Fig. 5. Thermal stability of amylase from soybean. (▲) Crude enzyme; (■) Starch-SPIO (starch-coated affinity superparamagnetic particles)-purified enzyme.

& Rao, 2006); and the amylase in fermented cassava flour, pH 6.0 (Ganiyu, 2005). However, two acidic α-amylases of *Bacillus stearothermophilus* exhibited pH optimum at 4.5 and 5.0, respectively.

The present study indicated that soybean amylase was stable up to 60 °C. In contrast, the animal amylases are more labile to high temperature. For example, the amylase from *Prostephanus truncates* was unstable when the temperature was over 40 °C (Olaya, Jiménez, Rodríguez, Frier, & Labra, 2000), the amylase activity of *Ruditapes variegates* was stable between 4 and 35 °C, but its activity was sharply lost when the temperature was higher than 40 °C (Hsu, 2003). The relative α-amylase activity of tilapia was reduced to 20% when the temperature was over 50–60 °C and the relative α-amylase activity of *Toxoplasma gondii* was <20% when the temperature was over 64 °C (Ferre, Hoebeke, & Bout, 1999). In comparison to the above results, the amylase from soybean is more stable at higher temperature than the animal amylase. Taking advantage of the temperature stability, we had used heating of the crude extract at 65 °C as the initial step in the process of purifying amylase protein.

The present study demonstrated that the affinity superparamagnetic particles could be used as carrier support to absorb and purify the soybean amylase and that the technology of affinity purification can be widely used in protein purification. Compared to the traditional chromatography used in the purification of proteins, this novel affinity superparamagnetic particles technology

is rapid, provides a low cost of operation with simple facility for separation and recovery of enzymes.

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Food Chemistry 117 (2009) 99–105

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Influence of grapevine leafroll associated viruses (GLRaV-2 and -3) on the fruit composition of Oregon *Vitis vinifera* L. cv. Pinot noir: Free amino acids, sugars, and organic acids

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ARTICLE INFO

Article history:
Received 4 November 2008
Received in revised form 13 February 2009
Accepted 23 March 2009

Keywords:
Vitis vinifera
Pinot noir
Viruses
Grape quality
YAN
Biotic stress
Free amino acids
FAN

ABSTRACT

Individual free amino acids, yeast assimilable amino acid (YAN) content, ammonia, organic acids, and simple sugars of berries from vines infected with GLRaV-2 or -3 were compared with paired vines free of these viruses. Samples were taken from two commercial vineyards during two growing seasons (2005 and 2006), with three different rootstock/scion combinations. Vines infected with GLRaV-2 did not differ significantly from their healthy counterparts in individual free amino acids, ammonia, or YAN content. Vines infected with GLRaV-3 were significantly lower in valine and methionine from *Vitis riparia* rootstock/'Pinot noir' clone 114 (VY2a) samples, and lower in glutamic acid from self-rooted/'Pinot noir' clone Pommard (VY2b) samples, compared to samples from their healthy counterparts. Samples from VY2b (self-rooted/'Pinot noir' clone Pommard) infected vines had significantly lower levels of malic acid and total organic acids compared to samples from their healthy counterparts. There were no significant differences between healthy and infected vines from all three rootstock/scion pairs in ammonia or free amino acids in samples taken during the weeks before ripening and at commercial harvest. This is the first study to report the influence of GLRaV-2 and -3 on 'Pinot noir' berries nitrogen (N) compounds significant to fermentation. Individual free amino acids may be inferior to phenolic compounds as indicators of GLRaV infection status.

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1. Introduction

Nitrogen-containing compounds found in grapes have been reported to vary, depending on cultivar, vine nutrition, vineyard management, soil type, soil moisture content, vine virus status, grape maturity and growing season (Bell & Henschke, 2005 references therein; Conde et al., 2007; Kliewer & Lider, 1976; Stines et al., 2000; Ueno, Kinoshita, Togawa, & Iri, 1985). Vineyard management practices and environmental factors that influence the amount of nitrogen (N) found in grapes and wine have been reported (Bell & Henschke, 2005).

Amino acids are primary metabolites that are important to grapevines' survival, but are also valuable nutrients for the yeasts and bacteria responsible for alcohol and malolactic fermentations (Bell & Henschke, 2005). Ammonia and certain free amino acids, which are primary amines, impact fermentation rate and the completion of fermentation, and these compounds are known as yeast assimilable nitrogen (YAN). Grape cultivar, must pH, yeast strain, and other factors have been reported to influence the utilisation of the available must N during fermentation (Bell & Henschke, 2005; Garde-Cerdan & Ancin-Azpilicueta, 2008; Monteiro & Bisson, 1991; Taillandier, Portugal, Fuster, & Strehaiano, 2007). Low grape YAN can lead to stuck or sluggish fermentation and potentially produce undesirable compounds in the resulting wine, such as sulfur and thiol-containing compounds like hydrogen sulfide, or fusel alcohols (Bell & Henschke, 2005; Beltran, Esteve-Zaraoso, Rozes, Mas, & Guillamon, 2005; Vilanova et al., 2007). Fermentation conditions (must temperature, oxygen level, timing of N addition, form of N addition, etc.) and juice components such as sugar and vitamin levels alter the yeast fermentation kinetics for different forms of available N (Arias-Gil, Garde-Cerdan, & Ancin-Azpilicuta, 2007; Beltran et al., 2005; Taillandier et al., 2007). It is still unknown if YAN compounds derived from grape are superior to N supplements from diammonium phosphate (DAP) for a healthy fermentation of N-deficient must into fine wine. Some amino acids are precursors to particular volatile compounds and recent studies have demonstrated that the addition of free amino acids increased a number of wine volatiles (Garde-Cerdan & Ancin-Azpilicueta, 2008; Hernandez-Orte, Cacho, & Ferreira, 2002).

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0308-8146/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2009.03.082

'Pinot noir' grapes and wines are major economic contributors to the state of Oregon, where 'Pinot noir' is the dominant cultivar grown, and made up more than 50% of the 38,000 tons of wine grapes produced in 2007. 'Pinot noir' production in Oregon is followed, in order of tons produced, by 'Pinot gris', 'Chardonnay', 'Riesling', and 'Cabernet Sauvignon' (National Agricultural Statistics Service, 2008a).

The presence of grapevine leafroll associated viruses (GLRaV) has been reported in Oregon vineyards (Martin, Eastwell, Wagner, Lamprecht, & Tzanetakis, 2005). GLRaV status has the potential to alter berry composition, beyond changes in their phenolic profiles (Lee & Martin, 2009; Singh Brar, Singh, Swinny, & Cameron, 2008), and N containing compounds (Kliewer & Lider, 1976; Ueno et al., 1985). To the best of our knowledge, there are only reports on the impact of GLRaVs on free amino acids of 'Burger' ('Burger' scion grafted onto 'Dogridge' rootstock), 'Zenkoji', and 'Koshu' grapes (Kliewer & Lider, 1976; Ueno et al., 1985), and none on the cultivar 'Pinot noir'.

GLRaVs are phloem-limited viruses that can result in reduced net leaf photosynthesis, decreasing % soluble solids and reducing vine productivity. Because of this, there is interest in exploring how GLRaV infections alter berry sugars and organic acids that contribute greatly to the taste and final alcohol content of the wine (Bertamini, Muthuchelian, & Nedunchezhian, 2004; Cabaleiro, Segura, & Garcia-Berrios, 1999; Christov et al., 2007; Guidoni, Mannini, Ferrandino, Argamante, & Di Stefano, 1997; Kliewer & Lider, 1976; Lee & Martin, 2009; Ueno et al., 1985).

It is well established that GLRaVs impact grape differently depending on cultivar, rootsock, scion, vine age, and causal virus (Cabaleiro et al., 1999; Golino, 1993; Guidoni et al., 1997; Kliewer & Lider, 1976; Kovacs, Hanami, Fortenberry, & Kaps, 2001; Krake, 1993; Lee & Martin, 2009; Singh Brar et al., 2008; Wolpert & Vilas, 1992). A recent report conducted in our laboratories (Lee & Martin, 2009) demonstrated that GLRaVs imparted a significant decrease in individual and total anthocyanins in 'Pinot noir' berries from *Vitis riparia* rootstock/'Pinot noir' clone 114 scion, with other minor differences in non-anthocyanin phenolics, but no significant difference in anthocyanins from GLRaV were observed in the other two rootstock/scion combinations examined (unknown rootstock/'Chardonnay' interstock/'Pinot noir' scion [clone unknown] and self-rooted/'Pinot noir' clone Pommard). It is important to understand how GLRaVs impact compounds beyond phenolics (i.e., N-containing compounds) that influence wine grape quality and healthy fermentations.

The objective of this study was to compare the compounds important for a healthy fermentation (i.e., simple sugars, organic acids, ammonia, and free amino acids) of 'Pinot noir' berries from vines infected by GLRaV-2 or -3 with berries from vines free of these viruses, to better understand how GLRaVs impact 'Pinot noir' grape quality.

2. Materials and methods

2.1. Plant material

Details of the samples, vineyards, vines, collection dates, and harvest dates were presented in Lee and Martin (2009), but for convenience, the information has been reproduced with modifications in Table 1, with the same abbreviations being used for this paper. All grapes were obtained from commercial fields. Briefly, four clusters were randomly taken from each presumptively identified healthy or infected vine (at each reported collection date; Table 1) and stored at −23 °C, until virus status was determined for each vine by RT-PCR in the laboratory. All clusters from a vine were grouped for chemical analysis. Vineyards one (VY1; one rootstock/scion combination) and two (VY2a and VY2b representing the two different rootstock/scion combination sampled from vineyard 2) were approximately 40 km apart. Both vineyards were located in Oregon's Willamette Valley (USA).

2.2. Reagents, chemicals, and standards

All chemicals for ammonia, organic acids, simple sugars, and free amino acid standards analyses were obtained from Sigma Chemical Co. (St. Louis, MO). Chemicals for the in-line derivatisation prior to HPLC injection were purchased from Agilent Technologies Inc. (Santa Clara, CA). All solvents and chemicals for this investigation were analytical and high performance liquid chromatography (HPLC) grade.

2.3. Virus detection

Virus status was determined as reported previously (Martin et al., 2005). Alterations to the detection procedure were described in Lee and Martin (2009).

2.4. Extraction and sample preparation

Berries were excised from the clusters (four clusters per vine from 5 to 11 vines depending on virus status; Table 2). Whole berries were puréed with a hand blender for 3 min (which macerated skin, pulp, and seeds), then centrifuged for 10 min at 4000 rpm. The resulting supernatants were then filtered with disposable 25 mm GD/X syringe filters (Whatman Inc., Florhan Park, NJ) prior to organic acids, simple sugars, and ammonia determination. Disposable Millex-FH syringe filters (Millipore, Bedford, MA) were used for supernatants prior to free amino acid determination.

2.5. HPLC conditions for organic acids, sugars, and free amino acids analyses

An Agilent HP1100 system (Agilent Technologies Inc.) equipped with a diode array detector (DAD) connected to refractive index detector (RID), and a Rezex ROA-organic acid H + (300 mm × 7.8 mm, 8 μm; Phenomenex, Torrance, CA) column were used for organic acid and sugar analyses. A ratio of 80% diluted sulfuric acid (2.5 mM sulfuric acid solution) to 20% acetonitrile was used as the mobile phase (m.p.) at a flow rate of 0.5 ml/min under isocratic conditions. The analytical column compartment was maintained at 55 °C during the 15 min analysis. Organic acids were detected at 210 nm with a DAD. RID was used for simple sugar identification and quantification. Tartaric acid, malic acid, fumaric acid, glucose, and fructose external standards were used. The malic acid standard was corrected for the fumaric acid contamination that occurs in all malic acid standards. Sugar and organic acid values were expressed as mg/100 ml.

HPLC/DAD was used for individual free amino acid analysis. Zorbax Eclipse AAA analytical (150 mm × 4.6 mm, 5 μm, Agilent Technologies Inc.) and guard (12.5 mm × 4.6 mm, 5 μm; Agilent Technologies Inc.) columns were used for free amino acid determination. Inline-derivatisation by *o*-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) was performed by the autosampler immediately prior to injection, as described in detail by Henderson, Ricker, Bidlingmeyer, and Woodward (2006). Mobile phase A was 40 mM sodium phosphate (adjusted to pH 7.8 with 10 N sodium hydroxide solution). Mobile phase B was a mixture of acetonitrile: methanol: water (45:45:10, v/v/v). HPLC mobile phase conditions were extended from the gradient program described by Schuster (1988) and Henderson et al. (2006) to suit our HPLC and column set-up. Three min extension of the gradient (m.p. B was 57–81% and the rest of the m.p. was made up by m.p. A

Table 1
Description of samples taken from two commercial vineyards in Oregon (Lee & Martin, 2009).

	Vineyard 1	Vineyard 2	Vineyard 2
Code	VY1	VY2a	VY2b
Location	Amity, OR	Yamhill, OR	Yamhill, OR
Cultivar	Pinot noir	Pinot noir	Pinot noir
Rootstock/scion	Unknown rootstock/'Chardonnay' interstock/'Pinot noir' scion (clone unknown)	*V. riparia* rootstock/'Pinot noir' clone 114	Self-rooted/'Pinot noir' clone Pommard
Virus tested for	GLRaV types 1–3 and RSPaV	GLRaV types 1–3 and RSPaV	GLRaV types 1–3 and RSPaV
Virus confirmation after testing	GLRaV-2 positive or negative; RSPaV positive	GLRaV-3 positive or negative; RSPaV positive	GLRaV-3 positive or negative; RSPaV positive
Sampling dates for both seasons. Codes used in Figs. 1–3. Dates in bold were final commercial harvest dates	VY1-1: 9/19/2005	VY2a-1: 9/28/2005	VY2b-1: 9/28/2005
	VY1-2: 9/26/2005	**VY2a-2: 10/3/2005**	**VY2b-2: 10/3/2005**
	VY1-3: 10/3/2005	VY2a-3: 9/15/2006	VY2b-3: 9/15/2006
	VY1-4: 10/11/2005	**VY2a-4: 9/22/2006**	VY2b-4: 9/22/2006
	VY1-5: 10/17/2005		**VY2b-5: 9/28/2006**
	VY1-6: 9/15/2006		
	VY1-7: 9/22/2006		
	VY1-8: 9/28/2006		

Table 2
Virus status, simple sugars, organic acids, ammonia, individual free amino acids, total free amino acids (FAN) and yeast assimilable nitrogen (YAN) at harvest from all locations are listed. All sampled vines tested positive for RSPaV. Different lower case letters indicate significant differences ($p \leqslant 0.05$) within the pair of samples (e.g., samples from GLRaV-2 positive versus negative vines from vineyard 1). Different upper case letters indicate significant differences ($p \leqslant 0.05$) among the three healthy 'Pinot noir' samples (comparison among VY1, VY2a, and VY2b). No lower or upper case letters after values indicate no significant difference. Values in parenthesis are standard errors.

Sample code from the two vineyards		VY1		VY2a		VY2b	
Harvest dates		10/17/2005 and 9/28/2006		10/03/2005 and 9/22/2006		10/03/2005 and 9/28/2006	
Virus status		GLRaV-2 positive	GLRaV-2 negative	GLRaV-3 positive	GLRaV-3 negative	GLRaV-3 positive	GLRaV-3 negative
Number of vines corresponding to the GLRaV results		7	8	5	11	6	10
Total simple sugars (g/100 ml)		24.4 (1.6)	24.8 (2.2) A	25.0 (0.7)	26.2 (0.7) AB	25.6 (0.9)	27.4 (0.7) B
Glucose		11.9 (0.3)	12.1 (0.4) A	12.4 (0.4)	12.9 (0.3) AB	12.5 (0.5)	13.4 (0.3) B
Fructose		12.5 (0.3)	12.7 (0.3) A	12.6 (0.3)	13.3 (0.4) AB	13.1 (0.4)	13.9 (0.3) B
Total organic acids (g/100 ml)		0.81 (0.03)	0.78 (0.02) B	0.70 (0.02)	0.71 (0.03) A	0.63 (0.03) a	0.70 (0.02) b A
Tartaric acid		0.43 (0.02)	0.43 (0.02)	0.40 (0.02)	0.45 (0.02)	0.37 (0.03)	0.40 (0.02)
Malic acid		0.38 (0.03)	0.35 (0.03) B	0.30 (0.02)	0.26 (0.01) A	0.25 (0.02) a	0.30 (0.01) b AB
Ammonia in juice (mg N/l)		43.3 (3.2)	49.2 (3.0) B	20.8 (1.3)	19.8 (1.7) A	19.0 (3.9)	19.9 (2.6) A
Total free amino acids (mg N/l) = FAN		421.4 (44.4)	393.9 (30.2) B	177.6 (13.3)	189.8 (13.7) A	150.4 (29.2)	157.3 (22.6) A
1	Aspartic acid (ASP)	2.6 (0.4)	2.4 (0.2)	3.0 (0.6)	3.3 (0.3)	3.3 (0.9)	2.8 (1.0)
2	Glutamic acid (GLU)	4.5 (0.6)	4.5 (0.5) B	1.5 (0.1)	1.8 (0.2) A	1.5 (0.3) a	2.3 (0.2) b A
3	Asparagine (ASN)	1.5 (0.8)	1.3 (0.3) B	1.0 (0.1)	1.1 (0.2) AB	0.7 (0.1)	0.9 (0.1) A
4	Serine (SER)	11.4 (2.5)	11.7 (1.7) C	7.6 (0.5)	8.2 (0.5) B	6.0 (0.6)	6.5 (0.6) A
5	Glutamine (GLN)	19.9 (5.1)	21.3 (2.9) B	12.2 (0.9)	14.3 (1.0) A	10.1 (1.7)	11.3 (1.8) A
6	Histidine (HIS)	13.8 (1.8)	11.5 (0.8) B	7.3 (0.6)	7.4 (0.6) A	6.0 (1.3)	6.2 (0.8) A
7	Glycine (GLY)	1.2 (0.1)	1.1 (0.1)	1.1 (0.2)	0.9 (0.1)	0.7 (0.1)	0.9 (0.1)
8	Threonine (THR)	27.0 (1.9)	24.9 (1.5) B	14.4 (1.2)	14.4 (0.8) A	14.0 (2.4)	12.5 (1.4) A
9	Citrulline (CIT)	2.8 (0.5)	2.6 (0.4) B	1.1 (0.1)	1.4 (0.1) A	0.9 (0.2)	1.2 (0.2) A
10	Arginine (ARG)	231.7 (28.6)	207.2 (19.8) B	73.6 (8.2)	74.1 (7.3) A	62.0 (16.9)	61.3 (11.5) A
11	Alanine (ALA)	26.0 (2.3)	26.1 (2.2) B	15.5 (1.3)	12.3 (1.1) A	10.8 (0.8)	13.0 (1.1) A
12	Tyrosine (TYR)	0.6 (0.1)	0.5 (0.1) B	0.3 (0.0)	0.3 (0.0) AB	0.2 (0.0)	0.2 (0.0) A
13	Valine (VAL)	2.9 (0.4)	3.1 (0.4) A	2.7 (1.6) a	6.6 (1.0) b B	3.6 (0.7)	2.6 (0.3) A
14	Methionine (MET)	0.4 (0.1)	0.5 (0.2) A	0.3 (0.2) a	1.5 (0.3) b B	0.6 (0.1)	0.4 (0.1) A
15	Tryptophan (TRP)	4.1 (0.6)	4.1 (0.4) B	2.0 (0.2)	2.7 (0.2) A	2.1 (0.2)	2.4 (0.2) A
16	Phenylalanine (PHE)	4.8 (1.0)	5.3 (0.7) B	2.0 (0.3)	2.8 (0.4) A	2.0 (0.2)	2.6 (0.3) A
17	Isoleucine (ILE)	4.6 (1.0)	5.2 (0.8) B	1.7 (0.3)	2.6 (0.4) A	1.8 (0.2)	2.4 (0.3) A
18	Leucine (LEU)	7.3 (1.4)	8.2 (1.2) B	3.2 (0.4)	3.7 (0.7) A	2.6 (0.4)	3.6 (0.4) A
19	Lysine (LYS)	1.4 (0.1)	1.3 (0.1)	0.8 (0.1)	1.1 (0.4)	0.7 (0.0)	0.7 (0.1)
20	Hydroxyproline (HYP)	31.3 (3.9)	28.1 (2.9) B	12.3 (1.7)	12.5 (1.6) A	9.4 (2.5)	11.4 (3.7) A
21	Proline (PRO)	21.8 (2.3)	22.9 (1.7) B	13.8 (2.8)	16.6 (1.8) A	11.4 (1.7)	12.1 (2.4) A
YAN (mg N/l)		411.7 (44.7)	392.1 (30.4) B	172.3 (12.8)	180.5 (12.8) A	148.7 (29.0)	153.6 (19.7) A

from 43% to 19%) was included at the end of the analytical conditions described by Henderson et al. (2006). OPA-derivatised amino acids were monitored at 338 nm and FMOC-derivatised amino acids were monitored at 262 nm. Purchased standards of each individual amino acid (Sigma Chemical Co.) were used for identification and quantification (external standard method). Two internal standards, norvaline for OPA-derivatised amino acids and sarcosine for FMOC-derivatised amino acids, were used. Individual free amino acid values were expressed as mg N/l.

2.6. Ammonia concentration and calculation of YAN values

Ammonia was quantified by an enzymatic assay (Sigma ammonia assay kit; Sigma Chemical Co.) using a spectrophotometer (SpectraMax M2 microplate reader; Molecular Devices Corp., Sunnyvale, CA). Manufacturer instructions were followed. One-centimeter path length disposable semi-micro cuvettes (Fisherbrand, Thermo Fisher Scientific Inc., Waltham, MA) were used for ammonia measurements. YAN content was calculated by combining the

ammonia concentration found in the berries with primary free amino acids. Primary free amino acid content was determined by excluding hydroxyproline and proline contents from total free amino acids found in the berry samples. Ammonia and YAN were expressed as mg N/l.

2.7. Statistical analysis

Statistica for Windows version 7.1 was used (StatSoft, Inc., Tulsa, OK) for *t*-test calculations and one-way analysis of variance (ANOVA) for the pair of samples from infected and healthy vines with the same rootstock/scion combination ($\alpha = 0.05$). Correlation between organic acid values determined by HPLC and titratable acidity (values from Lee and Martin (2009)), sugars determined by HPLC and % soluble solids (values from Lee and Martin (2009)), and between ammonia and primary amino acid values were calculated ($\alpha = 0.05$). Differences among the three different rootstock/scion the values were compared using Fisher's LSD (least significant difference; $\alpha = 0.05$).

3. Results and discussion

Again, details of the vineyard location and vine virus status were reported in the first part of this project (Lee & Martin, 2009). Briefly, all vines despite their GLRaV status in both vineyard locations tested positive for grapevine Rupestris stem pitting-associated virus (RSPaV). GLRaV-infected vines from VY1 had co-infection of RSPaV and GLRaV-2. VY2a and VY2b GLRaV-positive vines were co-infected with RSPaV and GLRaV-3. Vineyard and virus data are summarised in Table 1. The observed results in this study were assumed to be due to GLRaV status despite the co-infection with RSPaV. RSPaV has been reported as having little or no significant impact on the yield components of titratable acidity, and pH in five grape cultivars ('Kerner', 'Michurinetz', 'Okanagan Riesling', 'Madeleine Sylvaner', and 'Ortega'; Reynolds, Lanterman, & Wardle, 1997). Additional details of the vineyards and vines can be found in Lee and Martin (2009). There was no significant interaction between growing season and vine virus status, so the data of two seasons are pooled and presented in Table 2.

3.1. Simple sugars and organic acids

Two simple sugars, glucose and fructose, were found in all samples; glucose and fructose as reported by others (Diakou et al., 1997; Kliewer, 1967; Kliewer, Lider, & Schultz, 1967). In general, sugar content in all samples steadily increased during the sampling period up to harvest (Fig. 1). The ratio of glucose to fructose ranged from 0.91 to 1.02. There were no significant differences between fruit from infected and healthy vines in their glucose and fructose contents throughout the sampling periods (Fig. 1; Table 2). GLRaV infection status did not alter sugar composition or content.

Tartaric acid and malic acid were the two main organic acids identified in 'Pinot noir', which have been reported by others (Diakou et al., 1997; Kliewer et al., 1967). When both seasons were combined, there were no differences in tartaric and malic acids at commercial harvest between healthy and infected vines from VY1 or VY2a (Table 2). Samples from healthy vines in VY2b had significantly higher levels of malic acid and total organic acids compared to those from GLRaV-3 infected vines when both growing season values were compared (berry sizes were not significantly different; Lee & Martin, 2009). In 2006, samples from VY2a vines infected

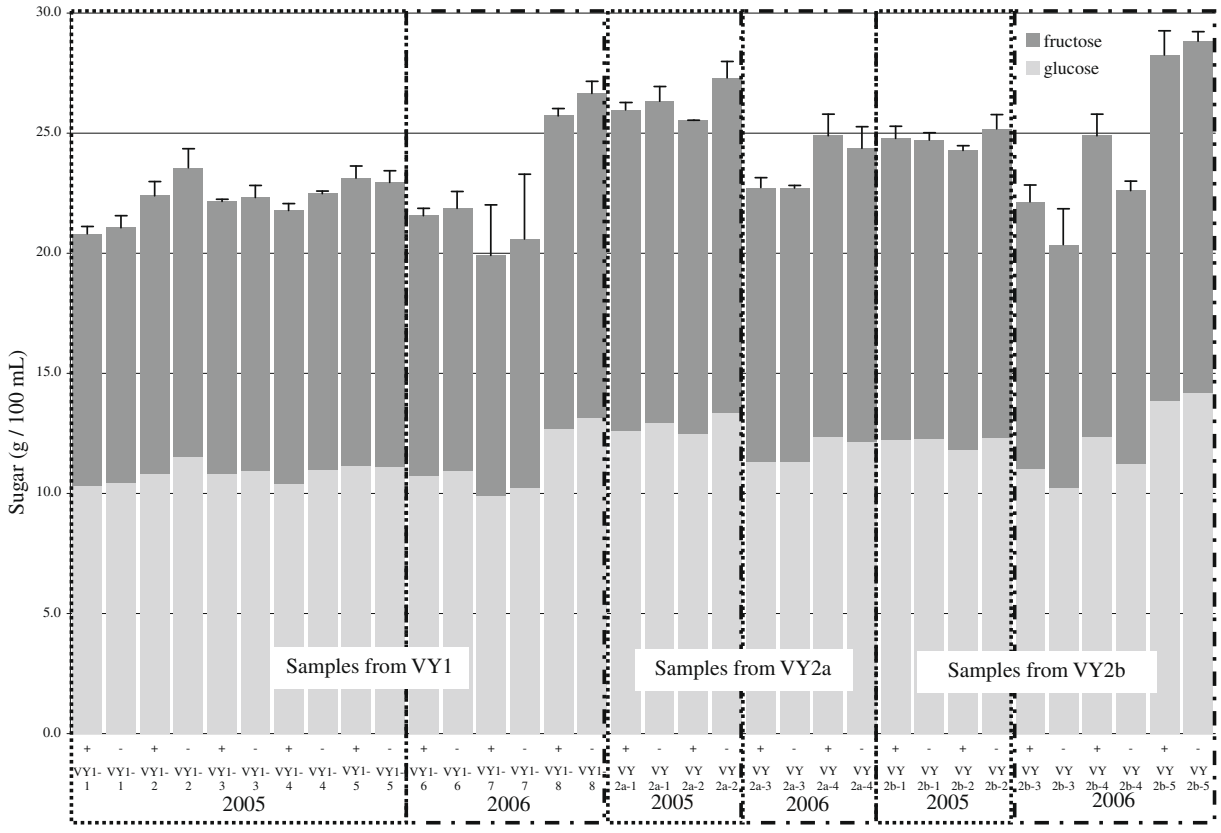

Fig. 1. Simple sugars during the sampling periods from VY1, VY2a, and VY2b. The '+' indicates GLRaV-positive and '–' indicates GLRaV-negative by RT-PCR. VY1-5, VY1-8, VY2a-2, VY2a-4, VY2b-2, and VY2b-5 indicate commercial harvest. The corresponding sampling dates (e.g., VY1-1 sampled 9/19/2005) are listed in Table 1. Sugar values were obtained by HPLC as g/100 ml. No pairs were significantly different at $p \leqslant 0.05$ (infected versus healthy). Error bars indicate standard errors.

with GLRaV-3 were significantly higher in malic acid at harvest compared to healthy vines (Fig. 2), which was not observed in 2005 and did not significantly alter total organic acid content.

Total sugar and total organic acid values obtained in this study were highly correlated (r values of 0.964 and 0.605, $p \leqslant 0.05$) to °Brix and titratable acidity values, respectively (Lee & Martin, 2009).

GLRaV (type not reported) eliminated (negative) 'Zenkoji' and 'Koshu' grapes had higher levels of glucose and fructose (Ueno et al., 1985) compared to their infected cohorts. Guidoni et al. (1997) reported no significant difference in malic and tartaric acids in 'Nebbiolo' healthy vines versus vines infected with GLRaV-3. GLRaV (type not reported) positive 'Burger' grapes (Kliewer & Lider, 1976) were reported to have higher levels of titratable acidity, malic acid, and tartaric acid compared to healthy vines.

A comparison among the healthy vines from the three rootstock/scion combinations were made, and VY2b samples were significantly higher in glucose, fructose, and total sugar compared to VY1 samples (Table 2) at the time of harvest. Healthy VY1 samples were significantly higher in malic acid and total organic acids, although tartaric acid was not different among the three different rootstock/scion combinations.

3.2. Ammonia, FAN (free amino acids), and YAN of grapes

Ammonia content (main mineral N source) ranged from 19.0 to 49.2 mg N/l at harvest (contributed to 10–14% of grape YAN; Table 2). Grapes from VY1 had overall higher levels (more than double) of ammonia (Table 2 and Fig. 3) than VY2a and VY2b samples. There were no significant differences in ammonia content between GLRaV-infected and healthy vines in the weeks prior to harvest or at commercial harvest in VY1, VY2a, and VY2b samples (Fig. 3).

Twenty-one free amino acids (Asp, Glu, Asn, Ser, Gln, His, Gly, Thr, Cit, Arg, Ala, Tyr, Val, Met, Trp, Phe, Ile, Leu, Lys, Hyp, and Pro; in the order of HPLC elution) were found in all samples, and are listed in Table 2. ARG was the main free amino acid, contributing to 30–62% of total free amino acids (FAN), in all 'Pinot noir' grapes, as reported by Huang and Ough (1991) and Stines et al. (2000).

Individual free amino acids, FAN, and YAN were not significantly different between the grapes from healthy and infected vines in VY1. Val and Met contents of VY2a healthy vines were significantly higher, compared to their infected counterparts. Only one amino acid, Glu from VY2b grapes, was significantly higher in grapes from healthy compared to infected vines. Overall, GLRaV infection did not impact N-containing compounds when the summation (YAN and FAN values) was compared in 'Pinot noir' grapes. The same statistical results were obtained when individual amino acid values were compared as mg of amino acids/l as well (data not shown). FAN and ammonia contents were not significantly different throughout the sampling period either (Fig. 3).

At harvest, all samples had an acceptable level of YAN (above 150 mg N/l) from the general guidelines of Bely, Sablayrolles, and Barre (1990), who recommends additional must nutrients (N supplementation in the must) if YAN is below 140 mg N/l for a healthy fermentation. Free amino acids were the major contributor to YAN values (86–90% of YAN was from free amino acids) in these samples at harvest as reported previously (Butzke, 1998).

Butzke (1998) performed a survey on commercial must of 'Pinot noir' grapes ($n = 88$) and found ammonia of 92 mg N/l, primary

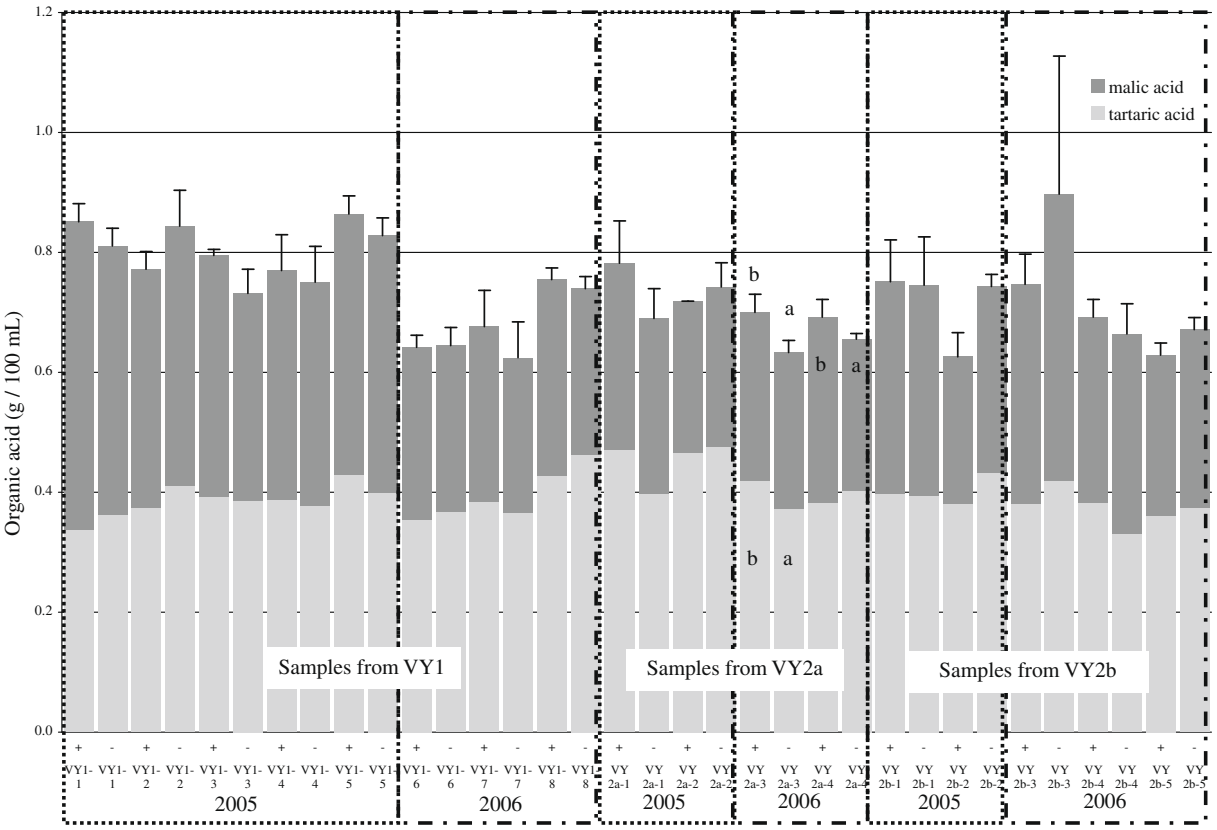

Fig. 2. Organic acids during the sampling periods from VY1, VY2a, and VY2b. The '+' indicates GLRaV-positive and '–' indicates GLRaV-negative by RT-PCR. VY1-5, VY1-8, VY2a-2, VY2a-4, VY2b-2, and VY2b-5 indicate commercial harvest. The corresponding sampling dates (e.g., VY1-1 sampled 9/19/2005) are listed in Table 1. Organic acids were obtained by HPLC as g/100 ml. Different lower case letter indicates significant differences at $p \leqslant 0.05$ for the pair of samples (infected versus healthy). No lower case letter indicates no significant difference between healthy and infected vines. Error bars indicate standard errors.

amino acids 142 mg N/l, and YAN content at 236 mg N/l on average. Samples from VY2a and VY2b, despite virus status, were within what Butzke (1998) reported, but VY1 samples were well above these values.

Individual free amino acids were grouped into the following five groups: Ala-Val-Leu family (Ala, Val, and Leu), aromatic amino acid family (Phe, Tyr, His, and Trp), Asp family (Asp, Thr, Asn, Met, and Ile), Ser family (Ser and Gly), Glu family (Glu, Gln, Cit, Arg, Hyp, and Pro), and Lys, based on chemical similarities and biosynthesis pathways. There were no significant differences between samples from healthy and infected vines for all three rootstock/scion combinations. It appears that GLRaV did not alter the profile of amino acids in these rootstock/scion combinations when values were compared as grouped amino acid families.

At harvest, GLRaV-infected 'Burger' berries had significantly more ARG and less PRO than those from healthy vines (Kliewer & Lider, 1976) when compared to cluster-thinned vines. In 'Zenkoji' and 'Koshu' grapes, N and the majority of the free amino acids were lower in fruit from virus-eliminated vines (Ueno et al., 1985). These two previous publications and the work presented here demonstrate the varying impacts from GLRaV infection among grape cultivars, GLRaV type, etc.

The individual amino acids, FAN, YAN, and ammonia were compared among the samples from the healthy vines from the three rootstock/scion combinations at harvest (Table 2). Berries from VY1 were significantly higher in individual free amino acids (except for Asp, Asn, Gly, Tyr, Val, Met, and Lys of VY2a samples or Asp, Gly, Val, Met, and Lys of VY2b samples), FAN, YAN, and ammonia than VY2a and VY2b samples. Berries from VY2a were significantly higher in Val and Met, compared to samples from VY1 or VY2b. These observed variations might be due to rootstock/scion combinations, as was found in 'Chardonnay' (Treeby, Holzapfel, Walker, & Nicholas, 1998) and 'Shiraz' (Holzapfel & Treeby, 2007), or the disparity in vineyard management practices (i.e., levels, timing, and rate of N application in the vineyard; Bell & Henschke, 2005; Holzapfel & Treeby, 2007).

There was a significant correlation between primary amino acids and ammonia content in the juice samples ($r = 0.86$ from all the juice samples and $r = 0.924$ from time of commercial harvest $p \leqslant 0.05$), which was not observed by Butzke (1998). He reported no correlation between primary amino acids and ammonia, and r of 0.29.

This preliminary work provides some insight into the minor differences in individual free amino acids, ammonia content, and YAN content of juice imparted by GLRaV infection. The lack of magnitude in the measured differences during this study might be due to decreased vine stress imposed by the Oregon vineyard practice of low crop load (average 2.8 tons per acre in Oregon for 2007; National Agricultural Statistics Service, 2008a) compared to other grape growing regions (e.g., on average 6–8 tons per acre in California for 2007; National Agricultural Statistics Service, 2008b).

Future work is needed to determine and clarify how vine age, virus infection period, rootstock/scion combination, and virus type influences the relationships among amino acids, sugars, and organic acids in an experimental vineyard setting, in order to eliminate the variability of commercial vineyard management. This future controlled study will allow us to understand the direct impact of GLRaVs.

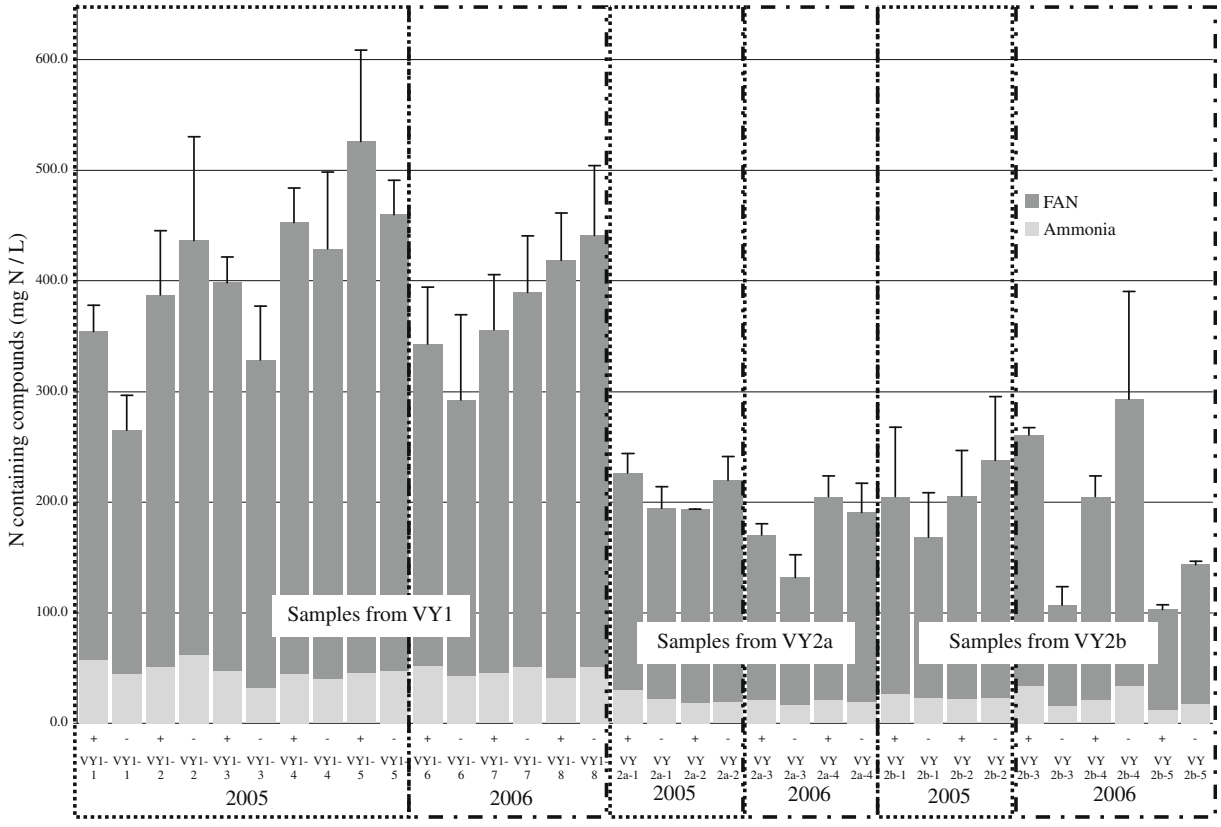

Fig. 3. FAN and ammonia values during the sampling periods from VY1, VY2a, and VY2b. The '+' indicates GLRaV-positive and '–' indicates GLRaV-negative by RT-PCR. VY1-5, VY1-8, VY2a-2, VY2a-4, VY2b-2, and VY2b-5 indicate commercial harvest. The corresponding sampling dates (e.g., VY1-1 sampled 9/19/2005) are listed in Table 1. FAN were obtained by HPLC as mg of N/l. Ammonia (mg N/l) were determined by enzymatic assay. None of the paired samples were significantly different from each other. Error bars indicate standard errors.

4. Conclusion

To the best of our knowledge, this is the first report upon the investigation of how GLRaVs impacted N-containing compounds, simple sugars, and organic acids in 'Pinot noir' berries from commercial vineyards in Oregon. In most comparisons, GLRaV did not significantly alter organic acids, simple sugars, ammonia, YAN, or FAN between fruit from healthy and infected vines. The exceptions were malic acid, total organic acid, Val + Met (in *V. riparia* rootstock/'Pinot noir' clone 114; VY2a), and Glu (in self-rooted/'Pinot noir' clone Pommard; VY2b) in GLRaV infected samples, compared to their GLRaV-negative cohorts. Among the healthy vines of the three rootstock/scion combinations, there was an apparent difference in the N-containing compounds, sugars, and acids, but this has been established for differing rootstock/scion combinations within a cultivar.

Acknowledgements

We thank Sam Kamo for technical assistance and the two vineyard managers for access to their vines. This work was funded by USDA-ARS.

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Food Chemistry 117 (2009) 106–113

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Effect of various levels of rosemary or Chinese mahogany on the quality of fresh chicken sausage during refrigerated storage

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ARTICLE INFO

Article history:
Received 18 July 2008
Received in revised form 18 March 2009
Accepted 23 March 2009

Keywords:
Chinese mahogany
Rosemary
Fresh sausage
Volatile compound

ABSTRACT

The purpose of this study was to evaluate the effect of rosemary or Chinese mahogany, at levels of 500, 1000 and 1500 ppm, of the phenolic compounds, on the quality of fresh chicken sausage stored at 4 °C for 14 days. The results showed that sausages with addition of Chinese mahogany or rosemary underwent less pH value reduction. The intense colour of Chinese mahogany or rosemary resulted in samples with lower *L* values and higher *a* values. Samples with more Chinese mahogany or rosemary added had higher total phenolic compounds. Lower TBA (thiobarbituric acid) and VBN (volatile basic nitrogen) values, and lower total plate counts were observed for the samples with Chinese mahogany or rosemary added. Samples with Chinese mahogany added had higher overall acceptance than had samples with rosemary added. Some volatile compounds, including alcohols, acids, esters, aldehydes, ethers and phenolic compounds, were isolated from the samples and identified.

1. Introduction

Sausage is one of the oldest known forms of processed meat products and is very popular in many areas. Fresh sausages, e.g. fresh pork sausage, country-style pork sausage, fresh kielbasa (Polish), Korr (Swedish), Italian sausage, bratwurst, bockwusrt, chorizo (fresh) and thuringer (fresh), are some common examples (Romans, Costello, Carlson, Greaser, & Jones, 1994). The cited authors indicate that fresh sausage is a sausage "made from selected cuts of fresh meat (not cooked or cured) and must be stored in a refrigerated (or frozen) state prior to being consumed." Therefore, adding "curing agents" (mainly nitrites and nitrates) to a formula, or not, is the major criterion used to judge whether the product belongs to "fresh sausage" or cured sausage. Also, raw materials of fresh sausage should not be cooked. No typical thermal treatments, such as drying, smoking or cooking, should be applied when making fresh sausages.

Lipid oxidation, resulting in rancidity, is one of the most important quality defects of meat or meat product during storage. Antioxidants can retard lipid rancidity in foods and prolong product shelf life. Since consumers have concerns regarding synthetic antioxidants, such as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) and propyl gallate (PG), natural antioxidants may be applied in foods (Aruoma, Halliwell, Aeschbach, & Löligers, 1992). Many herbs and spices contain phenolic compounds, which have some antioxidative properties.

Rosemary (*Rosmarinus officinalis* L.), like other aromatic herbs and spices, which has been planted in many areas and used in Mediterranean and other cuisine, is not only used to improve or modify flavours of foods, but also to provide some functionality. For example, its extract has been widely used as an antioxidant in the food industries. Carnosol, carnosic acid and rosmarinic acid have been identified as major constituents that contribute to the antioxidant activity of rosemary (Aruoma et al., 1992). Utilising DPPH and ABTS radical-scavenging assays, and the ferric thiocyanate test, Erkan, Ayranci, and Ayranci (2008) pointed out that rosemary extract had a higher phenolic content than had blackseed (*Nigella sativa* L.) essential oil, thus leading to a higher antioxidant activity. Many reports have indicated that rosemary extracts can retard lipid oxidation and prolong the shelf life of meat products (Georgantelis, Ambrosiadis, Katikou, Blekas, & Georgakis, 2007; Georgantelis, Blekas, Katikou, Ambrosiadis, & Fletouris, 2007; Sebranek, Sewalt, Robbins, & Houser, 2005). In addition, rosemary extracts have been shown to have some antimicrobial effect (Angioni et al., 2004).

Chinese mahogany, also known as *Toona sinensis* Roem, is a perennial tree that has become widely grown in Taiwan and China (Edmonds & Staniforth, 1998). Its leaves have a special aroma and are often consumed in Taiwan. Several reports regarding the medical uses of this plant, such as for treatment of enteritis, dysentery and itch (in the practice of oriental medicine) and for anticancer and hypoglycaemic effects have also appeared (Edmonds & Staniforth, 1998). Hseu et al. (2008) reported that *T. sinensis* aqueous extracts, at levels up to 100 μg/ml, showed some antioxidant activities, including the scavenging of free and superoxide anion

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doi:10.1016/j.foodchem.2009.03.083

radicals, reducing power and metal chelation. Methanol extracts of *T. sinensis* also demonstrated strong DPPH radical-scavenging activities and inhibitory effects on lipid peroxidation Cho et al. (2003). Similarly, some potent antioxidative components in the young leaves and shoots of *T. sinensis* let to a promising healthy-promoting food (Wang, Yang, & Zhang, 2007). Even though, *T. sinensis* was reported to have some antimicrobial activity (Shi, 2003), limited information regarding the antimicrobial effect of this plant is available.

Therefore, the aim of this study was to compare the effects of rosemary or Chinese mahogany on the quality of fresh chicken sausage during refrigerated storage. We also wanted to identify the volatile compounds from Chinese mahogany, rosemary and sausages with rosemary or Chinese mahogany added.

2. Materials and methods

2.1. Rosemary and Chinese mahogany preparation

Rosemary, which was obtained from a local spice company in Taiwan, was comminuted with a grinder (DIAX 600, Heidolph, Germany) into approximately 2 mm lengths, and stored in a moisture-proof cabinet (BK236, Bossmen, Taiwan). Chinese mahogany leaves, which were obtained from a local farm in Pingtung, Taiwan, were dried in an oven at 60 °C for 8 h, ground with the same grinder to approximately 2 mm in length, and then stored in the moisture-proof cabinet.

A pre-measurement of the total phenol contents in rosemary and Chinese mahogany was first conducted according to the Folin–Ciocalteu method of Tsau (2006) and is described briefly as follows. Two grams of ground rosemary or Chinese mahogany were mixed with 100 ml of distilled water, boiled and extracted for 20 min, cooled rapidly, and filtered. The filtered liquid was combined with phenol reagent (Sigma) and saturated Na_2CO_3 (Union Chemical Works Ltd., Hsinchu, Taiwan), vortexed, and then held for 1 h. The optical density values were determined using a spectrophotometer (U3210, Hitachi, Japan) at 700 nm wavelength. A standard curve was prepared with gallic acid added and regression determined as $Y = 2.35X - 0.0472$, where Y represents OD (optic density) and X represents the concentration of the total phenol contents of the solution (mg/ml). Total phenol contents of rosemary or Chinese mahogany were determined according to the formula: Total phenol content (mg/g) = $(X \times 10 \times 100)/2 \times 1000$. Based on the preliminary test results, 395 and 82 mg/g total phenol contents were determined for the Chinese mahogany and rosemary, respectively, in this study. Therefore, amounts of 1.265, 2.530, or 3.795 g of ground Chinese mahogany were added to 1 kg of sausage mixtures, respectively, in order to have 500, 1000 or 1500 ppm of phenolic compounds, respectively. Similarly, amounts of 6.1, 12.2, or 18.3 g of ground rosemary were added to 1 kg of sausage mixtures, respectively, in order to have 500, 1000, or 1500 ppm of phenolic compounds, respectively.

2.2. Sausage preparation

Fresh chicken tenderloin, chicken skin, pork backfat and salted natural pork casing were purchased from local markets in Nantou, Taiwan. Chicken meat, chicken skin and pork backfat were first frozen at −20 °C and then ground. Chicken meat was ground through a 9 mm plate, whereas chicken skin and pork backfat were ground through a 6 mm plate. Ground chicken (75%) was mixed thoroughly with salt (1.8%) and polyphosphates (0.15%) with a mixer (DITO, BM10) for 1.5 min, and then other spices and seasonings were added, including 0.5% sugar, 0.3% monosodium glutamate, 0.1% white pepper powder, 0.075% nutmeg powder, 0.03% parsley powder, 0.03% thyme powder, 0.03% onion powder, pre-assigned amounts of ground Chinese mahogany leaves or rosemary and non-lean tissue (25%, in which the ground chicken skin:ground pork backfat ratio = 1:2), then mixed for another 1.5 min. The mixtures were cured at 4 °C for 16 h, and then stuffed (Stuffer, Dick D-73779, Germany) into pork casings which were soaked in water prior to use. Raw sausages were manually linked, packed in a tray with PVC film and stored at 4 °C.

2.3. Proximate composition and pH

Samples were first ground (with a grinder, 31BL91, Blender, USA). Proximate compositions of samples including moisture, crude fat, crude protein and ash contents, were measured according to the AOAC (1990) method. Crude fat was measured using a fat extractor (Sotec System HT 1043 Extraction Unit, Tecator Co. Sweden). Crude protein was measured using the Kjeldahl method using a digester (Model 2006, Foss tecator, Sweden) and a distillation unit (Model 2100, Foss tecator, Sweden). Ten gram samples were blended with 90 ml of distilled water in a polyethylene bag for 1 min using a stomacher (Stomacher 400, Seward Ltd., England) at high speed for 2 min, and then the pH of the mixture was measured using a pH meter (Micro-Computer pH meter, Model 6210, Taiwan).

2.4. Instrumental colour measurement

Ground samples were placed in a measuring container, and then the Hunter *L* (lightness), *a (redness)* and *b* (yellowness) values of samples were measured with a colour meter (Spectrophotometer, Model TC1, Tokyo Co., Ltd., Japan). A standard plate, with "*Y*" = 86.53, "*X*" = 82.45, and "*Z*" = 91.28, was used as a reference.

2.5. Total phenol contents in products

Fifty grams of ground sausage samples were mixed with 100 ml of distilled water, boiled and extracted for 20 min, cooled rapidly, filtered, and then put through the same method as described in Section 2.1 to determine the total phenol contents in products.

2.6. Thiobarbituric acid (TBA) values and volatile basic nitrogen (VBN)

TBA values of the samples were determined according the methods described by Faustman, Specht, Malkus, and Kinsman (1992). TBA value was expressed as mg malonaldehyde/kg of meat. Volatile basic nitrogen was determined according to CNS (1982) by the Conway micropipette diffusion method.

2.7. Microbial evaluation

At a specified sample time, sausages were aseptically removed from the bags. Ten gram samples were placed in a sterile bag containing 90 ml of sterile water and homogenised with a stomacher (Stomacher blender, Model 400, Seward) for 2 min. Serial dilutions were then made. Plate count agar (PCA, Merck) was used for enumeration of total plate count, and the pour plate method was used for enumeration of bacteria. Total microflora were incubated at 37 °C for 48 h. Microbial counts in this study were expressed as $\log_{10}$ colony forming units (CFU) per gram of sample.

2.8. Sensory evaluation

At days 0, 7 and 14, during storage, sausages were first cooked on a grill at 160 °C for 15 min, cooled at room temperature (approximately 25 °C), sliced (approximately 0.25–0.30 cm thickness), and then served to a sensory panel which consisted of 12

meat science-majored faculty and students. Sensory attributes, including colour, aroma, off-odour, flavour and overall acceptance were determined using 1–7 point hedonic scale, with 1, 4 and 7 representing extremely dislike, neither like nor dislike and extremely like, respectively, for the attributes.

2.9. Volatile compounds analysis

Volatile compounds were analysed according to the methods of Wang, Liu, and Chen (1998), and are described briefly as follows: 500 g of ground sausage samples was treated with 1 l of saturated sodium chlorine solution, homogenised for 30 s, transferred to a 5 l round-bottom flask attached to a Likens–Nickerson apparatus, and extracted for 4 h. A mixture of pentane (Merck) and diethyl ether (Merck) at 1:1 ratio (v/v) was used as an extracting solvent. After adding anhydrous sodium sulphate (Merck) to the extracted solution, extracted solution was filtered (Whatman No. 1), and condensed to 1–2 ml. The Likens–Nickerson concentrates were analysed by injecting 0.2 μl into a gas chromatograph (Model 5890 II, Hewlett–Packard, Palo Alto, CA, USA) coupled to a gas chromatograph–mass spectrometer (GC–MS, Hewlett–Packard, USA). The GC was equipped with a capillary fused silica column (CP-Wax 52 CB, 60 m × 0.25 mm i.d., Chrompack Inc., The Netherlands). Carrier gas was hydrogen (1.0 ml min^{-1} flow rate) and the column temperature was initially maintained at 40 °C for 5 min and subsequently programmed from 40 to 250 °C at a rate of 5 °C min^{-1}. The mass spectra were obtained by electron impact at 70 eV. Identification of the volatiles was based on comparison of the spectra with the spectra of the Wiley Spectrum Library.

2.10. Statistical analyses

Data were analysed using the general linear model (GLM) of Statistical Analysis System's Procedures (SAS Institute Inc., Cary, NC) with a 5% level of significance. Means were separated using the Duncan's new multiple range test.

3. Results and discussion

3.1. Proximate composition and pH

The contents of moisture, crude protein, crude fat and ash, of the fresh chicken sausages, were 59.8–61.8%, 15.7–16.4%, 20.1–22.4% and 2.70–2.95%, respectively. Three samples for each replicate and, totally, three replicates, were analysed for the proximate composition in this study. The pH values of the sausage remained stable and were approximately 6.4–6.6 during the first 10 days of refrigerated storage, and significantly ($P < 0.05$) decreased thereafter. This pH reduction was probably due to the fact that some existing oxygen inside the package might trigger fat oxidation, thus resulting in the decrease of pH values. At day 14, the pH value of the control samples decreased dramatically to 5.90, whereas, in the samples treated with rosemary or Chinese mahogany, it ranged from 6.00 to 6.14. A comparatively smaller pH reduction was observed for the samples with more rosemary or Chinese mahogany added.

3.2. Instrumental colour measurement

Fig. 1 illustrates the *L* and *a* colour value changes of the samples during storage. It shows that addition of rosemary or Chinese mahogany significantly decreased sample *L* values and increased sample *a* values. The more rosemary or Chinese mahogany that was added into the formula, the lower were the *L* values and the higher were the *a* values of the sausages. The *a* values of the control samples decreased during refrigerated storage while those of the samples with either rosemary or Chinese mahogany added increased. More intense colours of rosemary or Chinese mahogany itself led the sausages to have lower *L* values and higher *a* values, which resulted in darker colours. A possible browning reaction, occurring in the rosemary or Chinese mahogany added, might also contribute to this colour change. Polyphenol oxidases (PPOs), which are widespread enzymes in many plants, are responsible for browning in plants (Dogan & Dogan, 2004). In the presence of oxygen, the cited authors explained that these PPOs would oxidise plant phenolic compounds to corresponding quinones, which then condense to form darkened compounds.

Sebranek et al. (2005) reported that frozen pork sausages with 2500 ppm rosemary extract added had better red colour retention than did BHA/BHT-treated sausages during 84 days of storage. In the same study, they also reported that there was no significant difference of the $a*$ values between BHA/BHT treated, rosemary extract treated and controls for the refrigerated sausages, while a significant difference of colour loss occurred during 14 days of refrigerated storage. In contrast, Georgantelis and Blekas, et al. (2007) reported that $a*$ values of both rosemary extract treated and control beef burgers decreased during frozen storage for 180 days and addition of rosemary extract improved colour stability when compared to the controls. In the same study, they reported that a combination of chitosan and rosemary extract significantly improved sample colour stability and red colour retention. Georgantelis and Blekas, et al. (2007) explained that many factors, such as differences in the oxidation pattern of oxymyoglobin under conditions of reduced enzymatic activity, storage temperature, packaging method, muscle type and light intensity and differences in the meat species studied, might contribute to the variations in rosemary colour retention efficiency between different studies. In addition, types of antioxidative components added, for example, dried rosemary but not extract in this study, could also influence its efficiency.

3.3. Phenolic compounds in sausages

In this study, a higher content of total phenolics in Chinese mahogany, of 395 mg/g was observed, when compared to 65.0 mg/g, as reported by Chen, Lin, and Hsieh (2007), and 139 mg/g, reported by Yang et al. (2006). This deviation was probably due to various measuring conditions (i.e. absorbance measured at 700 or 750 nm) applied in these studies. In addition, drying conditions of Chinese mahogany leaves might not be the same in these studies (60 °C for 8 h in this study, but not reported by Chen et al. (2007). The phenolic compounds in sausages decreased with storage time for all the treatments (Fig. 2). This reduction of phenolic compounds in sausages during storage is in agreement with other studies. Daood, Vinkler, Markus, Hebshi, and Biacs (1996) reported that some antioxidative compounds, existing in spices, such as α-tocopherol, ascorbic acid and β-carotene, would lose their antioxidative ability dramatically during drying and storage. A dramatic decrease in the concentration of antioxidant compounds and loss of active antioxidation performance was observed during the late stage of storage, which resulted in an increase of fat oxidation. This phenomenon of progressive lipid oxidation, due to loss of protection by antioxidants after storage for a period of time, was also indicated by the dramatic increase of TBA values. The samples treated with Chinese mahogany showed comparatively higher total phenolic compound residuals at the end of storage when compared to the samples treated with rosemary at the same level. This more advanced protection against oxidation might be the reason that the samples with Chinese mahogany added had comparatively lower TBA values.

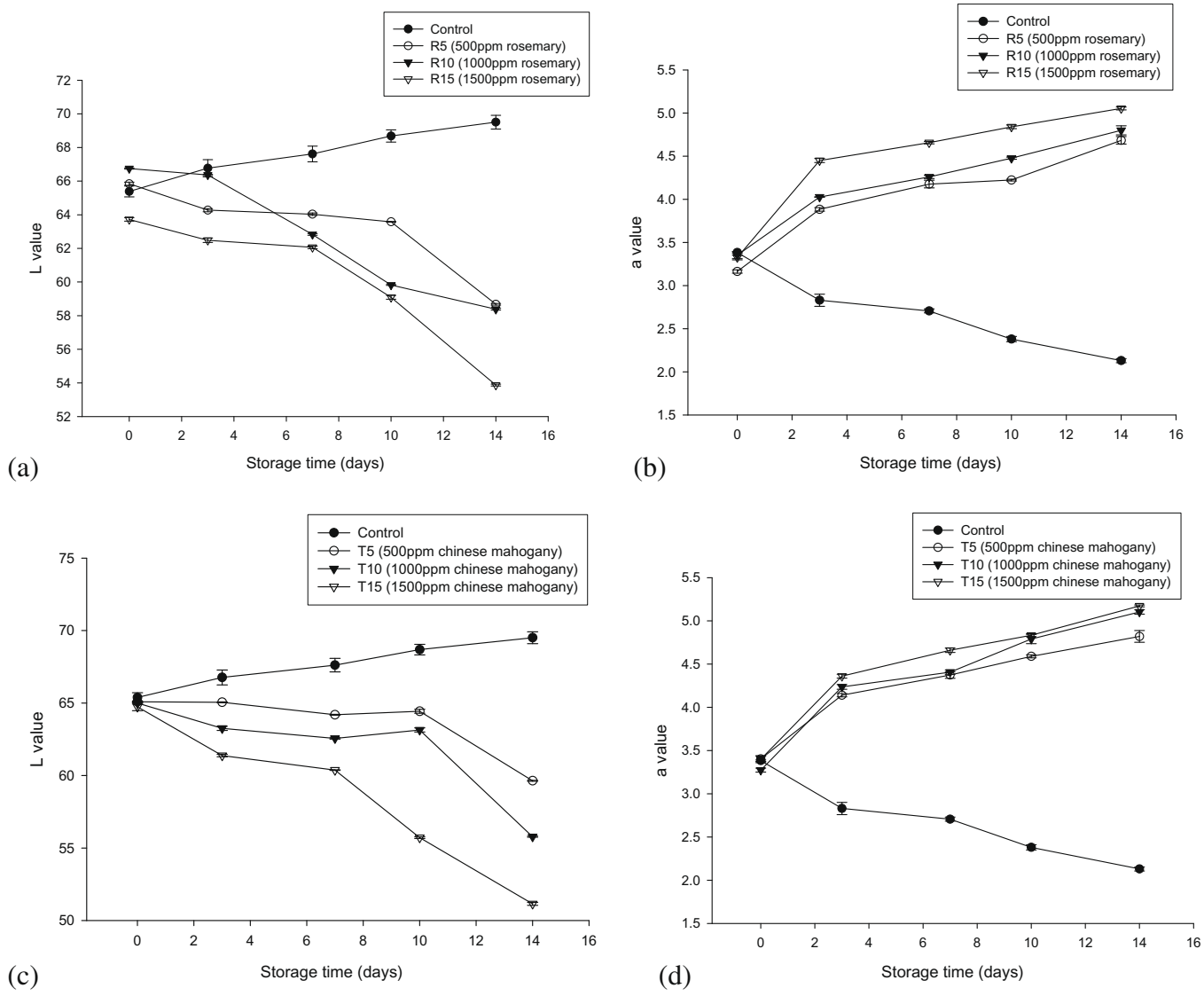

Fig. 1. Changes of (a) *L* values, (b) *a* values of fresh chicken sausages with addition of rosemary, (c) *L* values and (d) *a* values of fresh chicken sausages with Chinese mahogany during storage at 4 °C.

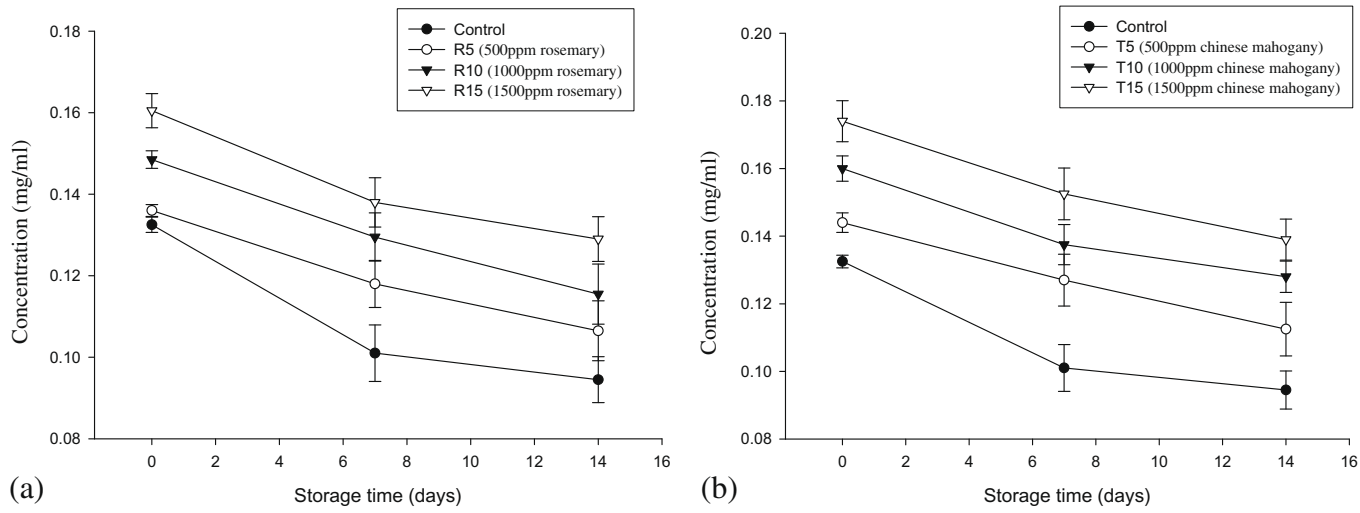

Fig. 2. Changes of residual amounts of phenolic compounds of fresh chicken sausages with addition of (a) rosemary or (b) Chinese mahogany during storage at 4 °C.

3.4. TBA and VBN

TBA values, which are indicators of lipid oxidation, are shown in Fig. 3. The TBA values of the sausage samples increased with refrigerated storage time during the first 3 days, indicating that lipid oxidation had occurred during this stage. This increase of TBA values of fresh sausages during storage has also been reported by other researchers. In this study, TBA values increased up to a maximum point at day 3, and then decreased gradually, and this tendency agreed with a study reported by Gokalp, Ockerman, Plimpton, and Harper (1983). Kuo, Dresel, Huang, and Ockerman (1987) explained that, during storage, malonaldehyde, which is an intermediate by-product during lipid oxidation, is further oxidised to other organic acids and alcohols that can not react with TBA agent. This might be the reason why the TBA values increased then decreased.

In this study, the TBA values of the samples with rosemary or Chinese mahogany added were lower than those of the controls. At day 3, the TBA values of the controls increased from 1.15 to 1.54 mg malonaldehyde/kg, while the TBA values of the Chinese mahogany treated samples were 1.30, 1.21, and 1.17 mg malonaldehyde/kg for T5 (500 ppm Chinese mahogany), T10 (1000 ppm Chinese mahogany), and T15 (1500 ppm Chinese mahogany) samples, respectively, and were significantly lower than the controls. Similarly, samples with rosemary added had significantly lower TBA values of 1.42, 1.40 and 1.25 malonaldehyde/kg for R5 (500 ppm rosemary), R10 (1000 ppm rosemary) and R15 (1500 ppm rosemary) samples, respectively. During storage, between day 3 and 14, the TBA values decreased for all treatments. The TBA value of the controls decreased to 1.10 mg malonaldehyde/kg at day 14, while the TBA values of the samples with rosemary added ranged from 1.00 to 1.05 mg malonaldehyde/kg for R5, R10, R15, and from 0.93 to 1.01 mg malonaldehyde/kg for T5, T10, and T15 samples, respectively. These decreases of TBA values were probably due to both the rosemary and Chinese mahogany, which are reported to have some antioxidative ability, and to retard fat oxidation. The results in this study demonstrate that the addition of rosemary or Chinese mahogany inhibited the lipid oxidation of fresh chicken sausages, and agree with other studies. In agreement with our findings, the positive effects of use of rosemary to prevent lipid oxidation have been well documented. Addition of 1000 ppm of rosemary extract was as effective as a combination of 100 ppm BHA plus 100 ppm BHT to maintain low TBARS values of precooked-frozen pork sausage (Sebranek et al., 2005). Yu, Scanlin, Wilson, and Schmidt (2002) reported that water-soluble rosemary extracts significantly decreased TBARS values of cooked turkey during 13 days of storage at 4 °C when compared to the controls, and higher levels of extracts added at 100, 250, or 500 ppm, were more effective in delaying lipid oxidation. The authors explained that free radical-scavenging and transition metal-chelating activities of water-soluble rosemary extract might contribute to the inhibitory effects on lipid peroxidation in cooked samples. Carnosol has been recognised as a major antioxidant in rosemary (Wei & Ho, 2006). Similarly, some phenolic diterpenes, such as carnosic acid, carnosol, rosmanol, rosmariquinone and rosmaridiphenol, which break free radical chain reactions by donation hydrogen atoms, have been reported to be associated with the antioxidant activity of rosemary extracts (Aruoma et al., 1992).

Hseu et al. (2008) reported that *T. sinensis* aqueous extracts, at 25–100 μg/ml, had some effective antioxidant activities, including the scavenging of free and superoxide anion radicals, reducing power, and metal chelation. Methanol extracts of *T. sinensis* have also demonstrated strong DPPH radical-scavenging activities and inhibitory effects on lipid peroxidation (Cho et al., 2003). Similarly, some potent antioxidative components in the young leaves and shoots lead *T. sinensis* to become a promising healthy-promoting food (Wang et al., 2007). Chen et al. (2007) reported that triterpenes and phenolic compounds, isolated from *Toona* species, were responsible for its antioxidant activity. In the DPPH radical-scavenging assay, Wang et al. (2007) reported that the 80% acetone extract of Chinese toon (the fresh young leaves and shoots of *T. sinensis*) had considerable antioxidant activity. Furthermore, the authors reported that gallic acid and its derivatives, gallotannins and flavonoids, were the main constituents contributing to its antioxidant ability. The antioxidative compounds contained in rosemary and Chinese mahogany might contribute to the antioxidative activity, thus retarding the fat oxidation of products during storage.

Increase amounts of volatile basic nitrogen (VBN), which is the result of decomposition of protein during storage by microorganisms, can be an index of meat product freshness. Fig. 4 illustrates that VBN values of the sausage samples increased as expected when storage time increased. In this study, VBN values, as well as the microbial counts, increased significantly during storage. In addition, samples that had more rosemary or Chinese mahogany added had significantly ($P < 0.05$) lower VBN values. At day 14, the control samples had significantly higher VBN value of 30.7 mg%, compared with those of the samples with rosemary added (24.4, 23.9 and 21.4 mg% for the R5, R10 and R15 samples,

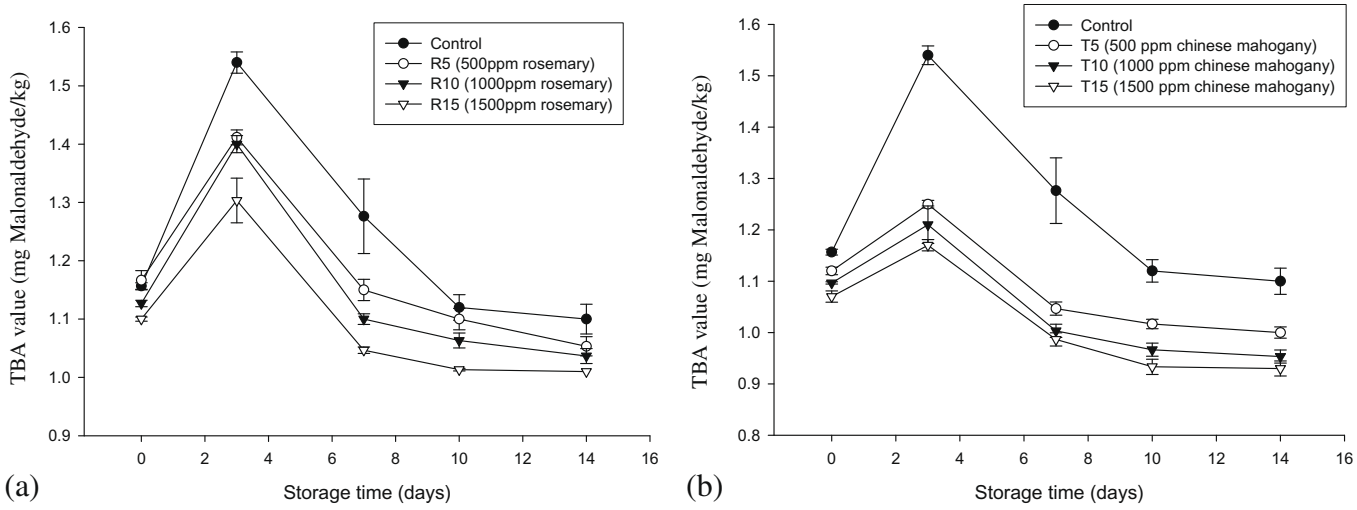

Fig. 3. Changes of TBA values of fresh chicken sausages with addition of (a) rosemary or (b) Chinese mahogany during storage at 4 °C.

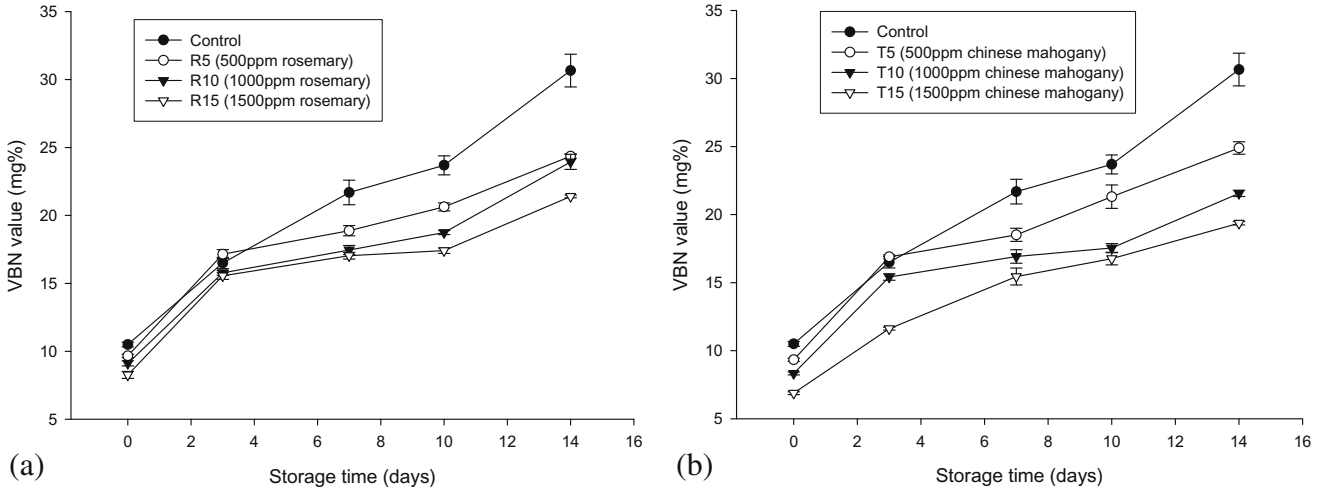

Fig. 4. Changes of VBN values of fresh chicken sausages with addition of (a) rosemary or (b) Chinese mahogany during storage at 4 °C.

respectively). Similarly, the samples with Chinese mahogany added had significantly lower VBN values (24.9, 21.6 and 19.4 mg% for the T5, T10 and T15 samples, respectively). The more rosemary or Chinese mahogany that was added to sausages, the lower were the VBN values of the samples observed. In this study, lower VBN values might suggest smaller bacterial populations for the samples with more rosemary or Chinese mahogany added, which is in agreement with the microbial counts shown in this study. These decreases of sample VBN values were probably because rosemary or Chinese mahogany contained some antimicrobial compounds. Carnosol and ursolic acid, which were the two antioxidant compounds extracted from rosemary, were demonstrated to inhibit the growth of 6 strains of food-associated bacteria and yeasts (Collins & Charles, 1987). Less microbial growth in sausages, due to the addition of rosemary or Chinese mahogany to the formula, thus led to less protein decomposition and lower VBN values.

3.5. Microbial qualities

The total plate counts of all samples increased during refrigerated storage (Fig. 5). Those with rosemary or Chinese mahogany added had comparatively lower microbial counts. At day 14, the total plate counts of sausages with the addition of rosemary at the levels of 500 ppm (R5), 1000 ppm (R10) and 1500 ppm (R15) were 5.99, 5.72 and 5.55 log CFU/g, respectively, which were significantly ($P < 0.05$) lower than that of the controls (6.08 log CFU/g). Similarly, total plate counts of 5.75, 5.59 and 5.30 log CFU/g were obtained for the sausages with 500 ppm (T5), 1000 ppm (T10) and 1500 ppm (T15) of Chinese mahogany added, respectively. In addition, samples with Chinese mahogany added had comparatively lower microbial counts than had the rosemary-treated sausages in this study. This difference was probably due to the total phenol content of Chinese mahogany which was higher than that of the rosemary (395 mg/g vs. 82 mg/g). Such antimicrobial effects due to the addition of rosemary or Chinese mahogany were also reported by other researchers.

Georgantelis and Ambrosiadis, et al. (2007), reported that the fresh pork sausages with addition of rosemary extract at level of 260 mg/kg had significantly lower ($P < 0.05$) Enterobacteriaceae, pseudomonad and yeast/mould counts than had the controls. Addition of carnosol, which was isolated from rosemary at levels of 50 μg/ml and 150 μg/ml, could significantly inhibit *Staphylococcus aureus* and *Escherichia coli* (Collins & Charles, 1987). Rosemary extracts have been demonstrated to have some inhibitory effects on the selected lactic acid bacteria and *Listeria* in an agar diffusion test (Fernández-Lopez, Zhi, Aleson-Carbonell, Pérez-Alvarez, &

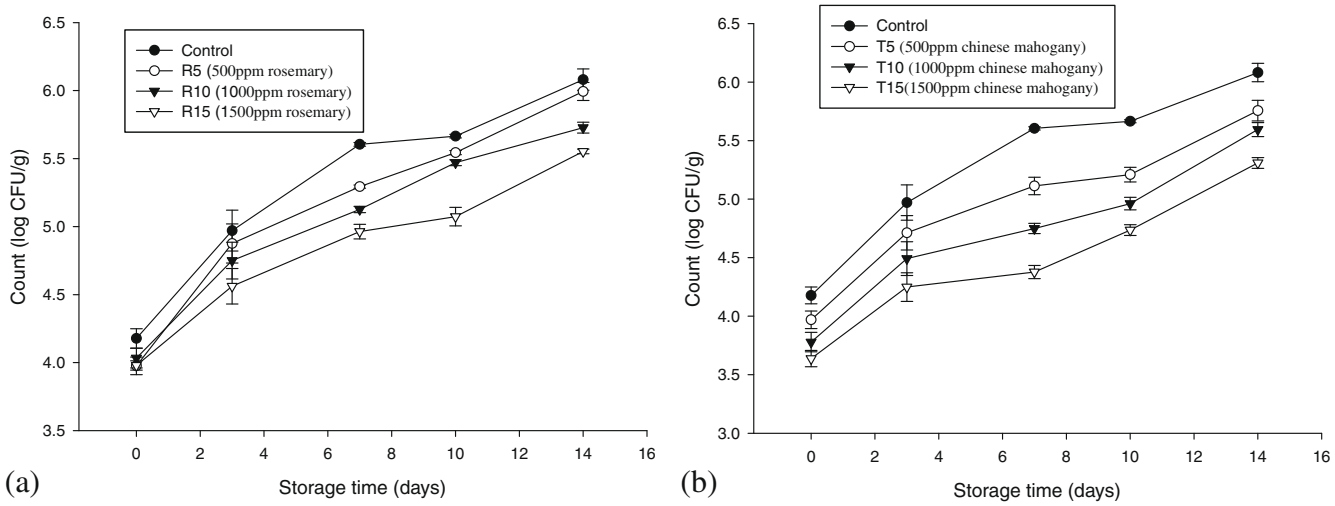

Fig. 5. Changes of total plate counts of fresh chicken sausages with addition of (a) rosemary or (b) Chinese mahogany during storage at 4 °C.

Kuri, 2005). Some non-polar components, such as phenolic diterpenes, which were isolated from rosemary, were indicated to be responsible for its antimicrobial properties, especially towards Gram-positive bacteria (Fernández-Lopez et al., 2005). Furthermore, when in combination with factors which can disturb cell membrane integrity and/or its permeability, such as lowered pH values, increased NaCl concentrations, combined with chitosan, phenolic diterpenes could further be used to inhibit Gram-negative bacteria (Georgantelis and Blekas, et al., 2007).

3.6. Sensory evaluation

Results of the sensory evaluation of the sausage during the 14-day storage period are presented in Table 1. A 7-point hedonic scale test was used in this study, with scores 1, 4 and 7, representing extremely dislike, neither like nor dislike, and extremely like, respectively. At day 0, colour scores of each treatment were less than 5, among which R15 (1500 ppm rosemary added) had the lowest colour score. In addition, the R15 (1500 ppm) colour score was significantly lower than those of R5 (500 ppm) and R10 (1000 ppm). No significant difference ($P > 0.05$) of aroma between treatments was observed. However, there was a significantly higher off-odour of 3.33 for the R15 (rosemary 1500 ppm added) than of 1.92 for the control sample. This higher off-odour of the samples might be due to a too condensed aroma due to higher level addition of rosemary. Samples with Chinese mahogany added had significantly higher flavour and overall acceptance scores than had the samples with rosemary added. At day 7, control samples had significantly higher colour scores than had those of the other treatments. This result was probably because colours of the samples with either rosemary or Chinese mahogany added became darker, which could have decreased sensory preference. On the other hand, samples with rosemary or Chinese mahogany added tend to have higher aroma scores, while the controls tended to have higher off-odours, even without significance. Addition of rosemary or Chinese mahogany seemed, not only to enhance the aroma of samples, but also to retard off-odour produced by fat oxidation. Samples with Chinese mahogany added (T5, T10 and T15) tended to have higher overall acceptance than did rosemary-added samples (R5, R10 and R15). At day 14, samples with 1500 ppm Chinese mahogany (T15) had significantly lower colour scores because of the darker colour of Chinese mahogany after being stored for a long time. This less preferred darker colour could also be evaluated subjectively by the lower *L* and higher *a* colour values. At the same level of addition, samples with Chinese mahogany had higher total overall acceptance.

3.7. Volatile component analysis

Flavour is one of the most important factors influencing the eating quality of meat and consumer decisions. Less aroma is sensed when meat is uncooked, while a complex series of thermally induced reactions occur between non-volatile components of lean and fatty tissues during cooking (Mottram, 1998). These thermally induced reactions and some volatile compounds formed during cooking thus contribute to specific aroma and flavour of meats (Mottram, 1998). In addition, not only enhancing colours, addition of some seasonings or spices, such as rosemary or Chinese mahogany, used in this study, also provides some unique aromas and flavours for meat products. Some volatile compounds, including esters (etheyl acetate), alcohols (ethanol) and acids (formic acid and acetic acid), were identified in fresh chicken sausage. Alcohols (ethanol, 1,8-cineole, L-terpineol, α-terpineol, mytenol, and ρ-cymen-8-ol), ketones (acetone and camphor), an ether (ε-anethole), alkenes (α-pinene, camphene, β-pinene, myrcene, and verbenene), acids (formic acid and acetic acid), and esters (etheyl acetate and methyl eugenol) and phenols (estragol, thymol and carvacrol), were identified from the fresh chicken sausage with 1500 ppm rosemary added. Alkenes (α-pinene, β-pinene, limonene, phellandrene and anethole), alcohols (ethanol, linalool, L-terpineol and α-terpineol), acids (formic acid and acetic acid), esters (etheyl acetate and ethyldicanoate), phenol (2-acetylfuran), and ethers (safrole and myristicin), were identified in the fresh chicken sausage with 1500 ppm Chinese mahogany added.

Unsaturated fatty acids are more readily oxidised than are saturated fatty acids. Therefore, a comparatively much higher proportion of unsaturated fatty acids leads phospholipids to become a more important source of volatile compounds during cooking than

Table 1
Sensory evaluation of fresh chicken sausages with addition of rosemary or Chinese mahogany during storage at 4 °C.

Characteristics[1]	Treatment[2]						
	C	R5	R10	R15	T5	T10	T15
	Day 0						
Colour	4.42 ± 0.23^{ab}	4.75 ± 0.28^{a}	4.58 ± 0.34^{a}	3.75 ± 0.30^{b}	4.75 ± 0.30^{a}	4.50 ± 0.15^{ab}	4.33 ± 0.14^{ab}
Aroma	4.33 ± 0.26^{a}	4.17 ± 0.30^{a}	3.83 ± 0.32^{a}	3.83 ± 0.34^{a}	4.67 ± 0.40^{a}	4.58 ± 0.26^{a}	4.50 ± 0.29^{a}
Off-odour	1.92 ± 0.29^{b}	1.92 ± 0.29^{b}	3.00 ± 0.43^{ab}	3.33 ± 0.56^{a}	2.08 ± 0.29^{ab}	2.58 ± 0.34^{ab}	2.58 ± 0.54^{ab}
Flavour	4.12 ± 0.31^{ab}	4.33 ± 0.26^{ab}	3.58 ± 0.34^{b}	3.75 ± 0.30^{b}	5.08 ± 0.26^{a}	4.83 ± 0.30^{a}	5.00 ± 0.25^{a}
Overall acceptance	4.83 ± 0.24^{ab}	4.42 ± 0.29^{bc}	3.83 ± 0.30^{cd}	3.17 ± 0.24^{d}	5.25 ± 0.25^{a}	5.00 ± 0.21^{ab}	5.08 ± 0.19^{ab}
Day 7							
Colour	5.50 ± 0.31^{a}	4.42 ± 0.26^{bc}	3.75 ± 0.18^{cd}	3.25 ± 0.18^{d}	5.00 ± 0.25^{ab}	4.17 ± 0.21^{c}	4.00 ± 0.12^{c}
Aroma	3.58 ± 0.29^{b}	4.00 ± 0.28^{ab}	4.25 ± 0.22^{ab}	4.25 ± 0.37^{ab}	4.83 ± 0.24^{a}	4.00 ± 0.25^{ab}	4.42 ± 0.23^{ab}
Off-odour	3.33 ± 0.38^{a}	2.75 ± 0.35^{a}	2.75 ± 0.43^{a}	2.25 ± 0.33^{a}	2.50 ± 0.38^{a}	2.25 ± 0.37^{a}	2.42 ± 0.36^{a}
Flavour	4.25 ± 0.25^{ab}	4.33 ± 0.22^{ab}	3.75 ± 0.22^{bc}	3.33 ± 0.22^{c}	4.75 ± 0.30^{a}	5.00 ± 0.33^{a}	4.67 ± 0.36^{a}
Overall acceptance	4.42 ± 0.26^{bc}	4.50 ± 0.19^{bc}	3.58 ± 0.19^{d}	3.83 ± 0.24^{cd}	5.17 ± 0.24^{ab}	4.92 ± 0.38^{ab}	5.33 ± 0.31^{a}
Day 14							
Colour	5.75 ± 0.39^{a}	4.83 ± 0.39^{ab}	4.00 ± 0.28^{b}	4.08 ± 0.36^{b}	4.83 ± 0.30^{ab}	4.00 ± 0.39^{b}	2.58 ± 0.26^{c}
Aroma	3.33 ± 0.53^{a}	3.58 ± 0.34^{a}	3.75 ± 0.39^{a}	3.75 ± 0.51^{a}	4.25 ± 0.48^{a}	4.25 ± 0.39^{a}	4.25 ± 0.58^{a}
Off-odour	4.17 ± 0.66^{a}	3.83 ± 0.47^{a}	4.00 ± 0.52^{a}	3.42 ± 0.62^{a}	3.58 ± 0.45^{a}	3.50 ± 0.42^{a}	3.00 ± 0.52^{a}
Flavour	4.33 ± 0.28^{a}	2.50 ± 0.31^{ab}	2.25 ± 0.28^{bc}	1.92 ± 0.31^{c}	3.83 ± 0.46^{a}	3.92 ± 0.48^{a}	3.17 ± 0.52^{a}
Overall acceptance	4.58 ± 0.42^{a}	2.25 ± 0.39^{b}	1.83 ± 0.30^{b}	1.92 ± 0.31^{b}	4.17 ± 0.37^{a}	4.33 ± 0.45^{a}	3.50 ± 0.36^{a}

Mean ± SD. $n = 12$.
$^{a-d}$Means within the same row for the same test day with different superscripts are significantly different ($P < 0.05$).
[1] A 7-point hedonic scale test, 1 = extremely dislike, 4 = neither like nor dislike and 7 = extremely like.
[2] C = control, R5 = 500 ppm rosemary, R10 = 1000 ppm rosemary, R15 = 1500 ppm rosemary, T5 = 500 ppm Chinese mahogany, T10 = 1000 ppm Chinese mahogany and T15 = 1500 ppm Chinese mahogany.

triglycerides (Mottram, 1998). In addition to verbenone, which was the major component (35.4%), Mata et al. (2007) identified several components, including α-terpineol (7.2%), camphor (5.5%), 4-terpineol (3.9%), 1,8-cineole (3.1%), caryophyllene oxide (2.4%), β-caryophyllene (2.3%), borneol (2.1%), α-bisabolool (2.1%), linalool (1.9%) and α-humulene (1.2%), in the essential oil of rosemary. Carvalho, Moura, Rosa, and Meireles (2005) explained that the chemical compositions of rosemary volatile oil and extracts varied widely, probably because of the difference in agricultural conditions of cultivation and the extraction technique used.

4. Conclusion

This study showed that smaller pH reduction was observed for the samples with more rosemary or Chinese mahogany added. Addition of rosemary or Chinese mahogany significantly decreased sample *L* values and increased sample *a* values. The phenolic compounds in sausages decreased during storage. Addition of rosemary or Chinese mahogany resulted in lower TBA, VBN values and microbial counts of the samples. Samples with addition of Chinese mahogany had significantly higher overall acceptance. The main volatile compounds of fresh chicken sausage were alcohols, acids, esters, aldehydes, ethers and phenolic compounds. In conclusion, Chinese mahogany and rosemary, applied in this study, have been demonstrated to improve meat products quality and function as a promising natural ingredient applied to food.

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Food Chemistry 117 (2009) 114–121

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Effects of drying and extrusion on colour, chemical composition, antioxidant activities and mitogenic response of spleen lymphocytes of sweet potatoes

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ARTICLE INFO

Article history:
Received 15 February 2006
Received in revised form 18 March 2009
Accepted 23 March 2009

Keywords:
Sweet potato chip
Drying
Extrusion
Antioxidant activity
Physiological property
Mitogenic response

ABSTRACT

Antioxidative and physiological property changes of freeze-dried, hot air-dried and extruded products made from two different colours of sweet potatoes (yellow and orange) were investigated. The results showed that there were no significant differences of dietary fibre content amongst all the treatments, except that the extruded products of orange sweet potatoes had higher dietary fibre contents. However, the water solubility index (WSI) values and antioxidant content were significantly different between yellow and orange sweet potatoes, and significantly different amongst freeze-dried, hot air-dried and extruded samples. The WSI values of the extruded samples were significantly higher than those of the hot air- and freeze-dried samples. The freeze-dried samples of orange sweet potatoes had more total phenolic compounds, β-carotene, and anthocyanin and had better scavenging effect on DPPH radicals than had freeze-dried yellow sweet potatoes. However, the scavenging effect on DPPH radicals was appreciably greater for hot air-dried than freeze-dried yellow sweet potatoes, but this trend was opposite for orange sweet potatoes. The extrusion process significantly increased the WSI values, scavenging effect on DPPH radicals and total phenolic compounds but decreased the β-carotene and anthocyanin for both yellow and orange sweet potatoes. At lower concentration (25–50 μg/ml), the methanolic extract of freeze-dried yellow sweet potatoes possessed a greater capacity of increasing the mitogenic response than did freeze-dried orange sweet potatoes; at higher concentration (100–200 μg/ml), the proliferation of the lymphocytes increased when stimulated with mitogen Con A for all the samples. This suggests that mitogen Con A selectively promotes T-cell-dependent proliferative activity.

1. Introduction

Sweet potatoes (*Ipomoea batatas*) represent the sixth most important food crop in the world. In Taiwan the total production of sweet potatoes harvested in 2006 was 235,203 metric tons, ranking first in the coarse grains (COA, 2006). To promote the consumption of sweet potatoes in Taiwan, interest has been focused on the development of new uses, particularly making sweet potatoes into bioactive functional foods. The high dietary fibre content of sweet potatoes is well known. Dietary fibre, one of the important bioactive functional food sources, can prevent coronary heart disease, hypertension and serum hyperlipidemia (Prosky & DeVries, 1992). Lund (1984) compared the cholesterol-binding capacities of 28 fibre samples from a variety of the common tropical fruits and vegetables and found that sweet potatoes were the most effective binder.

Several other investigators have reported the antioxidant activity and suppression of melanogenesis of mouse melanoma B16 by sweet potato extract, as well as the reducing effects of sweet potato juice against carbon tetrachloride-induced liver injury (Hayase & Kato, 1984; Shimozona, Kobori, Shimoto, & Tsushida, 1996; Suda, Furuta, Nishiba, Matsugano, & Sugita, 1997). Active oxygen species and free-radical-mediated reactions have been reported in degenerative or pathological processes such as aging, cancer, coronary heart disease and Alzheimer's disease (Ames, 1983; Ames, Shigena, & Hegen, 1993; Diaz, Frei, Vita, & Keaney, 1997; Smith, Perry, Richey, & Sayre, 1996). Antioxidants inhibit *in vivo* initiation and progression of a range of human diseases, such as coronary heart disease, carcinogenesis, neuronal disease, and age-related macular degeneration (Ames, 1989; Diplock et al., 1998). The antioxidative effects in vegetables were reported to be mostly due to phenolic compounds (Chu, Chang, & Hsu, 2000; Philpott, Gould, Markham, Lewthwaite, & Ferguson, 2003; Zhan, 1996). Hayase and Kato (1984) found that 70% methanol extract of sweet potatoes had a strong antioxidative activity. They also identified the major phenolic components contained in the extract. These were chlorogenic acid and isochlorogenic acid −1, −2 and −3. Additionally they found that the effective antioxidant activity of the sweet potato extract was due to the synergistic effect of phenolic components with amino acids. On the other hand, Hou et al.

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0308-8146/$ - see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2009.03.084

(2001) found that the 33 kDa trypsin inhibitor, one of the major sweet potato root storage proteins, had scavenging activity against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and the hydroxyl radical. Philpott et al. (2003) found that hydroxycinnamic acid was the major antioxidant component of sweet potatoes. Recently, anthocyanins from sweet potatoes have been shown to possess potent antioxidative activity *in vitro* (Oki et al., 2002; Philpott, Gould, Lim, & Ferguson, 2004; Stintzing, Stintzing, Carle, Frei, & Wrolstad, 2002) and the stoichiometric and kinetic values of phenols against DPPH· were also determined (Cevallos-Casals & Cisneros-Zevallos, 2003). A recent study found that phenols, flavonoids, and anthocyanins of sweet potato flours were significantly positively correlated with the DPPH radical scavenging effects (Huang, Chang, & Shao, 2006) but not β-carotene bleaching (Teow et al., 2007). Steam treatment also increased the DPPH radical scavenging effect of sweet potatoes (Huang et al., 2006).

A rapid colorimetric method based on the capacity of viable cells to cleave the tetrazolium salt MTT (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) to MTT formazan has been used to measure the proliferation and activation of cells (Chen, Campbell, & Newman, 1990; Mosmann, 1983). Several kinds of foods or their components have been analysed, utilising the MTT colorimetric method to identify antiproliferation activity, e.g. the adlay seed (Kuo, Shih, Kuo, & Chiang, 2001), phenolic compounds (Kasugai, Hasegawa, & Ogura, 1991), lycopene (Hwang & Bowen, 2004) and isoflavone (Kayisli, Aksu, Berkkanoglu, & Arici, 2002). According to a study of the antiproliferation activity of sweet potatoes, the water extract of storage roots showed the second highest antiproliferation activity, using human lymphoma N84 cells (Huang, Lin, Chen, & Lin, 2004).

Extrusion cooking has certain unique features compared to other heat processes because the material is subjected to intense mechanical shear (Harper, 1979). However, sweet potato extrusion researches have been extensively focused on chemical composition changes (Iwe, van Zuilichem, Ngoddy, & Lammers, 2001) and the effects of physical properties (Iwe, van Zuilichem, Stolp, & Ngoddy, 2004; Iwe, Wolters, Gort, Stolp, & van Zuilichem, 1998) but only sporadically on the bioactivity.

There are two very important cultivars of sweet potatoes in Taiwan–Tainong 57 (yellow sweet potato, YSP) and Tainong 66 (orange sweet potato, OSP). The former is whitish to yellowish in colour and suitable for producing sweet potato flakes. The latter is of an orange colour and suitable for producing sweet potato flakes and chips (Chen & Chiang, 1985). The effects of extrusion on the amount and activity of antioxidants contained in sweet potatoes has so far been only sporadically studied. This research was carried out to study the physicochemical and physiological properties and bioactivity of sweet potato dry products (chips and extruded products) made from yellow and orange sweet potatoes by using different processes (freeze-drying, hot air-drying, and extrusion).

2. Materials and methods

2.1. Materials

Two cultivars of sweet potatoes–Tainong 57 (yellow sweet potato, YSP) and Tainong 66 (orange sweet potato, OSP) – were obtained from a local market. The sweet potatoes were processed within 24 h after purchasing.

2.2. Sweet potato chip making process

The process procedure of the sweet potato chips was modified according to Chen (1987) and Chen and Chiang (1985). Washed sweet potatoes were cut into 1 mm thick slices with a conventional food slicing machine. To prevent discoloration of sliced sweet potatoes during dehydration, the slices were soaked in 0.5% sodium bisulfite ($NaHSO_3$) solution for 30 min, and then soaked in running water for 1 h. The prepared slices were dried in a tunnel drier (Si Dien Co., Taichung, Taiwan) at 80 °C for 1 h according to the commercial preparation procedure. The conditions of the tunnel drier were: total length 15 m, conveyor speed 0.25 m/min, air humidity less than 40% relative humidity, air speed 2.7 m/s, and water content of the final product less than 5%. The slices, without a $NaHSO_3$ solution soaking treatment, were freeze-dried. All processing treatments were done in triplicate.

2.3. Extrusion

Hot air-dried sweet potato chips were ground in a stainless grinder to pass a 100 mesh sieve (Geng Sin Co., Taipei, Taiwan). Extrusion was done by a twin screw extruder (Hungyang Co., Yulin, Taiwan), using a screw length of 120 cm (barrel length:diameter = 20:1) and a pitch of 36.7 mm. Each of the dies was 2.5 mm in diameter. Temperature settings for the feeding, kneading, compression, kneading, metering and die zones were room temperature, 100, 100, 110, 115, and 115 °C, respectively. Temperature settings were monitored and adjusted using a thermocouple. The extruder screw speed was 120 rpm, feed rate was 60 kg/h and feed moisture was 5.53 kg/h. Received samples were stored in polyethylene bags and used in the subsequent analyses. All processing treatments were done in triplicate.

2.4. Colour analysis

The sweet potato chips were ground into powder of less than 40 mesh with a commercial blender. Hunter colour of the sweet potato samples was determined for the colour analysis with a colorimeter (Nippon Denshoku Σ90, Kogyo Co., Tokyo, Japan). The reported values are the averages value of four duplicate tests for each sample. Results were expressed as tri-stimulus values (L: lightness (0 = black, 100 = white), a ($-a$ = greenness,+a = redness) and b ($-b$ = blueness,+b = yellowness)) of the Hunter colour scale. The instrument was set in reflectance mode. A white standard plate (X = 93.28; Y = 95.12; Z = 113.14) was used for calibration. Numerical total colour difference (ΔE), hue angle and chroma were calculated by

$$\Delta E = \left[(L - L_o)^2 + (a - a_o)^2 + (b - b_o)^2\right]^{1/2}$$

$$\text{Hue} = \left[\tan^{-1}(b/a)\right]$$

$$\text{Chroma} = \left[\left(a^2 + b^2\right)\right]^{1/2}$$

where L_o, a_o and b_o were the L, a, and b values of the reference sample which here is the freeze-dried sample.

2.5. Water solubility index (WSI)

The WSI of sweet potato samples was measured by the modified method of Anderson, Conway, Pfeifer, and Griffin (1969). A 2.5 g sample, with 30 ml of distiled water, was incubated in a 30 °C water bath for 30 min. The mixture was centrifuged at 3000 g for 10 min; the supernatant was then poured into a weighing bottle and dried at 105 °C, overnight, to determine the soluble solids weight:

$$\text{WSI (\%db)} = \text{soluble solids weight (g)} / \text{sample weight (g, dry mass)} \times 100\%$$

2.6. Dietary fibre analysis

Dietary fibres of sweet potato samples were determined according to the AOAC method (Prosky, Asp, Schweizer, DeVries, & Furda, 1988). A one gramme sample (dry matter) was treated with a heat-stable α-amylase (EC 3.2.1.1, Catalogue No. A3306, Sigma, St. Louis, MO) for 15 min in a boiling water bath, and then treated with protease (Catalogue No. P3910, Sigma, St. Louis, MO) and amyloglucosidase (Catalogue No. A3513, Sigma, St. Louis, MO) for 30 min at 60 °C. The residue, as water insoluble dietary fibre (IDF), was recovered by filtering with a glass crucible and dried at 105 °C overnight and weighed. The weight was corrected for ash and residual protein contents. Subsequently, 60 °C −95% alcohol, in the amount of four times the filtrate volume, was added to the filtrate and the filtrate was allowed to set and precipitate for 1 h. The precipitate, as water-soluble dietary fibre (SDF), was dried at 105 °C overnight and weighed after being recovered by filtering with a glass crucible. The weight was further corrected for ash content. The total dietary fibre (TDF) content was the sum of IDF and SDF.

2.7. β-Carotene analysis

Sweet potato sample powder (5 g) was mixed with approximately 2 g of calcium carbonate, 1 g of diatomaceous earth, and 25 ml of methanol. A hexane–acetone (1:1) mixture (50 ml) was added and the whole stirred (Chen, 1987). The mixture was filtered under vacuum through a funnel with a fritted disc. The residue in the funnel was washed twice more with 25 ml of methanol and then by 50 ml of the hexane–acetone mixture. All of the extracts were combined in a 250 ml separating funnel and washed with water. Drops of saturated sodium chloride solution were added to the funnel until phase separation appeared. The aqueous phase was discarded and the upper layer was evaporated to 15 ml in a vacuum evaporator. The concentrate was purified by pouring into a 30 ml glass filter with 10 cm height of absorbent (magnesium oxide: diatomaceous earth = 1:3) and washed with petroleum ether (with 3% acetone) until all of the orange colour had disappeared. The filtrate was diluted to give an absorbance of <1.2 at 436 nm. Commercial β-carotene (Catalogue No. C9750, Sigma, St. Louis, MO) was used as a standard.

2.8. Total anthocyanins assay

Content of anthocyanins was determined by following the procedures of Mancinelli, Huang Yang, Lindquist, Anderson, and Rabino (1975). The sweet potato flour (0.5 g) was treated with 20 ml of acidified methanol (1% HCl) in a rotary mixer at a 45° angle and 200 rpm for 30 min in the dark. After centrifuging at 450 g and 4 °C for 15 min, the supernatant was diluted to an appropriate concentration and the absorbance was read at 657 nm (A657) and 530 nm (A530). The anthocyanin content was calculated on the basis of the following equation:

$$\text{Anthocyanin content}(\mu g/g,\ \text{dry matter}) = (A530 - 0.33 \times A567) \times V \times DF/W$$

where V = volume of sample, DF = dilution factor, W = sample weight (g).

2.9. Preparation of the methanolic and ethanolic extracts

Twenty grammes of powder from each sample were extracted with 200 ml of methanol or ethanol, stirred on a stirring plate at room temperature for 24 h, and then filtered through #1 filter paper (Whatman Inc., Hillsboro, OR). The filtrate was concentrated *in vacuo* to dryness to obtain methanolic or ethanolic extract. The dried filtrates were weighed to determine the extraction ratio of soluble constituents.

$$\text{Extraction ratio}\ (\%) = \text{dried filtrate weight}(g)/\text{sample weight}(g, \text{dry mass}) \times 100\%$$

The methanolic or ethanolic extract was then stored at −20 °C to determine the scavenging effect on the α,α-diphenyl-β-picrylhydrazyl (DPPH) radicals. The methanolic extract was also used to determine the total phenolic compound and mitogen responses of spleen lymphocytes.

2.10. Determination of the scavenging effect on DPPH radicals

The experimental method in this study was that of Kuo et al. (2002). A 400 μM solution of DPPH was prepared in 100% methanol. Instead of reading samples spectrophotometrically, the assay was performed in a microplate. 50 μl of sample (methanolic or ethanolic extract, final concentration 0–3000 μg/ml) and 150 μl of DPPH· solution were added to a well in a 96-well flat-bottom EIA microtitration plate. After thorough mixing, the solutions were kept in the dark for 90 min. The absorbency of the samples was measured using an Optimax automated microplate reader (Molecular Devices, CA) at 517 nm against methanol without DPPH· as the blank reference. Each sample was tested four times and the values were averaged. For the determination of EC_{50} (which is the efficient concentration of antioxidant, defined as 50% of the initial DPPH concentration), each sample was measured at more than five different concentrations in the DPPH· test. EC_{50} was obtained by interpolation from linear regression analysis (Fig. 1).

2.11. Total phenolic compounds

Total phenolic compounds were measured with Folin–Ciocalteu reagent, using catechin as a standard (Maillard, Soum, Boivin, & Berset, 1996). 5 ml of Folin–Ciocalteu reagent (diluted tenfold in distiled water), 2 ml of 200 g/l sodium bicarbonate solution, and 2 ml of distiled water were added to 1 ml of the raw methanolic extract of sweet potato samples. After 1 h at 20 °C, the absorbance at 755 nm was read.

2.12. MTT colorimetric assay for detecting mitogen responses of spleen lymphocytes

The experimental method in this study was that of Kuo (2001). Eight to twelve week-old male BALB/c mice were used as spleen

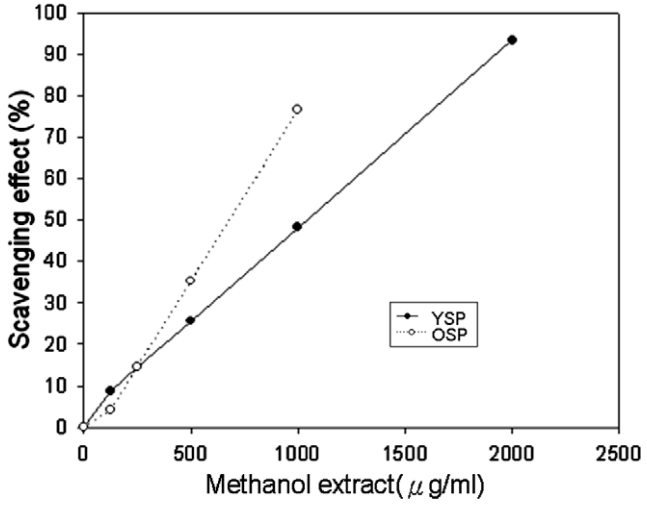

Fig. 1. EC_{50} of the methanolic extracts of two cultivars of raw sweet potato in scavenging effect on DPPH radicals. (YSP: yellow sweet potato; Tainong 57; OSP: orange sweet potato, Tainong 66).

donors. Mice were sacrificed by cervical dislocation. The spleen was removed aseptically and washed 3 times by RPMI-1640 medium. The spleen was gently teased into a single cell suspension with toothed forceps. The cell suspension was filtered with a nylon mesh screen (53 mesh). The murine spleen cells were put into a 96-well microplate and were adjusted to a concentration of 2×10^6 cells/ml in RPMI-1640 medium with 10% FCS, 1 μg/ml mitogen Con A and various concentrations (0–200 μg/ml) of test samples in a total volume 200 μl, then cultured in 5% CO_2 at 37 °C for 72 h. Following this initial incubation, 25 μl of MTT solution (5 mg/ml) were added to all wells and incubated at 37 °C for 4 h, and 100 μl of lysis buffer were then applied to each well and incubated at 37 °C overnight (about 16 h) to dissolve the dark blue crystals. The absorption of formazan solution at 570 nm was then measured using an Optimax automated microplate reader (Molecular Devices, CA). Index of mitogenic response was the absorbance percentage of control. Each sample was tested four times and the values were averaged.

2.13. Statistical analysis

Data were analysed by analysis of variance (ANOVA), using general linear model, or by the *t*-test model. Duncan's multiple range tests was used to determine the differences amongst samples. Significant levels were defined as probabilities of 0.05 or less. All processing treatments were done in triplicate.

3. Results and discussion

3.1. Change of colour

The *L*, *a*, *b*, Δ*E*, chroma (saturation) and hue values of sweet potato chips were compared (Table 1). The results showed that the colour change was greater between the two drying methods for yellow sweet potatoes than for orange sweet potatoes. For yellow sweet potatoes (YSP, Tainong 57), the *a* value, *b* value, hue and chroma were significantly different between two drying methods. For orange sweet potatoes (OSP, Tainong 66), only the *L* value was significantly different between the two drying methods. According to the results for yellow sweet potatoes, the colour of hot air-dried samples was yellowish orange whereas the colour of freeze-dried samples was yellowish green, and the colour of the former was more saturated (intensive) than was the latter. For orange sweet potatoes, the hot air-dried samples were darker than were freeze-dried samples.

3.2. WSI value and dietary fibre

Water solubility index (WSI) and content of dietary fibre (insoluble dietary fibre and soluble dietary fibre) of the sweet potato samples are shown in Table 2. The WSI values of the sweet potato samples were significantly different amongst the freeze-dried, hot air-dried and extruded samples for both cultivars of sweet potatoes. No differences were found in the contents of dietary fibre in terms of the insoluble dietary fibre (IDF), soluble dietary fibre (SDF), and total dietary fibre (TDF) between the hot air-dried and freeze-dried samples. The soluble dietary fibre (SDF) and total dietary fibre (TDF) of the extrudate of orange sweet potatoes (OSP–EXT, Tainong 66) were significantly higher than those of the hot air- and freeze-dried samples. The content of dietary fibre after extrusion cooking was either increased, decreased or unchanged, depending on the raw material condition and parameter of extrusion. Fornal, Soral-Smietana, Smietana, and Szpendowski (1987) investigated the chemical characteristic changes of an extruded mixture of buckwheat, barley and corn starch and found that the dietary fibre content decreased after extrusion. Shih and Chiang (1992) and Artz, Warren, and Villota (1990) investigated the chemical characteristic changes of an extruded mixture of corn and soy milk residue and corn fibre/corn starch blend, and found that the dietary fibre contents were similar before and after extrusion. Rinaldi, Ng, and Bennink (1999) used wet okara, combined with soft wheat flour, to produce two different formulations (33.3% and 40% okara) and extruded using four combinations of two screw configurations and two temperature profiles. Extrusion of the formulations resulted in decreased insoluble fibre (< = 25.5%) and increased soluble fibre (< = 150%) contents of the extrudate.

In general, the WSI value is related to the degradation of the starch (Gomez & Aquilera, 1984). The higher the WSI after processing, the higher is the soluble material, and the more severe is the starch decomposition. According to the results in Table 2, the hot air-drying and extrusion process significantly increased the decomposition of starch. The results also showed that the orange sweet potatoes (OSP, Tainong 66) had higher WSI value than had the yellow sweet potatoes (YSP, Tainong 57), regardless of the processing methods. But the delta WSI values between hot air- and freeze-dried samples (WSI value of hot air-dried sample minus WSI value of freeze-dried sample) of these two cultivars of sweet potatoes were similar (3.3 for the YSP and 3.7 for the OSP). Meanwhile, the delta WSI values between extruded and hot air-dried samples (WSI value of extrusion sample minus WSI value of hot air-dried sample) of these two cultivars of sweet potatoes were similar (26.6 for the YSP and 25.5 for the OSP). These values imply that the decompositions of starch during hot air-drying and extrusion were similar for these two sweet potatoes.

According to the correlation analysis, the WSI value was significantly ($p < 0.05$) positively correlated with soluble dietary fibre

Table 1
Colour analysis of different treatments of sweet potato chips.

Sample*	*L*	*a*	*b*	Δ*E***	Hue****	Chroma*****
YSP–FD	86.45	−1.44#	19.90#	–	−85.86#	19.95#
YSP–AD	85.13	0.50	26.20	6.72	88.91	26.20
OSP–FD	77.80#	13.43	25.67	–	62.38	28.97
OSP–AD	70.24	14.55	24.11	7.80	58.89	28.16

* YSP: yellow sweet potato (Tainong 57); OSP: orange sweet potato (Tainong 66); AD: sweet potato treated with 0.5% sodium bisulfite before hot air-drying; FD: freeze-dried sweet potato and no sodium bisulfite treated.
** $\Delta E = \left[(L-L_o)^2 + (a-a_o)^2 + (b-b_o)^2\right]^{1/2}$ where, L_o, a_o and b_o were the Lab value of freeze-dried samples.
*** Hue = $[\tan^{-1}(b/a)]$.
**** Chroma = $[(a^2 + b^2)]^{1/2}$.
\# Significantly different ($p < 0.05$) tested by *t*-test.

Table 2
Water solubility index (WSI) and dietary fibre content of different sweet potato samples.

Sample*	WSI** (%db)	IDF*** (%db)	SDF*** (%db)	TDF*** (%db)
YSP–FD	18.2[d]	8.73[a]	5.97[b]	14.70
YSP–AD	21.5[c]	7.25[a]	7.23[b]	14.48
YSP–EXT	48.1[a]	8.17[a]	7.74[b]	15.91
OSP–FD	22.8[c]	7.34[a]	6.25[b]	13.59
OSP–AD	26.5[b]	8.17[a]	6.95[b]	15.12
OSP–EXT	52.0[a]	8.16[a]	10.60[a]	18.76

* YSP: yellow sweet potato (Tainong 57); OSP: orange sweet potato (Tainong 66); AD: sweet potato treated with 0.5% sodium bisulfite before hot air-drying; FD: freeze-dried sweet potato and no sodium bisulfite treated; EXT: extrusion treatment.
** WSI: water solubility index, referred to dry weight sample (db).
*** IDF as insoluble dietary fibre; SDF as soluble dietary fibre; TDF as total dietary fibre, and TDF = IDF + SDF, all these values referred to dry weight sample (db).
[a–d] Within a column, results followed by the same letter do not differ at the 5% level by Duncan's multiple range test ($n = 3$).

(SDF) (r = 0.8521) and total dietary fibre (TDF) (r = 0.8560). This means that the increase of SDF may, in part, contribute to the increase of the WSI of the extruded products in our study.

3.3. Antioxidant components

Antioxidant components (total phenolic compounds (from the methanolic extract), β-carotene and anthocyanin) of sweet potato samples with different treatments are shown in Table 3. The total phenolic compounds of freeze-dried samples of orange sweet potatoes (OSP–FD, Tainong 66) were higher than those of yellow sweet potatoes (YSP–FD, Tainong 57). However, the effects of the drying process on the total phenolic compounds for these two cultivars of sweet potatoes were significantly different. There was almost no difference in the total phenolic compounds of yellow sweet potatoes (YSP, Tainong 57) between freeze-dried and hot air-dried samples, but there was a significant difference for the orange sweet potatoes (OSP, Tainong 66) between the two different drying treatments. The drying process could cause damage of cell structures of tubers and allowed easier extraction of antioxidant components from the tuber itself or those from the diffusing to the tuber, which increased the phenolic compounds of air-dried samples (Huang et al., 2006). Hot air-drying may also decrease the phenolic compounds of marionberries, strawberries and corn grown by conventional, organic, or sustainable agricultural practises compared with freezing and freeze-drying (Asami, Hong, Barrett, & Mitchell, 2003). In general, air-drying at temperatures >60 °C is regarded as unfavourable, due to the possibility of inducing oxidative condensation or decomposition of thermolabile compounds, such as (+)-catechin. Since the phenolic content of the two sweet potato hot air-dried samples (YSP–AD and OSP–AD) showed opposite results, this showed that the distributions of phenolic compounds between these two colours of sweet potato may be different.

For both sweet potatoes the phenolic contents of the extrudate were higher than that of the freeze-dried sweet potatoes. The effects of extrusion on the amount and activity of antioxidants contained in food have so far been only sporadically studied. A more thorough study has been carried out by Zieliński, Kozłowska, and Lewczuk (2001) and has revealed that the ferulic acid content may increase by three times due to extrusion. Gumul and Korus (2006) studied extruded rye and found, irrespective of rye cultivar and the parameters of processing, that the ferulic acid and diferulic acid contents increased two times, and the *p*-coumaric acid content increased by 70% due to extrusion. The increase in the levels of phenolic acids (particularly ferulic acid) after extrusion can be attributed to the release of the acid and its derivatives from the cell walls of the plant matter.

The β-carotene content in orange sweet potatoes (OSP, Tainong 66) is greater than that in yellow sweet potatoes (YSP, Tainong 57) (Table 3). For both of the sweet potatoes, the β-carotene contents of hot air-dried (YSP–AD and OSP–AD) and extruded samples (YSP–EXT and OSP–EXT) were significantly lower than that of freeze-dried samples (YSP–FD and OSP–FD) (Table 3). This indicates that the hot air-drying and extrusion treatments had greater impacts, than did freeze-drying, on the decrease of β-carotene content of sweet potatoes. There is general agreement that, amongst the several ways of processing foods, drying and dehydration involve the highest carotenoid losses (Arya, Natesan, Parihar, & Viijayaraghavan, 1979). Decaying affects colour, nutritive value, texture and flavour of vegetables during dehydration and storage. Pinheiro-Santana, Stringheta, Brandao, Paez, and Queiroz (1998) analysed the influence of dehydration and different preparation methods, during home-processing, related to α-carotene, β-carotene and total carotenoid stabilities in carrots and found that dehydration promoted the greatest degradation of all three. Marty and Berset (1988) found that extrusion cooking caused degradation of all-trans-beta -carotene.

Orange sweet potatoes (OSP, Tainong 66) have a higher content of anthocyanins than have yellow sweet potatoes (YSP, Tainong 57) (Table 3). However, there were no significant differences in the contents of anthocyanin amongst the extruded, hot air-dried and freeze-dried samples of yellowish sweet potatoes (YSP, Tainong 57). In contrast, the anthocyanin content of the extruded product of orange sweet potatoes (OSP, Tainong 66) was significantly lower than those of the hot air- and freeze-dried samples (Table 3). Lohachoompol, Srzednicki, and Craske (2004) examined the effect of cabinet drying on the anthocyanin content of blueberries (*Vaccinium corymbosum* L.) and found a 49% loss after drying. Sugawara (2001) used extrusion as a sterilization treatment to examine the change of sweet potatoes under various conditions and found that the anthocyanin count in the product was decreased by extrusion treatment. However, for the genotypes of sweet potatoes with low anthocyanin contents in our study, hot air-drying and extrusion treatments showed little effect except on the extruded product of orange sweet potatoes (OSP, Tainong 66).

3.4. The scavenging effect on DPPH radicals

Antioxidative activities of sweet potato samples with different treatments are shown in Table 4. The results showed that, for freeze-dried samples, the extraction ratio of methanolic and ethanolic extracts of the orange sweet potato (OSP–FD, Tainong 66) was higher than that of the yellowish sweet potato (YSP–FD, Tainong

Table 3
Antioxidant components of different sweet potato samples.

Sample*	Total phenolic compound** (mg equiv catechin/g db)	β-Carotene (μg/g db)	Anthocyanin (μg/g db)
YSP–FD	6.38[d]	9.7[d]	3.6[b]
YSP–AD	6.45[d]	7.5[d]	2.5[b]
YSP–EXT	8.18[c]	4.3[e]	2.0[b]
OSP–FD	10.9[b]	83.3[a]	8.5[a]
OSP–AD	5.67[d]	62.5[b]	7.3[a]
OSP–EXT	13.9[a]	34.6[c]	4.0[b]

* YSP: yellow sweet potato (Tainong 57); OSP: orange sweet potato (Tainong 66); AD: sweet potato treated with 0.5% sodium bisulfite before hot air-drying; FD: freeze-dried sweet potato and no sodium bisulfite treatment; EXT: extrusion treatment.
** Total phenolic compounds in methanolic extract.
[a–e] Within a column, results followed by the same letter do not differ at the 5% level by Duncan's multiple range test (n = 3).

Table 4
Extraction ratio and scavenging effect on DPPH radicals of methanolic and ethanolic extracts of different sweet potato samples.

Sample*	Methanolic extract		Ethanolic extract	
	Extraction ratio** (%db)	EC_{50}*** (μg/ml)	Extraction ratio (%db)	EC_{50} (μg/ml)
YSP–FD	8.14[d]	1084[a]	7.61[d]	2481[a]
YSP–AD	5.11[e]	728[b,c]	7.41[d]	2461[a]
YSP–EXT	11.38[c]	610[d]	9.44[c]	2005[b]
OSP–FD	15.02[b]	681[a]	12.70[b]	909[d]
OSP–AD	14.05[b]	803[b]	17.25[a]	1372[c]
OSP–EXT	18.08[a]	570[d]	18.39[a]	889[d]

* YSP: yellow sweet potato (Tainong 57); OSP: orange sweet potato (Tainong 66); AD: sweet potato treated with 0.5% sodium bisulfite before hot air-drying; FD: freeze-dried sweet potato and no sodium bisulfite treatment; EXT: extrusion treatment.
** Extraction ratio: dried filtrate weight (g)/total sample weight (g, dry mass) × 100%, all these values referred to dry weight sample.
*** EC_{50} (μg/ml): the efficient concentration of antioxidant decreasing initial DPPH· concentration by 50%.
[a–e] Within a column, results followed by the same letter do not differ at the 5% level by Duncan's multiple range test (n = 3).

57). The extrusion treatments showed the same results. However, the effects of the hot air-drying on the extraction ratio of methanolic and ethanolic extracts are quite different between these two cultivars of sweet potatoes. The extraction ratio of the methanolic extract of the yellow sweet potatoes (YSP, Tainong 57) was decreased after hot air-drying. On the other hand, the extraction ratio of the ethanolic extract of the orange sweet potatoes (OSP, Tainong 66) was increased after hot air-drying. In contrast, the extraction ratio of the methanolic extract of the orange sweet potatoes (OSP, Tainong 66) and the extraction ratio of ethanolic extract of the yellow sweet potatoes (YSP, Tainong 57) were not significantly different before and after hot air-drying. However, extrusion treatment will significantly increase the extraction ratio, no matter whether it is the methanolic or ethanolic extract.

Fig. 1 shows that the scavenging effect on DPPH radicals of the methanolic extract of the orange sweet potatoes (OSP–FD, Tainong 66) was higher than that of the yellow sweet potatoes (YSP–FD, Tainong 57) for freeze-dried samples. However, after hot air-drying, the scavenging effect on DPPH radicals of the yellow sweet potatoes (YSP–AD, Tainong 57) was significantly increased, but was decreased for the orange sweet potatoes (OSP–AD, Tainong 66) (Table 4). Hayase and Kato (1984) reported that a 70% methanol extract solution of sweet potatoes had the highest antioxidative activity amongst three different solvents –70% methanol, ethyl ether and hexane. Our study revealed that the antioxidative activity of the methanolic extract solution was higher than that of the ethanolic extract, as indicated in Table 4. Masuda et al. (2002) examined 36 edible and medicinal plant species from the Okinawa region of Japan and showed that ethanolic extracts from sweet potatoes had strong protective effects against oxidative cell death. Hayase and Kato (1984) observed the same antioxidative effect between sweet potatoes, Kintoki and Kokei No. 14, in another experiment. Oki et al. (2002) found that red sweet potatoes were radical scavengers. However, in our study, the antioxidative effect is significantly different between the two chosen colours of sweet potatoes. Therefore, the change of antioxidative activity during hot air-drying may be dependent on the types of sweet potatoes being chosen.

The extrusion process will significantly increase the scavenging effect on DPPH radicals for both sweet potato samples.

From the values of the scavenging effect on DPPH radicals, it was concluded that the hot air-dried samples of the yellow sweet potatoes (YSP–AD, Tainong 57) had higher antioxidative activity than had the freeze-dried samples (YSP–FD). This may be attributed to the browning reaction induced by the hot air-drying process. Since the browning reaction may produce the antioxidative activity, as reported by Bressa, Tesson, Rdsa, Sensidoni, and Tubaro (1996), other studies have demonstrated that thermal processing may increase the antioxidant activity of tomatoes (Dewanto, Wu, Adom, & Liu, 2002), sweet corn (Dewanto, Wu, & Liu, 2002) and sweet potatoes (Huang et al., 2006). In the study of Zieliński and Troszyńska (2000), the antioxidant activity of rye extrudates was substantially higher than that of raw grain. Similar conclusions were reached by Baublis, Clydesdale, and Decker (2000) and Miller, Rigelhof, Marguart, Prakash, and Kanter (2000), who found that extracts from extruded cereal grain or cereal bran exhibited a greater antioxidant activity than did those from unprocessed grain or bran.

As shown by another study (Zieliński & Troszyńska, 2000), the antioxidant activity of rye grain increased after extrusion. An earlier study, carried out by Grela, Jensen, and Jakobsen (1999), suggested that extrusion lowers the levels of natural antioxidants, as well as tocopherols and carotenoids, in plant material. Other investigations provided evidence to the contrary, indicating that extrusion may positively influence the phenolic content of cereals (Baublis, Lu, Clydesdale, & Decker, 2000; Baublis et al., 2000; Zieliński & Troszyńska, 2000; Zieliński et al., 2001).

It is well known that the amounts of phenolic compounds, β-carotene, and anthocyanin may affect the antioxidative activity of food. Hayase and Kato (1984) isolated six phenolic compounds from sweet potatoes by HPLC and concluded that the effective antioxidative activity of a sweet potato extract was mainly due to the synergistic effect of phenolic compounds with amino acids. Zhan (1996) found sweet potato extract had high antioxidative activity resulting from the presence of phenolic acids, including chlorogenic, isochlorogenic, 4-O-caffeoylquinic and neochlorogenic acids. Philpott et al. (2004) showed that anthocyanin acts as an antioxidant *in situ* within the sweet potato storage roots. Furuta, Suda, Nishiba, and Yamakawa (1998) reported that the purple-fleshed sweet potato cultivars had a higher radical-scavenging or antioxidant activity than had those with white, yellow or orange flesh. Furthermore, anthocyanins and phenols were the major DPPH radical-scavengers in purple-fleshed sweet potatoes (Oki et al., 2002). Similar results were found by Huang et al. (2006). Although it is well known that the amounts of phenolic compounds, β-carotene, and anthocyanin may affect the antioxidative activity of food, there was no correlation ($p > 0.05$) amongst the WSI, total content of phenolic compounds, β-carotene, or anthocyanin and the scavenging effect on DPPH radicals in our study.

3.5. Mitogenic effect of the methanolic extracts of sweet potato in in vitro murine splenocyte cultures

A methanolic extract is usually used for immunomodulatory activity research. Therefore, in this study the methanolic extract was selected to evaluate the immunomodulatory activity of sweet potatoes. The lectin concanavalin A (Con A) has been used extensively as a T-cell-specific mitogen and Con A-induced T-cell activation is one of the classical models for assessing materials with immunomodulatory activity. In order to determine whether the methanolic extracts of sweet potatoes had immunostimulatory effects, the mitogenic response of splenocytes from BALB/c mice was tested in *in vitro* cultures with various concentrations of the methanolic extracts of sweet potatoes. The results are shown in Fig. 2. All of the methanolic extracts from sweet potatoes increased the proliferation of Con A-stimulated splenocytes in a concentration-dependent manner. However, the methanolic extract of the yellow sweet potatoes (YSP, Tainong 57) had greater capacity to increase

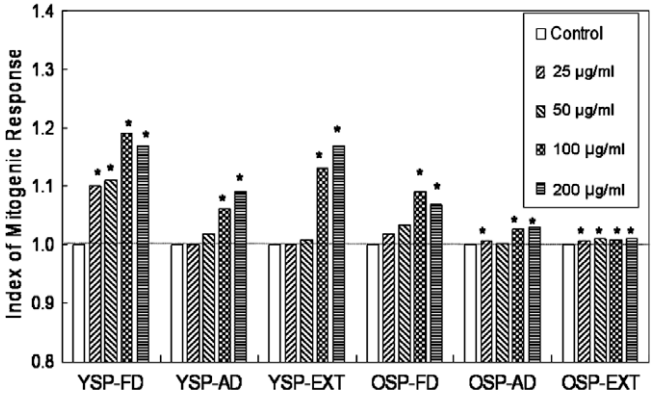

Fig. 2. Effect of the methanolic extracts from sweet potato on T-lymphocyte transformation assay. T-lymphocyte transformation was measured by mitogen Con A-induced proliferation of splenocytes from BLAB/c mice. Murine splenocytes were cultured in 96 well microtitre plates (3.6×10^5/ml), in the presence of Con A (1 μg/ml) and various concentration of test samples for 72 h. Mitogenic response was measured by the MTT colorimetric assay. Index of mitogenic response was indicated as absorbance percentage of control. ($n = 4$) ($^*p < 0.05$). YSP: yellow sweet potato (Tainong 57); OSP: orange sweet potato (Tainong 66); AD: sweet potato treated with 0.5% sodium bisulfite before hot air-drying; FD: freeze-dried sweet potato and no sodium bisulfite treated; EXT: extrusion treatment.

the mitogenic response than had that of the orange sweet potatoes (OSP, Tainong 66), in lower concentrations (25–50 μg/ml) for freeze-dried samples. In addition, the methanolic extract from freeze-dried sweet potatoes seems to preserve the most immunostimulatory constituents because of the highest mitogenic index as compared to that of samples prepared from hot air-drying and extrusion processing, for both yellow (YSP, Tainong 57) and orange (OSP, Tainong 66) sweet potatoes. However, all freeze-dried, hot air-dried and extruded sweet potato samples exhibit great ability in the proliferation of lymphocytes when stimulated with mitogen Con A. This suggests that these agents selectively promote T-cell-dependent proliferative activity. According to the study of Huang et al. (2004), the water extract of sweet potatoes showed the second highest antiproliferation activity amongst the storage roots used by human lymphoma N84 cells and some activity may be contributed by trypsin inhibitor (Huang, Sheu, Chen, Chang, & Lin, 2007). Chen et al. (2005) studied the immunological effects of physiological doses of purple sweet potato leaves and found that consumption of purple sweet potato leaves modulates various immune functions, including increased proliferation responsiveness of PBMC, secretion of cytokines IL-2 and IL-4, and the lytic activity of NK cells.

The T-lymphocyte is responsible for propagating both humoral and cell-mediated immunity via proliferation and subsequent secretion of a plethora of lymphokines, including interleukin-2 (IL-2), IL-4, IL-5 and IL-6. Several studies have used the *in vitro* murine splenocyte cultures method in food or its components, such as the adlay seed (Kuo, 2001; Kuo et al., 2001), EPA and DHA (Jolly, Jiang, Chapkin, & McMurray, 1997), whey protein (Hwang & Bowen, 2004) and bifidobacteria cells (Kado-Oka, Fujiwara, & Hirota, 1991). Because the aging process is associated with deterioration of the responsiveness of the immune system, several studies have reported a decline in T-cell-mediated immunity, including a compromised ability to proliferate in response to T-cell mitogens in laboratory animals. Thus, this study supported the idea that sweet potatoes may benefit elderly people and act as anti-aging immunomodulators.

It is believed that beta-carotene (Albanes, Heinonen, & Taylor, 1996), anthocyanins (Konczak-Islam, Yoshimoto, Hou, Terahara, & Yamakawa, 2003) and phenolic compounds (Kampa et al., 2004) have antiproliferative activity. Konczak-Islam et al. (2003) found that aqueous extracts of an anthocyanin-producing sweet potato line exhibited higher potency (antiproliferative and antimutagenic potential) than did extract from field-grown crops. The responsible determinants of sweet potatoes remain to be elucidated, as does the biological significance of the present observations.

Acknowledgement

Financial support for this study from the Council of Agriculture of the Republic of China (90 Agriculture-3.1.3-Food-Z1 (11)) is greatly appreciated.

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Food Chemistry 117 (2009) 122–126

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Effect of various anti-browning agents on phenolic compounds profile of fresh lettuce (*L. sativa*)

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ARTICLE INFO

Article history:
Received 29 December 2008
Received in revised form 12 February 2009
Accepted 23 March 2009

Keywords:
Lettuce (*L. sativa*)
Browning
Phenolic compounds
Enzymatic oxidation
Inhibitors

ABSTRACT

High performance liquid chromatography (HPLC) was used to characterise phenolic profile and to select the most effective anti-browning compound(s) on fresh lettuce. Four anti-browning agents, ascorbic acid, cysteine, citric acid and oxalic acid were tested for their effectiveness on preventing loss of phenolic compounds in lettuce during processing and storage. Aliquots of the reaction mixture were withdrawn at different times varying from 0 to 24 h, and directly analysed by HPLC. Protocatechuic acid, chlorogenic acid, ferulic acid, caffeic acid, *p*-coumaric acid and phloridzin were identified in fresh lettuce. Degradation of phenolic compounds followed a first-order kinetic pattern. The effect of anti-browning agents on first-order degradation rates of phenolic compounds was determined. Lettuce treated with oxalic acid and ascorbic acid maintained a higher level of phenolic compounds than citric acid and cysteine. Interestingly, cysteine had no positive effect for the prevention of oxidation of phenolic compounds even though it prevented browning in lettuce.

1. Introduction

Minimally processed lettuce has become an important area of potential growth in the rapidly expanding fresh-cut produce industry because of its fresh-like character and convenience. A major challenge faced by the produce industry is to manipulate the quality of fresh-cut produce so that the shelf-life is long enough to ensure efficient marketing (Gonzalez-Aguilar et al., 2005). Fresh-cut produce deteriorates faster than intact produce because of internal and external browning of the cut surface. Browning detracts from the appearance of the slices and reduces their marketability. Physical damage during the peeling and cutting process also causes an increase in respiration rates, biochemical changes and microbial spoilage, which often result in degradation of colour, texture, nutrient and flavour of the produce (Buta, Moline, Spaulding, & Wang, 1999).

Phenolic compounds are plant secondary metabolites synthesised mostly through the phenylproponaid pathway and are involved in the defence of plants against invading pathogens. Therefore, they are widely distributed in plant-derived foods significantly affecting their stability, colour, flavour, taste, nutritional and aesthetic value. In fact, plant secondary metabolites account for most antioxidant properties (Pati, Losito, Palmisano, & Zambonin, 2006). Today, it is believed that regular consumption of dietary antioxidants may reduce the risk of several diseases. Among natural antioxidants, plant polyphenols play a very important role. Most of the beneficial health effects of flavonoids are attributed to their antioxidant and chelating abilities; the protective effects can be ascribed to their capacity to transfer electron-free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce α-tocopherol radicals and inhibit oxidases (Heimler, Isolani, Vignolini, Tombelli, & Romani, 2007).

Being deprived of their natural protecting matrices, the phenolic compounds are exposed to oxygen during processing and may therefore be oxidised to quinones. Various classes of phenolic compounds, showing a great diversity of structures such as catechins, hydroxycinnamic acid derivatives and anthocyanins have been found to contribute to non-enzymatic and enzymatic browning of foods (Pati et al., 2006).

Basically, enzymatic browning can be defined as an initial enzymatic oxidation of phenolic compounds into slightly coloured *o*-quinones, catalysed by polyphenol oxidase (PPO). Although PPOs are localised in plastids, their phenolic substrates are mainly located in the vacuole so that enzymatic browning only occurs when this sub-cellular compartmentation is lost (Rigal, Gauillard, & Richard-Forget, 2000).

Various approaches to control the extent of browning have been investigated. In general, enzymatic browning can be avoided by thermal inactivation of PPO, but heat can cause unwanted softening of the tissues. Instead of blanching, chemical additives have been used to prevent enzymatic browning (Tortoe, Orchard, & Beezer, 2006). Reducing agents, antioxidants, and enzymatic inhibitors prevent browning by chemically reducing the *o*-quinones to

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doi:10.1016/j.foodchem.2009.03.085

colourless diphenols. Acidulants, such as citric, oxalic, malic, or phosphoric acid, can also inhibit PPO activity by reducing pH and/or chelating copper in a food product (Ibrahim, Osman, Saari, & Abdul-Rahman, 2004).

In agricultural products, phenolic compounds and PPO may react more or less during processing or storage resulting in a decrease of phenolic content. Phenolic compounds have a significant role in oxidation processes as antioxidants and as substrates in browning reactions (Robards, Prenzler, Tucker, Swatsitang, & Slover, 1999). In both roles, the key process is oxidation. Anti-browning agents were used to prevent or delay oxidation in different ways, but their effect on the phenol profile of fresh-cut produce is unclear. In this work, high-performance liquid chromatography (HPLC) has been used to investigate how various inhibitors influence the polyphenol profile of lettuce during enzymatic oxidation processes.

2. Material and methods

2.1. Material

Lettuce was obtained from a local market in Ankara. Oxalic acid (OA), ascorbic acid (AA) and cysteine were purchased from Merck, and citric acid (CA) from Carlo Erba. Caffeic acid, chlorogenic acid and gallic acid were purchased from Acros. Protocatechuic acid, chlorogenic acid, ferulic acid and caffeic acid were obtained from Sigma.

2.2. HPLC analysis of phenolic compounds in lettuce

Three grams of lettuce were homogenised in 9 ml of distilled water, AA (0.5%), cysteine (0.05%), CA (0.5%) and OA (0.5%) solutions, respectively. Aliquots were taken from the slurry at 0, 0.25, 0.5, 1, 2, 4, 6 and 24 h. The slurry was centrifuged at 15,000 g for 15 min. The supernatant was used for HPLC analysis.

Chromatographic analyses were performed on an Agilent 1200 HPLC system consisting of a photodiode array detector, quaternary pump, autosampler and column oven. Phenolic compounds were separated on a Waters Atlantis C18 column (250 mm × 4.6 mm, 5 μm) using a linear gradient elution programme with a mobile phase containing solvent A (formic acid/H_2O, 1:99, v/v) and solvent B (methanol) at a flow rate of 0.8 ml/min. The solvent gradient was programmed as follows: linear gradient elution from 10% B to 60% B, 0–15 min; isocratic elution of 60% B, 15–20 min; linear gradient elution from 60% B to 10% B, 20–25 min; isocratic elution of 10% B, 25–30 min. The chromatograms were recorded at 280 nm by monitoring spectra within a wavelength range of 190–400 nm. Identification of phenolic acids was accomplished by comparing the retention time and absorption spectra of peaks in samples to those of standard compounds. The quantitation of phenolic compounds was based on calibration curves built for each of the compounds identified in the samples.

3. Results and discussion

The profile of phenolic compounds in fresh lettuce extract was determined by HPLC analysis. Relative changes in the concentration of individual phenolic compounds in lettuce with time as influenced by the addition of 0.5% AA, 0.05% cysteine, 0.5% CA and 0.5% OA at 25 °C were compared to that of the control. Protocatechuic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid and phloridzin were identified by matching the retention time of the compounds determined in the lettuce extract to those of pure standards (Fig. 1). The chemical structures of the peaks identified in the lettuce extract were further confirmed by comparing their UV spectra to those of pure standards.

As a result of enzymatic oxidation, a sharp decrease in the concentration of phenolic compounds was observed in the control lettuce. Loss of individual phenolic compounds identified in lettuce was expressed in terms of the first order degradation rate constant (k). The results are summarised in Table 1. The results are in agreement with the results obtained by others (Nourian, Ramaswamy, & Kushalappa, 2003). Phenolic compounds and PPO originally exist separately in different organelles in plant cells. When the plant is damaged, phenolic compounds and PPO come into contact and react and parts of the phenolic compounds are isomerized (Rigal et al., 2000).

Adding of anti-browning agents is one of the typical methods used to inhibit the browning reactions. Treatments with AA, cysteine, CA and OA were more effective in delaying polyphenol oxidation in lettuce during storage. Fig. 2 illustrates relative changes in chlorogenic acid concentration in the presence or absence of various inhibitors. It was observed that the effectiveness of anti-browning compounds in reducing polyphenols content diminished with the length of storage (except for cysteine). These results are in agreement with our previous findings (Altunkaya & Gökmen, 2008).

3.1. Effect of ascorbic acid on lettuce enzymatic oxidation

The concentrations of phenolic compounds identified in the lettuce extract have been monitored up to 24 h in the presence of AA. Aliquots of the sample were withdrawn after 0, 1, 3, 6 and 24 h for HPLC analyses. AA showed effective protection against oxidation. Moreover, the rate of degradation of all identified phenolic compounds was decreased slowly in lettuce when 0.05% of AA was added (Table 1).

AA is an important nutrient and very likely to decompose during storage. It was reported that temperature, pH, oxygen concentration, metals (iron, copper) and light are important parameters for the destruction of AA (Jung, Kim, & Kim, 1995). Degradation of AA has been also implicated with colour changes as well as reduced nutritional quality.

AA is a reducing compound but it does not directly inhibit PPO. Following enzymatic oxidation, AA reduces the *o*-quinone formed by the enzyme to the original diphenol to limit browning through a process known as "reaction deactivation". Unfortunately, AA offers only temporary inhibition of browning (Altunkaya & Gökmen, 2008). During oxidation, it appears that AA is most likely converted to dehydroascorbic acid (DHAA) and further degraded to 2,3 diketo-gluconic acid (Gökmen, Kahraman, Demir, & Acar, 2000). AA content of lettuce treated with 0.5% AA have been previously shown to follow a similar pattern (Gonzalez-Aguilar et al., 2005).

AA acts as a protector of pigments preserving them from chemical and biochemical oxidation by competing amides and amine–carbonyl interactions that result in browning. An alternative mode of action may be suppression of free radical formation involved in the browning reaction. There is also evidence that AA has polyphenol-protective and -enhancing activities, probably due to the reduction of oxidised phenols and regeneration of polyphenols. A cooperative action between AA and polyphenols could be beneficial in enhancing the ability of the latter to rescue cells from damage induced by an oxidative stress (Gonzalez-Aguilar et al., 2005).

AA can react either with the radicals generated in chemical ways, or it can regenerate polyphenols from their oxidised forms due to its antioxidant potential. In order to obtain synergy *via* regeneration reactions between AA and phenolic compounds, these two reactions must compete efficiently with the other reactions in which AA is oxidised (Beer, Joubert, Gelderblom, & Manley, 2005). Based on standard reduction potentials, it has been predicted and confirmed by kinetic studies that ascorbate regenerates plant phenolic compounds in homogeneous solutions from their

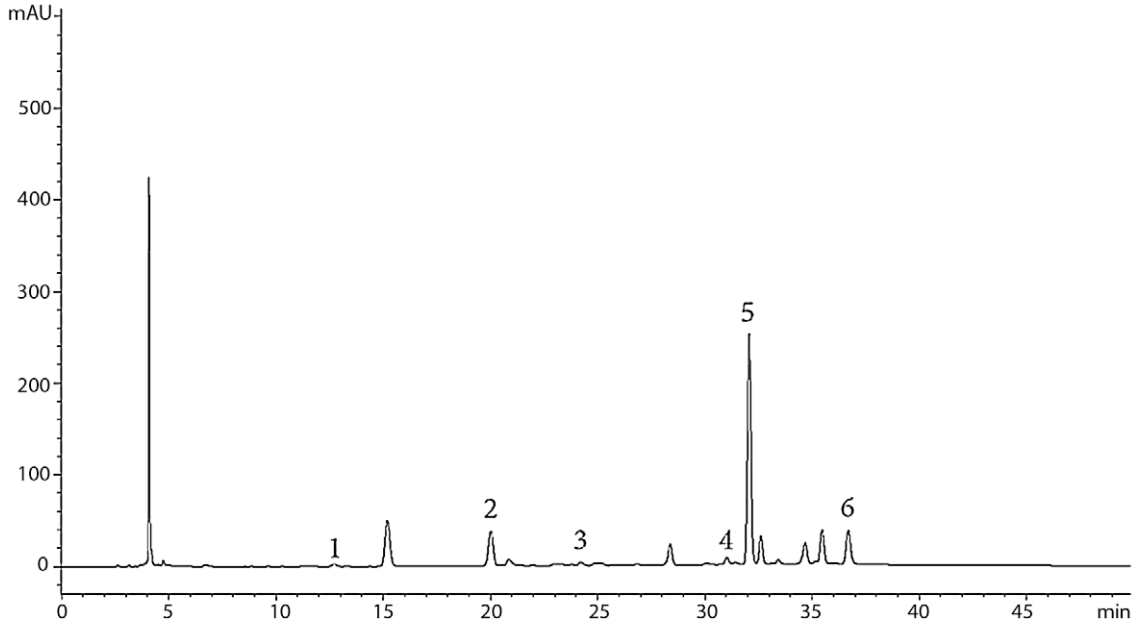

Fig. 1. HPLC profile of phenolic compounds in fresh lettuce. Peaks identified are: (1) protocatechuic acid; (2) chlorogenic acid; (3) caffeic acid; (4) *p*-coumaric acid; (5) ferulic acid; (6) phloridzin.

Table 1
First-order degradation rate constants of individual phenolic compounds identified in lettuce.

Phenolic compound	Rate constants (1/min)				
	Control	Ascorbic acid	Cysteine	Citric acid	Oxalic acid
Protocatechuic acid	0.557	0.056	0.576	0.447	0.083
Chlorogenic acid	0.333	0.036	0.667	0.434	0.055
p-Coumaric acid	0.668	0.030	0.386	0.183	0.080
Ferulic acid	0.890	0.016	0.929	0.862	0.396
Phloridzin	0.985	0.027	0.113	0.167	0.068

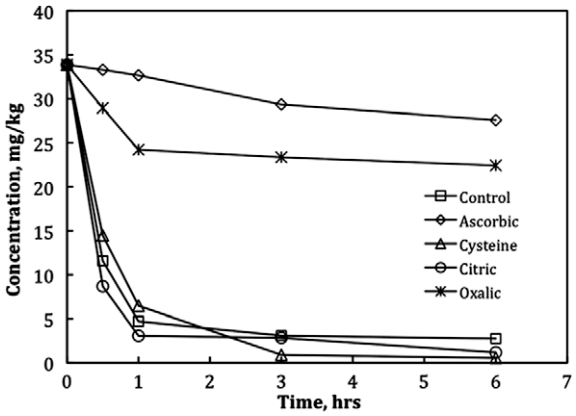

Fig. 2. Changes in chlorogenic acid concentration with time in the presence of various inhibitors (□: control, ◇: 0.5% ascorbic acid, Δ: 0.05% cysteine, ○: 0.5% citric acid, ∗: 0.5% oxalic acid).

oxidised form (Jovanovic, Steenken, Tosic, Marjanovic, & Simic, 1994). Polyphenols have a higher redox potential than ascorbate and consequently oxidise it to the ascorbyl radical (Bors, Heller, Michel, & Saran, 1990).

Lettuce also contains high levels of ascorbate oxidase activity which oxidise AA to DHAA after the lettuce is cut and exposed to oxygen. The amount of AA could be decreased during oxidation due to this reason (Yamaguchi et al., 2003). As a consequence, the assuring stability of AA during storage has been a major problem for preventing browning and phenol destruction.

3.2. Effect of cysteine on lettuce enzymatic oxidation

The concentrations of phenolic compounds were monitored in lettuce in the presence of 0.05% cysteine. It was determined that the degradation rates of individual phenolic compounds in the presence of cysteine were similar to those obtained in the control sample without anti-browning agent. It should be noted here that the lettuce sample with 0.05% of cysteine showed no enzymatic activity against catechol as determined by a spectrometer at 420 nm (data not shown).

The action of cysteine is complex. During enzymatic oxidation, it traps the *o*-quinone by forming cysteine adducts. These adducts are not substrate, but competitive inhibitors of PPO with a slightly higher affinity than their polyphenol precursors (Richard-Forget, Goupy, & Nicolas, 1992). In the presence of an excess amount of thiols such as cysteine (cysteine/phenol > 1), phenolic compounds are fully converted to their corresponding cysteine adducts, and in theory they are protected against enzymatic browning. The strong inhibition of polyphenol degradation by PPO in the presence of cysteine is explained primarily by the competitive inhibition of the enzyme, and by the secondary effect of the coupled oxidation by *o*-quinone, leading to phenol regeneration (Richard-Forget et al., 1992).

Gorny, Hess-Pierce, Cifuentes, and Kader (2002) have demonstrated that sulphites act as a reducing agent at a pH below 4. At a pH above 4, quinones form colourless adducts with sulfites, cysteine or glutathione. Nucleophilic attack of quinones by cysteine may be more effective at a neutral pH since the thiol group of cysteine has a pKa of 8.33. Because the vegetable tissue is at neutral pH values, cysteine alone is effective as an inhibitor of enzymatic browning.

A consideration of biochemical changes inside the lettuce samples may be just as important as appearance in selecting treatments to extend the shelf life of fresh-cut lettuce. It is important to mention that the concentrations of anti-browning agents that were used in the present study did not affect the sensorial characteristics of fresh-cut lettuce except for cysteine. Due to the excess amount of cysteine, lettuce samples treated with cysteine became yellow and did not turn pink.

3.3. Effect of citric acid and on lettuce enzymatic oxidation

CA was found to protect lettuce against browning. Moreover, the rate of degradation of individual phenolic compounds decreased in lettuce within 24 h of storage when 0.05% of CA was added (Table 1). Acidification of samples appeared to have an important function to stabilise phenolic compounds in lettuce.

CA has been reported extensively for its inhibitory effect on PPO, so it is recommended as a potential anti-browning agent in minimally processed fruits and vegetables (Ahvenaien, 1996). It lowers the pH and chelates the copper at the active site of the PPO. Its inhibitory effect could be related to the phenolase Cu-chelating power. Especially, at pH values below 4, the looser binding of copper at the active enzyme site causes the PPO activity to decrease further, permitting the CA to remove the copper (Ibrahim et al., 2004). The effect of CA on flavanols have been previously considered as both anti-browning and antioxidant agent (Wang, Kim, & Park, 2003). The acidification and chelating function of CA works together. Therefore, it could be correct to presume that CA has a protective effect on phenolic compounds.

3.4. Effect of oxalic acid on lettuce enzymatic oxidation

OA was found more effective than CA and cysteine in preventing browning and oxidation of individual phenolic acids in lettuce during storage (Table 1). It showed almost the same anti-browning effect as AA. OA seems to inhibit PPO by chelating copper from the active site of the enzyme since OA has a high affinity to form metal complexes with a copper ion. The extent of inhibition is influenced by both OA concentration and pH. OA has been previously shown to diminish catechol-quinone formation (Zheng et al., 2007).

OA has been reported as the most potent inhibitor among a number of dicarboxylic acids by Son and others (2001). The ability of carboxylic acids to inhibit browning depends on their chemical structure. Inhibitory effects of dicarboxylic acids decreases with increasing chain length and dissociation constants, signifying the possible role of steric interference in the interaction of the longer chains with the enzyme and of the degree of their ionisation in solution. OA has been shown to reduce the rate of phenol oxidation in the presence of hydrogen peroxide, iron and copper due to its chelating ability and lowering of pH (Kayashima & Katayama, 2002). When present in the human diet, OA may combine with essential minerals such as calcium, iron, magnesium, and potassium to form less soluble salts known as oxalates and hinder the bio-availability (Palaniswamy, Bible, & McAvoy, 2004). This fact, together with our study, suggests that treatment with OA is safe and seems to be a promising method for controlling browning in minimally processed fresh-cut lettuce.

4. Conclusion

The degradation of individual phenolic compounds during enzymatic oxidation followed a first-order kinetic model. The degradation rate constants obtained for individual phenolic compounds in the presence of cysteine and CA were found significantly higher than that of AA and OA. Higher k values indicated more rapid degradation of phenolic compounds in lettuce. AA and OA could be recommended to prevent enzymatic browning and loss of phenolic compounds.

Among the anti-browning agents tested, OA and AA had a great capability for the prevention of the degradation of phenolic compounds in fresh-cut lettuce. Hence, it appears that they have strong potential for practical applications.

Degradation rates of phenolic compounds with cysteine were almost the same as the control. It allowed permanent protection against enzymatic browning, but phenolic compounds could not be protected against oxidation using cysteine. Loss of phenolic compounds is undesirable from a nutritional point of view.

Two of the important properties of phenolic compounds involve antioxidant activity and oxidative browning. They are both good browning substrates and good antioxidants. In both systems, the potential oxidising agents are PPO and free radicals when phenolic compounds act as substrates and antioxidants (Robards et al., 1999).

Enzymatic browning in foods is reasonably well understood, but prevention of browning is not the only factor to take into account to be sure of quality. Besides the anti-browning property of inhibitors, protection of nutritional properties should be taken into consideration to maintain a fresh like quality. As a consequence, formulations including additives have to be optimised to succeed in the control of enzymatic browning and loss of the nutritional quality.

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Food Chemistry 117 (2009) 127–134

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Sterols, fatty alcohol and triterpenic alcohol changes during ripe table olive processing

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ARTICLE INFO

Article history:
Received 30 December 2008
Received in revised form 23 March 2009
Accepted 24 March 2009

Keywords:
Discriminant analysis
Fatty alcohols
Ripe table olives
Sterols
Triterpenic alcohols

ABSTRACT

The aim of the work was to study the effects of processing on the unsaponifiable matter, sterols and fatty and triterpenic alcohol in ripe olive fat (Manzanilla and Hojiblanca cultivars) and to disclose the most influential factors using GLM, PCA and DA. There were significant effects of cultivars or ps on unsaponifiable matter, β-sitosterol, Δ^5-avenasterol, total sterols, 1-docosanol, 1-tetracosanol (ps), erythrodiol and percentage erythrodiol + uvaol. The values of most of these parameters were within the limits established by the EU Directives for olive and pomace oils but classification of the respective oils was not conclusive. Predictive discriminant analysis using these variables permitted 100% success in the classification according to cultivars and ps (68% in the case of cross validation). Results revealed that some influential steps should be re-designed, particularly the storage phase, in order to minimise changes in the studied fat components during ripe olive processing.

1. Introduction

Table olives constitute an important part of the Mediterranean diet and its world production reached a total of 1,762,000 tons in the 2005/2006 season (IOOC (International Olive Oil Council), 2007). One of the most common styles is ripe olives (California style). In brief, fruits for producing ripe olives (by alkaline oxidation) are previously preserved in an aqueous solution (brine or acidic water) and darkened throughout the year according to demand. Darkening consists of several treatments of dilute NaOH solutions and water washes (with aeration between them), immersion in a lactate or gluconate iron solution and packing in light brine (Garrido-Fernández, Fernández-Díez, & Adams, 1997). The successive treatments may affect the composition of the olives.

Apart from moisture, oil is the major component of table olives (López, Montaño, García, & Garrido, 2006). However, studies on the composition of this fat are scarce (Borzillo, Iannotta, & Ucella, 2000; Ünal & Nergiz, 2003). The unsaponifiable matter of olive oil and its components are essential for its classification into diverse categories (Directives (CEE) 2568/91 and 1989/2003); it consists of groups of components, mainly phytosterols, of recognised biological effects like cytostatic activity (Saenz, García, Ahumada, & Ruiz, 1998), blood cholesterol control (Richelle et al., 2004) or cancer prevention (Normén et al., 2001). Triterpenic compounds from "orujo" olive oil elicit vasorelaxation in aorta from spontaneously hypertensive animals (Rodríguez-Rodríguez, Perona, Herrera, & Ruiz-Gutierrez, 2006).

Sterol and alcohol profiles are used to characterise virgin olive oils and to detect the adulteration of olive oil with hazelnut oil (Vichi, Pizzale, Toffano, Bortolomeazzi, & Conte, 2001) or virgin olive oil with olive-pomace oil (Reina, White, & Jahngen, 1997).

Chemometric studies have been used for the characterisation of varietal olive oils based on their sterols and other fatty components (fatty acids, tocopherols, diacylglycerols or triacylglycerols) (Matos et al., 2007) to classify Portuguese olive oils according to the Protected Denomination of Origin (Rui Alves, Cunha, Amaral, Pereira, & Oliveira, 2005) and to discriminate between virgin olive oils from different olive varieties (Sánchez Casas, Osorio Bueno, Montaño García, & Martínez Cano, 2004). Several hazelnut cultivars have also been characterised based on their chemical, fatty acid and sterol composition (Amaral et al., 2006).

The amount of unsaponifiable matter in olive oil is about 1% (Aparicio & Harwood, 2003). In oils extracted from commercial table olives, the proportion of unsaponifiable matter ranged from 2% to 5% and the greatest proportion was found in ripe olives (López-López, Montaño, Ruíz-Méndez, & Garrido-Fernández, 2008). The cause of such a high level is unknown but it is likely that the treatments involved in their processing may affect the proportion of unsaponifiable matter and their composition (particularly sterols, fatty alcohols and triterpenic alcohols) with the subsequent loss of their physiological activities and quality. However, there is no information on these changes.

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doi:10.1016/j.foodchem.2009.03.086

Nomenclature

DA	discriminant analysis
GC	gas chromatography
GLM	general linear model
MANOVA	multiple analysis of variance
PCA	principal component analysis
PCs	principal components
ps	processing step
PVC	polyvinyl chloride
TCL	thin layer chromatography

The aim of this work was (i) to study the changes caused by the successive processing steps in ripe olive oil, particularly in the content of unsaponifiable matter, sterols, fatty alcohols and triterpenic dialcohols, according to cultivars, (ii) to contrast the characteristics of this oil with the criteria established in the EU Directives for the classification of olive oil into categories, and (iii) to apply chemometric techniques to disclose relationships among these compounds, revealing the most influential steps and checking their potential to discriminate among processing steps and cultivars. Results from this research are useful for the identification and redesign of the most influential processing steps so that changes in olive fat and its components can be minimised.

2. Materials and methods

2.1. Cultivars

Olives were of the Hojiblanca and Manzanilla cultivars, which are the most popular cultivars for preparing ripe table olives in Spain.

2.2. Previous storage

The olives (15 kg each cultivar) were stored in 25 L PVC fermenters for six months. Initial brines consisted of an acidified solution (corrected to pH 4.2 with acetic acid) containing 9% NaCl. To compensate for salt absorption by the olives, the proportion of NaCl was raised periodically to reach equilibrium at 8% (w/v). Periodically (8 h/day) air was bubbled through the brine by means of a column introduced into the interior of the fermenters. A more detailed description of the storage system can be found elsewhere (Garrido-Fernández et al., 1997).

2.3. Darkening process

Olives were treated in a cylindrical stainless steel container with successive lye solutions of 1.5%, 1.0% and 1.0%, which progressively penetrated the flesh until the alkali reached the pit at the end of the last immersion. Between lye treatments, olives were suspended in tap water to remove the excess alkali and air was injected through the bottom of the container. When the skin was black, the washings were prolonged until the pH reached 8.0 and then a 0.1% ferrous gluconate solution with pH corrected to 4.5 was added to fix the colour. The fruits were then canned in a 3.5% NaCl solution acidified with acetic acid to pH 4.5, subjected to sterilisation for 20 min at 130 °C and cooled down to room temperature with tap water.

2.4. Equilibrium period for the packed product

Sterilised olives were stored at room temperature for 30 days to permit equilibrium to take place before cans were opened and analysed.

2.5. Fat extraction

Olives were pitted, mixed with a homogeniser Ultraturax T25 (IKA-Labortecnik, Staufen, Deutschland) and then boiling water (100 °C) was added to the paste. The resulting suspension was subjected to malaxation for 40 min at room temperature (22 °C ± 2) and the liquid was removed by centrifugation using ABENCOR equipment (Abengoa, Madrid, Spain), similar to that used for the estimation of olive oil yield in olive mills (Martínez, Muñoz, Alba, & Lanzón, 1975). The liquid phase was allowed to decant and the oil was obtained, filtered and subjected to analysis. This method was used to prevent changes in the oil quality as much as possible. During extraction, one duplicate sample corresponding to Manzanilla raw material was irreversibly lost.

2.6. Determination of the unsaponifiable fraction

The unsaponifiable matter was determined by saponification of the oil with potassium hydroxide in an ethanolic solution and extracted with diethyl ether (AENOR (Asociación Española de Normalización y Racionalización), 2001).

2.7. Determination of sterols and triterpenic dialcohols

This analysis was performed according to the method described by the Official Journal of the European Communities (European Union Commission, 1991). The lipid with added α-cholestanol and betulin as internal standards was saponified and the unsaponifiable matter was extracted as mentioned above. The bands corresponding to the sterols and triterpenic alcohol fractions were separated from the extract by TLC on a basic silica gel plate. The sterols and erythrodiol and uvaol recovered from the plate were transformed into trimethylsilyl ethers and the mixture was analysed by GC using an HP 5890 Series II gas chromatograph (Hewlett–Packard, Minnesota, USA) equipped with a flame ionisation detector and a 30 m × 0.32 mm i.d. Tracsil TRB-5 (95% dimethylpolysiloxane–5% diphenyl, film thickness 0.25 μm) capillary column (Teknokroma, Barcelona, Spain). The chromatographic conditions were: injector 300 °C, isothermal column 275 °C, and detector 300 °C. Split ratio was 1:50. Hydrogen carrier gas was used at 1.0 mL/min.

2.8. Determination of fatty alcohols

This analysis was performed according to the method described by the Official Journal of the European Communities (European Union Commission, 2002). The fatty substance, with 1-eicosanol added as internal standard, was treated as mentioned in the determination of lipid and unsaponifiable fraction section. The alcohol fraction was separated from the unsaponifiable matter by chromatography on a basic silica gel plate. The alcohols recovered from the silica gel were transformed into trimethylsilyl ethers and analysed using capillary gas chromatography. The equipment and chromatographic conditions were the same as those mentioned above

for sterols and triterpenic dialcohols, except that oven temperature was as follows: 215 °C (5 min); 3 °C/min increase to 290 °C and held for 2 min. All analyses were performed in duplicate.

2.9. Statistical analyses

Data from the previous determinations were arranged in a 28 × 20 matrix array, where rows were cases (cultivars × processing steps) and columns were variables (sterols, and fatty and triterpenic alcohols). Data were analysed first using GLM nested factorial design to estimate cultivar and processing step means as well as to detect significant differences between processing steps for each individual compounds within the cultivar.

Before being subjected to the chemometric analysis, data were standardised using the auto scale procedure, which is the most commonly used scaling technique (Kowalski & Bender, 1972). The procedure standardises a variable m according to:

$$y_{mj} = \frac{(x_{mj} - \bar{x}_m)}{s_m}$$

where y_{mj} is the value j for the variable m after scaling, x_{mj} is the value j of the variable m before scaling, $\bar{x}_m$ is the mean of the variable m and s_m is the standard deviation for the variable m. The result is a variable with zero mean and a unit standard deviation.

Standardised data were successively studied by multiple analysis of variance (MANOVA) to test overall differences between groups across the different variables, principal component analysis (PCA), and discriminant analysis (DA). PCA was carried out using a varimax rotation to detect the data structure and to determine the relationships among the different components and to derive common dimensions in order to classify the samples and to map the different population groups into these dimensions. For the selection of the number of Principal Components (PCs), the Kaiser criterion (Jolliffe, 1986) was followed and only factors with eigen values higher than 1.00 were retained. Then, the loadings of the original variables were projected onto the factorial plane formed by the first and second component.

The selection of variables containing the most powerful information for the correct classification of the olive samples from the two cultivars (Manzanilla and Hojiblanca) or the four elaboration steps for the ripe olive processing categories (raw material, storage, oxidation and packing-sterilisation) was carried out on the basis of the canonical analysis of data, using the backward stepwise analysis option, which first includes all the variables in the model and then, at each step, eliminates the variable that least contributes to membership prediction. The process continues until only the important variables that contribute most to discrimination between groups are in the model. The values of probability to enter or to remove were fixed at 0.05 and 0.10, respectively. The number of steps was fixed at 100, the minimum tolerance at 0.001 and no variable was forced to enter into any model. The scores of table olive samples were plotted on the canonical axes (discriminant coordinates, called Factors). These axes were determined in such a way that the rate of the variance between groups compared to the variance within groups is maximised (Rencher, 1995).

DA classification was achieved by means of the corresponding classification functions. For k groups, k linear combinations of variables are constructed, called classification functions. The calculation of the values of these functions for each sample makes it possible to allocate this sample to the group for which the probability of belonging is the highest. Prior probabilities were established in proportion to the number of samples in each group.

A leaving-one-out cross validation procedure was performed for assessing the performance of the classification rule. In this last step, the sample data minus one observation was used for the estimation of the classification functions and then the omitted variable was classified from them. The procedure was repeated for all samples. Consequently, each sample was classified by classification functions which were estimated without its contribution (Rencher, 1995).

The different statistical techniques used in this work were implemented using STATISTICA, release 6.0 (GLM and PCA analyses) and SYSTAT, release 10.2 (DA analysis).

3. Results and discussion

3.1. Changes in ripe olive oil components (unsaponifiable, sterols and fatty and triterpenic alcohols) due to processing step

The content in unsaponifiable matter in the oils from the different processing steps is shown in Table 1. The analysis of variance of the nested GLM showed significant differences among elaboration steps within cultivars. The initial content (raw material) was higher in Manzanilla (1.45 g/100 g oil) but, at the end (the sterilised product), it was higher in Hojiblanca (1.36 g/100 g oil). Apparently, the oxidation process had opposing effects, depending on cultivars; these changes were closely related to those observed during the storage/fermentation phase, which can be due to the activity of the microorganisms present in this step. However, average contents throughout the processing steps remained fairly constant (1.28 ± 0.04 and 1.24 ± 0.05 g/100 g oil for Hojiblanca and Manzanilla, respectively) but slightly higher than the average proportion of unsaponifiable matter found in olive oil, which is usually about 1% (Aparicio & Harwood, 2003). Apparently, this difference is due not only to the effect of the processing steps but also to cultivar (Manzanilla raw material) (Table 1).

The total content of sterols was always higher than fatty and triterpenic alcohols (Tables 2 and 3). On average, the proportion of sterols in the oil obtained from the diverse steps of ripe olive processing ranged from 1088 to 2543 mg/kg oil, which is approximately within the proportion ranges found in olive oil (Aparicio & Harwood, 2003). In the raw material, the concentrations of total sterols were higher in Hojiblanca than in Manzanilla (Table 2) whilst the levels of fatty and triterpenic alcohols were similar in both cultivars (Table 3) because the values of Manzanilla were always within the confidence limits of the Hojiblanca cultivar bearing in mind the standard error and t value for this cultivar. The concentrations of total sterols in the oils from the raw material (fresh fruits) were similar to those found by Sánchez Casas et al. (2004) in olive oils from Carrasqueña, Cacereña, Cornezuelo, Corniche, Morisca, Picual and Verdial cultivars. So, the presence of sterols in olive oil and table olive cultivars does not differ. In the raw material, the proportion of β-sitosterol was about 87% (Hojiblanca) and 84% (Manzanilla) of the total sterols and the next abundant sterol was Δ^5-avenasterol, which accounted for about 4.4% and 5.6% in Hojiblanca and Manzanilla, respectively; the concentration of campesterol was also remarkable (about 2.8% regardless of cultivar). The concentrations of β-sitosterol in the raw material oil were similar to those reported by Sánchez Casas

Table 1
Average (±standard error) unsaponifiable matter (g/100 g oil) throughout processing steps, according to cultivars.

Processing step	Hojiblanca	Manzanilla
Raw material	1.090 (0.031)	1.453 (0.086)
End storage	1.369 (0.058)	1.112 (0.109)
End oxidation	1.191 (0.062)	1.346 (0.050)
Sterilised product	1.357 (0.035)	1.140 (0.030)

Nested GLM analysis of variance showed significant differences among processing steps within cultivars ($F = 4.42$, with 6 and 20 degrees of freedom, respectively; $P = 0.005$).

Table 2
Mean values (mg/kg oil ± standard error) of sterols in ripe olives, according to cultivar and processing step.

Component	Hojiblanca				Manzanilla			
	Raw material	End storage	End oxidation	Sterilised olives	Raw material	End storage	End oxidation	Sterilised olives
Cholesterol	2.9 ± 2.9	13.2 ± 7.4	7.0 ± 0.0.9	5.5 ± 1.9	2.9 ± [a]	5.4 ± 0.8	7.5 ± 0.9	4.1 ± 0.7
Brassicasterol	10.4 ± 6.4	4.8 ± 3.1	4.3 ± 4.3	Nd	3.6 ± [a]	0.8 ± 0.8	0.4 ± 0.4	2.8 ± 0.8
Campesterol	40.1 ± 2.0	113.6 ± 74.0	50.6 ± 5.2	67.9 ± 5.1	30.5 ± [a]	43.9 ± 10.2	41.1 ± 5.8	35.3 ± 1.6
Campestanol	4.6 ± 4.6	3.1 ± 3.1	11.2 ± 1.9	12.1 ± 4.1	8.5 ± [a]	6.2 ± 2.1	9.5 ± 0.2	8.6 ± 0.4
Stigmasterol	16.3 ± 1.7	20.4 ± 8.3	18.4 ± 2.7	21.3 ± 1.6	15.5 ± [a]	32.4 ± 13.5	28.5 ± 8.3	20.9 ± 1.4
Δ^7-Campesterol	Nd	1.4 ± 1.4	6.0 ± 6.0	Nd	Nd	9.8 ± 5.5	4.6 ± 2.7	Nd
Clerosterol	36.3 ± 9.3	38.4 ± 5.2	34.0 ± 9.0	27.0 ± 5.6	34.5 ± [a]	13.7 ± 8.3	25.5 ± 0.8	30.0 ± 8.8
β-Sitosterol	1248 ± 46	1359 ± 102	1452 ± 287	2323 ± 224	896 ± [a]	1022 ± 55	1195 ± 45	947 ± 14
Δ^5-Avenasterol	63.5 ± 2.9	56.3 ± 5.4	51.8 ± 5.6	68.1 ± 3.8	59.2 ± [a]	40.0 ± 2.0	45.0 ± 2.3	39.2 ± 2.3
$\Delta^{5,24}$-Stigmastadienol	7.4 ± 0.4	5.7 ± 2.9	Nd	Nd	6.8 ± [a]	5.14 ± 1.8	9.2 ± 0.8	Nd
Δ^7-Stigmastenol	Nd	6.0 ± 6.0	Nd	8.7 ± 8.7	Nd	20.0 ± 17.6	17.6 ± 11.9	Nd
Δ^7-Avenasterol	2.2 ± 2.2	3.3 ± 3.3	2.7 ± 2.7	9.2 ± 5.5	4.2 ± [a]	4.3 ± 2.3	7.8 ± 4.7	Nd
Total	1432 ± 37	1625 ± 194	1637 ± 297	2543 ± 228	1062 ± [a]	1204 ± 95	1392 ± 81	1088 ± 20

[a] Data without replicates. Values are means of duplicate analyses of replicates.

Table 3
Mean values (mg/kg oil ± standard error) of fatty and triterpenic alcohols in ripe olives, according to cultivar and processing step.

Component	Hojiblanca				Manzanilla			
	Raw material	End storage	End oxidation	Sterilised olives	Raw material	End storage	End oxidation	Sterilised olives
Aliphatic alcohols								
1-Docosanol	132.38 ± 18.98	42.50 ± 5.63	13.30 ± 6.82	18.49 ± 6.43	79.00 ± [a]	6.44 ± 6.44	28.95 ± 10.98	8.22 ± 8.22
1-Tetracosanol	5.94 ± 1.74	55.79 ± 16.75	11.24 ± 5.62	35.84 ± 4.62	37.96 ± [a]	115.56 ± 23.32	16.10 ± 9.29	26.23 ± 17.12
1-Hexacosanol	29.69 ± 17.95	171.88 ± 69.05	18.63 ± 9.38	78.35 ± 22.21	31.58 ± [a]	476.27 ± 134.59	272.12 ± 162.82	132.34 ± 34.02
1-Octacosanol	97.94 ± 66.77	111.05 ± 43.38	93.09 ± 46.79	99.29 ± 18.77	54.75 ± [a]	255.80 ± 78.51	97.28 ± 27.74	122.04 ± 20.98
Total	265.95 ± 105.44	381.22 ± 123.31	136.26 ± 25.52	231.98 ± 47.49	203.29 ± [a]	854.08 ± 228.11	414.45 ± 173.08	288.83 ± 63.76
Triterpenic alcohols								
Erythrodiol	6.30 ± 6.30	31.16 ± 5.19	13.13 ± 6.65	40.29 ± 6.71	54.22 ± [a]	76.25 ± 1.96	66.59 ± 1.13	86.61 ± 5.76
Uvaol	33.04 ± 14.00	6.55 ± 6.55	Nd	Nd	Nd	7.45 ± 4.94	6.71 ± 5.15	Nd
Total	39.34 ± 7.70	37.71 ± 6.86	13.13 ± 6.65	40.29 ± 6.71	54.22 ± [a]	83.70 ± 6.56	73.30 ± 5.55	86.61 ± 5.76

[a] Data without replicates. Values are means of duplicate analyses of replicates.

et al. (2004) in the above-mentioned oils or Rui Alves et al. (2005) in oils from Portuguese cultivars. The proportion of β-sitosterol in virgin olive oils from Cornicabra had a mean of 84% (Rivera del Álamo, Fregapane, Aranda, Gómez-Alonso, & Salvador, 2004), which is similar to the values found in this work. Then, regardless of cultivars and olive destination, the most abundant phystosterol was β-sitosterol but it was 3% higher in Hojiblanca. The β-sitosterol content in the raw material was always higher than those of any food included in the phytosterol data base of fatty foods consumed in Sweden and the Netherlands (Normén et al., 2001). This fact emphasises the role played by table olives as a source of phytosterols. However, the levels Δ^5-avenasterol, on average (60.3 ± 2.9 and 42.8 ± 1.9 mg/kg oil for Hojiblanca and Manzanilla cultivars, respectively), were lower than those found by Sánchez Casas et al. (2004), Rui Alves et al. (2005) or Rivera del Álamo et al. (2004). Apparently, the levels of this compound were systematically lower in oils from cultivars devoted to table olives.

The content of cholesterol in the raw material, about 3.0 mg/kg oil, regardless of cultivar, was approximately of the same order as those found by Cañabate-Díaz et al. (2007) either in virgin olive oils or in pomace olive oils but was higher than those reported by Rivera del Álamo et al. (2004) in virgin olive oils from Cornicabra. So, cholesterol concentration in oils from table olives showed an intermediate position with respect to data found in the literature. In any case, the cholesterol contents found were lower than those reported for numerous plant oils (Behrman & Gopalan, 2005). Some compounds were not detected in the raw material (Δ^7-campesterol and Δ^7-stigmastenol) or in other samples (Δ^7-campesterol, Δ^7-stigmastenol and $\Delta^{5,24}$-stigmastadienol), a circumstance which prevented their use in the chemometric studies. The concentrations of the rest of sterols in the raw material were lower (Table 2) than the levels given for them in the above-mentioned references. This fact may be related to the table olive cultivars of this study.

Fatty alcohol contents (Table 3) in oils from the raw material were markedly lower than sterols and their overall averages were 266 and 203 mg/kg in Hojiblanca and Manzanilla cultivars, respectively. The most abundant was 1-docosanol (C22) (132 mg/kg oil in Hojiblanca, 79 mg/kg in Manzanilla), followed by 1-octacosanol (C28) and 1-hexacosanol (C26) (in Hojiblanca) or tetracosanol (C24) (in Manzanilla).

The total triterpenic alcohol content was higher in the Manzanilla raw material (54 mg/kg oil) than in Hojiblanca (39 mg/kg oil) whilst uvaol was not detected in this or in other samples, a circumstance which prevented its use in PC and DA. Levels were reversed in Hojiblanca (uvaol, 33.0 mg/kg and erythrodiol 6.3 mg/kg). The concentrations of these compounds were markedly higher than those reported by Rivera del Álamo et al. (2004) in virgin olive oils obtained from the Cornicabra cultivar during several seasons. Apparently, fat from table olive cultivars may be a better source for these compounds than that obtained from olive oil cultivars.

The processing steps produced changes in the contents of the different sterols, fatty alcohols and triterpenic alcohols, which were diverse according to cultivars (Tables 2 and 3). The overall content in sterols remained fairly constant throughout the diverse processing steps, except in the sterilised product from the Hojiblanca cultivar which showed the highest levels (Table 2), close to the values reported by Cañabate-Díaz et al. (2007) for pomace olive oil (2200–2900 mg/kg oil) and higher than the levels in virgin and refined olive oils. This behaviour was mainly due to the increase observed in β-sitosterol after the sterilisation process of Hojiblanca. However, levels of β-sitosterol in Manzanilla showed

an increase after oxidation. The trend in Δ^5-avenasterol was the opposite of that shown by β-sitosterol but similar changes were produced for this compound after packing. Table 2 shows the changes observed in the rest of the sterols.

Processing treatments produced diverse changes in fatty and triterpenic alcohols. For instance, 1-docosanol, had a marked decrease during processing regardless of cultivar and its level in the final product was always lower than in fresh fruits. On the contrary, the erythrodiol content increased with processing in both cultivars but always maintained higher levels in Manzanilla. The percentage of erythrodiol + uvaol showed a clear increase in Manzanilla during storage and oxidation and showed a slight decrease after sterilisation; in Hojiblanca, the level remained constant during the first three phases of elaboration but increased after sterilisation; however, Manzanilla kept higher contents in the final product. Table 3 shows the changes for other components of this group.

3.2. Comparison of ripe olive oil characteristics with the criteria established in the EU Directives for the classification of olive oil into categories

The characteristics of these oils (restricted to only those compounds determined in this work) were also compared with the parameters used to classify olive or pomace oils into categories. The comparison was carried out to identify the most influential steps on these parameters and to check the quality of the processed product oil as an approximation for the overall evaluation of the canned olives, bearing in mind that fat is the most abundant component in these fruits. The estimated percentages of cholesterol, brassicasterol, campesterol, stigmasterol, apparent β-sitosterol, Δ^7-stigmastenol, percentage of erythrodiol + uvaol, and total sterols for olives in the diverse processing steps and the final products vs. the limits established for them in the Directives (CEE) 2568/91 and 1989/2003 are shown in Table 4. The overall average and the contents in the final products of cholesterol, campesterol and stigmasterol were below such limits in both cultivars; however, the proportions of cholesterol was above its legal limit at the end of the storage period (Hojiblanca) and at the end of oxidation (Manzanilla) and the causes of these increases deserve future investigation. Initial contents of brassicasterol in Hojiblanca and Manzanilla were above the limits established for this parameter but storage and oxidation reduced its level to below limits, except in Manzanilla. The influence of these treatments should also be clarified. The level of apparent β-sitosterol (expressed by the sum of the content of β-sitosterol and another five chromatographically adjacent phystosterols) was within the limit in the final products of either cultivar but above them after storage (both cultivars) and oxidation (Manzanilla). The regulatory minimum limit established for apparent β-sitosterol is in fact an indication that the sum of the remaining sterols does not surpass 7%, thereby confirming the authenticity of the corresponding oil (Sánchez Casas et al., 2004). The percentage of erythrodiol + uvaol was always <4.5 (limit for virgin extra, virgin, refined olive oil, mixtures of virgin and refined and lampante, a classification which also depends on wax content) for Hojiblanca but was above this level for Manzanilla. The total concentration of sterols was always >1000 mg/kg oil regardless of cultivar and processing step, a circumstance that would permit its classification as virgin extra, virgin, refined olive oil, mixtures of virgin and refined olive oils and even lampante olive oil. In some cases, the samples could also be classified as crude or refined oil according to this parameter alone. However, even virgin olive oils can exceed some of the limits established by the EU. Rivera del Álamo et al. (2004) reported that the proportion of campesterol was higher than 4% in Cornicabra olive oils. As shown, processing steps affected classification variables in diverse directions which should be investigated in deeper detail. In addition, most of the components in the final product seam to be within the limits established by the EU for the classification of virgin oil and olive and pomace oils but results were not conclusive and, apparently, not representative of the actual sensations perceived by the consumer when the final product is eaten. Further research is still necessary to develop new methods for measuring such quality "on site". This research must take into account not only the fat but also its relationships with other compounds present in olives such as polyphenols, fibre, organic acids, minerals and proteins.

3.3. Chemometric study

Some of the studied variables were correlated among them. The highest correlation was found between the following pairs: β-sitosterol vs. Δ^5-avenasterol (0.79), cholesterol vs. Δ^5-campesterol (0.78) and 1-octacosanol vs. 1-hexacosanol (0.77) or 1-tetracosanol (0.74); other less correlated variables were 1-tetracosanol vs. 1-hexacosanol (0.65), Δ^5-avenasterol vs. erythrodiol (−0.63) and campesterol vs. clerosterol (−0.56). These correlations suggest that variables could be reduced to a lower number of factors which could represent most of the original variance. After subjecting data to PCA analysis, only four eigen values higher than 1.00 were obtained. Their values (and the percentage of total variance explained) were: 3.79 (27.08%), 2.94 (21.10%), 1.96 (14.03%) and 1.53 (10.74%), which accounted for a cumulative 73.06% of the

Table 4
Mean values of the percentages of cholesterol, brassicasterol, campesterol, stigmasterol, β-sitosterol, Δ^7-stigmastenol, erythrodiol and uvaol and total sterols (mg/kg) in ripe olives, according to olive cultivar and processing step. Comparison with the limits established by the Directives (CEE) 2568/91 and 1989/2003.

Component percentage	Hojiblanca				Manzanilla				
	Raw material	End storage	End oxidation	Sterilised olives	Raw material	End storage	End oxidation	Sterilised olives	CEE limits
Cholesterol	0.21 ± 0.21	0.77 ± 0.38	0.34 ± 0.13	0.20 ± 0.07	0.27 ± [a]	0.44 ± 0.04	0.55 ± 0.08	0.38 ± 0.07	⩽0.5
Brassicasterol	0.72 ± 0.43	0.32 ± 0.23	0.24 ± 0.24	0.00	0.33 ± [a]	0.07 ± 0.07	0.03 ± 0.03	0.26 ± 0.08	⩽0.1or 0.2[b]
Campesterol	2.80 ± 0.21	6.18 ± 3.44	3.07 ± 0.24	2.69 ± 0.08	2.87 ± [a]	3.53 ± 0.50	2.91 ± 0.22	3.24 ± 0.09	⩽4.0
Stigmasterol	1.14 ± 0.09	1.19 ± 0.38	0.90 ± 0.37	0.85 ± 0.05	1.46 ± [a]	2.51 ± 0.80	1.98 ± 0.44	1.91±0.10	<% Camp.
β-Sitosterol[g]	94.64 ± 0.39	90.85 ± 4.65	94.64 ± 1.04	95.02 ± 0.29	93.86 ± [a]	90.39 ± 2.67	91.89 ± 1.71	93.42 ± 0.18	⩾93.0
Δ^7-Stigmastenol	0.00	0.30 ± 0.30	0.00	4.57 ± 4.57	0.00	1.39 ± 1.18	1.14 ± 0.71	0.00	⩽0.5
Erythrodiol + uvaol[h]	2.66 ± 0.44	2.32 ± 0.53	0.59 ± 0.38	1.63 ± 0.33	4.86 ± [a]	6.61 ± 0.70	5.05 ± 0.48	7.35 ± 0.35	⩽4.5[c] >4.5[c]
Total sterols (mg/kg)	1432 ± 37	1625 ± 194	1738 ± 233	2543 ± 228	1062 ± [a]	1204 ± 95	1392 ± 81	1089 ± 20	⩾1000 ⩾2500[d] ⩾1800[fe] ⩾1600[f]

Values are means of duplicate analyses of replicates. [a]Data without replicates. [b]For crude pomace olive oil. [c]The classification depends also on the wax content. [d]For crude pomace olive oil. [e]For refined pomace olive oil. [f]For pomace olive oil. [g]This apparent β-sitosterol includes $\Delta^{5,23}$-stigmastadienol, clerosterol, β-sitosterol, sitostanol, Δ^5-avenasterol and $\Delta^{5,24}$-stigmasterol. [h]Obtained as (erythrodiol + uvaol) × 100/(total sterols + erythrodiol + uvaol).

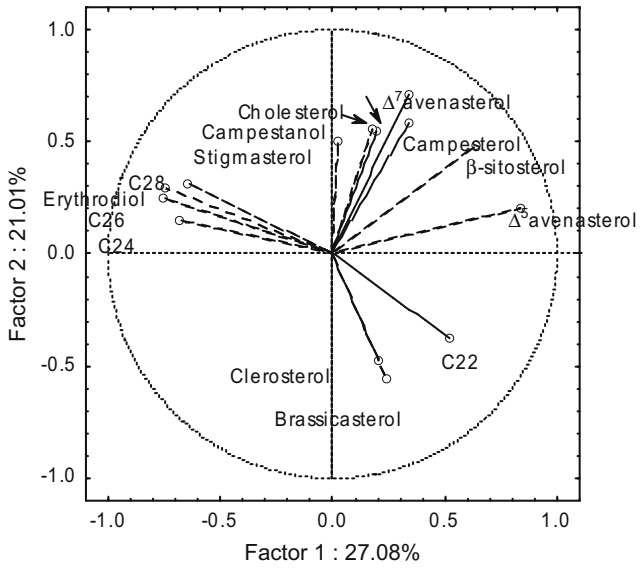

Fig. 1. Projection (loadings) of the variables subjected to chemometric analysis on the plane of the first two Principal Components.

variance. The loadings of each variable on these four principal components (PCs) can identify the main contributors to them. PC1 was mainly associated with Δ^5-avenasterol (0.84, factor variable correlation), 1-hexacosanol (−0.76), erythrodiol (−0.74), 1-octacosanol (−0.68) and 1-tetracosanol (−0.64); PC2 was related to Δ^7-avenasterol (0.71), campesterol (0.58) and brassicasterol (−0.56); PC3 was related to cholesterol (−0.59) and clerosterol (−0.54); PC4 was related to stigmasterol (−0.72) and campestanol (0.59). The projections of the loadings on the plane of the two first PCs are illustrated in Fig. 1. These projections allow for a visualisation of the position of the variables in the plane and the correlations among them and with the corresponding PCs. In this 2D graphical representation, the variables fall in a circle, called the correlation circle, with the pair of PCs as its axes. Correlations of variables and PCs can be deduced from their position with respect to the axes and the sign of the PC coordinates. Two distant variables (the angle between the corresponding variables is 90°) are not correlated because the correlation coefficient is the cosine of their angle (0, in this case) (Cichelli & Pertesana, 2004). Fig. 1 shows that clerosterol, brassicasterol and 1-docosanol (C22) are highly correlated as well as 1-tetracosanol (C24), 1-hexacosanol (C26), erythrodiol and 1-octacosanol (C28). There was, however, a high negative relationship between these two groups because they form an angle close to 180°. Stigmasterol, campestanol, cholesterol, Δ^7-avenasterol and, campesterol were strongly related but there was a low association among these and Δ^5-avenasterol. β-sitosterol was situated between these two last groups. The contribution of diverse variables to each PC (axes) can be easily deduced from Fig. 1. However, when the scores for the cases were plotted as a function of PC1 vs. PC2 no evident segregation among cases according to treatments and cultivars was observed (data not shown).

Then DA, a more powerful tool for discrimination, was used. The analysis of variance showed significant differences between cultivars and/or ps for unsaponifiable matter (ps), β-sitosterol, Δ^5-avenasterol, total sterols, 1-docosanol, 1-tetracosanol (ps), erythrodiol (ps), and percentage of erythrodiol + uvaol. The predictive DA was applied to the standardised data matrix to prevent the effect of scaling among variables. The multivariate analyses of the standardised data showed that β-sitosterol ($P = 0.01$), Δ^5-avenasterol ($P < 0.01$), 1-hexacosanol ($P = 0.024$) and erythrodiol ($P < 0.01$) showed significant differences among elaboration steps and cultivars whilst 1-docosanol ($P < 0.01$), 1-tetracosanol ($P < 0.01$) were significantly different between cultivars. Apparently data were thus appropriate for DA.

The selection of variables containing the most powerful information for the correct classification of olives was achieved by estimating the canonical discriminant functions (Table 5). The most discriminant variables among cultivars were: erythrodiol (*F* to remove, 40.64; tolerance, 0.444), β-sitosterol (34.87; 0.094), and campestanol (18.60, 0.190) as well as clerosterol, campesterol, Δ^7-avenasterol, and Δ^5-avenasterol with progressively lower *F* to remove values. The contribution of each one to discrimination can be deduced from the standardised coefficients for the variables vs. each factor (Table 6). The highest contribution was due to β-sistosterol (−2.79), followed by campestanol (1.745), and erythrodiol (1.311). Therefore, fatty alcohols did not contribute to discrimination between cultivars but mainly sterols and the triterpenic alcohol erythrodiol. The application of the classification functions led to a correct classification of all samples into the two cultivars. The validation procedure, cross classification, led to the same level of correct classifications (100%). These results are in agreement with data found in the literature; the content of sterols and the percentage of erythrodiol + uvaol in virgin olive oils have been successfully used for the classification of virgin olive oils from cultivars of Extremadura (Sánchez Casas et al., 2004). The sterol composition of monovarietal olive oils from the most important cultivars of north-eastern Portugal (Cobrançosa, Madural and Verdeal) and numerous samples of olive oils with Protected Denomination of

Table 5
Discriminant analysis. Variables retained in the canonical analysis, using the backward selection option (*P* to enter 0.05 and *P* to remove 0.10).

Component	Between cultivars		Among elaboration steps	
	F to remove	Tolerance	*F* to remove	Tolerance
Cholesterol			10.53	0.297
Brassicasterol				
Campesterol	14.55	0.366		
Campestanol	18.60	0.190	4.85	0.407
Stigmasterol	11.9	0.287		
Clerosterol	16.26	0.255	2.85	0.397
β-Sitosterol	34.87	0.094	20.11	0.031
Δ^5-Avenasterol	4.21	0.304	16.18	0.036
Δ^7-Avenasterol	7.48	0.234		
1-Docosanol			10.11	0.450
1-Tetracosanol			10.16	0.530
1-Hexacosanol				
1-Octacosanol				
Erythrodiol	40.64	0.444	3.12	0.334

Table 6
Discriminant analysis. Canonical discriminant functions, standardised by within variances.

Component	Between cultivars	Between elaboration steps		
	Function 1	Function 1	Function 2	Function 3
Cholesterol		1.435	0.536	0.544
Brassicasterol				
Campesterol	−1.185			
Campestanol	1.745	−0.713	−0.949	0.432
Stigmasterol	1.270			
Clerosterol	−1.459	0.925	−0.269	−0.417
β-Sitosterol	−2.785	5.179	0.004	−0.839
Δ^5-Avenasterol	0.867	−4.709	0.115	−0.753
Δ^7-Avenasterol	−1.218			
1-Docosanol		−1.270	−0.074	−0.156
1-Tetracosanol		−0.733	1.002	−0.204
1-Hexacosanol				
1-Octacosanol				
Erythrodiol	1.311	0.612	−0.141	−1.343

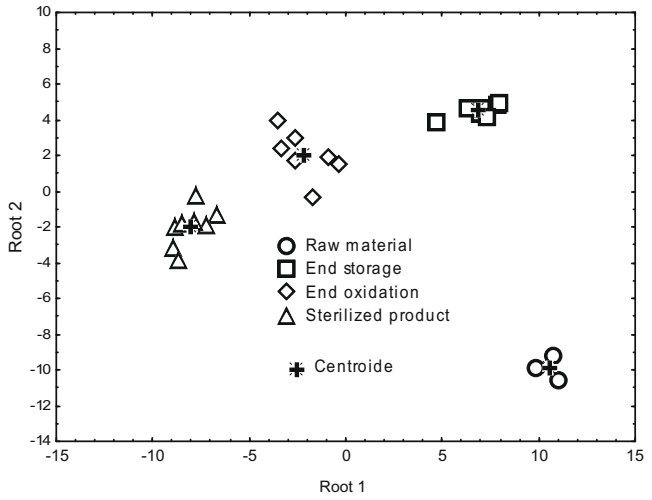

Fig. 2. Plot of the ripe olive sample scores as a function of the first two canonical discriminant functions, according to processing steps.

Origin from the same region was the base for their differentiation by chemometric methods (Rui Alves et al., 2005).

The most contributing variables for discrimination among processing steps (Table 5) were: β-sitosterol (*F* to remove, 20.11; tolerance, 0.031), Δ^5-avenasterol (16.18; 0.036), cholesterol (10.53; 0.297), 1-tetracosanol (10.16; 0.530), 1-docosanol (10.10; 0.450) and others with lower *F* values. The contribution of each variable depended on function. For Function 1, the most contributing variable was β-sitosterol (standardised coefficient, 5.179), Δ^5-avenasterol (−4.709), cholesterol (1.435) and 1-docosanol (−1.270) (Table 6). For Function 2, the most outstanding contributions were from: 1-tetracosanol (1.002), campestanol (−0.949), and cholesterol (0.536). Similarly, for Function 3 they were: erythrodiol (−1.343), β-sitosterol (−0.869), Δ^5-avenasterol (−0.753) and cholesterol (0.544). So cholesterol always had a marked effect whilst other variables contributed to specific functions: β-sitosterol to Function 1; 1-docosanol to Function 2 or erythrodiol to Function 3. Visualisation of the discrimination among elaboration steps on the plane of the first two functions led to a fairly good separation among the different groups (Fig. 2). Furthermore, the classification led to a hundred percent correct classification (Table 7). However, cross classification had a lower discriminant efficiency, mainly due to the inclusion of samples from sterilised products into those belonging to the end of oxidation and the end of storage, but its overall classification was still fairly good (68% correct). In any case, the classification must be taken with precaution due to the limitations derived from the application of the DA to a reduced number of samples. In addition, it must be emphasised that DA was mainly applied in this case to disclose possible differences introduced by processing and cultivars rather than with the aim of using the classification functions as a tool for the future assignation of new samples.

4. Conclusions

It was observed that storage increased the cholesterol content (and its proportion with respect to the other sterols) in processed Hojiblanca and Manzanilla oils, possibly due to the growth of microorganisms during this period. Modifications to the current system for preventing such changes should be studied. In Hojiblanca, β-sitosterol percentage markedly increased after sterilisation. Cultivar also had also a marked effect on the erytrodiol + uvaol levels, which was always higher in Manzanilla. At the end of the process (sterilised product), the unsaponifiable proportion (sterilised product) ranged from 1.14 to 1.36 g/100 g oil, the overall concentrations of phytosterols ranged from 1088 (Manzanilla) to 2543 (Hojiblanca) mg/kg oil, the most abundant sterol in the final product was β-sitosterol (average 947–2223 mg/kg oil), followed by Δ^5-avenasterol (average 39.2–68.1 mg/100 g oil) and the fatty alcohols were relatively scarce with total averages between 232 (Hojiblanca) and 289 (Manzanilla) mg/kg oil. Concentrations of erythrodiol were higher in Manzanilla whilst uvaol was not detected in all samples but the proportion of erythrodiol in the obtained oil significantly increased during some of the processing steps. These levels in the final product can be considered similar to those found in the literature for olive oil and for commercial table olives. In any case, after this work, it can be stated that ripe table olives are a very low cholesterol food and a good source of phytosterols.

Most of the parameters of the extracted oils were within the limits established by the EU Directives used for classification into the diverse categories of olive and pomace oils but the evaluation of the consubstantial quality of the fat in the intact table olive using this method was not conclusive. Chemometric methods were useful for disclosing relationships among variables and differences between cultivars and processing steps. PCA extracted four eigen values higher than 1 and showed the relationships among the different components on the plane of the first two components but did not properly separate the samples. However, predictive DA led to a hundred percent discrimination between cultivars and processing steps (68% for cross validation).

This work provides reference data related to the contents and changes in the unsaponifiable matter of ripe olive oil, particularly sterols and fatty and triterpenic alcohols (important biologically active compounds), during processing. It also represents a first approximation to the evaluation of the fat quality in the final product (actually eaten by consumers). In addition, the study was useful to disclose the effects of processing steps on the content and characteristics of these compounds and may help to minimise unfavourable transformations by re-designing the most influential phases.

Acknowledgements

This work was supported by the Spanish Government (AGL-2003–00779 and AGL-2006–03540/ALI, partially financed by European regional development funds, ERDF), and Junta de Andalucía (through financial support to group AGR-125).

Table 7
Classification matrix according to cultivars (Jackknified classification in parenthesis).

Actual group	Predicted group membership				
	Raw material	End storage	End oxidation	After sterilisation	% Correct
Raw material	3 (3)	0 (0)	0 (0)	0 (0)	100 (100)
End storage	0 (0)	7 (6)	0 (1)	0 (0)	100 (86)
End oxidation	0 (0)	0 (1)	7 (6)	0 (3)	100 (43)
After sterilisation	0 (0)	0 (1)	0 (2)	8 (5)	100 (63)
Total	3 (3)	7 (8)	7 (6)	8 (8)	100 (68)

Overall correct classification 100% (Jackknified classification, deduced from cross validation, 68%).

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Food Chemistry 117 (2009) 135–142

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Synthesis and antioxidant activity of selected 4-methylcoumarins

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ARTICLE INFO

Article history:
Received 21 January 2009
Received in revised form 18 February 2009
Accepted 24 March 2009

Keywords:
4-Methylcoumarins
Radical-scavenging activity
DPPH·
Reducing power
Trolox

ABSTRACT

A series of selected 4-methylcoumarins (4-methyl-2*H*-1-benzopyran-2-ones) were synthesised and tested for radical-scavenging ability using the stable 1,1-diphenyl-2-picrylhydrazyl radical, and for reducing power ability with a test based on the reduction of ferric to ferrous cation. All studied compounds showed activity comparable to Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), an already known antioxidant, which is used as standard in most of the testing methods. Observations from the study were made with regard to structural features that regulated the behaviour of the compounds. The antioxidant activity of some 4-methylcoumarin representatives is presented here for the first time and extends our knowledge of the range of valuable biological activities and possible roles in therapy and for food preservation associated with this group of compounds.

1. Introduction

Coumarins (2*H*-1-benzopyran-2-ones) occupy an important place in the realm of natural products and synthetic organic chemistry. They have been used as anticoagulants (Cravotto, Nano, Palmisano, & Tagliapietra, 2001), additives in food and cosmetics, and in the preparations of insecticides (O'Kennedy & Zhorenes, 1997).

As substitutions can occur at any of the six available sites of their basic molecular moiety, these compounds are extremely variable in structure and activity. This structural diversity leads to coumarins displaying multiple biological properties that promote human health and help reducing the risk of diseases. Coumarins comprise a vast array of biologically active compounds ubiquitous in plants, many of which have been used in traditional medicine for thousands of years. The medicinal properties of coumarins include inhibition of platelet aggregation, cytochrome P450, and steroid 5α-reductase, spasmolytic, anticoagulant, antibacterial, anticancer, and antiHIV activities (Hoult & Paya, 1996; Kostova, 2005, 2007). A number of coumarins were found to affect the formation and scavenging of reactive oxygen species (ROS), and reactive nitrogen species (RNS), exhibiting tissue-protective antioxidant properties (Bermejo, Pinero, & Villar, 2008; Surveswaran, Cai, Corke, & Sun, 2007; Wu, Huang, Lin, Ju, & Ching, 2007), which may include numerous different molecular mechanisms and are probably related to their structural analogy with flavonoids and benzophenones. Such studies are of importance in view of the presence in the human diet of many coumarins and other plant polyphenolics, some of which are attracting interest on account of their antioxidant properties and possible roles in therapy and for food preservation (Grace, 2005). Many coumarin structures can be found in dietary products, as flavouring additives or as natural components, such as wine and other alcoholic beverages, tobacco products, citrus fruits, vegetables, bakery products and cereals (Lake, 1999; Sproll, Ruge, Andlauer, Godelmann, & Lachenmeier, 2008).

Coumarins have been essentially found in green plants belonging to the family of Rutaceae and Umbelifferae. These compounds can be obtained from plants by different extraction methods. However, the extraction from plants is a time consuming job and needs sophisticated instruments to get the pure product. Therefore, the chemical synthesis of coumarin derivatives is done to fulfil their requirements in vast applications. Chemically, coumarins can be synthesised by several synthetic routes such as Pechmann, Perkin, Knoevenagel, Reformatsky and Wittig reactions (Sethna & Shah, 1945). However, due to simple and relatively inexpensive starting materials, the Pechmann reaction was widely used for the syntheses of coumarins.

The importance of free radicals, especially ROS in the pathogenicity of various diseases, including hepatic and vascular diseases has of late received greater attention. Antioxidants are now forged as the drug candidates to combat these diseases. Minor dietary constituents have been seriously considered to counter the ill effects of the oxygen radicals. In addition, some natural coumarins also affect the formation and scavenging of ROS and influence free radical-mediated oxidative damage (Grace, 2005). Very few systematic studies have been reported on structure-antioxidant activity correlations in coumarins. In a study of superoxide scavenging

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doi:10.1016/j.foodchem.2009.03.087

capacity, performed with sixteen synthetic or plant-derived coumarins with different substitution patterns, Paya found that only 7,8-dihydroxylated coumarins were active (Paya, Goodwin, De Las Heras, & Hoult, 1994). Similarly, it has been recently reported that DPPH· scavenging activity in furanocoumarins correlates with the number of phenolic hydroxyl groups present in their structures (Kogure et al., 2004; Piao et al., 2004). Tyiagi demonstrated that the amino group is an effective substitute for the hydroxyl group for antioxidant property of 4-methylcoumarins (Tyagi et al., 2005).

The purpose of this study was to compare the antioxidant activities of selected 4-methylcoumarins obtained by two different testing methods. Special attention was paid to the number and position of the functional groups attached to the benzenoid part of the coumarin molecule. We have examined 4-methylcoumarin possessing methyl-, methoxy- and hydroxy-, acetoxy-, and benzoxy-groups in the benzenoid ring. The majority of them are known as natural compounds. They have shown very good antioxidant and radical-scavenging properties, also comparable to Trolox.

2. Materials and methods

2.1. Chemicals

All applied chemicals and reagents were of the highest purity available and purchased from the Sigma–Aldrich Chemical Company.

2.2. Syntheses of 4-methylcoumarin and its derivatives

4-Methylcoumarin, and its derivatives, containing methyl-, methoxy-, and hydroxy-groups on the benzene ring of a molecule, (**2a**–**k**), were synthesised. The reaction route is given in Fig. 1. The syntheses of coumarins were carried out according to the slightly modified Pechmann method (Horning, 1955), which involves the condensation of phenols with β-keto esters in the presence of acidic condensing agents, such as concentrated sulphuric acid and anhydrous aluminium chloride. Hydroxy-derivatives of 4-methylcoumarin were acetylated and benzoylated in order to obtain the corresponding acetoxy- (**3f**–**k**) and benzoxy-derivatives (**4f**–**k**) of 4-methylcoumarin. Esterification reactions were carried out according to the slightly modified Fischer method (Fischer & Speier, 1895), involving the acetic anhydride and concentrated phosphoric acid, and benzoyl chloride in basic media.

Products were identified by determination of melting points (Kofler micro-hot stage apparatus), using elemental analysis (Perkin–Elmer 2400 CHN Analyzer), GC/MS (Hewlett–Packard GC 6890 series II and MSD 6890 series II) and NMR (Bruker DPX 300 NMR spectrometer) techniques.

2.3. Sample preparation

Stock solutions of synthesised 4-methylcoumarins were prepared in dimethyl-sulfoxide (DMSO) in concentration of 0.10 mol/l. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a positive probe for antioxidant testing, and it was prepared in same concentration as tested coumarins.

2.4. 1,1-Diphenyl-2-picrylhydrazyl radical-scavenging activity

The ability of the 4-methylcoumarin and its derivatives to a donate hydrogen atom or electron and scavenge a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined by the slightly modified method of Brand-Williams, (Brand-Williams, Cuvelier, & Berset, 1995). The concentrations of the tested samples ranged from 0.10 to 0.0001 mol/l. A portion of the sample solution (200 μl) was mixed with 3.0 ml of 5.25×10^{-5} mol/l DPPH· in absolute ethanol. Decreasing of absorbance of tested mixtures was monitored every 1 min for 30 min at 517 nm using a Perkin–Elmer Lambda 25 UV/Vis spectrophotometer. Absolute ethanol was used to zero the spectrophotometer, DPPH· solution was used as a blank sample, and Trolox was used as a positive probe. The DPPH· solution was freshly prepared daily, stored in a flask covered with aluminium foil, and kept in the dark at 4 °C between measurements. All experiments were carried out in triplicate.

The radical-scavenging activity of the tested samples, expressed as percentage inhibition of DPPH, was calculated according to the formula IC (%) = $[(A_0 - A_t)/A_0] \times 100$ (Yen & Duh, 1994), where A_t is the absorbance value of the tested sample and A_0 is the absorbance value of blank sample, in particular time. Percent inhibition after 30 min was plotted against concentration, and the equation for the line was used to obtain the IC_{50} value. A lower IC_{50} value indicates greater antioxidant activity.

2.5. Evaluation for reducing power

The reducing power test is based on reduction of ferric to ferrous by the potent antioxidant. In presence of cyanide ions, and adding a new amount of Fe^{3+}, blue colour of $Fe_4[Fe(CN)_6]_3$ develops.

The reducing power of samples was determined by a slightly modified method (Chung, Kurisawa, Kim, & Kobayashi, 2004; Duh & Yen, 1997), as described below. Sample of 1.0 ml of various dilutions (from 0.10 to 0.001 mol/l) was mixed with 2.50 ml of phosphate buffer (0.2 mol/l, pH 6.6) and 2.50 ml of 1% potassium-ferricyanide. The mixtures were incubated at 50 °C for 20 min. After incubation 2.50 ml of 10% trichloracetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer (0.5 ml) of solution is mixed with 2.5 ml of distilled water and 100 μl of 0.1% $FeCl_3$ and the absorbance was measured at 700 nm. The control sample contained 1.0 ml distilled water, 2.50 ml of phosphate buffer, 2.50 ml of 1% potassium-ferrocyanide and 2.50 ml of 10% trichloracetic acid. The blank sample contained 1.0 ml distilled water, 2.50 ml of phosphate buffer, 2.50 ml of 1% potassium-ferricyanide and 2.50 ml of 10% trichloracetic acid. Trolox was used as positive control.

The reducing power of samples was calculated by the following formula: RP (%) = $(A_B - A_A) \times 100$; where: RP – reducing power; A_B – absorption of controlling sample (100%); A_A – absorption of tested sample. Percent inhibition was plotted against concentration, and the equation for the line was used to obtain the RP_{50} value. The lower RP_{50} value indicates greater reducing power ability. All determinations were carried out in triplicate.

3. Results and discussion

3.1. Syntheses of 4-methylcoumarin and its derivatives

The coumarins (**2a**–**k**) were synthesised by the well-known Pechmann condensation in quantitative yield. The preparation of acetoxy- and benzoxy-derivatives of hydroxy 4-methylcoumarins is based on the methodology used by Fischer and Speier (1895). The synthetic routes are outlined in Fig. 1.

Structural confirmation was done using ^{1}H NMR and EI mass spectra. The ^{1}H NMR spectra showed characteristic peaks of 4-methyl position at δ 2.22–2.47, and peaks of protons from benzenoid part of molecule at δ 6.15–7.46, for all of the synthesised compounds. Series of functional groups attached at the aromatic part of coumarin molecules revealed peaks at δ 3.30 for methyl-group (**2b–c**), δ 3.84–3.99 for methoxy-group (**2d**–**e**), δ 9.13–9.81 for hydroxy-group (**2f**–**k**), δ 2.13–2.41 for acetoxy-group (**3f**–**k**), and at δ

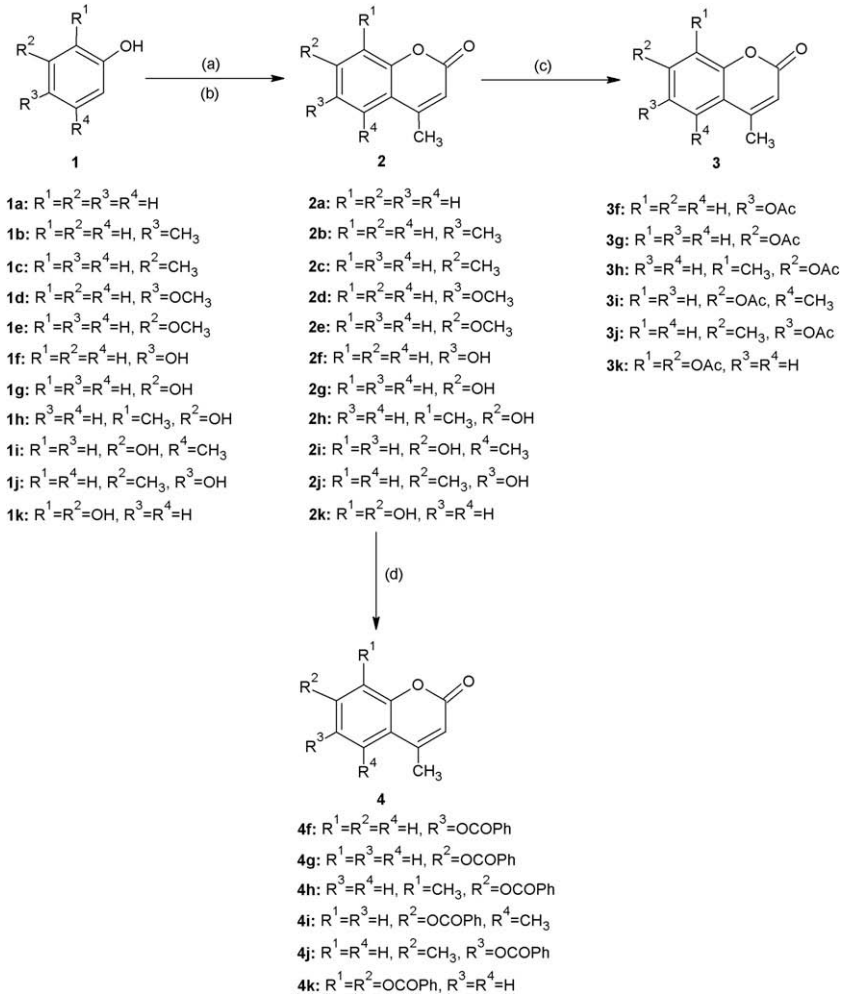


Fig. 1. Syntheses of selected 4-methylcoumarins. Reagents and conditions: (a) anhydrous aluminium chloride, nitrobenzene, ethylacetoacetate, 100–130 °C; (b) concentrated sulphuric acid, ethylacetoacetate, 0–10 °C; (c) acetic acid anhydride, concentrated phosphoric acid, 0–10 °C; (d) benzoyl chloride, 5% aqueous sodium hydroxide, 0–10 °C.

8.02–8.27 for benzoxy-group (**4f**–**k**). Finally, the structures of synthesised 4-methylcoumarins were supported by their EI mass spectra. All compounds showed characteristic molecular $[M]^+$peaks, *m*/*z* 160 (**2a**), *m*/*z* 174 (**2b**–**c**), *m*/*z* 190 (**2d**–**e**, **2h**,–**j**), *m*/*z* 176 (**2f**–**g**), *m*/*z* 192 (**2k**), *m*/*z* 218 (**3g**), *m*/*z* 232 (**3h**–**j**), *m*/*z* 276 (**3k**), *m*/*z* 280 (**4f**–**g**), *m*/*z* 294 (**4h**–**j**), and *m*/*z* 400 (**4k**). Moreover, all examined compounds revealed peaks characteristic for fragmentation of coumarin structure, such as $[M^+-CO]$, or $[M^+-CH_3]$.

3.2. 1,1-Diphenyl-2-picrylhydrazyl radical-scavenging activity

A number of methods are available for the determination of free radical-scavenging activity but the assay employing the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) has received most attention owing to its ease of use and its convenience. This assay is the most widely used *in vitro* test through which to assess free radical scavenger capacities. In the DPPH· assay, the antioxidant activity of a compound is evaluated spectrophotometrically by monitoring the decrease in absorbance at 517 nm as DPPH· (purple) is transformed to the reduced form DPPH-H (yellow).

Tested coumarins were able to reduce the stable violet DPPH· to the yellow DPPH-H, reaching 50% of reduction with IC_{50} values ranking from $(1.21 \pm 1.00) \times 10^{-5}$ mol/l, for 7,8-dihydroxy-4-methylcoumarin (**2k**), to $(9.72 \pm 2.10) \times 10^{-2}$ mol/l, for 4-methylcoumarin (**2a**), (Table 1). These values are comparable to Trolox, $(1.60 \pm 0.30) \times 10^{-4}$ mol/l, whose antioxidant properties are already well known (Aliaga, Rezende, & Arenas, 2009). Fig. 2 shows the reaction progress of some selected 4-methylcoumarins at the same conditions. From the mentioned figure it can be noticed that a substituent at the benzenoid moiety of coumarin molecule has an influence on the reaction kinetic progress, besides the influence on the antioxidant activity. This is probably due to the steric and resonance effects which will be discussed in the next sections.

3.3. Evaluation for reducing power

In order to compare results given above, we tested reducing ability of 4-methylcoumarins using a spectrophotometric method for determination of ferric ion content which were reduced by tested coumarins. Assayed compounds were able to reduce the ferric (Fe^{3+}) ions to corresponding ferrous (Fe^{2+}) ions, reaching 50% of reduction with RP_{50} values ranking from $(2.23 \pm 0.30) \times 10^{-4}$ mol/l, for 7,8-dihydroxy-4-methylcoumarin (**2k**), to $(1.42 \pm 0.04) \times 10^{-1}$ mol/l, for 4-methylcoumarin (**2a**), (Table 1). Gained results are comparable to Trolox, which RP_{50} value was $(3.06 \pm 0.60) \times 10^{-4}$ mol/l.

3.4. Comparison of two testing methods

In general, the antioxidative effectiveness of phenolic compounds depends on the reaction activity of the phenol towards

Table 1
Antioxidant activity of selected 4-methylcoumarins. IC_{50}, the concentration of coumarin required to inhibit radical formation by 50%; RP_{50}, the concentration of coumarin required to reduce ferric to ferrous ions by 50%.

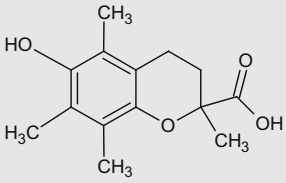
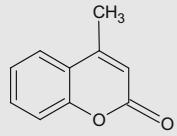
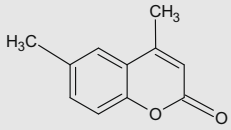
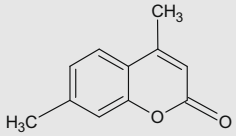
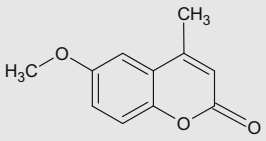
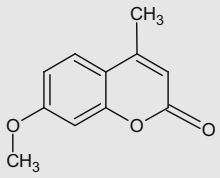
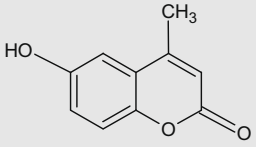
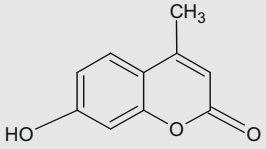
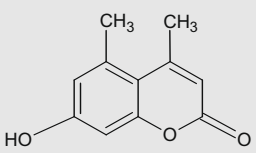
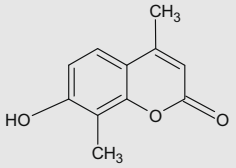
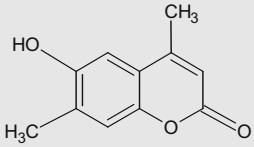
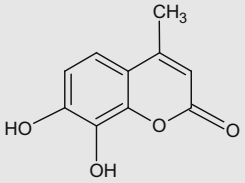
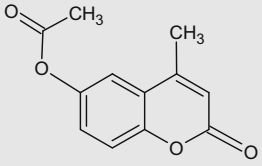
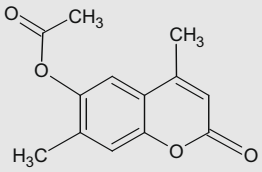
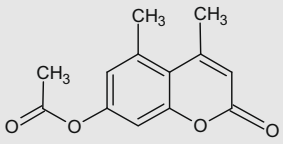
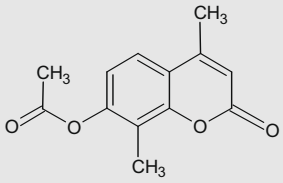
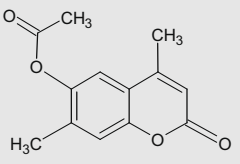
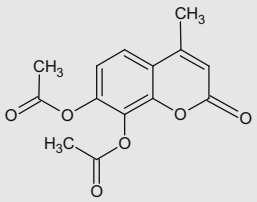
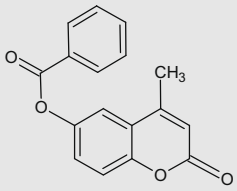
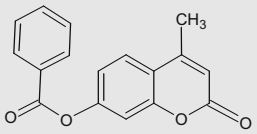
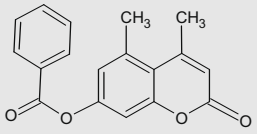
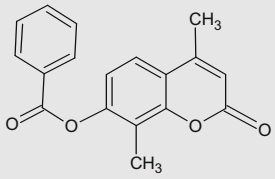
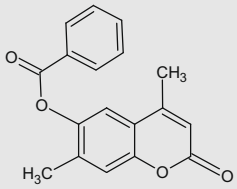
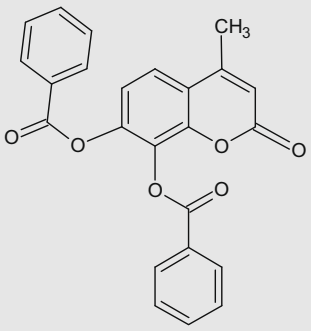
Entry	Chemical structure	Compound name	Antioxidant activity	
			$IC_{50} \times 10^3$ mol/l	$RP_{50} \times 10^3$ mol/l
Trolox		6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid	0.16 ± 0.03	0.31 ± 0.06
2a		4-Methyl-*2H*-benzopyran-2-one*	97.20 ± 0.21	142.00 ± 0.42
2b		4,6-Dimethyl-*2H*-benzopyran-2-one	77.30 ± 0.24	79.30 ± 0.36
2c		4,7-Dimethyl-*2H*-benzopyran-2-one	85.40 ± 0.33	88.20 ± 0.27
2d		6-Methoxy-4-methyl-*2H*-benzopyran-2-one*	13.80 ± 0.11	52.30 ± 0.17
2e		7-Methoxy-4-methyl-*2H*-benzopyran-2-one*	14.70 ± 0.15	55.30 ± 0.22
2f		6-Hydroxy-4-methyl-*2H*-benzopyran-2-one*	5.86 ± 0.19	7.23 ± 0.16
2g		7-Hydroxy-4-methyl-*2H*-benzopyran-2-one*	7.45 ± 0.16	9.45 ± 0.17
2h		4,5-Dimethyl-7-hydroxy-*2H*-benzopyran-2-one	7.17 ± 0.14	10.10 ± 0.18

Table 1 (*continued*)

Entry	Chemical structure	Compound name	Antioxidant activity	
			$IC_{50} \times 10^3$ mol/l	$RP_{50} \times 10^3$ mol/l
2i		4,8-Dimethyl-7-hydroxy-*2H*-benzopyran-2-one	6.99 ± 0.15	9.53 ± 0.16
2j		4,7-Dimethyl-6-hydroxy-*2H*-benzopyran-2-one	2.40 ± 0.09	4.17 ± 0.14
2k		7,8-Dihydroxy-4-methyl-*2H*-benzopyran-2-one*	0.01 ± 0.01	0.22 ± 0.03
3f		6-Acetoxy-4-methyl-*2H*-benzopyran-2-one*	2.17 ± 0.09	8.73 ± 0.123
3g		7-Acetoxy-4-methyl-*2H*-benzopyran-2-one*	3.77 ± 0.11	7.46 ± 0.16
3h		7-Acetoxy-4,5-dimethyl-*2H*-benzopyran-2-one	76.20 ± 0.43	84.30 ± 0.38
3i		7-Acetoxy-4,8-dimethyl-*2H*-benzopyran-2-one*	63.50 ± 0.13	75.10 ± 0.19
3j		6-Acetoxy-4,7-dimethyl-6-*2H*-benzopyran-2-one*	15.30 ± 0.54	39.70 ± 0.37

(*continued on next page*)

Table 1 (*continued*)

Entry	Chemical structure	Compound name	Antioxidant activity	
			$IC_{50} \times 10^3$ mol/l	$RP_{50} \times 10^3$ mol/l
3k		7,8-Diacetoxy-4-methyl-*2H*-benzopyran-2-one*	1.14 ± 0.10	2.29 ± 0.08
4f		6-Benzoxy-4-methyl-*2H*-benzopyran-2-one	24.90 ± 0.63	31.90 ± 0.54
4g		7-Benzoxy-4-methyl-*2H*-benzopyran-2-one	32.30 ± 0.27	66.60 ± 0.43
4h		7-Benzoxy-4,5-dimethyl-*2H*-benzopyran-2-one	48.50 ± 0.43	61.10 ± 0.35
4i		7-Benzoxy-4,8-dimethyl-*2H*-benzopyran-2-one	37.60 ± 0.37	48.50 ± 0.72
4j		6-Benzoxy-4,7-dimethyl-6-*2H*-benzopyran-2-one	26.80 ± 0.42	38.90 ± 0.32
4k		7,8-Dibenzoxy-4-methyl-*2H*-benzopyran-2-one	21.30 ± 0.43	25.10 ± 0.25

* Coumarins found in nature according to the available literature data.

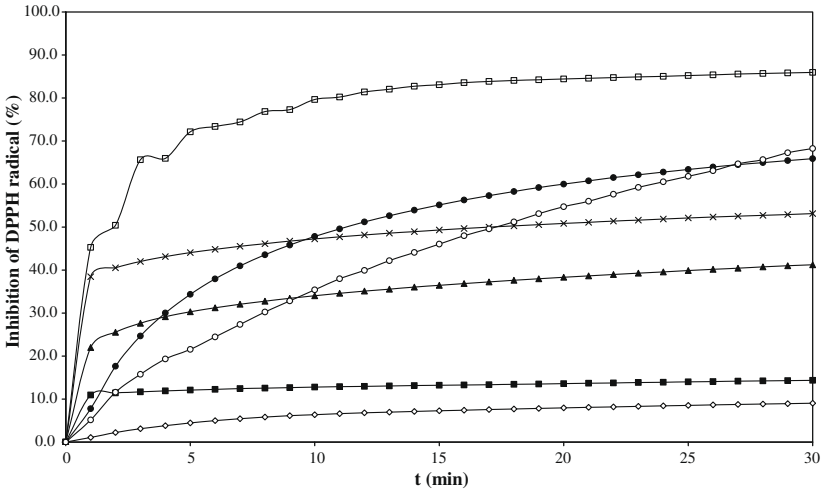


Fig. 2. Inhibition of DPPH· by some 4-methylcoumarins (0.001 mol/l). □, trolox; ◇, 4,6-dimethyl-*2H*-benzopyran-2-one (**2b**); ■, 6-methoxy-4-methyl-*2H*-benzopyran-2-one (**2d**); ○, 6-hydroxy-4-methyl-*2H*-benzopyran-2-one (**2f**); ●, 4,5-dimethyl-7-hydroxy-*2H*-benzopyran-2-one (2 h); ▲, 6-acetoxy-4,7-dimethyl-6-*2H*-benzopyran-2-one (**3j**); ×, 7-benzoxy-4,8-dimethyl-*2H*-benzopyran-2-one (**4i**).

the chain-carrying peroxyl radicals and on the stability of the phenoxyl radical formed in the reaction.

The reaction mechanism is based on an electron transfer (ET) reaction whilst the hydrogen atom abstraction is a marginal reaction pathway, because it occurs slowly in strong hydrogen-bond accepting solvents, such as methanol and ethanol. As occurs in other ET-based assays, the scavenging capacity against DPPH· is strongly influenced by the solvent and the pH of reaction (Magalhães, Segundo, Reis, & Lima, 2008). The steric accessibility of the DPPH· is a major determinant of the reaction, since small molecules that have better access to the radical site have relatively higher antioxidant capacity. On the other hand, many large antioxidant compounds that react quickly with radicals may react slowly or may even be inert in this assay. Then this might be the reason why introducing a benzoxy group into coumarin molecule displays relatively weak antioxidant properties against the DPPH·.

The examined compounds showed weaker activity in reducing Fe^{3+} ions compared to the scavenging radicals. This might be explained by the presence of aqueous media, where coumarins were less soluble and resonance structures of resulting phenoxyl radicals are less stable. However, coumarins still showed antioxidant activity, comparable with DPPH radical-scavenging method (Fig. 3).

As shown in Table 1 and Fig. 3, 7,8-dihydroxy-4-methylcoumarin posses the strongest antioxidant activity, both in radical-scavenging and reducing power tests. Foti and co-workers (1996) indicated that coumarins containing a catechol moiety were stronger than the others to scavenge the peroxyl radical, which was supported by the observations from other groups (Yu, Zai, & Liu, 1999). Since a large number of experimental and theoretical studies revealed that the catechol group was beneficial in enhancing the radical-scavenging activity of natural antioxidants, it is not surprising to see the catechol group playing a key role in enhancing the antioxidant activity of coumarins.

Our results show that the position and type of the substituent attached at the aromatic part of coumarin molecule have an influence on the radical-scavenging ability. In general, substitution at the six-position gives stronger feature to coumarin for scavenging

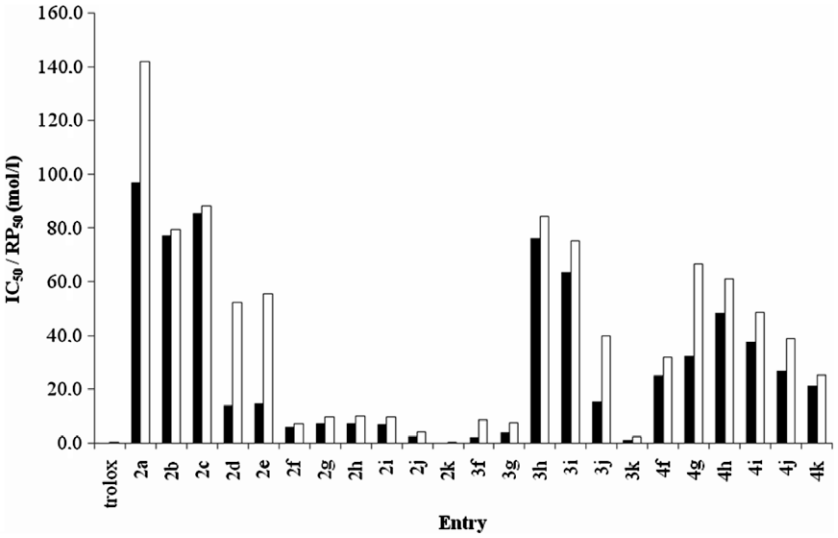


Fig. 3. Antioxidant activity of selected 4-methylcoumarins. ■, IC_{50}, the concentration of coumarin required to inhibit radical formation by 50%; □, RP_{50}, the concentration of coumarin required to reduce ferric to ferrous ions by 50%.

free radical than substitution at the seven-position. The results are consistent with a previous report that proved that resonance structures of the radicals derived from coumarins with substitution at the six-position are especially stable because of the *ortho*-quinone form (Lin et al., 2008). In addition, the type of substituent has an intense influence on the action of coumarins as antioxidants. Our results indicate that the hydroxy group has the strongest influence, while the methyl group has the smallest. A similar correlation was also reported for flavonoids (Heim, Tagliaferro, & Bobilya, 2002). Previously published data indicate that the pyrone ring of the coumarin moiety has little influence on the antioxidant activity of coumarins, due to its rather weak electronic effect (Zhang & Wang, 2004).

To the best of our knowledge, there is no published experimental data about structure elucidation of products for these kinds of reactions. Few authors gave proposed mechanisms of action (Foti et al., 2005) and theoretical elucidation of structure–activity relationships (Zhang & Wang, 2004) for coumarins to scavenge radicals. This issue will be subjected to our further research.

4. Conclusions

Antioxidants may be classified according to their mode of action as being free radical terminators, chelators of metal ions involved in catalysing lipid oxidation or oxygen scavengers that react with oxygen in closed systems. In this study we presented the antioxidant activity of selected 4-methylcoumarins using two comparable methods, 1,1-diphenyl-2-picrylhydrazyl radical-scavenging activity test and assay for evaluation of reducing power. The synthesised compounds scavenged the DPPH radical and reduced Fe^{3+}cations in a concentration and a time-depend manner.

These experiments were prompted, first, by the upsurge of interest in the contribution of reactive oxygen species and oxygen-derived free radicals to tissue damage and human diseases, and, second, by the increasing awareness that antioxidants that counteract oxidative stress may exert valuable protective actions. It was also noted that several of the plant-derived phenolic coumarins might play a role as dietary antioxidants because of their consumption in the human diet in fruits and vegetables.

Acknowledgment

We thank to contract grant sponsor, Ministry of Higher Education, Science and Technology, the Republic of Slovenia.

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Food Chemistry 117 (2009) 143–151

Contents lists available at ScienceDirect

Food Chemistry

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The purification and characterisation of polyphenol oxidase from green bean (*Phaseolus vulgaris* L.)

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ARTICLE INFO

Article history:
Received 15 December 2008
Received in revised form 17 March 2009
Accepted 24 March 2009

Keywords:
Green bean
Polyphenol oxidase
Enzymatic browning
Characterisation

ABSTRACT

Four isoforms of polyphenol oxidases (PPOs) were characterised in purified extracts of coats (PPOIa and PPOIb) and pods (PPOIIa and PPOIIb) of green bean (*Phaseolus vulgaris* L.). The molecular weights of four isoforms have been estimated to be from 57.5 to 39.0 kDa by SDS–PAGE. The PPOII (mixture of PPOIIa and PPOIIb) was used to characterise the PPO of green bean pods. All isoforms activities were stable between pH 6.8 and pH 7.2. PPOIa and PPOII have similar thermal inactivation profiles, and PPOIb has higher thermal stability than that of PPOIa and PPOII. PPOs showed the highest affinity to pyrogallol in all selected substrates. Although activities of PPOs were markedly inhibited by L-ascorbic acid, the activity of PPOI (mixture of PPOIa and PPOIb) was significantly activated by $MnSO_4$ and $CaCl_2$.

1. Introduction

Polyphenol oxidase (PPO: EC 1.10.3.1) is a copper-containing enzyme responsible for the hydroxylation of monophenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-diquinones. PPO is compartmentalised in plastids and their phenolic compounds are located in the vacuoles for most plant tissues. Action of PPO only occurs when this compartmentation is disrupted after tissues are wounded, as observed in diseased tissues or cell disruption caused by processing and storage (Mazzafera & Robinson, 2000). The production of the browning reaction may modify plant proteins and be more toxic to potential phytopathogens (Aydemir, 2004). The reactions also produce undesirable blackening in the products during food processing, or in post-harvest of plant products resulting in a reduction of their sensory properties and nutritional value. For this reason, the PPO have been characterised in several plants, such as butter lettuce (Gawlik-Dziki, Zlotek, & Swieca, 2008), broccoli (Gawlik-Dziki, Szymanowska, & Baraniak, 2007), coffee (Mazzafera & Robinson, 2000), potato (Marri, Frazzoli, Hochkoeppler, & Poggi, 2003), *Ferula* sp. (Erat, Sakiroglu, & Kufrevioglu, 2006), avocado (Gomez-Lopez, 2002), Chinese cabbage (Nagai & Suzuki, 2001), and Marula fruit (Mdluli, 2005).

Green beans (*Phaseolus vulgaris*, L.) are among the most important vegetables produced in China. They are generally harvested seasonally, and the outdoor varieties are excellent in nutrition, colour, texture, and flavour (Martins & Silva, 2004). Consumption of canned green beans has decreased in the last years, but that of fresh bean products has increased continuously (Cano, Monreal, Ancos, & Alique, 1998). Therefore, fresh green beans are more appreciated by consumers due to their sensorial and nutritional characteristics. The quality and shelf life of green beans varies depending on cultivars, maturity, processing, and storage conditions. It is a common phenomenon that browning decreases the storage period and commercial value of green beans. In green beans, the relation between green bean quality and PPO activity is not well known, although it has been described that PPO is one of several reasons causing green bean blackness during storage (Lian et al., 2006). In the literature, there are few studies reported on the purification and characterisation of PPO from green beans. This work covers investigations of purification of PPOs, effects of pH and temperature on PPO activity and stability, effects of inhibitors on PPO activity, and substrate specificity of PPO from green bean coats and pods. The research can lead to understanding the properties of the PPO that catalyses the browning reaction during storage. The results would provide information and effective methods for controlling browning and extending shelf-life.

2. Materials and methods

2.1. Samples and chemicals

Mature green beans of a major cultivar, *P. vulgaris* L., were obtained from a local farm (Heilongjiang Academy of Agricultural

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doi:10.1016/j.foodchem.2009.03.088

Science, Harbin, China). The green bean samples were transported to the laboratory within 2 h on the day of harvest and stored at 8 °C until extraction.

Catechol, L-dopa, 4-methylcatechol, pyrogallol, polyvinylploypyrolidone (PVPP), L-ascorbic acid, L-tyrosine, L-cysteine, sodium metabisulphite, Sephadex G-100, and DEAE-cellulose were obtained from Sigma Chemical Co (St. Louis, USA). Molecular Markers (M.W., 14.4–116.0 kD) were purchased from Fermentas Inc. (Glen Burnie, MD, USA). Acrylamide, bis-acrylamide, TEMED, ammonium persulfate and SDS were obtained from Amersham Pharmacia Biotech AB (Björkgatan, Uppsala, Sweden). Other reagents were all of analytical grade.

2.2. Enzyme extraction and partial purification from green bean

One hundred gram fresh green bean pods (or 20 g fresh green bean coats) were homogenised in 300 ml (or 50 ml) of 0.1 M phosphate buffer (pH 6.8, 10% PVPP) for 20 s at 4 °C (HR1724, Philips, Netherlands). The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000*g* for 20 min at 4 °C (Anke TGL-16G, Anting Scientific Instrument Factory, Shanghai, China). The supernatants were treated with $(NH_4)_2SO_4$ to obtain 60% saturation at 0 °C and then centrifuged at 15,000*g* for 20 min at 4 °C. The crude enzyme extracts were used for further purification.

For further purification of PPO from green bean coats, the precipitate, which was extracted and centrifuged from green bean coats, was dissolved in 0.01 M phosphate buffer (pH 6.8). The crude extract enzyme was extensively dialysed against the same buffer at 4 °C for 24 h with four changes of the buffer during dialysis. The dialysed enzyme solution was fractionated by a Sephadex G-100 column (1.6 cm × 50 cm). The column was equilibrated with 0.01 M phosphate buffer (pH 6.8). The 1.0 ml dialysed enzyme solution was passed through the column and was eluted by same phosphate buffer. The elution rate was adjusted to 30 ml/h. An elution of 5 ml was collected each time. The elution process was continued until obtaining zero absorbance at 280 nm. Protein was qualitatively detected by Protein Nucleic Acid Analyzer (HD-93–1, Kingdom Biochemical Instrument Co., Shanghai, China) at 280 nm. Each isolated PPO sample was then determined for their activity. Thus, values obtained were plotted against the sample numbers. The fractions having PPO activity were collected and purification degrees were determined by measuring specific activity before and after purification. Then the fractions were stored at 0 °C for subsequent analysis.

For the further purification of the PPO extracted from the green bean pods, the extracted and centrifuged precipitate was dissolved in 0.01 M Tris–HCl buffer (pH 7.1). The crude extract enzyme was extensively dialysed against the same buffer at 4 °C for 24 h with four changes of the buffer during dialysis. The dialysed enzyme solution was fractionated by a DEAE-cellulose column (1.6 cm × 10 cm). The column was equilibrated with 0.01 M Tris–HCl buffer (pH 7.1). The 2.0 ml dialysed enzyme solution was passed through the column and was eluted by 0.01 M Tris–HCl buffer (pH 7.1) NaCl at a flow rate of 30 ml/h. Enzyme activity was eluted with a linear gradient of 0–0.5 M NaCl in a total 600 ml of buffer. Fractions of 2.5 ml were collected and assayed for PPO activity and protein content. The fractions having PPO activity were collected and purification degrees were determined by measuring specific activity before and after purification. Then the fractions are stored at 0 °C for subsequent analysis.

2.3. PPO activity assay

PPO activity was determined according to the method of Yamamoto, Yoshitama, and Teramoto (2002) with some modification. The catechol was used as a substrate, and the quinone formation was measured on a spectrophotometer at 410 nm (Spectrum 754PC, Spectrum Instruments Co., Ltd, Shanghai, China). The sample contained 0.5 ml of 0.1 M catechol, 2.0 ml of 0.1 M phosphate buffer (pH 6.8) and 0.5 ml of the enzyme solution. The blank sample contained only 3.0 ml of substrate solution. The sample and blank were kept at ambient temperature for 30 min. PPO activity was assayed in triplicate measurements. One unit of enzyme activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 per min. The initial rate was calculated from the slope of the absorbance–time curve.

2.4. Estimation of molecular weight

The purified PPO was subjected to determine molecular weight based on SDS–polyacrylamide gel electrophoresis (SDS–PAGE) under denaturation conditions by a vertical electrophoresis unit (DYCZ-24D, Liuyi Instrument Factory, Beijing, China). The PPO preparation was followed by heating the PPO in boiling water for 3 min with 2% SDS and β-mercaptoethanol. The SDS–PAGE test employed 12% polyacrylamide gels and the buffer system. Electrophoresis was run at 10–25 mA for 2.0 h at ambient temperature. Gels were stained with Coomassie Brilliant Blue R-250. Molecular markers used were β-galactosidase (*M*r, 116.0 kDa), bovine serum albumin (*M*r, 66.2 kDa), ovalbovine (*M*r, 45.0 kDa), lactate dehydrogenase (*M*r, 35.0 kDa), restriction endonuclease Bsp981 (*M*r, 25.0 kDa), and β-lactoglobulin (*M*r, 18.4 kDa). The Gel-pro Analyzer 4.0 was applied to determine the molecular weight.

2.5. Effect of pH on PPOs activities and stability

The purified PPO (0.5 ml) was mixed with 2.5 ml of 0.1 M citrate buffer (pH 3.0–6.0) and 0.1 M phosphate buffer (pH 6.1–8.0) at the appropriate pH with 0.2 pH unit intervals. The PPO activity, as a function of pH, was determined at different pH values.

To determine pH stability, 0.5 ml purified PPO fractions which were originally isolated from green bean pods and green bean coats were mixed with 2.5 ml of 0.1 M citrate buffer (pH 3.0–6.0) and 0.1 M phosphate buffer (pH 6.1–8.0) at the appropriate pH with 0.2 pH unit intervals. After mixing of the sample and buffer, the pH was re-checked. The mixtures were kept at ambient temperature for 24 h, and then the mixtures were adjusted to pH 7.0. The residual PPO activity was determined.

2.6. Effect of temperature on PPOs activities and stability

A quantity of 2.5 ml substrate solution (0.1 M catechol in 0.1 M, pH 6.8 phosphate buffer) was pre-heated from 0 to 80 °C (at 10 °C intervals) in a water bath for 5 min. Then 0.5 ml PPO fraction was added into the substrate. PPO activity was determined as a function of temperature. PPO activity was calculated in the form of percent residual PPO activity at the optimum temperature.

To determine the thermal stability of PPO (0–95 °C), the test was divided into two temperature ranges. In first temperature range, each 0.5 ml PPO fraction (PPOIa, PPOIb and PPOII) was added into the 2.5 ml substrate solution (0.1 M catechol in 0.1 M, pH 6.8 phosphate buffer). After the mixtures were incubated 30 min at 0–80 °C (at 10 °C intervals), the samples were rapidly cooled in an ice bath for 5 min. Then residual PPO activity was determined at ambient temperature. In second temperature range, 0.5 ml PPOI (mixture of PPOIa, PPOIb) or PPOII (mixture of PPOIIa and PPOIIb) was treated same as above, but the differences were that samples were incubated 5 min at 85–95 °C (at 5 °C intervals). The residual activity was calculated as a percentage of unheated enzymes.

The kinetic data analysis of thermal inactivation can be described by the first-order reaction (Rapeanu, Loey, Smout, & Hendrickx, 2006):

$$\frac{dA}{dt} = -kA \quad (1)$$

where A is the enzyme at treatment time t and k is the reaction rate constant at the temperature studied. For constant extrinsic/intrinsic factors, in the case of a first-order reaction, the kinetics can be described by the following equations:

$$\log A = \log A_0 - \frac{t}{D} \quad (2)$$

or

$$\ln A = \ln A_0 - kt \quad (3)$$

where A_0 is the initial activity, and A is the activity at time t, and D is the decimal reduction time. At each temperature, the decimal reduction time D and the rate constant k were estimated using linear regression analysis on, respectively, Eqs. (2) and (3). The temperature-dependence of the D-value is characterised by the z_T value, which is the temperature increase necessary to induce a 10-fold reduction in D,

$$\log D = \log D_0 - \frac{T - T_0}{z_T} \quad (4)$$

The z_T value was estimated using linear regression analysis of Eq. (4). The temperature-dependence of the inactivation rate constants can be estimated using the Arrhenius model (5).

$$\ln(k) = \ln(k_0) + \left[\frac{E_a}{R} \cdot \left(\frac{1}{T_0} - \frac{1}{T}\right)\right] \quad (5)$$

where T and T_0 are the absolute and the reference temperatures (K), k_0 is the rate constant at T_0, E_a is the activation energy (kJ/mol) and R is the universal gas constant (8.314 J/mol/K). The activation energy was estimated using linear regression analysis of Eq. (5).

2.7. Substrate specificity

PPO activity was measured with six substrates at various concentrations, depending on their solubility; 0.1 M for catechol, 4-methylcatechol, L-dopa, pyrogallol and tannin, and 2.5 mM for L-tyrosine. Substrates were dissolved in 100 mM (pH 7.0) phosphate buffer. At each substrate concentration, the PPO activity was determined at each optimum wavelength. The Michaelis–Menten constant (K_m) and maximum reaction velocity (V_m) were determined using four substrates (catechol, 4-methylcatechol, pyrogallol and L-dopa) at five different concentrations and under standard conditions. Data were plotted as $1/V$ and $1/[S]$ concentration according to the method of Lineweaver and Burk.

2.8. Effect of inhibitors on PPO activity

The inhibitors, L-ascorbic acid, citric acid, L-cysteine, sodium metabisulphite, NaCl, $CaCl_2$, $MnSO_4$ and thiourea, were employed at three different concentrations. When 0.5 ml of catechol (0.1 M), 0.5 ml PPO fraction and 0.3 ml stated inhibitor were added into 1.7 ml phosphate buffer (0.1 M, pH 6.9), the PPO activity was measured in the standard reaction medium in the presence or absence of the stated concentration of inhibitor.

2.9. Statistical analysis

All analytical values represent the means of triplicate measurements. One-way analysis of variance (ANOVA) was used to compare the means. Differences were considered significant at $P < 0.05$. All statistical analyses were performed with SAS 8.0 (SAS Institute, Cary, NC).

3. Results and discussions

3.1. Purification of PPOs

The crude enzyme extracts from green bean coats and pods were precipitated by ammonium sulphate. The precipitates were dialysed by the buffer. The dialysed enzymes were purified 3.5-fold for coat and 3.7-fold for pod (Table 1) comparing with crude enzyme extracts. The dialysed enzyme extract from green bean coat was applied to a Sephadex G-100 column and eluted with phosphate buffer. The dialysed enzyme was eluted and four peaks observed that indicated four forms of PPO were isolated in fresh green bean coats (Fig. 1a). However, only the first two fractions had PPO activities (designated PPOIa and PPOIb). Compared to the crude enzyme extract of green bean coats, the purification degrees of PPOIa and PPOIb were 17.0-fold and 9.4-fold respectively (Table 1).

The dialysed enzyme from green bean pods was applied to a DEAE-cellulose column and eluted with a linear gradient of NaCl (0–0.5 M). The results showed that four forms of PPO were isolated from the dialysed enzyme (Fig. 1b), however, only the last two peaks indicated that the fractions had PPO activities (designed PPOIIa and PPOIIb). Comparing with a crude enzyme extract of green bean pods, the purification degrees of PPOIIa and PPOIIb were 6.6-fold and 3.8-fold, respectively (Table 1). The activities of PPOIIa and PPOIIb were significantly lower than the activity of PPOIa and PPOIb indicated that the green bean coats are easier to brown than the pods, because PPO activity and phenolic compounds mainly contribute to the enzymatic browning of fruits and vegetables. Gao and Li (2008) reported that PPO activity in the peel of sweet potato was higher than that in the tissue of sweet

Table 1
Purification of polyphenol oxidase from green bean.

Purification steps	Volume (ml)	Activity (u/ml)	Total activity (u)	Protein (μg/ml)	Specific activity (u/mg protein)	Purification (fold)	Yield (%)
Green bean coats							
Crude extract	50	184.7 ± 3.0	9235.0 ± 150.5	730.0 ± 24.5	253.0 ± 6.1	1	100
$(NH4)_2SO_4$	16	190.9 ± 3.9	3054.4 ± 62.2	217.0 ± 9.8	879.4 ± 23.6	3.5	33.1 ± 1.2
Sephadex G100							
PPOIa	6	228.0 ± 3.8	1368.0 ± 22.6	53.0 ± 2.2	4301.9 ± 78.9	17.0	14.8 ± 0.6
PPOIb	14	60.3 ± 1.0	844.2 ± 14.6	29.0 ± 1.6	2079.3 ± 50.7	9.4	9.1 ± 0.4
Green bean pods							
Crude extract	300	13.4 ± 0.6	4020.0 ± 174.0	3020.0 ± 84.7	4.5 ± 0.3	1	100
$(NH4)_2SO_4$	25	70.6 ± 1.8	1765.0 ± 45.7	4215 ± 98.4	16.8 ± 0.8	3.7	43.9 ± 1.8
DEAE-cellulose							
PPOIIa	9	20.0 ± 0.9	180.0 ± 7.7	673.4 ± 20.3	29.7 ± 1.3	6.6	4.5 ± 0.2
PPOIIb	6	18.5 ± 0.7	111.0 ± 4.4	1070.0 ± 33.5	17.3 ± 0.8	3.8	2.8 ± 0.1

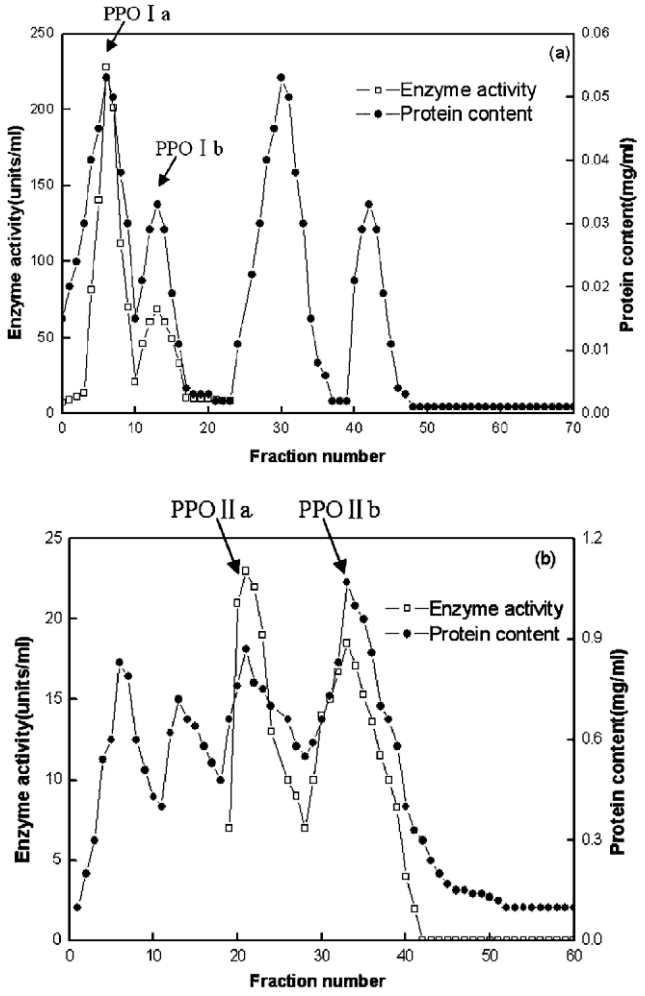

Fig. 1. Purification of PPO from green bean coats (a) by Sephadex G-100 and green bean pods and (b) by DEAE-cellulose.

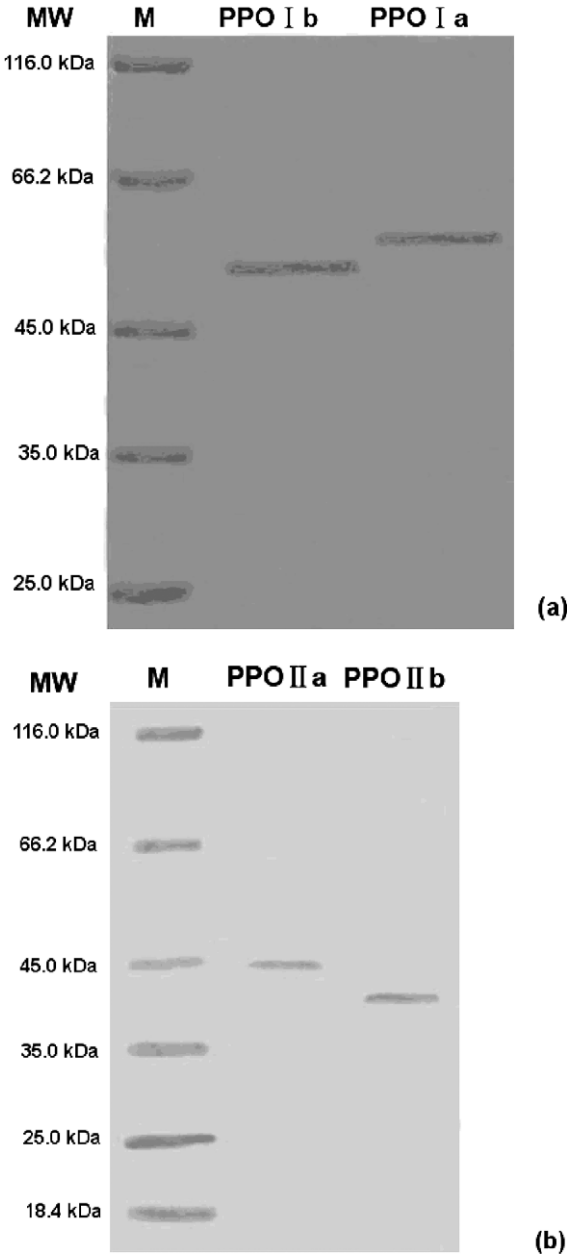

Fig. 2. SDS–PAGE of PPOI from green bean coats (a) and PPOII from green bean pods (b): (M) marker protein; (MW) molecular weight.

potato, and the peel was easier browning than that of tissue. Dong, Wang, Gong, Zhang, and Wang (2007) detected the PPO activities and browning degree of pericarp, sarcocarp and core in three varieties pears. The results demonstrated that the PPO activity in the core was significantly higher than that of pericarp and sarcocarp of pears, and then the browning degree of the core was significantly higher than that of the pericarp and sarcocarp of pears. For green bean, it was demonstrated that green bean coat was easier browning than that of pod during storage (Guo & Ma, 2008).

Both green bean coats and pods with two active PPO fractions were identified. Partington and Bolwell (1996) reported two types of PPO in potato tuber. Three types of PPO have been identified in peach (Flurkey & Jen, 1980) and artichoke (Aydemir, 2004). Park and Luh (1985) observed four forms of PPO as isoenzymes in kiwi fruit, and Ho (1999) found similar results in aerial roots. It is possible that the numbers of PPO isoenzymes are different among varieties (Marri et al., 2003; Yamamoto et al., 2002).

3.2. Estimation of molecular weight

The molecular weight of the purified fractions PPOIa, PPOIb, PPOIIa and PPOIIb were determined to be 57.5, 54, 46.0 and 39.0 kDa, respectively, by SDS–PAGE (Fig. 2a and b). The molecular weights of two PPO isoforms in green bean pods were smaller than that in the green bean coat. The results suggest that the activity of PPO may depend on its molecular weight. The high molecular weight fractions of PPO showed a higher activity. To our knowledge, there has never been a detailed comparative investigation of isoenzymes and molecular weights of PPO from the green bean coats and pods in the same plant. But similar results can be found in many various plants. Mazzafera and Robinson (2000) found that the molecular weights of PPO in coffee leaves and fruit endosperm were 46 and 50 kDa, respectively. The molecular weights of potato PPO were found to be 60 and 69 kDa (Partington & Bolwell, 1996). Four PPO fractions were purified from the aerial roots of an orchid, and their molecular weights were estimated to be 68, 48, 68, and 48 kDa, respectively (Ho, 1999). The molecular weight of PPO from other plants has been reported as follows: William pears, 43 kDa (Gauillard & Richard, 1997); butter lettuce, 60 kDa (Gawlik-Dziki et al., 2008); broccoli, 51.3–57 kDa (Gawlik-Dziki et al., 2007); and marula fruit, 71 kDa (Mdluli, 2005). Others reports of purified PPO are 30–70 kDa (Fujita et al., 1995; Marri et al., 2003; Nagai & Suzuki, 2001).

3.3. Effect of pH on PPO activity and stability

3.3.1. Effect of pH on PPO activity

The pH is one of the key factors that greatly influence the enzyme activity. The pH profiles of PPO activity in green beans were determined by using catechol as a substrate at a wide range of pH (3.0–8.0) with 0.2 pH unit intervals. For green bean coats, the optima pH of PPOIa and PPOIb were 6.8 and 7.0, respectively (Fig. 3a). The natural pH of green bean coat is 6.8. It means that green bean coat easy brown at its natural pH, because PPOIa and PPOIb have their higher activities between pH 6.8 and pH 7.0. The relative enzymatic activities were significantly ($p < 0.05$) decreased below pH 6.6 (72.7%) and above pH 7.0 (85.2%) for PPOIa, and below pH 6.8 (90.6%) and above pH 7.2 (76.1%) for PPOIb. The results indicated that PPOIa and PPOIb have narrow optimum pH ranges. These results are similar to the optimum pH range between 6.8 and 7.3 for PPO in kiwi (Park & Luh, 1985) and in marula fruit (pH 7.0) (Mdluli, 2005).

The PPO's concentration in green bean pods was very much lower, and thus it was hard to get the factions of PPOIIa and PPOIIb. Therefore, the eluted mixture of PPOIIa and PPOIIb (designed PPOII) was used to determine its pH profile. Two maximal activities of PPOII occurred at pH 4.0 and 7.0 (Fig. 3a). The results may be explained as an effect of a mixture of isoenzymes because PPO II was a mixture of PPOIIa and PPOIIb. Similar results have been found by other researchers, e.g. about pH 4.4, 7.5 and about pH 4.7, 7.5 for two varieties of avocado crude PPO extract (Gomez-Lopez, 2002), and pH 5.5 and 7.0 for crude enzyme extracts of Anamur banana (Unal, 2007). Gomez-Lopez (2002) thought that those two optima pH were contributed by isoenzymes. For PPOII, the relative enzymatic activities were significantly reduced ($p < 0.05$) in the range of pH 4.5–6.8 for both activity peaks. The relative enzymatic activities were also reduced above pH 7.2 (66.9%) for the secondary activity peaks. Therefore, PPOs of green bean coats and pods have narrow optimum pH ranges. Although different plants have different optima pH, the narrow optimum pH (6.8–7.0) range can be found in several plants (Erat et al., 2006; Ho, 1999; Jiang, Zauberman, & Fuchs, 1997; Mazzafera & Robinson, 2000).

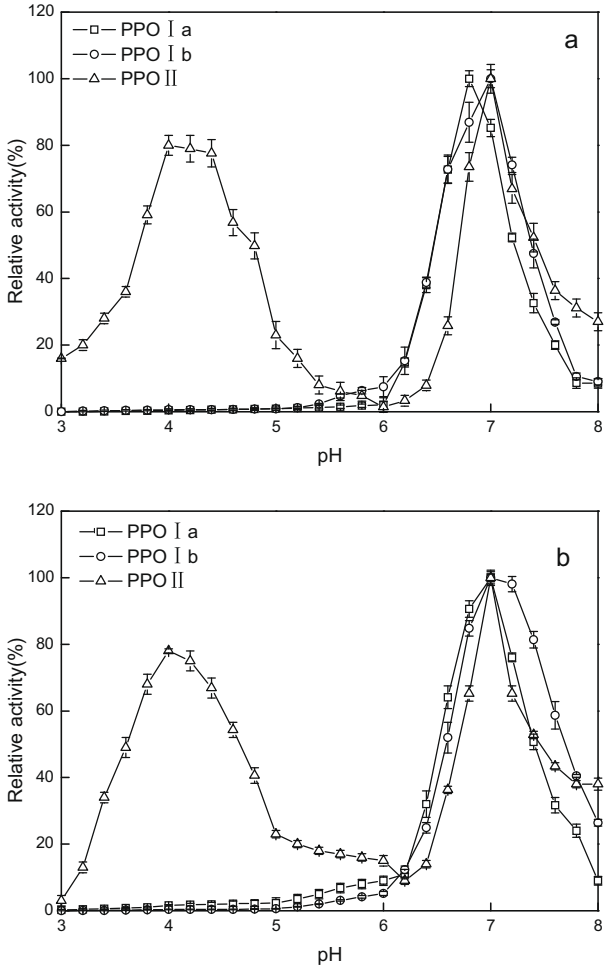

Fig. 3. pH-activity profiles (a) and pH-stability profiles (b) for green bean coat PPOIa and PPOIb and green bean pod PPOII.

3.3.2. Effect of pH on PPO stability

The stabilities of PPOs were determined by keeping preparations of PPOIa, PPOIb and PPOII in various pH states at ambient temperature for 24 h. As shown in Fig. 3b, PPOIa and PPOIb retained more than 95% of their original activities between pH 6.8 and pH 7.0 and in the range of pH 7.0–pH 7.2, respectively, but both lost 90% of their original activities when adjusted to a pH below 6.2 or above 8.0. Like their pH-activity profile, PPOIa and PPOIb have a narrow stable pH range. The results indicate that the change of pH would greatly influence the stability of PPO in green beans. Several researchers found that PPOs were stable near neutral pH and were unstable in acidic media, such as for longan fruit and yali pear (pH 7.0) (Jiang, 1999; Zhow & Feng, 1991), artichoke (pH 6.0–7.0) (Aydemir, 2004), and marula fruit (pH 6.0) (Mdluli, 2005).

For green bean pod, isolated PPOII retained more than 95% of its original activity at pH 4.0–4.2 and pH 7.0. However, there was an 80% loss in its original activity between pH 5.4 and pH 6.4, and below pH 3.2 (Fig. 3b).

The natural pH of green bean coats and pods are 6.8 and 5.8, respectively. In green bean coats, PPOIa and PPOIb have their maximum activities and the most pH stability at pH 6.8–7.0, so PPOIa and PPOIb keep their highly enzymatic activity in natural state. In green bean pods, the activity and pH stability of PPOII were 4.8% and 16% of its original activity, respectively, at pH 5.8. These results can be used to explain the reason that green bean coats are more susceptible to browning than that of the pods.

3.4. Effect of temperature on PPO activity and stability

3.4.1. Effect of temperature on PPO activity

The effects of temperatures between 0 and 80 °C on the PPOs' activities showed that optima temperatures for PPOIa, PPOIb and PPOII were 20, 30, and 50 °C, respectively (Fig. 4a). The high temperature may cause inactivation of PPO in green beans. For plants' PPO activity, optima temperatures between 20 and 35 °C were reported by other authors (Dincer, Colak, Aydin, Kadioglu, & Guner, 2002; Gawlik-Dziki et al., 2008; Jiang, 1999; Rapeanu et al., 2006; Unal, 2007). Higher optima temperatures for the PPO activities of strawberry have been reported (Serradell et al., 2000) and for litchi (Jiang et al., 1997) were 50 and 70 °C, respectively. The optima PPO for two varieties of hybrid sunflowers were 55 and 60 °C (Singh, Singh, Kaur, & Singh, 1999). The activity loss of PPOIa and PPOIb were 72.4% and 53.3% at 40 °C, respectively. However, the PPOIa and PPOIb were completely inactivated at 70 °C. The results suggested that PPOIa and PPOIb are sensitive to heat, and that their activities decreased with elevating temperature. Whereas the activity loss of PPOII was 66.7% at 70 °C, PPOII still retained 32.9% of its original activity even at 80 °C. PPOII retained more than 50% of its original activity between 20 and 65 °C. Therefore, PPOII tolerated changes in temperature as compared with PPO isolated from green bean coats.

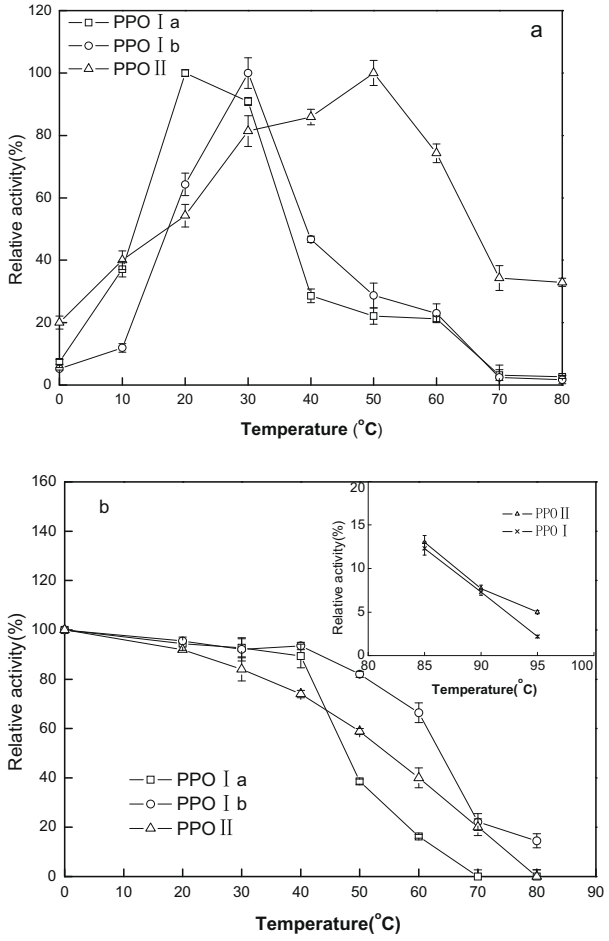

Fig. 4. Thermal activity profiles (a) and thermal stability profiles (b) for green bean coat PPOIa and PPOIb and green bean pod PPOII.

3.4.2. Effect of temperature on PPO stability

Thermal stability profiles for PPOs, presented in the form of the residual percentage activity, were investigated in two temperature ranges (0– 80 °C for 30 min and 85–95 °C for 5 min). In 0–80 °C, PPOs were stable between 0 and 40 °C (Fig. 4b). The enzyme activities were rapidly lost at higher temperatures. For instance, the temperature was increased from 40 °C to 80 °C, the residual relative activities of PPOIa and PPOII decreased from 89.4% to 0% and 93.5% to 0%, respectively. However, the residual relative activity of PPOIb decreased from 93.5% to 14.5% and from 40 °C to 80 °C. In 85–95 °C, thermal stability profile of PPOI (mixture of PPOIa and PPOIb) and PPOII was shown in Fig. 4b (small figure). At 95 °C (5 min), the residual relative activities of PPOI and PPOII were original 5.0% and 2.2%, respectively. But PPOI and PPOII were completely inactivated at 95 °C and 6 min. In consideration of industrial processing of green bean, at storage temperature (0 °C and 8 °C) of fresh green bean, PPOs have the highest stability, but the activities of PPOs are very low (Fig. 4a). When green bean was pre-treated by hot air of 50 °C, the relative activities of PPOs were decreased (PPOIa, 38.5%; PPOIb, 82.1%; PPOII, 44.3%). However, more than 95% PPOs were inactivated when green bean was blanched at 95 °C and 5 min.

Based on the results of thermal stability, kinetics of PPOs was studied in 50– 80 °C (Table 2). The higher inactivation temperature the inactivation proceeds faster. In the temperature range of 50–80 °C, the *D*-value of PPOs linearly decreased with increasing temperature. The *D* values of PPOIb are significantly higher than that of PPOIa and PPOII, and the range of temperatures required for the inactivation of PPOIb also was higher than those required for PPOIa and PPOII. Therefore, these results indicate that PPOIb is more thermostable than PPOIa andPPOII.

Considerable PPOs' inactivation at higher temperature is probably the result of changes in the enzyme tertiary structure (Dincer et al., 2002; Duangmal & Owusu Apenten, 1999). It appears that isoenzymes PPOIa and PPOIb have different thermal properties and stabilities. These results are consistent with some thermal stability profiles that have been found in tobacco isoenzyme PPOs (Dai, Shi, Tang, & Liu, 2001) and *Lentinus edodes* isoenzyme laccases (Wang et al., 2007). In addition, different molecular forms from the same source may have different thermostabilities (Zhow & Feng, 1991). The thermostability of PPOs varies considerably from plant to plant. However, several researchers have indicated that plant PPOs were stable between 20 and 40 °C (Arslan, Temur, & Tozlu, 1997; Aydemir, 2004; Duangmal & Owusu Apenten, 1999; Fujita et al., 1995; Gawlik-Dziki et al., 2008; Jiang, 1999). The higher thermal stabilities were found in the PPO from bananas at 60 °C for 30 min (Yang, Fujita, Ashrafuzzaman, Nakamura, & Hayashi, 2000), and leaves and fruit endosperm of coffee at 50 °C for 30 min (Mazzafera & Robinson, 2000).

3.5. Substrate specificity and enzyme kinetics

The substrate specificities of PPOs (PPOIa, PPOIb and PPOII) were examined using monophenols, diphenols, and triphenol as substrates. The Michaelis–Menten constant (K_m) and maximum reaction velocity (V_m) were determined using four substrates (catechol, 4-methylcatechol, pyrogallol, and L-dopa) at five different concentrations and under optimum analysis conditions. Data were plotted as $1/V$ and $1/[S]$ concentration according to the method of Lineweaver and Burk (Table 3). Catalytic efficiency and substrate affinity were the highest for pyrogallol, followed by catechol, 4-methylcatechol, and L-dopa. With pyrogallol, the V_m values of PPOIa and PPOIb were similar, but the K_m of PPOIa was nearly twice that of PPOIb. This may be indicative of their differences in affinity with pyrogallol. PPOIa and PPOIb were very similar in relation to their K_m and V_m with catechol, 4-methylcatechol and L-dopa. The V_m and K_m of PPOIa and PPOIb were higher than those for PPOII with these same substrates. When the activities of PPOs

Table 2
Kinetic data of thermal inactivation of green bean PPOs.

T (°C)	PPOIa		PPOIb		PPOII	
	D-values (min)	k (min^{-1})	*D*-values (min)	k (min^{-1})	*D*-values (min)	k (min^{-1})
50	72.37 ± 3.20	0.006 ± 0.001	348.08 ± 5.72	0.0016 ± 0.001	150.14 ± 3.08	0.003 ± 0.001
60	38.08 ± 1.80	0.011 ± 0.012	168.70 ± 3.13	0.003 ± 0.001	75.39 ± 2.31	0.006 ± 0.004
70	7.80 ± 1.73	0.056 ± 0.016	45.76 ± 2.65	0.009 ± 0.001	42.92 ± 1.80	0.010 ± 0.004
80	–	–	35.77 ± 3.14	0.012 ± 0.002	6.50 ± 0.21	0.067 ± 0.025
	r^2 = 0.9436		r^2 = 0.9453		r^2 = 0.9148	
	z_T-value (°C)	E_a (kJ/mol)	z_T-value (°C)	E_a (kJ/mol)	z_T-value (°C)	E_a (kJ/mol)
	20.33 ± 2.45	102.15 ± 4.04	28.33 ± 1.98	77.25 ± 5.14	23.04 ± 2.00	93.94 ± 4.01

Table 3
Kinetic constant and relative activities of PPOIa and PPOIb from green bean coat and PPOII in green bean pod.

Substrates Wavelength assay (nm)[a]		Catechol 400	4-Methylcatechol 410	L-dopa 500	Pyrogallol 334	L-Tyrosine 472	Tannins 420
PPOIa	K_m (mM)	10.6 ± 0.6	14.5 ± 0.5	35.2 ± 1.1	7.6 ± 0.2	–	–
	V_m(U/ml min)	11700 ± 553	10050 ± 495	4570 ± 196	17100 ± 319	–	–
	Relative activity (%)	100 ± 4.8	63 ± 3.1	10.3 ± 0.5	958 ± 43.5	2.3 ± 0.1	0
PPOIb	K_m (mM)	10.7 ± 0.3	15.8 ± 0.8	34.7 ± 1.0	4.1 ± 0.2	–	–
	V_m(U/ml min)	10570 ± 382	9750 ± 177	4834 ± 134	19900 ± 778	–	–
	Relative activity (%)	100 ± 4.5	37 ± 2.2	8.5 ± 0.3	718 ± 21.2	1.0 ± 0.04	0
PPOII	K_m (mM)	37.7 ± 1.8	39.0 ± 1.7	50.6 ± 2.0	15.1 ± 0.6	–	–
	V_m(U/ml min)	4260 ± 210	4015 ± 168	3720 ± 155	9140 ± 466	–	–
	Relative activity (%)	100 ± 3.9	73 ± 4.1	38 ± 2.4	733 ± 25.7	0	0

"–" no detect the value.
[a] The values of the wavelength were previously reported by Jiang (1999).

using catechol as substrate were set at 100%, the relative activities of PPOs using the substrate pyrogallol were 958% (PPOIa), 718% (PPOIb), and 733% (PPOII), respectively (Table 3). Therefore, it can be summarised that the isolated PPOs from green beans have substrate-binding sites with highly affinity for pyrogallol and catechol. Pyrogallol is the best substrate for green bean PPOs. The green beans PPOs showed no activity towards L-tyrosine or tannins. These results suggested that PPOs in green beans, lack monophenolase (cresolase) activity. Catechol and 4-methylcatechol are substrates that are often chosen for determining the activity of polyphenol oxidase isolated from food and plant sources (Aydemir, 2004; Gawlik-Dziki et al., 2007; Gawlik-Dziki et al., 2008; Gomez-Lopez, 2002; Mdluli, 2005). However, PPOs from litchi and longan have high affinities for pyrogallol (Jiang, 1999; Jiang et al., 1997). Edible burdock PPO strongly oxidised 1,2,3-trihydroxybenzene (pyrogallol), but no activity toward monophenols or diphenols (Murao, Oyama, Nomura, Tono, & Shin, 1993). Martinez and Whitaker (1995) indicated that all of the PPOs discovered have the ability to convert *o*-dihydroxyphenols to *o*-benzoquinones, using O_2 as the second substrate (catecholase activity), but not all PPOs hydroxylate monophenols. Considering their substrate specificity, the green bean PPOs seem inclined toward *o*-diphenols and 1,2,3-trihydroxybenzenes.

3.6. Effect of inhibitors on PPO activity

Eight inhibitors with three different concentrations were employed to determine their potential for inhibition of PPOI (PPOIa + PPOIb) and PPOII (Table 4). These inhibitors include metal ions and reducing agents. Relative activity was calculated as a percentage of the residual activity occupying original activity (without any inhibitor). Of all inhibitors used in the study, L-ascorbic acid was the most effective for inhibition of PPOIand PPOII, followed by sodium metabisulphite and L-cysteine, since the three compounds induced a high degree of inhibition even at low concentration. The results are similar to the results with Victoria grape obtained by Rapeanu et al. (2006) and medlar fruit PPOs (Ayaz, Demir, Torun, Kolcuoglu, & Colak, 2008; Dincer et al., 2002).

The mechanism of L-ascorbic acid inhibition involves the reduction of quinones generated by PPO. PPO catalyses the oxidation of phenolic substrate to *o*-quinones whilst L-ascorbic acid converts *o*-quinones back to phenolic compounds (Duangmal & Owusu Apenten, 1999). L-ascorbic acid has also been reported to cause irreversible enzyme inhibition (Aydemir, 2004; Rapeanu et al., 2006). Two other mechanisms for inhibition involving direct interaction with the enzyme have been reported: chelation of the copper at the active site and reduction of Cu^{++} to Cu^{+} (Gawlik-Dziki et al., 2007; Gomez-Lopez, 2002).

The action of sulphite in the prevention of enzymatic browning can be explained by the actions on *o*-quinones and PPO. The formation of a quinone–sulphite complex prevents quinone polymerisation. A further action of metabisulphite on PPO is directly on the enzyme structure, leading to the inactivation of PPO (Duangmal & Owusu Apenten, 1999). It has been suggested that sulphite reacts with disulphide bound within PPO. This leads to a change in the tertiary structure of the enzyme and its inactivation (Duangmal & Owusu Apenten, 1999). Because L-cysteine can easily form complexes with *o*-quinones, PPO was inhibited by the formation of additional products. L-cysteine has been suggested to prevent enzymatic browning in processed fruit products (Jiang, 1999). The effectiveness of an inhibitor depends on the variety (Gomez-Lopez, 2002). L-ascorbic acid was the most efficient inhibitor for *Allium* sp. PPO (Arslan et al., 1997), Taro PPO, potato PPO (Duangmal

Table 4
Effects of various compounds on the activities of PPOI from green bean coat and PPOII from green bean pod.

Compounds	PPOI		PPOII	
	Concentration (mM)	Relative activity (%)	Concentration (μM)	Relative activity (%)
Ascorbic acid	0.1	55.9 ± 1.0[b]	1	82.8 ± 1.3
	1.0	0.70 ± 0.04	10	6.1 ± 0.5
	10.0	0.70 ± 0.04	100	2.2 ± 0.3
L-Cysteine	0.1	74.5 ± 1.1	1	94.1 ± 1.9
	1.0	1.7 ± 0.3	10	94.0 ± 1.9
	10.0	0.7 ± 0.1	100	4.3 ± 0.1
Sodium metabisulphite	0.1	84.3 ± 6.6	1	71.0 ± 1.27
	1.0	1.0 ± 0.3	10	59.1 ± 0.99
	10.0	0.7 ± 0.1	100	4.2 ± 0.18
Thiourea	0.1	102.8 ± 0.9	1	77.1 ± 1.64
	1.0	97.2 ± 0.3	10	76.8 ± 1.34
	10.0	70.6 ± 0.6	100	74.7 ± 1.13
Citric acid	0.1	96.7 ± 1.6	1	71.4 ± 1.59
	1.0	90.0 ± 1.6	10	70.7 ± 1.41
	10.0	88.0 ± 1.9	100	69.3 ± 1.58
$CaCl_2$	0.1[a]	102.4 ± 1.3	1[a]	97.8 ± 1.22
	1.0[a]	102.4 ± 1.4	10[a]	91.1 ± 1.43
	10.0[a]	209.4 ± 2.1	100[a]	87.3 ± 1.46
NaCl	0.1[a]	99.7 ± 1.5	1[a]	93.1 ± 1.7
	1.0[a]	101.4 ± 1.3	10[a]	92.8 ± 1.1
	10.0[a]	98.6 ± 1.7	100[a]	91.0 ± 1.1
$MnSO_4$	0.1[a]	110.5 ± 2.4	1[a]	100.0 ± 1.2
	1.0[a]	174.8 ± 2.7	10[a]	89.1 ± 1.3
	10.0[a]	215.4 ± 4.3	100[a]	86.9 ± 1.5

[a] Final concentrations of various ion.
[b] Standard deviation.

& Owusu Apenten, 1999), butter lettuce (Gawlik-Dziki, Zlotek, & Swieca, 2008), cabbage (Fujita et al., 1995) and banana (Unal, 2007; Yang et al., 2000). But L-ascorbic acid was a median inhibitor for longan PPO (53% inhibition at 1.0 mM) (Jiang, 1999), and litchi (69% inhibition at 1.0 mM) (Jiang et al., 1997). It has been reported by several authors that L-cysteine and metabisulphite were strong inhibitors for plant PPO (Aydemir, 2004; Duangmal & Owusu Apenten, 1999; Erat et al., 2006; Gomez-Lopez, 2002; Jiang, 1999; Jiang et al., 1997), However, L-cysteine was a medium inhibitor for butter lettuce PPO (16.8% inhibition at 1.0 mM) (Gawlik-Dziki, Zlotek, & Swieca, 2008).

In this study, NaCl, thiourea, and citric acid were poor inhibitors. Several workers have reported that the inhibitory effectiveness on PPO activity is quite low at low NaCl concentrations (Erat et al., 2006; Fujita et al., 1995; Jiang, 1999; Rapeanu et al., 2006; Unal, 2007; Yang et al., 2000). On the other hand, higher concentrations of NaCl can achieve higher inhibition. It was shown that the inhibition of PPOs from two varieties of avocado by 0.8 M NaCl were 37% and 39% (Gomez-Lopez, 2002). It is believed that the action of NaCl is due to its interaction with the copper at the active centre of the enzyme (Duangmal & Owusu Apenten, 1999; Gomez-Lopez, 2002).

The relative activities of PPOI were significantly promoted by $MnSO_4$ and $CaCl_2$, but the relative activities of PPOII were not significantly changed, compared with the original activity. The relative activities of PPOI influenced by 10 mM $MnSO_4$ and $CaCl_2$ were increased to 174.8% and 209.4% respectively. Similar results have been found in longan, cabbage and litchi (Fujita et al., 1995; Jiang, 1999; Jiang et al., 1997). But Aydemir (2004) found that 30–32% inhibition of PPO activity was observed at 10 mM of $MnCl_2$ and $CaCl_2$. The two compounds also inhibited PPO in medlar fruit (Ayaz et al., 2008) and Japanese pear (Aydemir, 2004).

4. Conclusion

This study describes the isolation and characterisation of four isoforms of PPO from green beans and also compares their properties. In some properties, the four isoforms of PPOs present significant differences in molecular weights, pH-profiles, temperature profiles, enzyme kinetics, substrate specificity and effect of inhibitors. The molecular weights of four isoforms were 57.5 kDa (PPOIa), 54 kDa (PPOIb), 46.0 kDa (PPOIIa) and 39.0 kDa (PPOIIb), respectively. The optimal pH of PPOs were 6.8 (PPOIa), 7.0 (PPOIb), 4.0 and 7.0 (PPOII), respectively. All four isoforms activities were stable between pH 6.8 and pH 7.2. The natural pH of green bean coats and pods are 6.8 and 5.8, respectively. Therefore, in natural sate, PPOIa and PPOIb from green bean coats keep their highly enzymatic activity and PPOII from green bean pods was greatly inhibited at its lower pH value. The optimal temperatures for PPOIa, PPOIb and PPOII were 20, 30 and 50 °C, respectively. PPOIa and PPOII have similar thermal inactivation curves. PPOs are stable at 0–40 °C, but PPOIb has higher thermal stability than that of PPOIa and PPOII from 50 to 80 °C. So the higher temperatures can efficiently inactivate PPO activity. PPOs of green bean have substrate-binding sites with highly affinity for pyrogallol and catechol. Pyrogallol is the best substrate for green bean PPOs. The green beans PPOs lack monophenolase (cresolase) activity. Although activities of PPOI and PPOII were markedly inhibited by L-ascorbic acid, the activity of PPOI was significantly activated by $MnSO_4$ and $CaCl_2$.

Further investigation of the molecular structure and origin of different PPOs and the role of an individual PPO in cellular metabolism could help in understanding their functions in green beans, especially in understanding the browning mechanism.

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Food Chemistry 117 (2009) 152–159

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Ethanol modified supercritical fluid extraction and antioxidant activity of cajaninstilbene acid and pinostrobin from pigeonpea [*Cajanus cajan* (L.) Millsp.] leaves

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ARTICLE INFO

Article history:
Received 3 February 2009
Received in revised form 19 February 2009
Accepted 24 March 2009

Keywords:
Supercritical fluid carbon dioxide extraction
Cajaninstilbene acid
Pinostrobin
Pigeonpea leaves
Antioxidant activity

ABSTRACT

Supercritical fluid carbon dioxide (SF-CO_2) extraction (SFE) of cajaninstilbene acid (CSA) and pinostrobin (PI) from pigeonpea leaves and antioxidant activity were investigated for the first time. SFE gave higher extraction yields than conventional heat-refluxing extraction (HRE) because of the severe breakage of cell morphology caused by SF-CO_2, which was observed by scanning electron microscopy (SEM). CO_2 flow rate, modifier composition and liquid to solid ratio were firstly optimised, 12 kg/h, and 80% EtOH with a liquid to solid ratio of 10 ml/g raw material exhibited better extraction performance. Then a central composite design (CCD) combined with response surface methodology was used to study the effects of extraction pressure, temperature and time on the extraction yields of CSA and PI, the optimal parameters were 30 MPa, 60 °C and 2 h. An antioxidant activity investigation showed SFE extracts exhibited better free radical-scavenging activity than HRE extracts with an IC_{50} value of 0.2259 mg/ml.

1. Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.], also known as red gram, Congo pea, Dhal, no eye pea and pois caja, etc., is a famous and multi-use grain legume crop in semi-tropical and tropical developing countries. It has been used for centuries in intercropping systems, and ranks sixth in area and production in grain legumes worldwide. It is an ideal source of protein and Vitamin B in human diet, especially in the vegetarian population (Salunkhe, Chavan, & Kadam, 1986). Besides its usage as food, green vegetables, fodder, firewood, soil improvement in agriculture (Sharma, Sreelatha, & Dayal, 2006), pigeonpea has many uses as traditional folk medicinal plants. It has been used for many years for treating diabetes, expelling bladder stones, stabilising the menstrual period, applying to sores, skin irritations, hepatitis, measles, jaundice, dysentery and many other illnesses (Grover, Yadav, & Vats, 2002; Morton, 1976). In China, pigeonpea leaves are used as expectorant, against sore gums, in child delivery, as anhelminthic, etc. (Morton, 1976). It was also found that pigeonpea leaves exhibited notable vulnerary, anti-biotic, anti-sickling activities and inhibited *Leishmania* and capillary permeability.

A chemical composition investigation indicated that there are a number of phenolic phytoalexins in pigeonpea leaves, including flavonoids (Fu et al., 2008) and stilbenes (Cooksey, Dahiya, Garratt, & Strange, 1982; Inman & Hoppe, 2002), etc. In preliminary investigations on active constituents in pigeonpea leaves, cajaninstilbene acid (CSA) and pinostrobin (PI), which belongs to stilbenes and flavonones, respectively, have been found in higher content with remarkable pharmacological activities. CSA is stilbene-2-carboxylic acid, which rarely exists in plants (Cooksey et al., 1982). It has hypoglycemic, hypotriglycerimic activity (Inman, 2002, 2003) and potential action in the treatment of postmenopausal osteoporosis (Zheng, Yang, Chen, & Sun, 2007), it was also found to be a good antioxidant in our previous study (Wu et al., 2009). PI, a potent flavonoid inducer, shows an extraordinarily high ability to induce mammalian phase 2 detoxication enzymes and antioxidant enzymes, it has a concentration required to double the quinone reductase induction of 0.5 μM in a microtiter plate assay with murine hepatoma cells (Fahey & Stephenson, 2002). It also possesses the ability to inhibit the human placental aromatase and decrease the proliferation of MCF-7 cells induced by dehydroepiandrosterone sulphate and 17β-estradiol (Le Bail, Aubourg, & Habrioux, 2000). It is known that many stilbenes and flavonoids are natural antioxidants (Alén-Ruiz, García-Falcón, Pérez-Lamela, Martínez-Carballo, & Simal-Gándar, 2009). Pigeonpea leaves are

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0308-8146/$ - see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2009.03.091

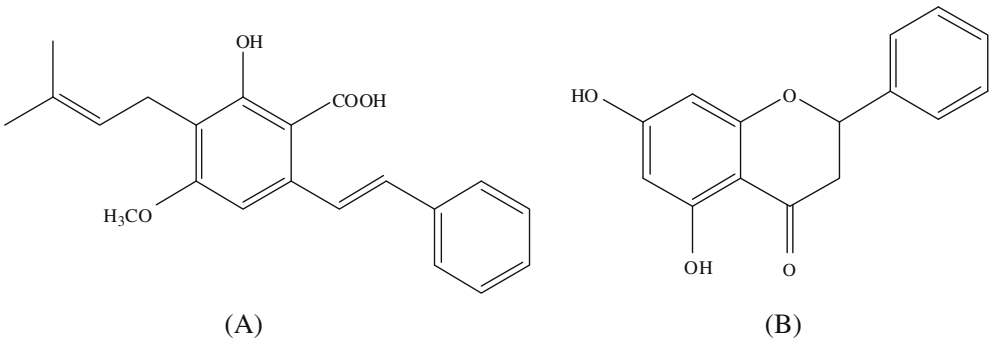

Fig. 1. Structures of CSA (A) and PI (B).

abundant as a natural resource, and the constituents CSA and PI with good antioxidant activity, are of high content in pigeonpea leaves, therefore, it is of great interest to investigate the extraction of CSA and PI from pigeonpea leaves as natural antioxidants.

The structures of CSA and PI are shown in Fig. 1. They are relatively non-polar or slightly polar compounds, and are difficult to dissolve in water. Therefore, higher concentrations of organic solvent and higher temperature are needed to guarantee a better extraction performance. PI has been extracted with ether, ethanol and methanol (Cannon & Martin, 1977; Duker-Eshun, Jaroszewski, Asomaning, Oppong-Boachie, & Brogger-Christensen, 2004; Mohamad et al., 2004). While for the extraction of CSA, methanol and ethanol (Cooksey et al., 1982) have been used. However, organic solvent is quite harmful to human health and environment, so therefore, its use in food, cosmetic and pharmaceutical industries has many restricts. Regarding the extraction of antioxidants, supercritical fluid extraction with CO_2 is an alternative method for replacing organic solvent, it has received considerable attention recently (Abbas, Mohamed, Abdulamir, & Abas, 2008). CO_2 is abundant, inert, non-toxic, environmentally friendly solvent and acceptable in food industry. The extracts obtained by SFE are of outstanding quality and the yields are comparable with those of organic solvent extraction methods (He et al., 2005). SFE extracts were generally recognised as safe (GRAS) to be used in food products. Therefore, SFE may serve as a promising technology in food and pharmaceutical processing (Abbas et al., 2008).

In the present study, SFE of CSA and PI from pigeonpea leaves was studied for the first time. Extraction yields of SFE and HRE were compared and the morphological changes of leaves before and after extraction were observed by SEM. The effects of modifier composition, liquid to solid ratio, and CO_2 flow rate were studied by single factor test, while three operating parameters including extraction pressure, temperature and time on the extraction yields were investigated and optimised by central composite design combined with response surface methodology. Furthermore, the antioxidant activity of extracts, obtained under optimised SFE conditions was determined by means of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay.

2. Materials and methods

2.1. Plant material

Pigeonpea leaves were collected from Hainan province, China, and authenticated by Professor Shao-quan Nie from the Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University, PR China. Voucher specimens were deposited in the herbarium of this Laboratory. The leaves were dried in the airy shade for 30 days. After that, the dried leaves were pulverised, parts of them were sieved to sizes of 40–60 mesh. The sieved leaves were dried to a constant weight, then, kept away from light in a desiccator at room temperature until used.

2.2. Chemical and reagents

Carbon dioxide (purity 99.99%, Liming gas Corp., China), ethanol (99.7%, A.R., Harbin Xinchun chemical reagents Corp., China), methanol (J & K Chemical Ltd., China), formic acid (96%, DIMA Technology Inc., USA), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ascorbic acid (VC) were purchased from Sigma–Aldrich, Steinheim, Germany. The water used in all experiments was bi-distilled water. Employed reference compounds CSA ($\geqslant$98%, HPLC grade) and PI ($\geqslant$95%, HPLC grade), were separated and purified in the same Key Laboratory. The structures were confirmed by comparing their UV, MS, ^{1}H NMR and ^{13}C NMR data with the data from literatures (Cooksey et al., 1982; Cuong, Sung, Kamperdick, & Adam, 1996).

2.3. Heat-refluxing extraction

A conventional method of heating-refluxing extraction using ethanol–water (80:20, v/v) was performed to compare the extraction performances with SFE. According to the preliminary investigation, target compositions were extracted by adding 20 g of pigeonpea leaves into 400 ml of solvent in a round bottom flask. The extraction was employed to optimum condition of 65 °C for 2 h under magnetic stirring at 500–700 rpm. The extracting solution was filtered by membrane filtration and analysed by HPLC. The extraction results were compared with that using SFE.

2.4. SF-CO_2 extraction

The SF-CO_2 extraction experiments were performed using an HA121-50-01 SFE device (Hua'an Supercritical Fluid Extraction Corp., Nantong, China), the schematic flow diagram was described in detail in a previous study (Wang, Pan, Sheng, Xu, & Hu, 2007). Briefly, the operating methodology was as follows: liquid CO_2 supplied from a gas cylinder was cooled by ethanol to −5 °C. The cooled CO_2 and aqueous ethanol from the modifier tank were pressurised, respectively, to the desired pressure, then mixed, heated to a specified temperature and passed to the device, the entire device was also pre-pressurised. For each experiment, approximately 100 g pigeonpea leaves were filled into a steel cylinder equipped with mesh filters (6.5 μm) on both ends, the loaded cylinder was then introduced into the extraction vessel, which was placed in a heating muff, the desired extraction temperature and pressure were adjusted, after that, the CO_2 feed was started. The extracts were deposited in the separators and collected together in plastic bottles loop.

A 2^3 factorial portion central composite design (CCD) combined with response surface methodology was used for optimising

extraction pressure (X_1), temperature (X_2) and time (X_3). In the CCD test, 14 experiments and six replicates at the centre were employed to fit the full quadratic equation model. The general equation is:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum \sum_{i<j} \beta_{ij} X_i X_j \quad (k = 3)$$

where Y is the predicted response, β_0, β_j, β_{jj} and β_{ij} are the regression coefficients for intercept, linearity, square and interaction, respectively, while X_i and X_j are the independent coded variables. The variables of each factor were transferred to range between −1 and 1 for the appraisals, while the dependent variable was the extraction yield of CSA and PI. Coded variables were used according to the equation:

$$X_i' = \frac{X_i - X_{0i}}{\Delta X_i} \quad (i = 1, 2, 3)$$

where X_i' is the coded value of the independent variable. X_i is the real value of the independent variable. X_{0i} is the real value of the independent variable at the centre point. ΔX_i is the step change of the real value of variable 'i'. The actual and coded levels of the independent variables used in the experimental design are shown in Table 1.

During all of the experimental process, CO_2 flow rate ranged from 3.8 to 14.0 kg/h. The extraction pressure varying from 15 to 40 MPa and temperature varying from 35 to 70 °C were controlled by adjusting valves according to the meter on the front panel. Aqueous ethanol was delivered by a high pressure liquid pump. Once the scheduled time was achieved, the extraction vessel was depressurised and the extracts were collected from the separation vessel. The accumulated product samples were collected and concentrated to give the extracts mass, which was used for the following tests.

2.5. Scanning electron microscopy

The morphological changes of samples before and after extraction were observed by SEM. After removing the solvent, the remaining pigeonpea leaf samples were plunged into liquid nitrogen and cut with a cold knife. The sectioned particles were fixed on a specimen holder with aluminium tape and marked, then sputtered with gold in a sputter-coater. All the samples were examined with a Quanta-200 SEM (FEI Company, USA) under high vacuum and at an accelerating voltage of 15.0 kV (20 μm, 1000 × magnification).

2.6. Determination of CSA and PI

The Agilent 1200 reversed-phase HPLC system was employed for CSA and PI determination. An HIQ sil C_{18}W column (150 mm × 4.6 mm i.d., 5 μm) fit with an Alltech C_{18} guard column (8 mm × 4.6 mm i.d., 5 μm) was used. The column temperature was set at 25 °C. The mobile phase was chosen as solvent A (0.1% formic acid in water, v/v) and B (methanol) at a flow rate of 1.0 ml/min. A linear gradient lasted for 60 min: 0–10 min, keep A with 67%; from 10 to 30 min, changing A from 67% to 35% in 20 min; then from 30 to 40 min, changing A from 35% to 10% in 10 min and keep this scale for 10 min; finally, changing A to 67% in 10 min for the balance.

The CSA at wavelength of 259.2 nm and PI at 280.8 nm was verified and used for the RP-HPLC quantification. Eight experimental points were employed for establishing a calibration curve. The calibration curves for CSA and PI were $Y = 80876\ X - 771.79$, $Y = 36591\ X + 703.22$, respectively, where Y is the peak area of the analyte, and X is the concentration of the analyte (mg/g). The correlation coefficients (R^2) were 0.9996 and 0.9932, respectively. The recovery of CSA and PI varied between 94.3% and 106.8%, and RSD were less than 5%.

2.7. Antioxidant activity

The DPPH radical-scavenging activity (anti-free-radical activity) is based on the determination by 2,2-diphenyl-1-picrylhydrazyl (DPPH) at a steady state in ethanol solution after adding the mixture of antioxidants. Following the detailed method of Liyana-Pathiranan and Shahidi (2005), the extract was dissolved in 10 ml of absolute ethanol to give a final concentration of 4 mg/ml, then 2 ml of 0.004% DPPH (0.2 mM) in ethanol was added to 1 ml of the extract solution. After shaking the mixture vigorously, the decrease of absorbance was measured at 517 nm until the

Table 1
Coded and real levels of the operational parameters, and observed and predicted values for different levels of experimental design. *P*, the extraction pressure (MPa); temp., the extraction temperature (°C); *T*, the extraction time (h). Pred., predicted value; exp., experimental value.

Runs	Factors			Extraction yield of CSA (mg/g)		Extraction yield of PI (mg/g)	
	X_1 (P, MPa)	X_2 (temp., °C)	X_3 (T, h)	Pred.	Exp.	Pred.	Exp.
1	−1(25)	−1(45)	−1(1.5)	5.800	5.885	1.349	1.156
2	−1(25)	−1(45)	1(2.5)	8.555	8.123	2.437	2.310
3	−1(25)	1(65)	−1(1.5)	9.198	9.084	2.291	2.013
4	−1(25)	1(65)	1(2.5)	9.935	9.891	2.946	2.716
5	1(35)	−1(45)	−1(1.5)	6.690	6.239	2.362	2.156
6	1(35)	−1(45)	1(2.5)	9.723	9.342	2.806	2.648
7	1(35)	1(65)	−1(1.5)	11.190	11.127	3.245	2.935
8	1(35)	1(65)	1(2.5)	12.204	11.624	3.255	3.011
9	−1.682(21.59)	0(55)	0(2)	7.054	7.115	2.122	2.403
10	1.682(38.41)	0(55)	0(2)	9.710	10.347	3.234	3.569
11	0(30)	−1.682(38.18)	0(2)	6.773	7.235	1.930	2.126
12	0(30)	1.682(71.82)	0(2)	11.718	11.953	3.101	3.521
13	0(30)	0(55)	−1.682(1.16)	8.743	8.825	2.237	2.613
14	0(30)	0(55)	1.682(2.84)	11.913	12.526	3.161	3.401
15	0(30)	0(55)	0(2)	11.912	10.985	3.369	3.512
16	0(30)	0(55)	0(2)	11.912	12.038	3.369	3.358
17	0(30)	0(55)	0(2)	11.912	12.054	3.369	3.429
18	0(30)	0(55)	0(2)	11.912	12.415	3.369	3.501
19	0(30)	0(55)	0(2)	11.912	12.082	3.369	3.314
20	0(30)	0(55)	0(2)	11.912	11.794	3.369	2.996

reaction reached a plateau. Absolute ethanol was used as the control and ascorbic acid, a stable antioxidant, was used as synthetic reference. The DPPH radical-scavenging activity in percentage of sample was calculated according to the following equation: DPPH scavenging activity (%) = $(1 - A_{517}$ sample$/A_{517}$ DPPH solution$) \times 100$.

2.8. Statistical analysis

All data collected from central composite design SFE experiments were centred by using three parallel measurements of mean ± SD. Then, the data was analysed using a response surface analysis procedure (Statistica 6.0). Analysis of variance was performed for calculations and modelling the optimum conditions for SFE of CSA and PI from pigeonpea leaves. P values <0.05 were regarded as significant and P values <0.01 as very significant.

3. Results and discussion

3.1. Comparison of SFE with HRE

The comparison of SFE with HRE was conducted with the same experimental condition of 65 °C for 2 h, 80% EtOH as the solution. In SFE, the pressure was 30 MPa, with a CO_2 flow rate of 14.0 kg/h, while in HRE, magnetic stirring was employed at 500–700 rpm.

The extraction yields of CSA and PI obtained using SFE were 11.17 ± 0.58 mg/g and 2.73 ± 0.61 mg/g, which were higher than those of HRE (8.31 ± 0.94 and 1.99 ± 0.61 mg/g, respectively). The amount of extract was weighed after removing the solvent to determine the yields, the extracts yield obtained by SFE was 81.42 ± 7.49 mg/g, which was lower than using HRE (208.51 ± 16.43 mg/g). The contents of CSA and PI in SFE extracts

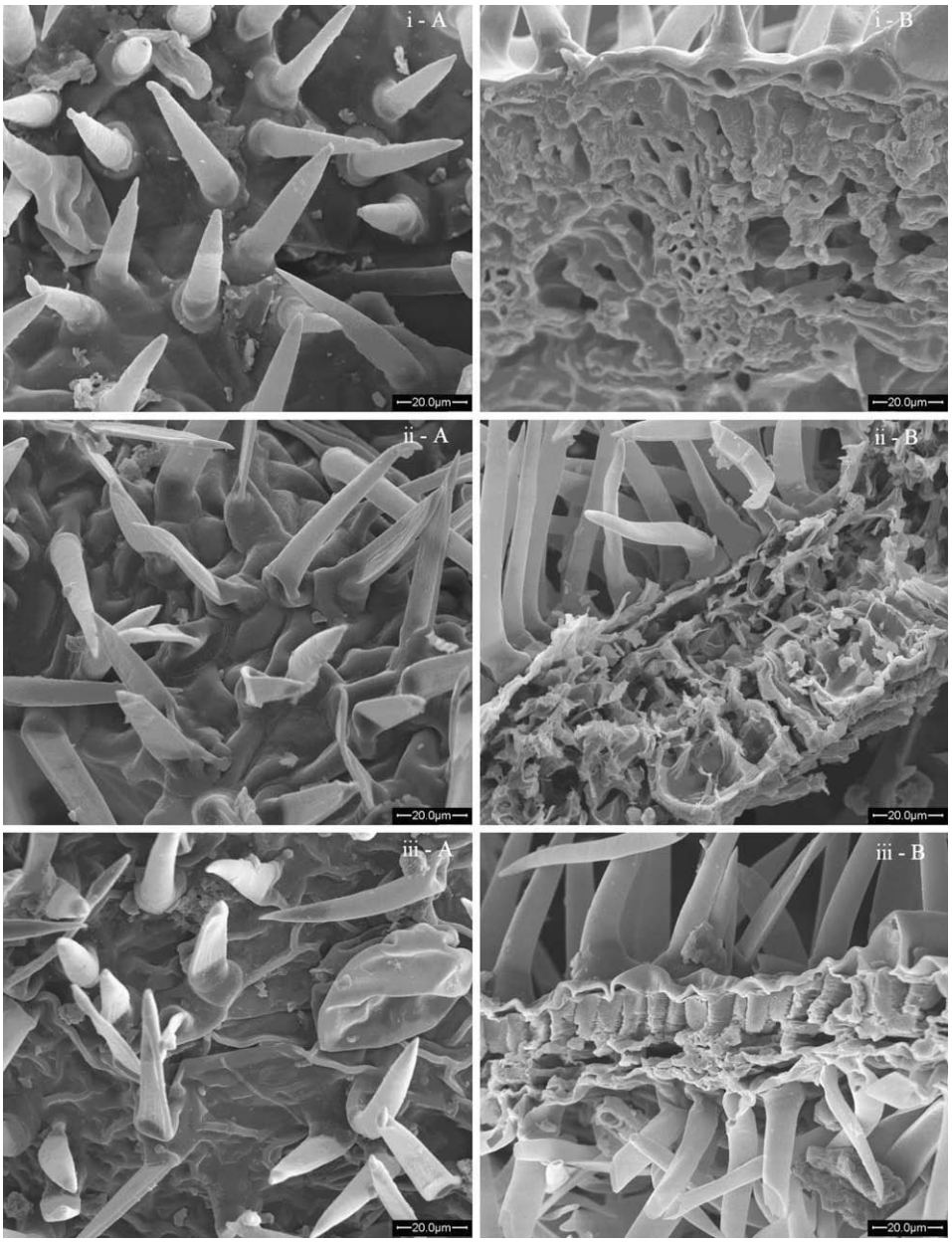

Fig. 2. Scanning electron micrographs of untreated pigeonpea leaves sample (i), sample after HRE (ii) and sample after SFE (iii); (A) surface-observation of sample, (B) cutting plane-observation of sample. Each figure was scanned at the same accelerating voltage of 15.0 kV (20 μm, 1000× magnification).

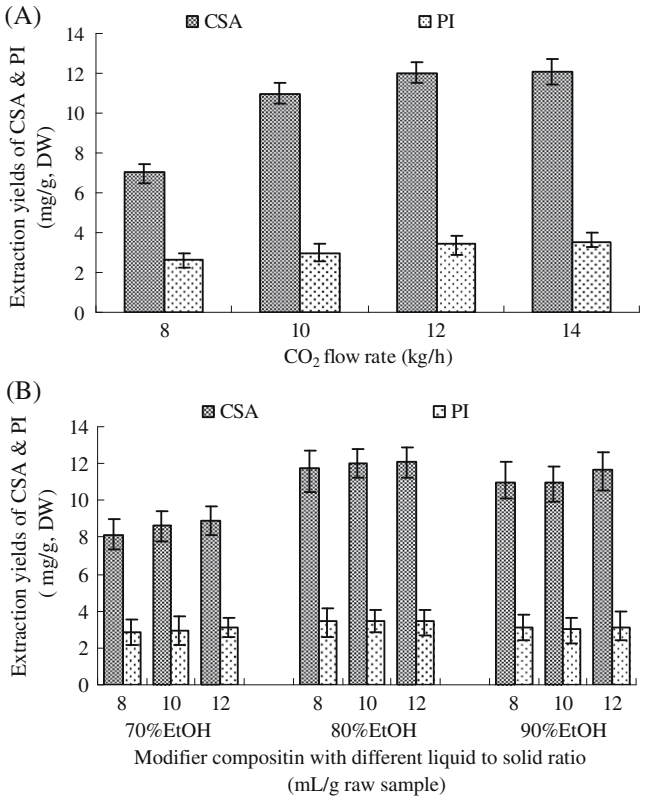

Fig. 3. Effect of CO_2 flow rate (A), modifier composition with different liquid to solid ratio (B) on extraction yields of CSA and PI ($n = 3$).

reached 138.24 ± 9.51 and 33.12 ± 3.47 mg/g, which were 3.47- and 3.53-fold to those in HRE extracts. The result demonstrated that SFE could selectively extract CSA and PI, because SFE is suitable for non-polar or slightly polar compounds and less volatile compounds (Brunner, 2005).

Moreover, SEM was used to elucidate the morphological changes during each extraction procedure. Fig. 2 shows the micrographs of the untreated sample, HRE sample and SFE sample, respectively. As shown in Fig. 2i-A, ii-A and iii-A, the surface morpha observed in HRE were hardly wrinkled from that of the untreated sample. However, obvious wrinkling took place on the surface of sample after SFE. When compared with the profiles shown in Fig. 2i-B, ii-B and iii-B, the palisade parenchyma and spongy parenchyma of pigeonpea leaf sample were slightly changed after HRE, but greatly changed or destroyed after SFE. The observation suggested that SFE influences the structure of mesophyll cells, because the supercritical fluids have the diffusivity of a pressure–temperature dependent density and modifier sensitivity (Chakraborty, Kumbhar, & Sarkar, 2007), the pressurised solvent in supercritical or, more generally, near-critical state flows continuously through the bed of particles and dissolves out the solute (Sovová, 2005).

The above result presented a clear advantage of SFE in enriching CSA and PI. The optimisation of SFE and quality evaluation of the optimum SFE extracts in terms of antioxidant activity is discussed below.

3.2. Optimisation of SFE procedure

3.2.1. Effect of CO_2 flow rate, modifier composition and liquid to solid ratio

Fig. 3 shows the effects of CO_2 flow rate, modifier composition and liquid to solid ratio. The effect of CO_2 flow rate was studied with the following conditions: 100 g pigeonpea leaves were extracted at a temperature of 55 °C, pressure 30 MPa, 80% EtOH with liquid to solid of 10 ml/g raw material and extraction time of 2 h, and a CO_2 flow rate were 8, 10, 12, 14 kg/h, respectively. It can be seen from Fig. 3A, at the range of 8–14 kg/h, the extraction yields obviously increased with the CO_2 flow rate. After a CO_2 flow rate of 12 kg/h was achieved, the extraction yields did not increase much further with the increase of CO_2 flow rate. At the range of this trial, the mass transfer parameter increased with the supercritical fluid flow rate until it reached a constant value. Salgın (2007) reported similar results in the SFE of seed oil. Soybean isoflavones recovery tended towards the same value (Zuo, Zeng, Yuan, & Yu, 2008) when higher amounts of carbon dioxide were used. In this study, extraction at the CO_2 flow rate of 12 kg/h gave the highest extraction yields for the targets.

The effects of modifier composition (70–90% EtOH) and liquid to solid ratio (8–10 ml/g raw material) were investigated at a temperature of 55 °C, pressure of 30 MPa, CO_2 flow rate of 12 kg/h and extraction time of 2 h. The results are shown in Fig. 3B. The modifier of 80% EtOH with liquid to solid ratio of 10 ml/g raw material gave the highest extraction yields of CSA and PI, followed by 90% and 70% EtOH. As shown in this figure, as EtOH concentration was higher than 80%, the extraction yield decreased. Lower EtOH concentration yielded stronger polarity of the modifier, which favored the extraction of polar compounds. On the contrary, CSA and PI are slightly polar compounds, and much lower EtOH concentration limited their solubility, resulting in declined extraction yields. In addition, water could swell the plant material, which increases the contact area between solvent and plant sample, therefore proper water in solvent could enhance the extraction ratio (Hemwimon, Pavasant, & Shotipruk, 2007). Hence, 80% ethanol solution with liquid to solid ratio of 10 ml/g raw material was selected for the following tests.

3.2.2. Optimisation of SFE operating parameters

The objective of the present study is to optimise the operating conditions to achieve an efficient extraction of CSA and PI from pigeonpea leaves. Because various parameters potentially influence the SFE process, the optimisation of the operating conditions plays a critical role in the development of a SFE method. Central composite design (CCD) combined with response surface methodology was used for optimising. According to the preliminary test, material size was sieved to 40–60 mesh, CO_2 flow rate was 12 kg/h, and a modifier of 80% EtOH with liquid to solid ratio of 10 ml/g raw material was employed. Three parameters including extraction pressure, temperature and time were optimised by a 2^3 factorial portion central composite design. All trial results obtained from 20 runs of the experiment and their predicted data from the model based on the experimental data were summarised in Table 1.

As shown in Table 1, the extraction yields of CSA and PI in the trial were similar to those expected and the results demonstrated the accuracy of predictive model. According to the analysis of variance (ANOVA), correlation coefficients (R^2) of 0.942 and 0.939, respectively, were obtained for CSA and PI with the calculated model. In addition, high significant levels for these ($P < 0.001$) were obtained by statistical analysis.

Eqs. (I) and (II) were obtained by statistical analysis, and show the relationship between the extraction yields of CSA and PI with extraction pressures, temperatures and time, respectively

$$Yc = 11.9123 + 0.7897X_1 + 1.4698X_2 + 0.9424X_3 + 0.2754X_1X_2 + 0.0694X_1X_3 - 0.5046X_2X_3 - 1.2478X_1^2 - 0.9426X_2^2 - 0.5601X_3^2 \quad (I)$$

$$Yp = 3.3686 + 0.3307X_1 + 0.3479X_2 + 0.2746X_3 - 0.0151X_1X_2 - 0.1611X_1X_3 - 0.1084X_2X_3 - 0.2441X_1^2 - 0.3016X_2^2 - 0.2366X_3^2 \quad \text{(II)}$$

where Yc is the extraction yield of CSA (mg/g) and Yp is the extraction yield of PI (mg/g); X_1 the extraction pressure (MPa), X_2 is the extraction temperature (°C) and X_3 is the extraction time (h).

By solving the Eqs. (I) and (II), the maximum predictive extraction yield of CSA 12.82 mg/g and PI 2.96 mg/g were obtained. The

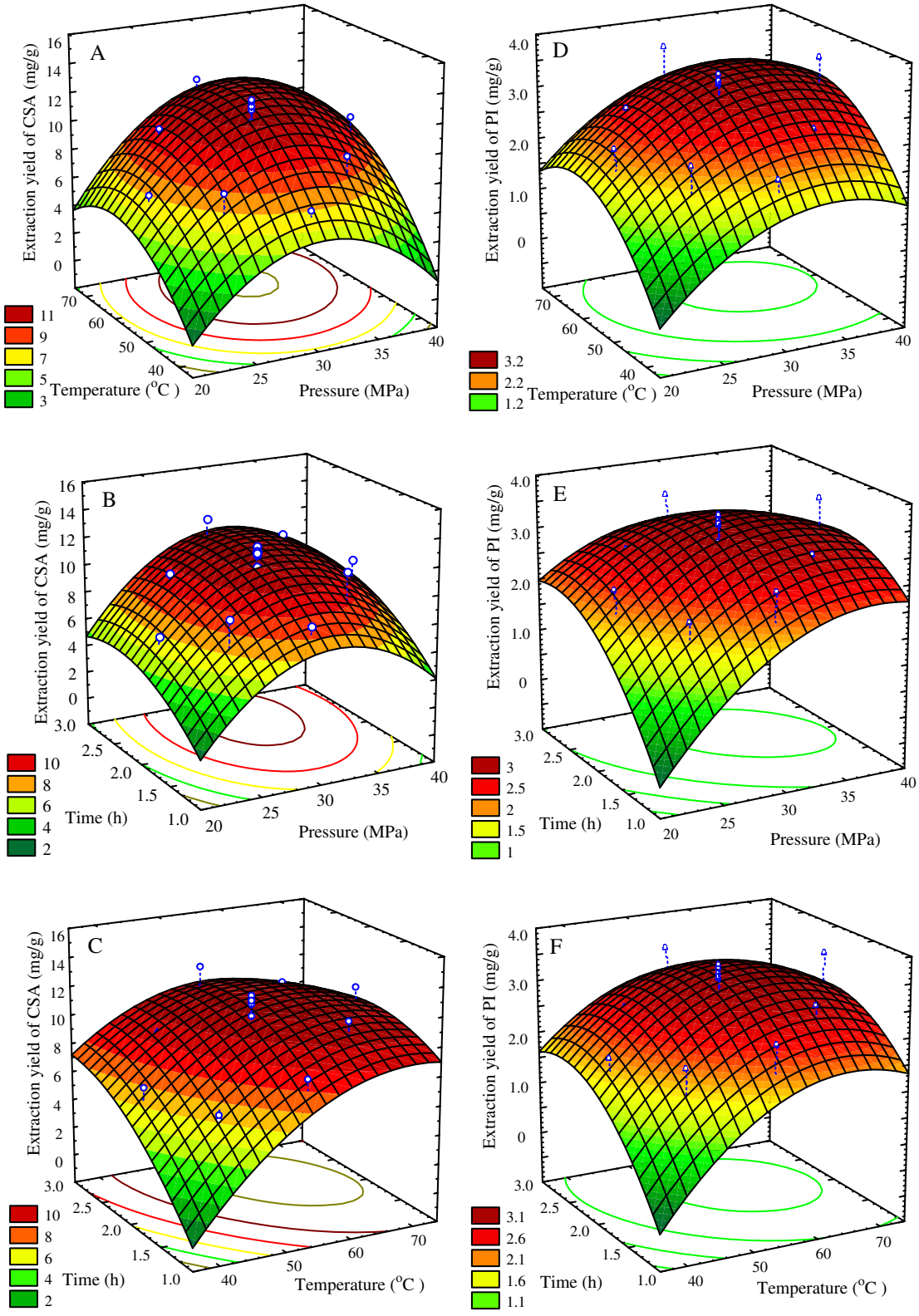

Fig. 4. Response surfaces for CSA (A–C) and PI (D–F) from pigeonpea leaves: (A and D) varying extraction temperature and pressure; (B and E) varying extraction pressure and time; (C and F) varying extraction temperature and time.

predictive values of relevant parameters for CSA were pressure 31.90 MPa, temperature 60.90 °C and time 2.24 h, while for PI they were: pressure 40.12 MPa, temperature 61.06 °C and time 1.74 h.

Meanwhile, Fig. 4A–F of three-dimensional profiles of multiple non-linear regression models were used to depict the interactive effects of operational parameters for CSA and PI, respectively.

As shown in Fig. 4A and D, increase of temperature from 35 to 60 °C with extraction pressure increasing from 20 to 30 MPa enhanced the extraction yields of CSA and PI simultaneously. It was expected that CO_2 density increased with pressure, and therefore increased the solvent power to dissolve the solute. Once the extraction pressure was higher than 30 MPa at 65 °C, the extraction became difficult, due to the interactive effect of density and solute vapour pressure for the extraction yield (Machmudah et al., 2008; Wang, Yang, Dua, & Yi, 2008). It was also observed that as temperature increased, the solubility changed accordingly, which would enhance the extraction efficiency. From Fig. 4A and D, an approximate temperature of 60 °C produced the highest extraction yields of CSA and PI (suggesting 60 °C could offer the suitable surface tension of extraction temperature for CSA and PI enriched extract). Wang et al. (2008) reported a similar result for flavonoid enriched extracts.

Response surfaces for CSA and PI with varying extraction pressure and time are shown in Fig. 4B and D. For increased pressure from 25 to 35 MPa with an increase of extraction time from 1 to 3 h, the CSA extraction yield was slightly enhanced, while for PI, there was nearly no effect. The result suggested, during experimental procedure or statistic analyses, that the interactive effect between extraction pressure and time on the extraction efficiency of CSA and PI could be ignored.

Fig. 4C and F describe the interactive effect of extraction temperature and time on CSA and PI. It can be seen that with an increase in temperature from 35 to 75 °C along with an increase in extraction time from 1 to 2 h, the CSA and PI extraction yields were enhanced. It is indicated that an increase in temperature could accelerate the mass transfer ratio from plant material to solvent, thus increasing the extraction yield. However, the extraction yields of CSA and PI decreased after 2 h extraction with a temperature of 65 °C. This could be because as the extraction time prolongs, the chemical decomposition of bioactive compounds presented in extracts may occur, resulting in the decrease of extraction yields (Huang, Xue, Niu, Jia, & Wang, 2008). It was also observed in the experiment that as temperature increased, the solvent capability changed, which extracted more impurities.

In summary, the optimal parameters of pressure 30 MPa, temperature 60 °C and time 2 h were selected on the basis of response surface. Those were within the investigation ranges. In comparison with the predictive values from Eqs. (I) and (II), the extraction parameters for CSA were accorded with predictive values. For PI the extraction temperature was a little lower, because some non-significant effects from response surface were eliminated but they were involved in the predictive equations. Accordingly, the better SFE process was obtained as follows: modifier composition 80% EtOH solution with liquid to solid ratio 10 ml/g raw material, CO_2 flow rate 12 kg/h, extraction pressure 30 MPa, temperature 60 °C and extraction time 2 h. Under the optimised conditions, the extraction yields of CSA and PI reached 12.17 ± 0.88 and 3.22 ± 0.56 mg/g, respectively, which were increased 1.46- and 1.77-fold to those by HRE. Furthermore, the contents of CSA and PI in the SFE extracts were 149.64 ± 8.26 and 39.08 ± 1.88 mg/g, which were 3.76- and 4.17-fold to the contents of HRE extracts. The above results were attributed to SF-CO_2 efficiently enhancing the release of lower polarity CSA and PI from the insoluble mesophyll tissue of pigeonpea leaves, therefore higher extraction yields and higher contents were obtained.

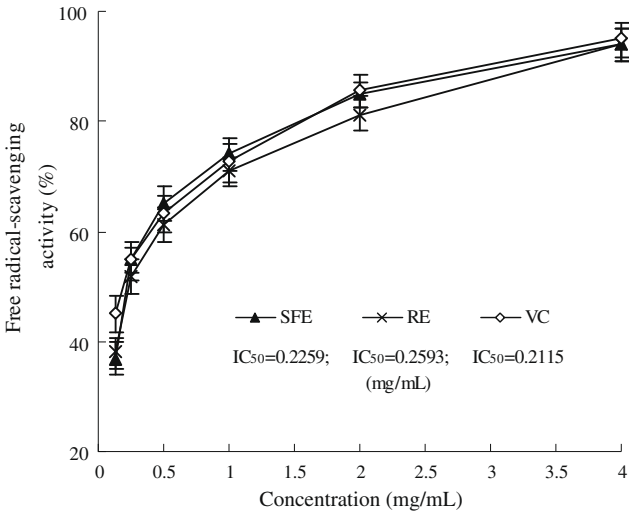

Fig. 5. Free radical-scavenging activity of SFE extracts, HRE extracts and VC (n = 3).

3.3. Antioxidant activity

Antioxidant activities of the SFE extracts and HRE extracts were tested by 1,2-diphenyl-2-picrylhydrazyl (DPPH) radicals. The reduction of the DPPH absorbance at 517 nm after 70 min incubation with the sample in darkness was measured, the results are presented in Fig. 5. The concentration of sample producing a 50% reduction of the radical absorbance (IC_{50}) was used as an index to compare the antioxidant activity.

The samples were assayed over a range of dilutions. It was observed that SFE extracts from pigeonpea leaves exhibited notable DPPH radical-scavenging activity, with an IC_{50} value of 0.2259 mg/ml nearly equal to that of the reference VC (0.2115 mg/ml), and superior to that of HRE extracts (0.2593 mg/ml). The values for SFE extracts ranged from 94.06 ± 2.85% to 36.79 ± 2.80%, with concentrations varying from 4 to 0.25 mg/ml, the decrease in the extract concentration resulted in the reduction of its free radical-scavenging activity. In the authors' previous paper (Wu et al., 2009), IC_{50} values of CSA of 0.3012 and PI with more than 0.500 mg/ml have been reported. These results combined with the data in this test, indicated that CSA should be one of the most efficient antioxidants in pigeonpea leaves. In comparison with HRE, SFE extracts with higher CSA and PI content showed higher antioxidant activity. Although PI did not present *in vitro* antioxidant activity by DPPH (Wu et al., 2009), it strongly induced mammalian phase 2 detoxication enzymes and antioxidant enzymes (Fahey & Stephenson, 2002). Hence, further studies on the antioxidant activity of SFE extracts *in vivo* are urgently needed. The present result provides evidence that the SFE extracts of pigeonpea leaves could be used in the field of food industries and other fields because of its excellent antioxidant activity.

4. Conclusions

In the present study, the SFE operating conditions were optimised to achieve an efficient extraction of CSA and PI from pigeonpea leaves, and the antioxidant activity of the resulting extracts have been evaluated *in vitro* by DPPH. The optimal performance was obtained under the operating pressure 30 MPa, temperature 60 °C, time 2 h, and 80% EtOH solution as the modifier with a liquid to solid ratio of 10:1 and CO_2 flow rate of 12 kg/h. Compared with HRE, the SFE method obtained higher extraction yields (12.17 ± 0.88 and 3.22 ± 0.56 mg/g for CSA and PI, respectively) and higher contents (149.64 ± 8.26 and 39.08 ± 1.88 mg/g for CSA

and PI, respectively), and the SFE extracts showed better antioxidant activity with an IC_{50} value 0.2259 mg/ml. SFE could be a prospective alternative to the traditional HRE for the efficient extraction of natural constituents CSA and PI from pigeonpea leaves. Meanwhile, the present research indicates that SFE extracts of pigeonpea leaves might be a valuable natural antioxidant resource and have potential use in food industries and other fields for its excellent antioxidant activity. However, further studies should be conducted *in vivo* for screening the antioxidants of SFE extracts from pigeonpea leaves.

Acknowledgements

The authors gratefully acknowledge the financial supports by National Natural Science Foundation of China (30770231), Heilongjiang Province Science Foundation for Excellent Youths (JC200704), Key Project of Chinese Ministry of Education (108049), National Key Technology R & D Program (2006BAD1 8B0405), Innovative Program for Importation of International Advanced Agricultural Science and Technology, National Forestry Bureau (2006-4-75) and Research Foundation for Science and Technology Innovation Talents of Harbin (2006RFXXS001).

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Food Chemistry 117 (2009) 160–168

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Antioxidative activity and emulsifying properties of cuttlefish skin gelatin modified by oxidised phenolic compounds

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ARTICLE INFO

Article history:
Received 20 December 2008
Received in revised form 16 February 2009
Accepted 24 March 2009

Keywords:
Gelatin
Phenolic compounds
Protein–phenolic complex
Antioxidative activity
Emulsifying properties

ABSTRACT

Antioxidative activity and emulsifying properties of cuttlefish skin gelatin modified by different oxidised phenolic compounds including caffeic acid, ferulic acid and tannic acid at different concentrations were investigated. Oxidised phenolic compounds were covalently attached to gelatin as indicated by the decrease in amino groups. Fourier transform infrared spectroscopic studies indicated the presence of an aromatic ring and a hydroxyl group in gelatin after modification. Modified gelatin had the increased antioxidative activity but the decreased surface hydrophobicity. Gelatin modified with 5% oxidised tannic acid had no change in emulsifying properties. Emulsion stability and oxidative stability of menhaden oil-in-water emulsion stabilised by 0.5% and 1.0% gelatin without and with modification by 5% oxidised tannic acid were studied. Both gelatins at a higher concentration (1.0%) yielded an emulsion with a smaller particle size. Modified gelatin inhibited the formation of TBARS in the emulsion more effectively than the control gelatin throughout the 12 days of storage.

1. Introduction

Proteins are widely used as emulsifiers in food products because of their ability to improve the stability of oil-in-water emulsions (McClements, 2004). Although low molecular mass surfactants, such as lecitin, mono- and diglycerides etc., are more effective than protein in reducing the interfacial tension, foams and emulsions formed by such surfactants are mostly unstable (Damodaran, 2005). In addition to lowering interfacial tension, proteins can form a continuous viscoelastic membrane-like film around oil droplets, whereas the low molecular mass surfactants cannot form such a viscoelastic film (Damodaran, 1997). A major potential advantage of proteins as emulsifiers in foods is their ability to protect lipids from iron catalysed oxidation (Hu, McClements, & Decker, 2003). At pH values below their isoelectric point (pI), proteins form positively charged interfacial membranes around oil droplets, which electrostatically repel any Fe^{2+} and Fe^{3+} ions present in the aqueous phase, thereby preventing oxidation of polyunsaturated lipids within the droplets (Surh, Decker, & McClements, 2005). Moreover, amino acids in protein can scavenge free radical and chelate prooxidative metals (Djordjevic, Cercaci, Alamed, McClements, & Decker, 2008).

Gelatin is commercially made from skins and skeletons of bovine and porcine. It has been used extensively in the medical, food, photographic and other industries (Derkatch, Petrova, Izmailova, & Tarasevitch, 1999). Gelatin is surface-active and is capable of acting as an emulsifier in oil-in-water emulsions (Lobo, 2002). However, the occurrence of bovine spongiform encephalopathy (BSE) and foot/mouth diseases has led to the increasing interest of other gelatin sources, especially fish skin and bone from seafood processing. Nevertheless, gelatin from marine resources had the poorer emulsifying properties, compared with mammalian gelatin (Aewsiri, Benjakul, Visessanguan, & Tanaka, 2008).

Plant phenolics are defined as compounds possessing one or more aromatic ring bearing a hydroxyl substituent(s), and can be found in many foods and drinks from plant origin, e.g. fruits, vegetables, coffee (Parr & Bolwell, 2000). Phenolic compounds can interact with proteins through non-covalent and covalent interaction. However, covalent bonding seems to play an important role in protein-phenol interaction, which is used to improve functional properties of proteins. Foam formation and the stability of a Tween 20/β-lactoglobulin mixed system was improved by (+)-catechin due to (+)-catechin-induced cross-linking of proteins in the

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.03.092

adsorbed layer (Sarker, Wilde, & Clark, 1995). Additionally, antioxidative activity of proteins can be modified by phenolic compounds (Rohn, Rawel, & Kroll, 2004). Nevertheless, little information regarding the modification of gelatin with phenolic compounds and its impact on stability and lipid oxidation of emulsion systems has been reported. Therefore, the objectives of this study were to investigate the effect of cuttlefish skin gelatin modified by oxidised phenolic compounds, including ferulic acid, caffeic acid and tannic acid, on stability and lipid oxidation of fish oil emulsion.

2. Materials and methods

2.1. Chemicals

Ferulic acid (FA), tannic acid (TA), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-triazine (TPTZ), 2,4,6-trinitrobenzenesulfonic acid (TNBS), Trolox and menhaden oil were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Caffeic acid was purchased from Fulka (Buchs, Switzerland). Hydrogen peroxide (H_2O_2), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), Folin–Ciocalteu's phenol reagent, sodium sulphite and ferric chloride were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

2.2. Preparation of cuttlefish skin

The ventral skin of cuttlefish (*Sepia pharaonis*) was obtained from a dock in Songkhla, Thailand. Cuttlefish skin was stored in ice with a skin/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, cuttlefish skin was washed with tap water and cut into small pieces (1 × 1 cm), placed in polyethylene bags and stored at −20 °C until use. Storage time was not longer than 2 months.

2.3. Extraction of gelatin from cuttlefish skin

Cuttlefish skin was treated with 10 volumes of 0.05 N NaOH for 6 h with a gentle stirring at room temperature (26–28 °C). The solution was changed every 1 h for up to 6 h. Alkali treated skin was then washed with distilled water until the neutral pH of wash water was obtained. The prepared skin was subjected to bleaching with 10 volumes of 5% H_2O_2 at 4 °C for 48 h at room temperature and then washed with 10 volumes of water 3 times. Gelatin was extracted from the bleached skin using distilled water (60 °C) for 12 h with a sample/water ratio of 1:2 (w/v). During extraction, the mixture was stirred continuously. The extract was centrifuged at 8000×*g* for 30 min using a refrigerated centrifuge (Sorvall Model RC-B Plus, Newtown, CT, USA) to remove insoluble material. The supernatant was collected and freeze-dried using a freeze dryer (Model Dura-Top™ μP/Dura Dry™ μP, FTS® System, Inc., Stone Ridge, NY, USA).

2.4. Modification of cuttlefish skin gelatin by phenolic compounds

Cuttlefish skin gelatin was dissolved in distilled water containing 0.02% sodium azide (NaN_3) to obtain a final concentration of 1.2% protein. The pH of the gelatin solution was adjusted to 9 using 1 M NaOH. To prepare the solutions of phenolic compounds, caffeic acid, ferulic acid and tannic acid at the concentration of 2% (w/v) were dissolved in distilled water, followed by a pH adjustment to 9 with 1 M NaOH. Solutions were then bubbled with oxygen at 40 °C for 1 h to convert the phenolic compounds into oxidised form. To 75 ml of the gelatin solution, oxidised phenolic solutions at different concentrations (5%, 10%, 25% and 50%, based on protein content) were added. The final volume was raised to 90 ml using distilled water (pH 9) to obtain a final concentration of 1% protein. The mixture was stirred continuously using a magnetic stirrer (RO 10 power IKAMAG®, IKA LABORTECHNIK, Selangor, Malaysia) at a speed of 200 rpm at room temperature for 12 h. Thereafter, the samples were dialysed for 24 h against 20 volumes of water to remove free phenolic compounds (unbound to proteins). The control was prepared in the same manner except that phenolic compound was excluded. Gelatin–phenolic complexes were subjected to analyses.

2.5. Determination of free amino group content

The free amino group content of samples was determined according to the method of Benjakul and Morrissey (1997). To diluted samples (125 μl), 2.0 ml of 0.2125 M phosphate buffer, pH 8.2 and 1.0 ml of 0.01% TNBS solution were added. The solution was mixed thoroughly and placed in a temperature-controlled water bath (Model W350, Memmert, Schwabach, Germany) at 50 °C for 30 min in dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulphite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and free amino group content was expressed in terms of L-leucine.

2.6. Determination of total phenolic content

The total phenolic content of samples was measured according to the method of Slinkard and Singleton (1977) with some modifications. The sample solution (1 ml) was 100-fold diluted with deionised water prior to mixing with 200 μl of the freshly prepared Folin–Ciocalteu reagent using a vortex mixer. After 3 min, 3 ml of sodium carbonate (15%, w/v) were added and the mixture was allowed to stand for 30 min at room temperature. The absorbance at 760 nm was measured using a UV–vis spectrophotometer (UV-160, Shimadzu, Kyoto, Japan). The blank was prepared by using deionised water instead of Folin–Ciocalteu reagent. The total phenolic content was expressed as ΔA_{760} after blank substraction.

2.7. Determination of surface hydrophobicity

Surface hydrophobicity (S_0ANS) was determined as described by Benjakul, Seymour, Morrissey, and An (1997) using 1-anilinonaphthalene-8-sulphonic acid (ANS) as a probe. Gelatin solution (4 mg/ml) was diluted in 10 mM phosphate buffer (pH 6.0) containing 0.6 M NaCl to obtain the protein concentrations of 0.1%, 0.2%, 0.3% and 0.5%. The diluted protein solution (2 ml) was mixed with 20 μl of 8 mM ANS in 0.1 M phosphate buffer (pH 7.0). The fluorescence intensity of ANS-conjugates was immediately measured at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. The initial slope of the plot of fluorescence intensity versus protein concentration was referred to as S_0ANS.

2.8. Fourier transform infrared (FTIR) spectroscopy

The spectra of gelatin modified without and with oxidised phenolic compounds were obtained using a Bruker Model EQUINOX 55 FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with a deuterated l-alanine tri-glycine sulphate (DLATGS) detector (Bruker, Ettlingen, Germany). The Horizontal Attenuated Total Reflectance Accessory (HATR) was mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), which was made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at a resolution of 4 cm^{-1} and the measurement range was 4000–600 cm^{-1} (mid-IR region) at room temperature. Automatic signals were collected in

16 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean, empty cell at 25 °C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

2.9. Determination of emulsifying properties

Emulsion activity index (EAI) was determined according to the method of Pearce and Kinsella (1978) with a slight modification. Soybean oil (2 ml) and gelatin solution (1% protein, 6 ml) were homogenised using a homogeniser (model T25 basic, IKA LABORTECHNIK, Selangor, Malaysia) at a speed of 20,000 rpm for 1 min. Emulsions were pipetted out at 0 and 10 min and 100-fold diluted with 0.1% SDS. The mixture was mixed thoroughly for 10 s using a vortex mixer. A_{500} of the resulting dispersion was measured using a spectrophotometer (UV-160, Shimadzu, Kyoto, Japan). EAI at 0 and 10 min were calculated by the following formula:

$$\mathrm{EAI}(m^2/g) = (2 \times 2.303\,A)\mathrm{DF}/l\phi C$$

where A, A_{500}; l, path length of cuvette (m), DF, the dilution factor (100); ϕ, oil volume fraction and C, protein concentration in aqueous phase (g/m^3).

2.10. Determination of antioxidative activities

2.10.1. DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Binsan et al. (2008) with a slight modification. To the diluted sample (1.5 ml), 1.5 ml of 0.10 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) in 95% ethanol was added. The mixture was then mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a UV–vis spectrophotometer (UV-160, Shimadzu, Kyoto, Japan). The standard curve of Trolox (60–600 μM) was prepared in the same manner. The activity was expressed as μmol Trolox equivalent (TE)/mg protein.

2.10.2. ABTS radical scavenging activity

ABTS free radical scavenging assay was determined according to the method of Re et al. (1999) with a slight modification. 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radical was generated by reacting 7.4 mM ABTS and potassium persulfate (2.6 mM) at a ratio of 1:1 (v/v) and leaving at room temperature in the dark for 12 h. ABTS free radical solution was diluted by mixing 1 ml of ABTS solution with 50 ml of methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using an UV–vis spectrophotometer (UV-160, Shimadzu, Kyoto, Japan). To 150 μl of sample, 2850 μl of ABTS**.**+ solution was added and mixed thoroughly using a vortex mixer (Vortex genie® 2, Scientific Industries, Bohema, NY, USA). The extent of quenching of the ABTS**.**+ was measured at 734 nm after 2 h incubation at room temperature in dark. The standard curve of Trolox (60–600 μM) was prepared in the same manner. The activity was expressed as μmol TE/mg protein.

2.10.3. Ferric reducing antioxidant power (FRAP)

FRAP was determined as described by Benzie and Strain (1996) with a slight modification. 2.85 ml of freshly prepared FRAP solution (2.5 ml of a 10 mM TPTZ solution in 40 mM HCl plus 2.5 ml of 20 mM $FeCl_3 \cdot 6H_2O$ solution and 25 ml of 300 mM acetate buffer, pH 3.6) was incubated at 37 °C for 30 min before mixing with 150 μl of sample. The mixture was allowed to react in dark at room temperature. The absorbance was measured at 593 nm after 30 min of reaction. The standard curve of Trolox (60–600 μM) was prepared in the same manner. FRAP was expressed as μmol TE/mg protein.

2.10.4. Determination of Fe^{2+} chelating activity

Chelating activity towards Fe^{2+} was measured by the method of Boyer and McCleary (1987) with a slight modification. The diluted sample (4.7 ml) was mixed with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine. The reaction mixture was allowed to stand for 20 min at room temperature. The absorbance was then read at 562 nm. The blank was prepared in the same manner except that distilled water was used instead of the sample. The chelating activity was calculated as follows:

$$\text{Chelating activity} = [(B - A)/B] \times 100$$

where A is A_{562} of sample and B is A_{562} of the blank.

2.11. Effect of gelatin modified without and with oxidised tannic acid on emulsion stability and lipid oxidation of menhaden oil emulsion

Menhaden oil-in-water emulsions were prepared by homogenising the mixture of menhaden oil and gelatin solution (1:9, v/v) at a speed of 10,000 rpm for 2 min (IKA LABORTECNIK, model T25 basic, Selangor, Malaysia). Gelatin modified without and with oxidised tannic acid (5% of protein) at concentrations of 0.5% and 1% protein were used. These coarse emulsions were then passed through a two-stage high-pressure valve homogeniser (M-110P, Microfluidizer, Newton, MA, USA) at 10,000 psi 2 times. NaN_3 (0.02%) was added to the emulsions as an antimicrobial agent. The fish oil emulsions were then stored at room temperature (28–30 °C) for 12 days. The samples were taken every two days for analyses.

2.11.1. Measurement of zeta potential and particle size

Zeta potential and particle size of emulsions were determined with ZetaPlus zeta potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA) at room temperature. Prior to analysis, the samples were properly diluted with deionised water.

2.11.2. Measurement of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were determined as described by Buege and Aust (1978). Two ml of fish oil emulsion sample were dispersed in 10 ml of thiobarbituric acid solution (0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl). The mixture was heated in boiling water for 10 min, followed by cooling in running tap water. The mixture was centrifuged at 3600×g for 20 min at room temperature. The absorbance of the supernatant was measured at 532 nm. The standard curve was prepared using malonaldehyde (2–10 ppm) and TBARS were expressed as mg malonaldehyde/l.

2.12. Protein determination

Protein content was determined by the Biuret method (Robinson & Hodgen, 1940) using bovine serum albumin as a standard.

2.13. Statistical analysis

The experiments were run in triplicate. All data were subjected to Analysis of Variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test. For pair comparison, T-test was used (Steel & Torrie, 1980). The SPSS statistic program (Version 10.0) (SPSS Inc., Chicago, IL, USA) was used for data analysis.

3. Results and discussion

3.1. Modification of cuttlefish skin gelatin by oxidised phenolic compounds

3.1.1. Changes in free amino group content of gelatin

The free amino group contents of cuttlefish skin gelatin modified by different oxidised phenolic compounds at various levels

Table 1
Free amino group content and emulsifying properties of cuttlefish skin gelatin modified with different oxidised phenolic compound at various levels.

Oxidised phenolic compound	Concentration (%w/w of protei)	Free amino group content (mM)	Emulsion activity index (m^2/g)	
			0 min	10 min
Control (unmodified)		3.84 ± 0.12i*	20.86 ± 1.24f	10.58 ± 0.08 h
Ferulic acid	5	3.61 ± 0.19gh	19.93 ± 0.48def	10.67 ± 0.50 h
	10	3.61 ± 0.02gh	18.63 ± 0.63bcd	10.15 ± 0.47 h
	25	3.55 ± 0.14 g	18.63 ± 0.24bcd	8.95 ± 0.81 fg
	50	3.34 ± 0.03f	18.75 ± 0.95bcd	7.92 ± 0.99def
Caffeic acid	5	3.03 ± 0.05e	18.72 ± 0.57bcd	8.82 ± 0.61efg
	10	2.73 ± 0.02d	18.48 ± 0.87bcd	7.77 ± 0.59de
	25	1.98 ± 0.05b	18.04 ± 0.68bc	7.23 ± 0.80 cd
	50	1.46 ± 0.13a	17.99 ± 0.60bc	6.57 ± 0.38bc
Tannic acid	5	3.79 ± 0.17hi	20.25 ± 0.87ef	9.89 ± 0.28gh
	10	3.08 ± 0.05e	19.38 ± 0.97cde	7.19 ± 0.62 cd
	25	2.68 ± 0.02d	17.44 ± 0.60b	5.63 ± 0.97b
	50	2.43 ± 0.02c	13.04 ± 0.68a	4.37 ± 0.68a

Mean ± SD from triplicate determinations.
* Different letters in the same column indicate significant differences ($p < 0.05$).

are shown in Table 1. Gelatin modified by oxidised phenolic compounds had the lower free amino group content than the control gelatin (without oxidised phenolic compound) ($p < 0.05$). It indicated that nucleophilic amino groups might interact with electrophilic quinone, an oxidised form of phenolic compounds. A reaction of protein with oxidised phenolic compounds led to a decrease in free amino group content (Rawel, Kroll, & Rohn, 2001). Furthermore, the oxidised phenolic compound can react with other nucleophilic groups, such as tryptophan, cysteine, methionine, histidine, tyrosine and *N*-terminal proline of the protein molecule (Kroll, Rawel, & Rohn, 2003). The rate of loss in free amino group of gelatin depended on the type and concentration of oxidised phenolic compounds used. At the same level of oxidised phenolic compounds, the higher loss in free amino groups was observed in gelatin modified by oxidised caffeic acid ($p < 0.05$), followed by that found in gelatin modified with oxidised tannic acid and oxidised ferulic acid, respectively. In general, the loss in free amino groups was increased when the concentration of oxidised phenolic compounds used increased ($p < 0.05$). Oxidised caffeic acid (MW ~ 180) exhibited the higher interaction with amino groups of gelatin, compared with oxidised tannic acid (MW ~ 1,701). The smaller molecules might disperse uniformly and could interact with amino groups of gelatin more effectively. Although oxidised ferulic acid (MW ~ 194) had the similar molecular mass to oxidised caffeic acid, the loss in free amino groups of gelatin modified with oxidised ferulic acid was lower. Ferulic acid, with one hydroxyl of caffeic acid replaced by $-OCH_3$, is considerably less reactive. Rawel et al. (2001) reported that ferulic acid cannot be oxidised to its corresponding quinone derivative. The formation of a semiquinone radical with lower reactivity was presumed, compared with oxidised caffeic acid and oxidised tannic acid. Quinone is known as an electrophilic compound, which can interact with nucleophilic amino group of proteins (Kroll et al., 2003).

3.2. Changes in total phenol content

Total phenol contents of cuttlefish skin gelatin modified by different oxidised phenolic compounds at various levels determined by Folin–Ciocalteu method are expressed as A_{760} (Fig. 1A). The increases in A_{760} were observed in gelatin modified with all oxidised phenolic compounds when the levels of introduced compounds increased ($p < 0.05$). This result indicated that oxidised phenolic compounds were incorporated with gelatin molecules to a higher extent when the higher level of oxidised phenols was introduced. At the same level, no differences in A_{760} were noticeable between

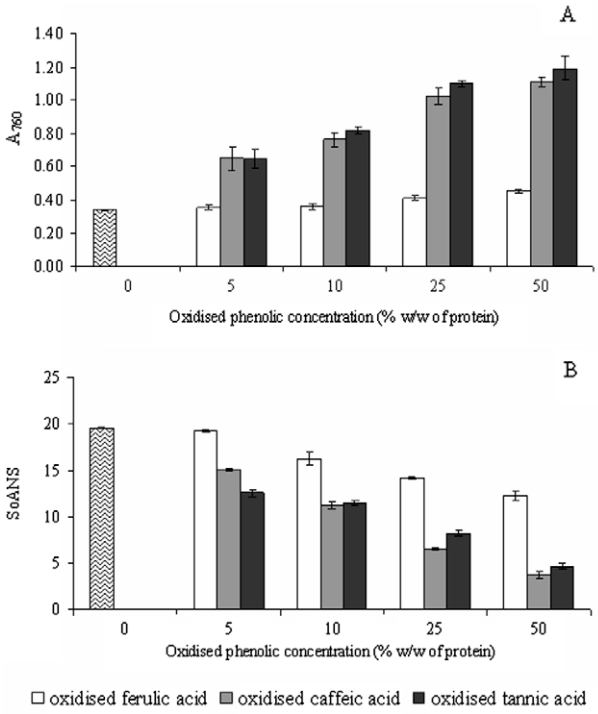

Fig. 1. A_{760} (A) and surface hydrophobicity (B) of cuttlefish skin gelatin modified with different oxidised phenolic compounds at various levels. Bars represent the standard deviation from triplicate determinations.

gelatin modified with oxidised caffeic acid and oxidised tannic acid ($p > 0.05$). A_{760} of gelatin modified by oxidised ferulic acid was lowest ($p < 0.05$). This result was associated with the lowest loss in the free amino groups of gelatin modified by oxidised ferulic acid. The result suggested that oxidised ferulic acid could incorporate with amino group of protein to some extent. Although the loss in free amino groups of gelatin modified with oxidised tannic acid was less than that of gelatin modified by oxidised caffeic acid (Table 1), no differences in A_{760} were observed between gelatin modified with both oxidised compounds. Tannic acid possessed a greater number of hydroxyl groups attached to the aromatic benzene ring as compared to caffeic acid. Those hydroxyl groups were converted to quinone, which was able to interact with the amino groups of gelatin molecules. During dialysis to remove unbound oxidised

phenols, the reduction of free quinone groups to hydroxyl groups of phenolic compounds attached to gelatin could probably take place to some degree. The similar number of reduced free hydroxyl groups between gelatin modified by oxidised caffeic acid and oxidised tannic acid more likely contributed to the similar reducing ability of Folin–Ciocalteu reagent, as indicated by the similar A_{760} between both modified gelatins. A_{760} observed in the control gelatin solution was more likely due to the presence of amino acid residues including tyrosine, tryptophan, cysteine, histidine and asparagines (Lowry, Rosebrough, Farr, & Randall, 1951). Those amino acids were able to reduce the Folin–Ciocalteu reagent as evidenced by the increase in A_{760}.

3.3. Changes in surface hydrophobicity

Changes in surface hydrophobicity of cuttlefish skin gelatin modified with different oxidised phenolic compounds at various levels are shown in Fig. 1B. Generally, gelatin modified with oxidised phenolic compounds showed a decrease in surface hydrophobicity, compared with the control gelatin ($p < 0.05$). The surface hydrophobicity of gelatin modified with all oxidised phenolic compounds decreased with increasing concentrations of compounds used ($p < 0.05$). ANS, a fluorescence probe, has been found to bind to hydrophobic amino acids containing an aromatic ring, such as phenylalanine and tryptophan, and can be used to indicate the surface hydrophobicity of protein (Benjakul, Seymour, Morrissey, & An, 1997). The decrease in surface hydrophobicity of the resulting gelatin indicated that phenolic compounds bound with gelatin most likely contributed to the increased hydrophilicity at the surface of gelatin molecules. This result was in agreement with Rawel, Czajka, Rohn, and Kroll (2002) who reported that the surface hydrophobicity of soy protein decreased when reacted with phenolic compounds. The decrease in surface hydrophobicity of gelatin derivatives was possibly caused by hydroxyl and carboxyl groups of phenolic compounds attached to gelatin. Kroll et al. (2003) reported that the covalent attachment of the phenolic compound to proteins causes the blocking of the hydrophilic groups like amino and thiol groups. On the other hand, there is also an increase in the amount of apolar groups (benzene ring) and polar groups (hydroxyl and carboxyl groups as in the case of the phenolic acids) being introduced. Amongst all phenolic compounds introduced, gelatin modified with oxidised caffeic acid or oxidised tannic acid had the lower surface hydrophobicity than that modified by oxidised ferulic acid ($p < 0.05$). At the same amount of oxidised phenolic compounds introduced, gelatin modified with oxidised tannic acid and oxidised caffeic acid exhibited similar surface hydrophobicity ($p > 0.05$). Thus, the introduction of oxidised phenolic compounds resulted in the altered hydrophobicity of resulting gelatin.

3.4. Changes in emulsifying properties

Emulsion activity index (EAI) of cuttlefish skin gelatin modified with oxidised phenolic compounds at various levels expressed as the turbidity of emulsion at wavelength of 500 nm is shown in Table 1. At the lowest level of oxidised phenolic compounds introduced (5 wt.% of protein), gelatins modified with oxidised caffeic acid showed a decrease in EAI both at 0 and 10 min when compared with the control ($p < 0.05$). Nevertheless, no change in EAI of gelatin modified with oxidised tannic acid and oxidised ferulic acid was observed ($p > 0.05$). However, at the concentration of oxidised phenolic compounds introduced above 5% (w/w of protein), EAI of gelatin modified with all oxidised phenolic compounds decreased for both at 0 and 10 min. The decrease was more pronounced as the concentration of oxidised phenolic compounds increased. This was possibly associated with the decreases in surface hydrophobicity of gelatin modified with oxidised phenolic. Kato and Nakai (1980) reported that the surface hydrophobicity of protein is generally associated with a better surface activity, in which the reduction in interfacial tension and the increase in emulsifying activity are achieved. The decrease in surface hydrophobicity of gelatin modified with oxidised phenolic compounds might lower the ability of gelatin to localise at the oil–water interface. Moreover, interactions of oxidised phenolic compounds with gelatin probably led to the aggregation of gelatin molecules. As a result, gelatin might not unfold at the oil–water interface and form the film around the oil droplet effectively (McClements, 2004). However, the improvement of foam and emulsion stability of proteins by phenolic compounds has been reported (Viljanen, Halmos, Sinclair, & Heinonen, 2005). The addition of blackberry and raspberry juices in oil-in-water emulsion using whey proteins as an emulsifier resulted in the enhanced stabilisation of the interface formed during emulsification. Sarker et al. (1995) reported that (+)-catechin, at 0.1 M ratio, could improve foam formation and stability of Tween 20/β-lactoglobulin mixed system, because of (+)-catechin-induced cross-linking of proteins in the adsorbed layer. Therefore, the incorporation of oxidised phenolic compounds into gelatin to a high level showed the detrimental effect on the emulsifying property of modified gelatin.

3.5. Changes in antioxidative activities

3.5.1. DPPH radical scavenging activity

DPPH radical scavenging activities of gelatin modified with different oxidised phenolic compounds at various levels are depicted in Fig. 2A. DPPH radical scavenging activity of the control gelatin was 249.87 μmol TE/mg protein. When gelatin was incorporated with 5% oxidised ferulic acid, caffeic acid and tannic acid, the activity was increased to 292.57, 420.69 and 491.68 μmol TE/mg protein, respectively. These results indicated that phenolic compounds introduced to gelatin contributed to the increased antioxidative activity of gelatin. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen-donating ability (Binsan et al., 2008). The increase in DPPH radical scavenging activity was observed in all gelatins modified with all oxidised phenolic compounds when the higher concentrations of oxidised phenolic compound were used ($p < 0.05$). The maximum activity was observed when gelatin was modified with 50% oxidised phenolic compound ($p < 0.05$). This was in agreement with Rohn et al. (2004) who found that the antioxidative activity of bovine serum albumin (BSA) derivatised by covalent attachment of quercetin increased with increasing concentration of quercetin.

At the same concentration of oxidised phenolic compounds used, gelatin modified with oxidised tannic acid had the highest DPPH radical scavenging activity, followed by those modified with oxidised caffeic acid and oxidised ferulic acid, respectively. The result revealed that DPPH radical scavenging activity of gelatin modified by oxidised phenolic compounds was governed by the type of phenolic compound used and the degree of incorporation of phenolic compounds into gelatin. The result indicated that free quinones of oxidised phenolic compounds were mostly reduced to hydroxyl groups. Those hydroxyl groups could donate hydrogen to DPPH radicals.

3.5.2. ABTS radical scavenging activity

Gelatin modified with different oxidised phenolic compounds showed different ABTS radical scavenging activities (Fig. 2B). A similar result was found when comparing with DPPH radical scavenging activity (Fig. 2A). The gelatin modified with oxidised tannic acid and oxidised caffeic acid showed an increase in ABTS radical scavenging activities with increasing concentration used. This result suggested that tannic acid and caffeic acid attached with gel-

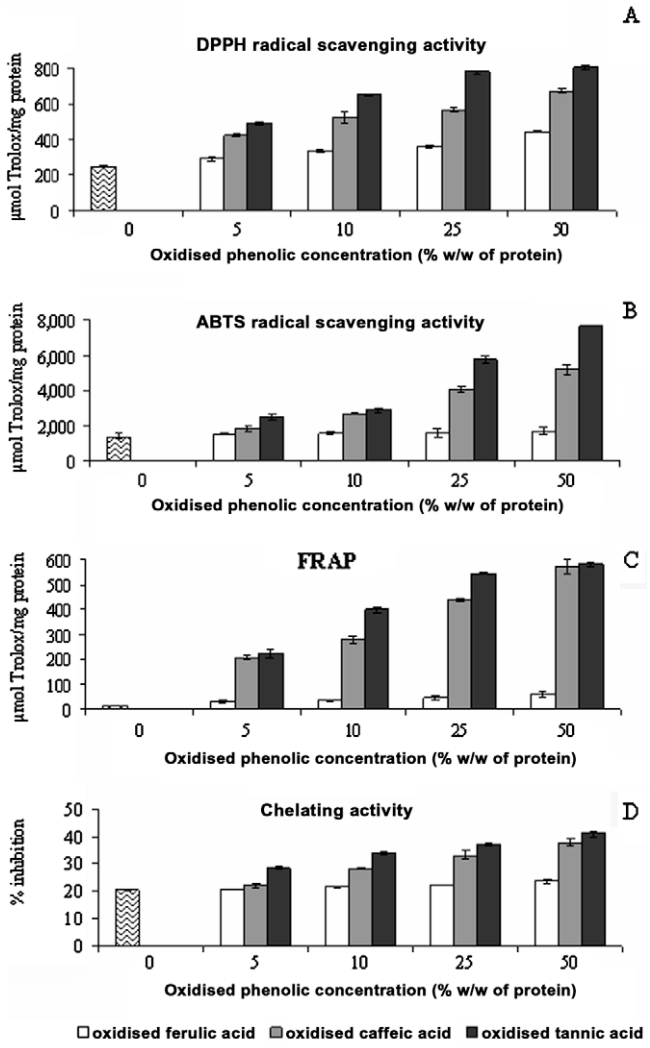

Fig. 2. DPPH radical scavenging activity (A), ABTS radical scavenging activity (B), ferric reducing antioxidant power (FRAP) (C) and chelating activity (D) of cuttlefish skin gelatin modified with different oxidised phenolic compounds at various levels. Bars represent the standard deviation from triplicate determinations.

atin could scavenge the radicals, forming a more stable product, thereby terminating the radical chain reaction. Nevertheless, gelatin modified with oxidised ferulic acid had no changes in ABTS radical scavenging activity even when a higher level of oxidised ferulic acid was used. The result suggested that ferulic acid attached to gelatin might not be able to quench ABTS radicals. ABTS radical assay is an excellent tool for determining the antioxidative activity, in which the radical is quenched to form ABTS radical complex (Binsan et al., 2008). It reconfirmed that the radical scavenging activity of gelatin was determined by the type of phenolic compounds attached.

3.5.3. Ferric reducing antioxidant power

Ferric reducing antioxidant power (FRAP) generally measures the reducing capacity of the ferric ion and has been correlated to the radical scavenging capacity (Moure, Domínguez, & Parajó, 2006). The FRAP of gelatin modified with different oxidised phenolic compounds at various levels is shown in Fig. 2C. The FRAP of gelatin modified with oxidised ferulic acid, oxidised caffeic acid and oxidised tannic acid at a level of 5% was 28.08, 206.71 and 222.72 µmol TE/mg protein, respectively, which was higher than that of the control gelatin (13.20 µmol TE/mg protein). As the concentration of oxidised phenolic compounds increased, FRAP activities increased ($p < 0.05$). A marked increase in FRAP of gelatin was observed in gelatin modified with oxidised caffeic acid and oxidised tannic acid, in comparison with the control gelatin, especially at the high concentration used. It was noted that the FRAP of gelatin modified with oxidised ferulic acid slightly increased. The antioxidative activity of phenolic acids depends on the number and position of hydroxyl groups bound to the aromatic ring (Sroka & Cisowski, 2003). The result was generally in agreement with those of DPPH and ABTS radical scavenging activities (Fig. 2A and B).

3.5.4. Chelating activity

The ability of gelatin modified with different oxidised phenolic compounds at different levels in metal chelating is depicted in Fig. 2D. Generally, the metal chelating activity of gelatin increased as the concentration of oxidised phenolic compounds increased ($p < 0.05$). At the same level of oxidised compounds introduced, gelatin modified with oxidised tannic acid exhibited the highest metal chelating activity, followed by those modified by oxidised caffeic acid and oxidised ferulic acid, respectively. Ferrous ion (Fe^{2+}) is the most powerful prooxidant amongst various species of metal ions, which can catalyse the generation of reactive oxygen species, hydroxyl radical ($OH^{\cdot}$), by which the lipid peroxidation chain reaction is accelerated (Stohs & Bagchi, 1995). Thus gelatin attached with phenolic compounds had the enhanced ability in metal chelation, in which prooxidative metals can be sequestered, leading to the lowered oxidation.

3.6. Changes in FTIR spectra

Due to the high antioxidative activity of gelatin modified with 5% oxidised tannic acid without any change in EAI, gelatin modified with 5% oxidised tannic acid was characterised by FTIR. FTIR spectroscopy at 4000–400 cm^{-1} was used to monitor the changes in the secondary structure of gelatin with and without the modification with 5% oxidised tannic acid (Fig. 3A). A spectra of cuttlefish skin

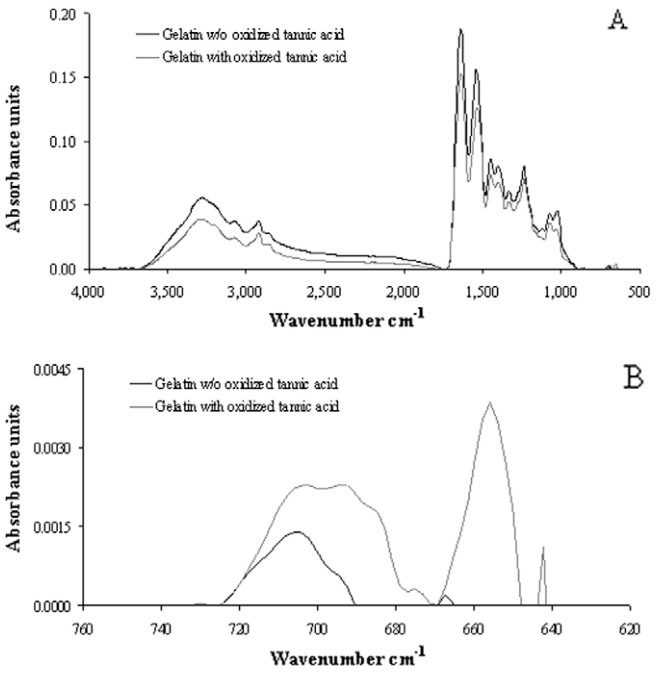

Fig. 3. Fourier transform infrared (FTIR) spectroscopic spectra of gelatin modified with 5% oxidised tannic acid.

gelatin displayed the major bands at 3278 cm^{-1} (amide A, representative of NH-stretching, coupled with hydrogen bonding), 1635 cm^{-1} (amide I, representative of C=O stretching/hydrogen bonding coupled with COO^-), 1535 cm^{-1} (amide II, representative of NH bending coupled with CN stretching) and 1236 cm^{-1} (amide III, representative of NH bending). Similar spectra were observed between gelatin with and without modification with 5% oxidised tannic acid. However, decreases in the amplitude of amide A, I, II and III bands was observed in gelatin modified with oxidised tannic acid. These changes are indicative of greater disorder (Friess & Lee, 1996) in gelatin and are associated with the loss of the triple helix state (Muyonga, Cole, & Duodu, 2004). The shift to a lower wavenumber of amine I, II and III peaks was observed from 1637, 1541 and 1238 cm^{-1} in the control gelatin to 1635, 1535 and 1236 cm^{-1} in gelatin modified with oxidised tannic acid, respectively. A shift of peaks to lower wavenumbers is associated with a lower molecular order (Payne & Veis, 1988). Muyonga et al. (2004) reported that the amide I and II peak of collagen extracted from adult Nile perch was at a higher frequency than the young fish skin collagen, due to more intermolecular cross-links in the adult fish collagen (Muyonga et al., 2004). On the other hand, the shift to a higher wavenumber of amine A peaks from 3278 cm^{-1} to 3296 cm^{-1} was observed when gelatin was modified with oxidised tannic acid. The peaks at wavenumber of 3400–3300 cm^{-1} were attributable to the OH stretch of alcohols and phenols (Smith, 1999). The result indicated the incorporation of tannic acid with a OH group into gelatin molecule. Moreover, the obvious increases in the amplitude of the peak at wavenumber 1080 cm^{-1} was found in gelatin modified with oxidised tannic acid, suggesting the stretching of the C–O bonds in secondary and tertiary alcohols (Smith, 1999) in modified gelatin. These results suggested that oxidised tannic acid might induce the changes in secondary structure and functional groups of the resulting gelatin.

The bands situated at 720–680 cm^{-1} and 655 cm^{-1} were observed in gelatin modified with oxidised tannic acid, whilst the band with a wavenumber of 720–690 cm^{-1} was found in the control gelatin. (Fig. 3B). The wavenumber at ~690 ± 10 and ~655 cm^{-1} were attributable to the bending of the C–C bonds in the aromatic and the out of plane bending of OH group in phenol, respectively (Smith, 1999). This result indicated that oxidised tannic acid could bind with the gelatin molecules and the unbound quinone groups were more likely reduced during dialysis as indicated by the additional OH band in phenol found in gelatin modified by oxidised tannic acid. Thus the modification could introduce the aromatic ring and hydroxyl group in gelatin.

3.7. Effect of gelatin modified with oxidised tannic acid on emulsion stability and lipid oxidation of menhaden oil-in-water emulsion

3.7.1. Zeta potential and particle size of emulsion

When tested at pH 7, zeta potential of menhaden oil emulsion droplets stabilised by control gelatin (without modification) and gelatin modified with oxidised tannic acid was −38.98 ± 0.77 and −32.16 ± 1.2 mV, respectively (Table 2). The negative charge of the emulsion droplets might be due to the negatively charged amino acid in the gelatin surrounding the oil droplet. At a pH lower than pI, the carboxyl groups are negatively charged (–COO^-), whereas most of the amino groups are neutral (–NH_2) (Onsaard, Vittayanont, Srigam, & McClements, 2006). The emulsion droplets were covered by a biopolymer with an appreciable electrical charge. Electrostatic repulsion may play an important role in stabilising them against droplet aggregation (McClements, 2005). The lower negative charge was observed in emulsion stabilised by gelatin modified with oxidised tannic acid, when compared with that of emulsion stabilised by control gelatin. These differences might be due to the differences in the number of ionisable amino acids in the gelatin. It was suggested that the occupation of amino groups by oxidised tannic acid might introduce the positive charge of phenolic compound to gelatin. As a consequence, the lower negatively charged complex was obtained. Moreover, the hydroxyl groups of tannic acid might interact with the carboxyl groups of protein *via* hydrogen bonding, leading to a loss of negative charge of the resulting complex.

Table 2
Particle size and zeta potential of menhaden oil-in-water emulsion stabilised by 0.5% and 1.0% gelatin with and without the modification with 5% oxidised tannic acid at pH 7.

Gelatin	Concentration (% protein)	Particle size (μm)	Zeta potential (mV)
Control (unmodified)	0.5	2.24 ± 0.19c*	–
	1.0	0.33 ± 0.02a	−38.98 ± 0.77a
Modified with 5% oxidised tannic acid	0.5	2.65 ± 0.09d	–
	1.0	0.37 ± 0.02b	−32.16 ± 1.2b

Mean ± SD from triplicate determinations.
* Different letters in the same column indicate significant differences ($p < 0.05$).

The particle diameter of oil droplets of 10% menhaden oil-in-water emulsion stabilised by 0.5% and 1.0% gelatin modified without and with oxidised tannic acid is shown in Table 2. The particle size of emulsion stabilised by 0.5% and 1.0% control gelatin was 2.24 ± 0.19 and 0.33 ± 0.02 μm, respectively, whereas that of emulsion stabilised by gelatin modified with oxidised tannic acid was 2.65 ± 0.09 and 0.37 ± 0.02 μm, respectively. Droplet size of the emulsion decreased markedly when the higher concentration of gelatin either with or without modification was used. A sufficient amount of proteins at the oil–water interface is required to cover oil droplet completely. This result was in agreement with Surh, Decker, and McClements (2005) who found that an increase in fish gelatin concentration from 0.5% to 4.0% protein could increase the fraction of small droplets and decrease the fraction of large particles. When the protein amount is limited, there is no longer sufficient protein to fully stabilise the droplet interface, and therefore larger particles may be formed as a result of coalescence or bridging flocculation. The increase in protein concentration enhanced protein adsorption and surface coverage of oil droplets, which effectively inhibited droplet aggregation or coalescence (Sun & Gunasekaran, 2009). The larger particle size was observed in the emulsion stabilised by gelatin modified with oxidised tannic acid when compared with that found in the emulsion stabilised by the control gelatin. The decreased surface hydrophobicity might not favour the migration of gelatin modified by oxidised tannic acid to the oil droplets, which were lipophilic. Thus, the amount of modified gelatin occupied at oil droplet might be slightly lower. This led to the lower emulsifying property of modified gelatin.

3.7.2. Changes in particle size of emulsion during storage

Changes in particle size of emulsions stabilised by 1.0% gelatin and gelatin modified with oxidised tannic acid during storage at room temperature for 12 days are shown in Fig. 4A. Particle size of both emulsions gradually increased with increasing storage time. For emulsions stabilised by the control gelatin, the particle size increased from 0.33 ± 0.02 to 0.44 ± 0.02 μm within 12 days of storage, whereas that of the emulsion stabilised by gelatin modified with oxidised tannic acid increased from 0.37 ± 0.02 to 0.46 ± 0.03 μm. This result might be due to individual droplet growth (Ostwald ripening) or droplet aggregation (flocculation or coalescence) during storage (Djordjevic et al., 2008). At day 12 of storage, no difference in the size of the oil droplet was noticeable between both emulsions though a smaller size of droplet was found in the emulsion stabilised by control gelatin at day 0. This indicated that gelatin modified by oxidised tannic acid yielded

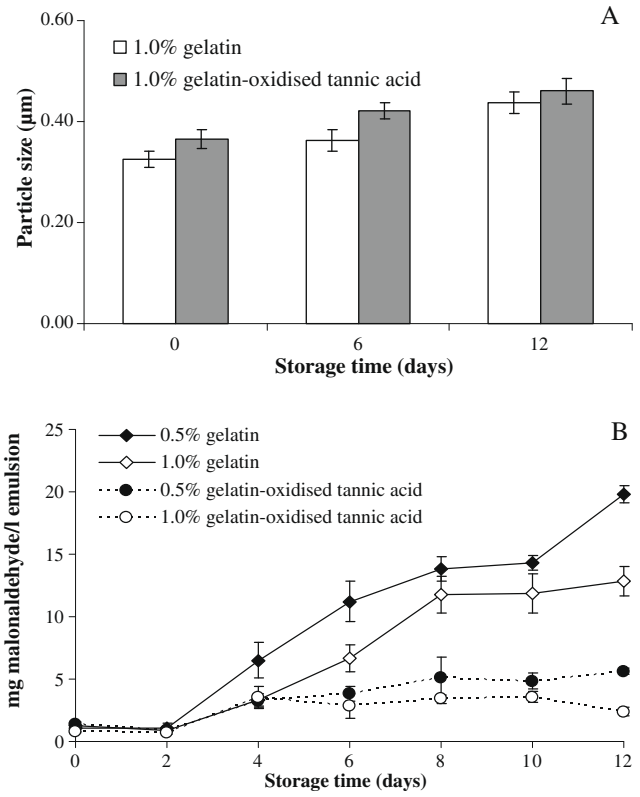

Fig. 4. Particle size (A) and TBARS value (B) of menhaden oil-in-water emulsion stabilised by 0.5% and 1.0% gelatin with and without the modification with 5% oxidised tannic acid during storage at room temperature for 12 days. Bars represent the standard deviation from triplicate determinations.

the similar emulsion stability to gelatin without modification at the extended storage.

3.7.3. Changes in lipid oxidation of emulsion during storage

Lipid oxidation of menhaden oil-in-water emulsion stabilised with gelatin modified with and without oxidised tannic acid was monitored and expressed as TBARS values during the 12 days of storage (Fig. 4B). Generally, no TBARS were found in the oil-in-water emulsion within the first 2 days of storage. Thereafter, the marked increase in TBARS was observed, especially in emulsion stabilised by control gelatin ($p < 0.05$). Emulsion stabilised by gelatin modified with oxidised tannic acid had the lower TBARS ($p < 0.05$). This result was in accordance with the antioxidative activity of gelatin modified with oxidised tannic acid (Fig. 2). Antioxidative activity of gelatin modified with or without oxidised tannic acid increased with increasing concentration used. Additionally, the preventive effect of gelatin at the higher level used on lipid oxidation might be associated with the thicker film around the oil droplet. This could prevent the penetration of oxygen into an oil droplet more effectively. Therefore, gelatin modified with oxidised tannic acid had the ability to retard TBARS formation in the oil-in-water emulsion system. This result was in agreement with Almajano and Gordon (2004) who reported that interaction of BSA and epigallocatechin gallate (EGCG) could induce the formation of a BSA-antioxidant adduct and cause a synergistic increase in the oxidative stability of sunflower oil-in-water emulsion effectively, when compared with BSA or EGCG alone.

4. Conclusion

Attachment of 5% oxidised phenolic compounds to cuttlefish skin gelatin enhanced antioxidative activity with no detrimental effect on emulsifying properties of resulting gelatin. Use of gelatin modified with oxidised tannic acid could inhibit lipid oxidation of menhaden oil-in-water emulsion effectively. The efficacy was dose dependent. Therefore, cuttlefish skin gelatin modified with oxidised tannic acid could be used as emulsifier possessing antioxidative activity in emulsion systems.

Acknowledgement

The authors would like to express their sincere thanks to Strategic Scholarship for Frontier Research Network of Thailand Commission of Higher Education to Tanong Aewsiri for the financial support.

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Food Chemistry 117 (2009) 169–173

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Composition of subcutaneous adipose tissue of dry-cured pork forelegs as affected by desalting and boiling: The effects of vacuum-packaging

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ARTICLE INFO

Article history:
Received 28 July 2008
Received in revised form 18 February 2009
Accepted 25 March 2009

Keywords:
Meat products
Subcutaneous fat
Culinary treatment
Sous-vide
Fatty acids

ABSTRACT

The effects of desalting and boiling, with or without vacuum-packaging, on the composition of the subcutaneous adipose tissue of dry-cured pork forelegs were investigated. The adipose tissue contained 7.1% water and 91.0% lipids. The main fatty acids in glycerides (about 89% of total lipids) were oleic (39.6%), palmitic (23.9%), linoleic (13.7%) and stearic (12.6%) acids. The main fatty acids in free fatty acids (about 11% of total lipids) were oleic (36.0%), linoleic (28.4%) and palmitic (13.1%). The culinary treatment (desalting and boiling) caused significant decreases in dry matter and lipid contents. These decreases were not as great in vacuum-packaged samples because the lipid losses were prevented during the boiling stage. No significant changes in lipid fraction proportions (glycerides and free fatty acids) were observed. The samples boiled with vacuum-packaging showed higher contents of polyunsaturated fatty acids than did the samples boiled without vacuum-packaging.

1. Introduction

The subcutaneous adipose tissue is an important component of dry-cured products and is normally eaten by consumers. Its fatty acid composition and its changes (lipolysis and oxidation) during the manufacturing process influence the acceptance of these products by consumers (Gandemer, 2002). The dry-cured pork foreleg ("Lacón Gallego") is manufactured in the northwest of Spain (Galicia). This product is made by following the same steps as in dry-cured ham, although the length of the process is shorter. The studies on the composition of dry-cured pork forelegs have been done only on muscles (Veiga, Cobos, Ros, & Díaz, 2003) or on whole pieces with no bones or skin (Lorenzo, García Fontán, Franco, & Carballo, 2008; Lorenzo, Prieto, Carballo, & Franco, 2003; Rodríguez, Carballo, & López, 2001). However, there are no studies on the composition of the subcutaneous adipose tissue of dry-cured pork forelegs. The lipid composition and the changes that take place during processing are different, in subcutaneous fat and muscle lipids, in dry-cured pork products. During the processing of dry-cured hams, the hydrolysis of glycerides in the subcutaneous fat is very important (Coutron-Gambotti & Gandemer, 1999; Flores, Nieto, Bermell, & Miralles, 1985; Narváez-Rivas, Vicario, Constante, & León-Camacho, 2007). In muscles of dry-cured pork forelegs, meat lipids undergo lipolysis and oxidation; the former affects only the phospholipid fraction (Veiga et al., 2003). However, the levels of phospholipids in subcutaneous fat are very low (Flores et al., 1985; Perona & Ruiz-Gutierrez, 2005); so, the lipolysis of this fraction is negligible.

"Lacón Gallego" is usually consumed after a process in which it is desalted and boiled in a restaurant or at home and people normally eat subcutaneous adipose tissue and muscles together with other ingredients, mainly vegetables. This culinary treatment can be done on the whole forelegs (Cobos, Veiga, & Díaz, 2004). However, the desalting and cooking of dry-cured pork foreleg pieces decreases the total time necessary to desalt and boil them; moreover, the *sous-vide* cooking technology (boiling the pieces after vacuum-packaging) can be used. This technology excludes oxygen; thus, it might prevent or reduce the oxidation process during the boiling of the dry-cured pork foreleg pieces. The effects of this culinary treatment on the chemical and lipid composition of dry-cured pork forelegs have been studied in muscles (Cobos, Veiga, & Díaz, 2008) and only the ash contents were significantly different between samples boiled with and without vacuum-packaging. However, the effects in subcutaneous adipose tissue might be different from those in muscle due to their different compositions and/or to their oxygen access.

One objective of this study was to know the composition of the subcutaneous adipose tissue of dry-cured pork forelegs. Furthermore, the effects of desalting and boiling, with or without vacuum-packaging, on the composition of the subcutaneous adipose tissue of deboned pieces of dry-cured pork forelegs were studied.

2. Materials and methods

2.1. Processing of forelegs and sampling

Four dry-cured pork forelegs were manufactured, as described by Cobos et al. (2004), by a local industry. Three deboned portions

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doi:10.1016/j.foodchem.2009.03.093

of similar size and shape (ca. 500 g), including the skin, subcutaneous adipose tissue and *pectoralis profundus*, *coracobrachialis*, *biceps brachii* and *triceps brachii* muscles, were obtained from each dry-cured pork foreleg. From one portion, a sample of subcutaneous adipose tissue was taken, while the other two whole portions were desalted. The desalting was done in water at 4 °C for 24 h (4 l of water/kg piece); the water was changed after 10 h. Afterwards, one of the desalted portions was vacuum-packed in a polypropylene/polyamide laminated pouch, using a Stephan Alval vacuum-packer (mod. 190). Both desalted portions were inserted directly into boiling water (11 l of water/kg piece) and cooked for 2.5 h. After they were cooled, samples of subcutaneous adipose tissue were taken from each portion.

2.2. Analytical methods

The subcutaneous adipose tissue samples were finely minced in a blender (Polytron PT 10–35). AOAC methods (1995) were used for dry matter and ash determinations. The lipids were extracted and purified from the former homogenised sample according to the Bligh and Dyer method (Hanson & Olley, 1963). The total lipids were gravimetrically determined; 50 mg of lipids were taken for the determination of cholesterol content by an enzymic method, using the laboratory kit from Sigma Diagnostics. The extent of lipid oxidation was assessed by the thiobarbituric acid (TBA) method described by Pikul, Leszczynski, and Kummerow (1983).

Total lipids (100 mg) were separated into neutral lipids, free fatty acids and phospholipids in NH_2-aminopropyl minicolumns according to the method described by Kaluzny, Duncan, Merritt, and Epps (1985). The neutral lipid fraction is mainly composed of glycerides and this term will be used throughout the text. Amounts of glycerides, phospholipids and free fatty acids were quantified by weighing (Vaghela & Kilara, 1995) and the results were expressed as a percent of the total weight obtained. The fatty acid compositions of glycerides and free fatty acids were determined by gas liquid chromatography of methyl esters prepared under basic conditions (KOH: methanol) for glycerides and acidic conditions (H_2SO_4: methanol) for the free fatty acids. The gas chromatograph was a Hewlett–Packard apparatus (HP 5890) equipped with a dual flame ionisation detector. The fused silica capillary column (30 m, internal diameter 0.25 mm) was packed with OV-225 (0.1 μm). Analyses were performed using an initial isothermic period (150 °C, 2 min); thereafter the temperature was raised to 210 °C at an increasing rate of 4 °C/min, and finally held at 210 °C for 15 min. The injector and detector were maintained at 250 °C. For quantitative analyses, a Hewlett–Packard HP3394A integrator was used. The identification of different fatty acid methyl esters was performed by comparison of the retention times with those of authentic standards (Sigma Chemical Co., St. Louis, MO). Amounts of fatty acids from glycerides and free fatty acids were expressed as a percent of the total area of injected methyl esters.

2.3. Statistical analyses

The means were compared by *t*-test for dependent samples. A significance level of $p < 0.05$ was used for all means evaluations (SPSS version 10.1. for Windows, 2000).

3. Results and discussion

3.1. Chemical and lipid composition

Table 1 shows the chemical and lipid composition of subcutaneous adipose tissue from dry-cured pork foreleg pieces (DF) and from dry-cured foreleg pieces subjected to culinary treatment by desalting and boiling (BDF) or by desalting and vacuum-packed boiling (VBDF).

Table 1
Chemical and lipid composition (means ± SD) of subcutaneous adipose tissue from dry-cured foreleg pieces (DF) and from dry-cured pieces subjected to culinary treatment by desalting and boiling (BDF) or by desalting and vacuum-packed boiling (VBDF) ($n = 4$).

	DF	BDF	VBDF
Dry matter (g/100 g tissue)	92.89 ± 3.16a	81.11 ± 3.28b	87.32 ± 2.47b
Ash (g/100 g tissue)	1.51 ± 0.66ab	0.80 ± 0.42a	1.26 ± 0.20b
Ash (g/100 g dry matter)	1.64 ± 0.75	0.99 ± 0.57	1.45 ± 0.23
Lipids (g/100 g tissue)	91.02 ± 3.41a	75.22 ± 3.82b	85.94 ± 2.41c
Lipids (g/100 g dry matter)	97.99 ± 1.16a	92.73 ± 2.48b	98.42 ± 0.57a
Cholesterol (g/100 g lipids)	0.11 ± 0.02	0.08 ± 0.04	0.11 ± 0.05
Cholesterol (mg/100 g tissue)	92.5 ± 15.07	58.9 ± 27.55	95.4 ± 37.56
TBA value (mg MDA/kg lipids)	10.2 ± 5.01	5.23 ± 3.18	8.42 ± 2.83
TBA value (mg MDA/kg tissue)	9.22 ± 4.36ab	3.85 ± 2.11a	7.21 ± 2.36b
Glycerides (g/100 g lipids)	88.7 ± 2.68	90.6 ± 0.84	92.4 ± 2.08
Free fatty acids (g/100 g lipids)	11.1 ± 2.69	9.44 ± 0.72	7.60 ± 2.08

Means with different letters in the same row differ significantly ($p < 0.05$).
TBA: thiobarbituric acid; MDA: malonaldehyde.

The subcutaneous adipose tissue of dry-cured pork forelegs contained 7.11% water, 91.02% lipids and 1.51% ash. Cholesterol accounted for 0.11% of total lipids. The cholesterol content was close to those obtained for intermuscular fat in pork (Chizzolini, Zanardi, Dorigoni, & Ghidini, 1999).

The mean value of TBA was 10.2 mg malonaldehyde/kg lipids. This value was higher than that observed by Coutron-Gambotti and Gandemer (1999) in subcutaneous adipose tissue of dry-cured hams (1.49 mg malonaldehyde/kg lipids). Rodríguez et al. (2001) have reported that dry-cured pork forelegs show a very intense oxidation, higher than those of other traditional meat products, due to their higher sodium chloride content and to their greater surface area.

The glycerides represented 89% and the free fatty acids 11% of the total lipid content in subcutaneous fat of dry-cured forelegs. The content of phospholipids was negligible. Very low levels of phospholipids have also been reported in subcutaneous fat of dry-cured hams (Flores et al., 1985). The high content of free fatty acids revealed that lipolysis is important during the processing of this product although the length of the manufacturing process is short (approximately 35 days). The level of free fatty acids was similar to that observed in subcutaneous adipose tissue of dry-cured hams after several months of processing (Coutron-Gambotti & Gandemer, 1999; Flores et al., 1985); these authors reported that these fatty acids come mainly from triglyceride hydrolysis. Veiga et al. (2003) also observed a high level of free fatty acids (10.6%) in muscle lipids of "Lacón Gallego" but lipolysis only affected the phospholipids fraction of muscle lipids. Timón, Ventanas, Carrapiso, Jurado, and García (2001b) observed that lipolytic processes occur more intensively in subcutaneous fat than in intermuscular fat of dry-cured Iberian hams. Therefore, glycerides of subcutaneous fat could be more susceptible than glycerides of muscular lipids to lipases.

The culinary treatment (desalting and boiling) caused significant decreases in the dry matter, ash and lipid contents of subcutaneous adipose tissue. The decreases in the dry matter content can be due to increases in water content or to ash and lipid losses during culinary treatment. In VBDF, these decreases are only due to increases in water content of subcutaneous adipose tissue during culinary treatment. The vacuum-packaging of the pieces before boiling prevented the ash and lipid losses (as can be observed when the results are expressed in g/100 g dry matter). Nevertheless, the culinary treatment caused water loss in the muscles (Cobos et al., 2008). This was related to the fact that, during wet heating, collagen was solubilised and denatured; it absorbed water

and turned to gelatine. It is also possible that part of the water loss in the muscles might migrate to the subcutaneous adipose tissue. In BDF, the decrease in dry matter content is probably due to both reasons: increases in the water content of the subcutaneous adipose tissue and the loss of ash and lipids in cooking water during boiling.

In the samples studied, no significant differences were found in the cholesterol contents of the subcutaneous fat of dry-cured pork forelegs (DF, BDF and VBDF). However, the cholesterol content in BDF was lower than those in DF and VBDF. The culinary treatment of whole dry-cured pork forelegs caused a significant decrease in the cholesterol content in muscles (Cobos et al., 2004). This loss of cholesterol might be related to the formation of cholesterol oxide products. The vacuum-packaging of the pieces before boiling (VBDF samples) could probably help to prevent the oxidation of cholesterol due to the exclusion of oxygen during heating.

The TBA values were lower in BDF and VBDF than in DF. Decreases in TBA values have also been observed in meat lipids after the culinary treatment of the pieces of dry-cured forelegs (Cobos et al., 2008), after boiling beef muscles (Badiani et al., 2002) and after grilling pork muscles (Nuernberg et al., 2006). The explanation of these results might be the degradation or denaturation of aldehydes that make the TBA-reactive substances less assessable for measurement (Nuernberg et al., 2006). The losses of malonaldehyde could also be due to their volatility when heated (Hernández, Navarro, & Toldrá, 1999). The TBA values were lower in BDF than in VBDF. This could be due to the fact that the malonaldehyde was included in lipid losses of the subcutaneous adipose tissue during boiling without vacuum-packaging; so, the possible effects against oxidation of vacuum-packaging of samples before boiling were not reflected in TBA values.

There were no significant differences in the levels of glycerides and free fatty acids between the different batches (DF, BDF and VBDF). There are no studies about the effects of the heating process in lipolysis of glycerides from adipose tissue. It is possible that more intensive heat could cause an increase in free fatty acids, due to thermal hydrolysis, as occurs in pork meat lipids, during boiling (Hernández et al., 1999). In this way, while there is no increase in the content of free fatty acids in muscle lipids during the culinary treatment of the deboned pieces of "lacón gallego" (Cobos et al., 2008), during the culinary treatment of dry-cured pork forelegs (a longer process), there is an increase in free fatty acids (Cobos et al., 2004). However, this increase was related to phospholipid hydrolysis since no changes were observed in the content of glycerides.

3.2. Fatty acid composition

Table 2 shows the fatty acid profiles (expressed as% of total fatty acids) in DF, BDF and VBDF of the glycerides and the free fatty acids fraction of subcutaneous fat.

In glycerides, the monounsaturated fatty acids were the most abundant (46.0%), followed by the saturated fatty acids (38.2%) and the polyunsaturated fatty acids (15.8%). Oleic (39.6%), palmitic (23.9%), linoleic (13.7%) and stearic (12.6%) acids were the main fatty acids. These results differ from those reported in triacylglycerols of abdominal adipose tissues of Iberian pigs (Perona & Ruiz-Gutierrez, 2005) and subcutaneous fat from dry-cured Iberian hams (Timón, Martín, Petrón, Jurado, & García, 2001a; Timón et al., 2001b) where the contents of C-18:1 were higher (51–54%) and the percentages of C-18:2 were lower (8–9%). These differences could mainly be due to the breed, the diet or to the localisation of the deposit of fat in the carcass; it is known that the fatty acid profiles of the subcutaneous fat of pigs are influenced by the breed (Lo Fiego, Santoro, Macchioni, & De Leonibus, 2005b), by the diet (Lo Fiego, Macchioni, Santoro, Pastorelli, & Corino, 2005a; Martin, Antequera, Gonzalez, Lopez-Bote, & Ruiz, 2007; Ruiz et al., 1988; Timón et al., 2001a) and by the localisation of the subcutaneous fat in the carcass (Franco, Escamilla, García, García Fontán, & Carballo, 2006; Monziols, Bonneau, Davenel, & Kouba, 2007). A higher content of C-18:2 and total polyunsaturated fatty acids in the subcutaneous adipose tissue from the shoulder than in that from the ham has been reported (Monziols et al., 2007).

The fatty acid composition of the glycerides in the subcutaneous fat from dry-cured pork forelegs shows some differences from that reported in glycerides in muscle lipids from dry-cured forelegs from pigs fed with the same diet (Cobos et al., 2008). The contents of saturated fatty acids of muscle lipids were higher (42.7%) and the percentages of polyunsaturated fatty acids of muscle lipids were lower (11.9%) than those found in subcutaneous fat. These differences were due to a higher content of C-16:0 (28.6%) and a lower content of C-18:2 (10.2%), respectively. In accordance with

Table 2
Fatty acid profiles (% of total fatty acids) (means ± SD) of the glyceride and free fatty acid fractions of subcutaneous fat from dry-cured pork foreleg pieces (DF) and from dry-cured pieces subjected to culinary treatment by desalting and boiling (BDF) or by desalting and vacuum-packed boiling (VBDF) ($n = 4$).

	Glycerides			Free fatty acids		
	DF	BDF	VBDF	DF	BDF	VBDF
C-14:0	1.36 ± 0.08	1.51 ± 0.36	1.56 ± 0.40	2.34 ± 0.35	2.18 ± 0.28	2.24 ± 0.20
C-16:0	23.9 ± 0.81	24.6 ± 1.98	24.4 ± 1.74	13.1 ± 1.65	11.6 ± 1.68	12 ± 1.12
C-16:1 $n-9$	0.54 ± 0.09	0.49 ± 0.17	0.53 ± 0.06	0.91 ± 0.13a	0.79 ± 0.15b	0.84 ± 0.06ab
C-16:1 $n-7$	2.14 ± 0.44	2.45 ± 0.62	2.37 ± 0.46	4.16 ± 0.94	4.27 ± 0.95	4.10 ± 0.64
C-17:0	0.30 ± 0.04ab	0.26 ± 0.06a	0.31 ± 0.05b	0.14 ± 0.02	0.13 ± 0.02	0.14 ± 0.02
C-17:1 $n-7$	0.29 ± 0.04ab	0.28 ± 0.05a	0.32 ± 0.04b	0.37 ± 0.03	0.31 ± 0.11	0.40 ± 0.05
C-18:0	12.6 ± 1.63	11.4 ± 0.97	11.2 ± 0.62	3.93 ± 0.45a	4.35 ± 0.36b	3.62 ± 0.14a
C-18:1 $n-9$	39.6 ± 1.55	41.7 ± 4.06	39.6 ± 2.44	36 ± 2.08	38.3 ± 3.63	34.9 ± 0.95
C-18:1 $n-7$	2.65 ± 0.31	2.94 ± 0.31	2.80 ± 0.17	3.99 ± 0.19ab	4.65 ± 0.59a	3.89 ± 0.23b
C-18:2 $n-6$	13.7 ± 1.24ab	11.9 ± 2.52a	14.1 ± 1.34b	28.4 ± 1.39ab	26.3 ± 2.88a	30.6 ± 0.67b
C-18:3 $n-3$	1.04 ± 0.10ab	0.90 ± 0.20a	1.10 ± 0.11b	3.49 ± 0.37	3.21 ± 0.41	3.77 ± 0.34
C-20:1 $n-9$	0.78 ± 0.13	0.73 ± 0.12	0.73 ± 0.09	0.69 ± 0.18	0.85 ± 0.33	0.61 ± 0.09
C-20:2 $n-6$	0.59 ± 0.05	0.54 ± 0.10	0.59 ± 0.05	1.02 ± 0.15	1.15 ± 0.22	1.06 ± 0.12
C-20:3 $n-6$	0.07 ± 0.03	0.07 ± 0.03	0.09 ± 0.01	0.23 ± 0.05a	0.31 ± 0.11ab	0.33 ± 0.01b
C-20:4 $n-6$	0.17 ± 0.07	0.21 ± 0.05	0.18 ± 0.06	0.83 ± 0.08a	1.13 ± 0.09b	1.00 ± 0.10b
C-20:5 $n-3$	0.19 ± 0.02	0.14 ± 0.04	0.15 ± 0.05	0.49 ± 0.15	0.47 ± 0.19	0.54 ± 0.06
SFA	38.2 ± 2.50	37.8 ± 2.72	37.4 ± 2.31	19.5 ± 1.68	18.2 ± 1.67	18 ± 0.92
MUFA	46 ± 1.83	48.6 ± 3.93	46.4 ± 2.25	46.1 ± 1.33	49.2 ± 3.72	44.8 ± 0.29
PUFA	15.8 ± 1.31ab	13.7 ± 2.87a	16.2 ± 1.49b	34.5 ± 1.43ab	32.6 ± 2.88a	37.3 ± 1.05b

Means of the same fraction with different letters in the same row differ significantly ($p < 0.05$).
SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

these results, Franco et al. (2006) have reported that the intramuscular fat of pigs shows higher values of saturated fatty acids and a lower content of polyunsaturated fatty acids than do subcutaneous dorsal and ventral fat.

No significant differences between fatty acid composition of glycerides in the subcutaneous fat of dry-cured pork forelegs (DF) and the subcutaneous fat in samples subjected to culinary treatment (BDF and VBDF) were observed. However, there were significant differences between BDF and VBDF. The samples of pieces boiled with no vacuum-packaging (BDF) showed a significantly lower content of C-18:2 and C-18:3 and total polyunsaturated fatty acids than did the samples which were vacuum-packed before boiling (VBDF). This result could be due to two reasons. First, it could be related to the oxidation of fatty acids. The vacuum-packaging of the pieces probably protects against the oxidation of polyunsaturated fatty acids. Secondly, the lipid losses observed in BDF in relation to VBDF could contain glycerides in adipose tissue with relatively more polyunsaturated fatty acids than saturated fatty acids. However, an increase in polyunsaturated fatty acids in total lipids has been reported in the culinary treatment of dry-cured pork forelegs (Cobos et al., 2008) and in the cooking of meat and meat products (Ono, Berry, & Paroczay, 1985; Ramamurti, 1986; Rodriguez-Estrada, Penazzi, Caboni, Bertacco, & Lercker, 1997; Scheeder et al., 2001) and it was related to drip losses, containing mainly triglycerides with relatively more saturated fatty acids than unsaturated fatty acids. Thus, a more plausible explanation for the higher content of polyunsaturated fatty acids in VBDF is related to the effects of vacuum-packaging on the pieces; it reduces and/or prevents the oxidation of these fatty acids. In contrast to subcutaneous fat, when comparing BDF to VBDF no significant differences were observed in polyunsaturated fatty acids of glycerides in meat lipids in the pieces of dry-cured forelegs (Cobos et al., 2008). Hence, it is possible that boiling with no vacuum-packaging affects the polyunsaturated fatty acids of subcutaneous fat but does not affect the polyunsaturated fatty acids of muscle lipids. These differences are probably due the fact that this is a better chance for the oxidation of the subcutaneous fat. The idea that the hydrolysis of phospholipids might protect the polyunsaturated fatty acids from oxidation in dry-cured hams has also been reported (Gandemer, 2002). In subcutaneous fat, this protection is not possible, due to the low levels of phospholipids.

In free fatty acids in the subcutaneous fat from dry-cured pork forelegs, the monounsaturated fatty acids were the most abundant (46.1%), followed by the polyunsaturated fatty acids (34.5%) and the saturated fatty acids (19.5%). Oleic (36.0%), linoleic (28.4%) and palmitic (13.1%) were the main fatty acids. These results differ from those reported in free fatty acids of adipose tissues from dry-cured hams (Coutron-Gambotti & Gandemer, 1999; Timón et al., 2001a, 2001b), where the contents of C-18:1 were higher (46–52%) and the percentages of C-18:2 were lower (7–13%). As in the fatty acid composition of glycerides, these differences were probably due to the diet or to the localisation of the deposit of fat in the carcass.

The fatty acid composition of free fatty acids in subcutaneous fat is very different from those reported for fatty acid composition of free fatty acids in muscles of dry-cured forelegs from pigs fed the same diet (Cobos et al., 2008) and, as has previously been commented, this could be due to the fact that the free fatty acids in the muscles of dry-cured pork forelegs probably come from phospholipids (Veiga et al., 2003) while the free fatty acids in subcutaneous fat probably come mainly from glycerides, as has been reported for the subcutaneous adipose tissue of dry-cured hams (Coutron-Gambotti & Gandemer, 1999; Flores et al., 1985). Furthermore, the fatty acid compositions of free fatty acids and glycerides in subcutaneous fat show important differences. The proportion of palmitic and stearic is lower in FFA and the contents of linoleic and total polyunsaturated fatty acids are higher than in glycerides. The increase in polyunsaturated fatty acids suggests a preferential hydrolysis of these fatty acids in glycerides in subcutaneous fat. This result has also been observed by Coutron-Gambotti and Gandemer (1999) in dry-cured hams. These authors indicated that the physical state of triacylglycerols might affect their rate of lipolysis; the triacylglycerols containing saturated fatty acids (palmitic and stearic) are solid at the temperature of dry-cured pork forelegs ripening, which limits their hydrolysis by lipases. In contrast, the triacylglycerols containing polyunsaturated fatty acids are in the liquid state, which would favour the action of lipases at the water-oil interface.

As in glycerides, the samples of pieces which were vacuum-packed before boiling (VBDF) showed a significantly higher content of polyunsaturated fatty acids than did the samples boiled with no vacuum-packaging (BDF); the vacuum-packaging of the pieces before boiling probably protected the fatty acids from oxidation.

4. Conclusions

The desalting and boiling of pieces of dry-cured forelegs caused a decrease in dry matter, ash and lipid contents of the subcutaneous adipose tissue. These decreases were not as great in vacuum-packaged samples because the lipid and ash losses were prevented during the boiling. The culinary treatment did not produce changes in the glyceride and free fatty acid proportions. The samples boiled with vacuum-packaging showed a higher content of polyunsaturated fatty acids than did the samples boiled without vacuum-packaging and this might be because the vacuum-packaging helps to reduce and/or prevent the oxidation of these fatty acids. Thus, vacuum-packaging might be useful in order to protect polyunsaturated fatty acids of subcutaneous fat.

Acknowledgements

This study was financially supported by projects AGL 2001–3007 (cofounded by Ministerio de Ciencia y Tecnología and FEDER) and PGIDIT02PXIC26201PN (Secretaría Xeral de Investigación e Desenvolvemento da Xunta de Galicia). Our thanks go to Dr. JoDee Anderson for the linguistic support that she provided.

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Food Chemistry 117 (2009) 174–180

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Chemical analysis, antioxidant, antiinflammatory and anticholinesterase activities of *Origanum ehrenbergii* Boiss and *Origanum syriacum* L. essential oils

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ARTICLE INFO

Article history:
Received 21 January 2009
Received in revised form 25 March 2009
Accepted 26 March 2009

Keywords:
Origanum ehrenbergii
O. syriacum
Lamiaceae
Essential oil
GC–MS
Antioxidant
Inhibition of NO production
Acetylcholinesterase and butyrilcholinesterase inhibition

ABSTRACT

The essential oils of *Origanum ehrenbergii* and *O. syriacum* collected in Lebanon were analysed by GC and GC–MS and evaluated for their anticholinesterase, NO production inhibitory activities, and antioxidant properties. *O. ehrenbergi* essential oil was characterised by the presence of 37 components, representing 94.9% of the total oil of which thymol (19%) and *p*-cymene (16.1%) were the main abundant compounds. Thirty-six compounds characterised the *O. syriacum* essential oil, representing 90.6% of the total oil. The most abundant components were thymol (24.7%) and carvacrol (17.6%). *O. ehrenbergii* demonstrated interesting scavenging effects on DPPH with an IC_{50} value of 0.99 μg/ml. In addition, both *O. ehrenbergii* and *O. syriacum* oils inhibited oxidation of linoleic acid after 30 min of incubation, as well as after 60 min of incubation with IC_{50} values of 42.1 and 33.6 μg/ml, and 46.9 and 58.9 μg/ml, respectively. Interestingly, *O. ehrenbergii* oil inhibited NO production in the murine monocytic macrophage cell line RAW 264.7 with an IC_{50} value of 66.4 μg/ml. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition was assessed by modifications of the Ellman's method. *O. ehrenbergii* exhibited a strong activity against both cholinesterases with IC_{50} values of 0.3 μg/ml. The data suggest that *O. ehrenbergii* and *O. syriacum* oils could be used as a valuable new flavour with functional properties for food or nutriceutical products with particular relevance to supplements for the elderly.

1. Introduction

The functional role of herbs and spices and their constituents is a hot topic in food related plant research. Spices, in fact, are actually not only evaluated for their seasoning properties, but are also appreciated for their bioactive efficacy as antioxidants, and nutrients (Hirasa & Takemasa, 1998). In recent years, there has been growing interest in finding natural antioxidants, including volatile chemicals, in plants because they inhibit oxidative damage and may consequently prevent inflammatory conditions (Khanna et al., 2007), ageing and neurodegenerative disease (Fusco, Colloca, Lo Monaco, & Cesari, 2007). The market constantly addresses its attention to new and underutilised spices, to check their properties and to evaluate their use in the industry. Most of the chemical constituents of essential oils are terpenoids, characterised by a low molecular weight which allows easy transport across cell membranes to induce different biological activities, including antioxidant, antiinflammatory and anticholinesterase effects (Chao et al., 2005; Kulisić, Krisko, Dragović-Uzelac, Milos, & Pifat, 2007; Loizzo et al., 2007).

The genus *Origanum* (Lamiaceae) is characterised by a large morphological and chemical diversity. Forty-nine taxa divided into 10 sections belong to this genus, most of them having a very local distribution around the Mediterranean. The essential oils of *Origanum* species vary in respect of the total amount produced by plants as well as in their qualitative composition. *Origanum* essential oils are characterised by a number of main components which are implicated in the various plant odours. A wide chemical diversity is found even within a single *Origanum* species e.g. the widely used *O. vulgare* where the pattern of variation of quantitative and qualitative essential oils depends on geographical distribution or on the time of plant collection (Kokkini, 1996). Today, *O. vulgare* (oregano) can be considered one of the most important spices both in

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doi:10.1016/j.foodchem.2009.03.095

Mediterranean countries and elsewhere. The overall market of oregano is about 350–500 tonnes in France, 600 tonnes in Germany, 500 tonnes in the UK and 150 tonnes in the Netherlands (Maftei, 1992). The popularity of oregano is increasing as a result of scientific developments achieved in the area of its cultivation and utilisation and its flavour is almost irreplaceable in several food preparations or as a herbal tea. Dried *Origanum* species are also used for the production of essential oil and aromatic water (Dundar et al., 2008) and they are used as stimulants, analgesics, antitussives, expectorants, sedatives, antiparasitisc and antihelminthics in folk medicine; but mostly for gastrointestinal complaints (Başer, 2002). Lebanon, located on the Mediterranean littoral, presents a climatic and ecological diversity that is unique to the eastern Mediterranean region and the whole country is recognised as a centre of plant diversity. An estimated 2600 plant species are recorded in Lebanon, of which 221 are broad endemics and 90 are narrow endemics (Khouzami, Bassil, Fortunat, & Hayek A., 1996). *O. ehrenbergii* Boiss. is endemic in Lebanon and it is used as a condiment, although the less strongly flavoured *O. syriacum* L. is preferred (Figuérédo, Chalchat, & Pasquier, 2005).

Alzheimer's disease (AD) is the most common form of neurodegenerative disorders, neurochemically characterised by a consistent deficit in cholinergic neurotransmission, particularly affecting cholinergic neurons in the basal forebrain. Symptoms of AD and other forms of dementia can be treated by the use of agents which restore the level of acetylcholine through inhibition of both the two major forms of cholinesterase: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). In late stages of AD, levels of AChE decline by up to 85% and BChE represents the predominant cholinesterase in the brain. BChE, primarily associated with glial cells but also with specific neuronal pathways, cleaves ACh in a manner similar to AChE to terminate its physiological action. Such studies, together with a statistically slower decline in the cognitive performance of dementia patients possessing specific BChE polymorphisms that naturally lower BChE activity, have targeted BChE as a new approach to intercede in the progression of AD (Ballard, Greig, Guillozet-Bongaarts, Enz, & Darvesh, 2005). Recently, Hodges (2006) demonstrated that the inhibition of AChE holds a key role not only to enhance cholinergic transmission in the brain but also to reduce the aggregation of β-amyloid and the formation of the neurotoxic fibrils in AD.

In recent years, oxidative stress has been described in the pathological changes that occur in AD (Pratico & Delanty, 2000). Nitric oxide (NO) is a diatomic free radical produced from L-arginine by constitutive and inducible nitric oxide synthase (cNOS and iNOS) in numerous mammalian cells and tissues. NO, superoxide (O_2^-) and their reaction product peroxynitrite ($ONOO^-$) may be generated in excess during the host response against infections and inflammatory conditions, contributing to some pathogenesis by promoting oxidative stress, tissue injury and even neurodegenerative disease (De Palma et al., 2008). The mechanism of inflammation injury is attributed, in part, to release of reactive oxygen species from activated neutrophils and macrophages. This over production leads to tissue injury by damaging macromolecules and lipid peroxidation of membranes (Gutteridge, 1995). In addition, ROS propagate inflammation by stimulating release of mediators such as NO and cytokines such as interleukin-1, tumour necrosis factor-α and interferon-γ, which stimulate recruitment of additional neutrophils and macrophages. Thus free radicals are important mediators that provoke or sustain inflammatory processes and, consequently, their neutralisation by antioxidants and radical scavengers can attenuate inflammation (Geronikaki & Gavalas, 2006). Nizri, Hamra-Amitay, Sicsic, Lavon, and Brenner (2006) showed that cholinergic up-regulation obtained with the use of AChE inhibitors was associated to an antiinflammatory effect through the suppression of lymphocyte proliferation and pro-inflammatory cytokine production. Following this view, in recent years, an increased interest has emerged directed to finding products from edible plants able to inhibit both of these events. The present study was planned to evaluate Lebanese *O. ehrenbergii* and *O. syriacum* for antioxidant, antiinflammatory, and cholinesterase inhibitory activities taking into account the chemical composition of the oils.

2. Materials and methods

2.1. Chemicals

Dimethylsulphoxide (DMSO), chloroform, ethanol were obtained from VWR International s.r.l. (Milano, Italy). Phosphate buffered saline (PBS), β-carotene, anhydrous sodium sulphate, ascorbic acid, propyl gallate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), linoleic acid, limonene, Tween 20, sodium phosphate buffer, sodium potassium tartrate tetrahydrate, acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI), 5,5′-dithiobis [2-nitrobenzoic-acid] (DTNB), physostigmine, acetylcholinesterase from *Electrophorus electricus* (EC 3.1.1.7, Type VI-S) and butyrylcholinesterase from equine serum (EC 3.1.1.8), Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine, foetal bovine serum (FBS), penicillin/streptomycin, trypan blue, sulphonamide, Griess reagent (1% sulfanamide and 0.1% *N*-(1-naphtyl) ethylenediamine dihydrochloride in 2.5% H_3PO4), MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), LPS and indomethacin were purchased from Sigma–Aldrich S.p.a. (Milano, Italy). Murine monocytic macrophage cell line RAW 264.7 were purchased from European Collection of Cell Cultures, London, UK.

2.2. Plant material

Origanum ehrenbergii Boiss and *O. syriacum* L. were collected on Baskinta Mountain at an altitude of up to 1500 m, Lebanon, in June 2004 and authenticated by Prof. S. Safi, Biology Department, Faculty of Sciences II, Lebanese University. A voucher specimen (AS32; AS36) was deposited in the Herbarium, Faculty of Sciences II, Lebanese University.

2.3. Oil Isolation

The flowering aerial parts (1 kg) of *O. ehrenbergii* Boiss and *O. syriacum* L. were subjected to hydrodistillation for 3 h using a Clevenger-type apparatus (Loizzo et al., 2007). Each oil was dried over anhydrous sodium sulphate to remove traces of moisture. Samples of essential oil were stored in a brown bottle in a freezer prior to further analysis.

2.4. Gas chromatography–mass spectrometry

To determine the composition of the essential oils from *O. ehrenbergii* and *O. syriacum*, analysis was carried out using a GC system (Hewlett–Packard Co., model 6890 N) with a fused capillary column (30 m length; 0.25 mm i.d.; 0.25-m film thickness; static phase methylsilicone SE-30) directly coupled to a selective mass detector (Hewlett Packard 5973 N). Electron impact ionisation was carried out at an energy of 70 eV. Helium was used as carrier gas. Injector and detector were maintained at 250 °C and 280 °C, respectively. Column temperature was initially kept at 50 °C for 5 min, then gradually increased to 250 °C at 5 °C/min rate and finally held for 10 min at 250 °C. The mass range from 50 to 550 amu was scanned at a rate of 2.9 scans/s. Analyses were also run by a HP-Innowax capillary column (30 m length, 0.25 mm i.d., 0.25 μ film thickness) under the same GC conditions. For

analysis, essential oils were dissolved in dichloromethane (1 mg/ml) and aliquots (1 μl) were directly injected. Most constituents were identified by gas chromatography by comparison of their GC retention indices with those of the literature or with those of standards available in our laboratories. The retention indices were determined in relation to a homologous series of *n*-alkanes (C8–C24) under the same operating conditions. Further identification was made by comparison of their mass spectra on both columns with those stored in the Wiley 138 library or with mass spectra from literature (Table 1) (Adams, 2001).

2.5. Gas chromatography

A Shimadzu model GC 17 gas chromatograph equipped with a FID detector and a capillary column (30 m length; 0.25 mm i.d.; 0.25 μ film thickness; static phase methylsilicone SE-30) and controlled by Borwin software was employed. The carrier gas was nitrogen. The percentage composition of *O. ehrenbergi* and *O. syriacum* oils was computed by the normalisation method from the GC peak areas related to GC peak area of an external standard injected into the GC equipment in isothermal conditions at 100 °C. The percentage of total area was obtained by their addition. All determinations were performed in triplicate and averaged.

2.6. Determination of hydrogen donation ability (DPPH Test)

Free radical scavenging activity of essential oils was performed according to the procedure of Conforti, Statti, Uznov, and Menichini (2006). The absorbance was measured using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer at 517 nm against a blank, which was without DPPH. All tests were run in triplicate and averaged. Ascorbic acid was used as a positive control. A decrease of DPPH solution absorbance indicates an increase of DPPH radical scavenging activity. This activity is given as% DPPH radical-scavenging that is calculated by the following equation:

$$\%\ \text{DPPH radical-scavenging} = [1 - (\text{sample absorbance with DPPH-sample absorbance without DPPH/control absorbance})] \times 100$$

2.7. β-Carotene Bleaching Test

Antioxidant activity was determined using a β-carotene bleaching test (Conforti et al., 2006). Briefly 1 ml of β-carotene solution (0.2 mg/ml in chloroform) was added to 0.02 ml of linoleic acid and 0.2 ml of 100% Tween 20. After evaporation of chloroform and dilution with water, 5 ml of the emulsion was transferred into

Table 1
Chemical composition (%) of *O. ehrenbergi* and *O. syriacum* essential oils.

Component	RI[a]	RI[b]	Abundance (%)[c]		ID method[d]
			O. ehrenbergi	*O. syriacum*	
α-Thujene	926	1035	2.3 ± 0.4	1.7 ± 0.2	*I*, MS
α-Pinene	936	1032	1.2 ± 0.2	1.6 ± 0.03	*I*, MS, Co-GC
Camphene	953	1076	0.1 ± 0.03	0.1 ± 0.03	*I*, MS
Sabinene	973	1138	0.5 ± 0.03	0.3 ± 0.02	*I*, MS, Co-GC
β-Pinene	978	1118	0.2 ± 0.03	0.2 ± 0.03	*I*, MS
3-Octanone	983		1.3 ± 0.03	tr	*I*, MS
β-Myrcene	986	1174	3.2 ± 0.7	1.9 ± 0.4	*I*, MS, Co-GC
3-Octanol	995		1.4 ± 0.4	1.3 ± 0.1	*I*, MS
δ-3-Carene	1012	1159	0.2 ± 0.03	0.2 ± 0.01	*I*, MS
α-Terpinene	1016	1188	2.9 ± 0.5	2.5 ± 0.8	*I*, MS
p-Cymene	1028	1281	16.1 ± 0.8	8.7 ± 0.6	*I*, MS
Limonene	1032	1203	0.9 ± 0.03	0.4 ± 0.02	*I*, MS, Co-GC
γ-Terpinene	1059	1255	11.8 ± 0.9	12.6 ± 0.6	*I*, MS, Co-GC
cis-Sabinene hydrate	1075	1450	0.7 ± 0.03	0.2 ± 0.03	*I*, MS
Linalool	1098	1554	1.8 ± 0.03	2.0 ± 0.2	*I*, MS, Co-GC
Terpinolene	1089	1290	0.2 ± 0.01	0.3 ± 0.03	*I*, MS, Co-GC
trans-Sabinene hydrate	1101	1474	0.2 ± 0.04	0.3 ± 0.03	*I*, MS
Borneol	1165	1715	0.2 ± 0.05	0.3 ± 0.06	*I*, MS
α-Terpineol	1189	1683	0.3 ± 0.01	0.7 ± 0.07	*I*, MS
Carvone	1242	1713	0.1 ± 0.03	0.3 ± 0.03	*I*, MS
Thymol methyl ether	1265	1604	1.1 ± 0.8	0.6 ± 0.01	*I*, MS
Carvacrol methyl ether	1275	1614	1.5 ± 0.6	0.9 ± 0.05	*I*, MS
Thymol	1278	2113	19.6 ± 0.6	24.7 ± 0.6	*I*, MS
2-Isopropyl-1-methoxy-4-methylbenzene	1284		14.9 ± 0.9	7.9 ± 0.5	*I*, MS
Carvacrol	1319	2225	6.7 ± 0.1	17.6 ± 0.7	*I*, MS
α-Copaene	1377	1497	0.2 ± 0.03	0.3 ± 0.03	*I*, MS
β-Bourbonene	1382	1546	0.1 ± 0.02	-	*I*, MS
Eremophilene	1486		0.3 ± 0.05	0.1 ± 0.01	*I*, MS
Aromadendrene	1439	1628	0.1 ± 0.03	0.2 ± 0.03	*I*, MS
trans-β-Farnesene	1441	1662	0.4 ± 0.06	0.1 ± 0.01	*I*, MS
α-Humulene	1454	1690	0.6 ± 0.01	0.3 ± 0.02	*I*, MS, Co-GC
Ledene	1492	1708	0.2 ± 0.03	0.1 ± 0.02	*I*, MS
β-Bisabolene	1508	1741	2.7 ± 0.2	1.5 ± 0.6	*I*, MS
γ-Cadinene	1515	1765	tr	0.1 ± 0.02	*I*, MS
δ-Cadinene	1524	1772	0.2 ± 0.01	0.2 ± 0.01	*I*, MS
Caryophyllene oxide	1576	1989	0.4 ± 0.01	0.3 ± 0.05	*I*, MS
Spathulenol	1579	2144	0.3 ± 0.01	0.1 ± 0.03	*I*, MS
Identified compounds			94.9	90.6	

[a] Retention index on SE-30 MS column.
[b] Retention index on HP-Innowax column.
[c] Mean value ± standard error, $n = 3$. Compositional values <0.1% are denoted as traces (tr).
[d] *I*, Retention index; MS, mass spectrum; Co-GC: co injection with authentic compound.

different test tubes containing 0.2 ml of samples in 70% ethanol at different concentrations. A standard (propyl gallate) at the same concentration as the samples was used for comparison. The tubes were then gently shaken and placed at 45 °C in a water bath for 60 min. The absorbance of the samples, standard and control was measured at 470 nm using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer against a blank, consisting of an emulsion without β-carotene. The measurement was carried out at initial time (t = 0) and successively at 30 and 60 min. All samples were assayed in triplicate and averaged. The antioxidant activity (AA) was measured in terms of successful bleaching of β-carotene by using the following equation:

$$AA = \{1 - [(A_0 - A_t)/(A_0^{\circ} - A_t^{\circ})]\} \times 100$$

2.8. Bioassay for Anticholinesterase Activity

Inhibition of AChE and BChE were assessed by a modified colorimetric Ellman's method (Loizzo et al., 2007), which is based on the reaction of released thiocoline to give a coloured product with chromogenic reagent *E. electricus* (EC 3.1.1.7, Type VI-S) AChE and equine serum (EC 3.1.1.8) BChE were used, while acetylthiocholine iodide (ATCI) and butyrylthiocholine iodide (BTCI), respectively, were used as the substrates of the reaction. 40 μl of AChE or BChE (0.36 U/ml in buffer pH 8) and oils at different concentrations (20 μl) were added to 2 ml of buffer pH 8 and pre-incubated in an ice bath at 4 °C for 30 min. Tested oils and control oils were dissolved in 5% MeOH. Duplicate tubes were also treated this way with 20 μl of physostigmine (0.1 mM) to allow interference of the test substances in the assay to be assessed, and to control any hydrolysis of acetylcholine or butyrylcholine not due to enzyme activity. The reaction was started by adding 5,5′-dithiobis [2-nitrobenzoic-acid] (DTNB) solution (20 μl of 0.05 mM in buffer pH 7) and (ATCI) or (BTCI) (20 μl 0.018 mM in buffer pH 7) and tubes were allowed to stand in a water bath for 20 min at 37 °C. The reaction was halted by placing the assay solution tubes in an ice bath and adding physostigmine (20 μl 0.018 mM in buffer pH 7). The hydrolysis of acetylthiocholine and butyrylthiocholine was monitored by the formation of the yellow 5-thio-2-nitrobenzoate, immediately recorded on a spectrophotometer (Jenway 6300) at 405 nm and the percentage inhibition was calculated. All the reactions were performed in triplicate. The inhibition rate (%) was calculated by equation:

$$\text{Inhibition}\,\% = \frac{[(\text{Blank} - \text{Blank positive control}) - (\text{Experiment} - \text{Experiment control})]}{(\text{Blank} - \text{Blank positive control})}$$

2.9. Cell culture

The murine monocytic macrophage cell line RAW 264.7 was grown in a plastic culture flask in DMEM with L-glutamine supplemented with 10% FBS and 1% penicillin/streptomycin solution under 5% CO_2 at 37 °C. After 4–5 days cells were removed from the culture flask by scraping and centrifuged for 10 min at 1500 rpm. The medium was then removed and the cells were re-suspended with fresh DMEM. Cells counts and viability were performed using a standard trypan blue cell counting technique. The cell concentration was adjusted to 1×10^6 cells/ml in the same medium. Hundred microlitre of the above concentration were cultured in a 96-well plate for one day to become nearly confluent. Concentrations ranging from 10–200 μg/ml of the samples were prepared from the stock solutions by serial dilution in DMEM to give a volume of 100 μl in each well of a microtiter plate (96-well). Then cells were cultured with the vehicle and *Origanum* oils in the presence of 1 μg/ml LPS for 24 h.

2.10. Assay for cytotoxic activity

Cytotoxicity was determined using the MTT assay (Tubaro et al., 1996) with some modification. The assay for each concentration of samples was performed in triplicate and the culture plates were kept at 37 °C with 5% (v/v) CO_2 for one day. After 24 h of incubation, 100 μl of medium was removed from each well. Subsequently, 100 μl of 0.5% w/v MTT, dissolved in phosphate buffer saline, was added to each well and allowed to incubate for a further 4 h. After incubation, 100 μl of DMSO was added to each well to dissolve the formazan crystals. Absorbance values at 550 nm were measured with a microplate reader (GDV DV 990 B/V, Roma, Italy). Cytotoxicity was expressed as CD_{50} which is the concentration to reduce the absorbance of treated cells by 50% with reference to the control (untreated cells).

2.11. Inhibition of nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells

The presence of nitrite, a stable oxidised product of NO, was determined in cell culture media by Griess reagent (1% sulfanamide and 0.1% *N*-(1-naphtyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4) (42). 100 μl of cell culture supernatant was removed and combined with 100 μl of Griess reagent in a 96-well plate followed by spectrophotometric measurement at 550 nm using a microplate reader (GDV DV 990 B/V, Roma, Italy). Nitrite concentration in the supernatants was determined by comparison with a sodium nitrite standard curve.

2.12. Statistical analysis

Data were expressed as means ± SD. Statistical analysis was performed by using Student's t test. Differences were considered significant at $P \leqslant 0.05$. The inhibitory concentration 50% (IC_{50}) was calculated from the Prism dose–response curve (GraphPad, Prism version 4.0 for Windows, GraphPad Software, San Diego, CA, USA) obtained by plotting the percentage of inhibition versus the concentrations.

3. Results and discussion

3.1. Composition of the essential oils

The yields of *O. ehrenbergii* and *O. syriacum* essential oils were 0.6% (w/w) and 0.5% (w/w), respectively. In order to identify putative active compounds present within the essential oils, gas chromatography systems were employed. The chemical composition of the oils is reported in Table 1. Both oils are characterised by a very high content of monoterpenes. *O. ehrenbergi* essential oil was characterised by the presence of thirty-seven components, representing 94.9% of the total oil. Thymol (19.6%), *p*-cymene (16.1%), 2-isopropyl-1-methoxy-4-methylbenzene (14.9%), γ-terpinene (11.8%), carvacrol (6.7%), β-myrcene (3.2%), α-terpinene (2.9%) and β-bisabolene (2.7%) were the major constituents. Two populations of *O. ehrenbergii* harvested in Lebanon were previously studied by Figuérédo et al. (2005). The oils of one of the populations were uniform and rich in carvacrol, while the other population displayed a variable chemical composition, including thymol, thymol-carvacrol and *p*-cymene/γ-terpinene chemotypes.

Thirty-six compounds characterised the *O. syriacum* essential oil, representing 90.6% of the total oil. The most abundant components were thymol (24.7%), carvacrol (17.6%), γ-terpinene (12.6%), *p*-cymene (8.7%), 2-isopropyl-1-methoxy-4-methylbenzene (7.9%) and α-terpinene (2.5%). Alma, Mavi, Yildirim, Digrak, and Hirata (2003) reported γ-terpinene, carvacrol, *p*-cymene and β-caryo-

phyllene as main constituents of Turkish *O. syriacum* essential oil. Previously, it was reported that *O. syriacum* var. *bevanii* growing in Turkey contained carvacrol (64.1%) and *p*-cymene (12.3%) as major components (Başer, Kürçüolu, Demirci, & Özek, 2003). On the other hand, while essential oil from *O. syriacum* from Israel contained thymol (59.9%) and carvacrol (80.2%) as a major component, the essential oil from the same plant from Egypt contained only carvacrol (76.7%) as the main constituent (Fleisher & Fleisher, 1991).

3.2. Antioxidant activity of the essential oils

The antioxidant potential of an essential oil or compound can be mainly attributed to its reducing power, radical scavenging ability and singlet oxygen quenching ability. Therefore, to be able to evaluate the total antioxidant activity of an essential oil, two activities were measured i.e. the DPPH radical scavenging assay and β-carotene bleaching test. The effect of antioxidants on the DPPH radical is thought to be due to their hydrogen donating ability. The scavenging effects of *O. ehrenbergii* and *O. syriacum* on DPPH were examined at different concentrations. As shown in Table 2, both essential oils exhibited a significant activity. In particular *O. ehrenbergii* oil showed the higher activity (IC_{50} 1.0 μg/ml) while *O. syriacum* oil had IC_{50} 1.7 μg/ml. Antioxidant activity, which was demonstrated by the ability of the samples to inhibit the bleaching of β-carotene, was measured and compared to that of the control which contained no antioxidant component. Both *O. ehrenbergii* and *O. syriacum* inhibited oxidation of linoleic acid after 30 min of incubation as well as after 60 min of incubation with IC_{50} of 42.1 and 33.6 μg/ml, and 46.9 and 58.9 μg/ml, respectively, indicating that its activity was not correlated with the time of heating. Previously, Alma et al. (2003) reported that *O. syriacum* oil from Turkey exhibited a considerable reducing power that was higher than those of essential oil from the leaves of another common spice *Laurus nobilis*. The antioxidant activity may be attributed primarily to the high content of phenolic components of the essential oil (Ruberto, Baratta, Sari, & Kaabache, 2002). The chemical composition of *O. syriacum* essential oil indicates that compounds such as carvacrol, carvacrol methyl ether and thymol methyl ether are relatively rich in the essential oil. In fact, recently it has been reported that these compounds have antioxidant ability (Mastelić et al., 2008). No previous studies were done on the antioxidant activity of *O. ehrenbergii*.

3.3. Inhibition of NO production

Inhibition of NO production may result in anti-inflammatory activity and was studied *in vitro* by analysing the effect of *O. ehrenbergii* and *O. syriacum* essential oils on chemical mediators released from macrophages. Once activated by a pro-inflammatory stimulus, macrophages produce a large number of cytotoxic molecules. Treatment of RAW 264.7 macrophages with LPS (1 μg/ml) for 24 h induces NO production, as assessed by measuring the accumulation of nitrite, a stable metabolite of NO, in the media by a colourimetric procedure based on the Griess reaction. NO, a macrophages-derived mediator, is considered to play a key role in inflammatory response, based on its occurrence at inflammatory sites and its ability to induce many of the hallmarks in the inflammatory response. The beneficial effect of *O. ehrenbergii* on the inhibition of production of inflammatory mediators in macrophages can be mediated through oxidative degradation of products of phagocytes, such as O_2^- and HOCl. As shown in Table 3, incubation of RAW 264.7 cells with *O. ehrenbergii* induced a significant inhibitory effect on the LPS-induced nitrite production in a dose-dependent manner, with an IC_{50} value of 66.37 μg/ml. This effect, which is comparable to the positive control indomethacin, is probably due to the presence of thymol, one of the major components of the oils which has a phenolic structure, and has been credited with a series of pharmacological properties, including antimicrobial, antioxidant and anti-inflammatory effects (Braga et al., 2006). Carvacrol showed anti-inflammatory properties (Sosa et al., 2005). The high content of this compound in *O. ehrenbergii* oil is probably the reason for its high activity.

Table 2
Free radical scavenging and antioxidant activity of *Origanum* essential oils (IC_{50} μg/ml).

Essential oil	DPPH assay	β-carotene bleaching test	
		30 min	60 min
O. ehrenbergii	1.0 ± 0.05	42.1 ± 0.9	46.9 ± 1.0
O. syriacum	1.7 ± 0.01	33.6 ± 0.8	58.9 ± 1.2
Ascorbic acid	2.0 ± 1.2		
Propyl gallate		1.0 ± 0.01	1.0 ± 0.01

Data are given as means ± S.D. (n = 3). Ascorbic acid and propyl gallate were used as positive control.

Table 3
Inhibition of NO production in LPS induced RAW 264.7 macrophages by *Origanum* essential oils.

Essential oil	IC_{50} (μg/ml)
O. ehrenbergii	66.4 ± 1.6
O. syriacum	>200
Indomethacin	52.8 ± 1.2

Data are given as means ± S.D. (n = 3). Indomethacin was used as positive control.

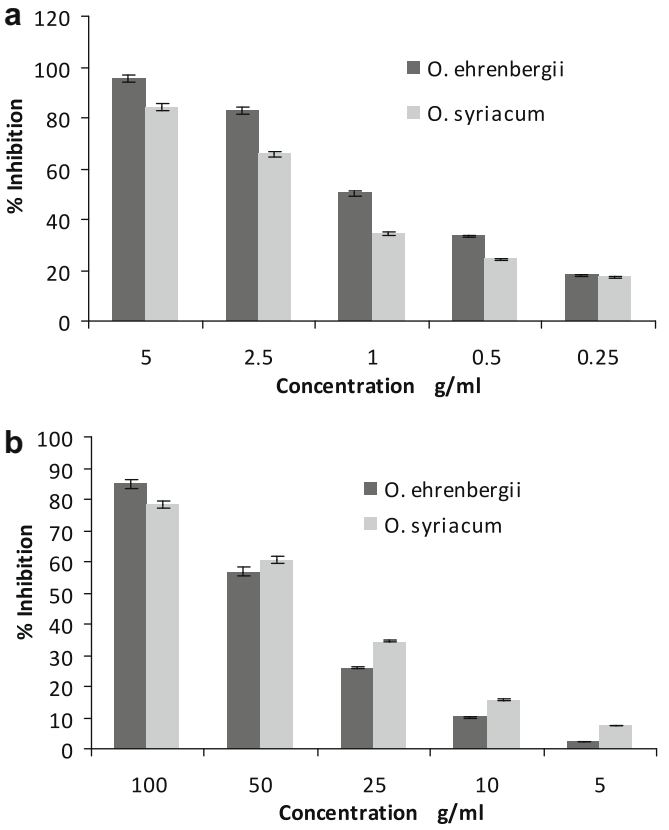

Fig. 1. (a) Free radical scavenging and (b) antioxidant activity after 30 min of incubation of *Origanum* essential oils. Data are given as means ± S.D. (n = 3).

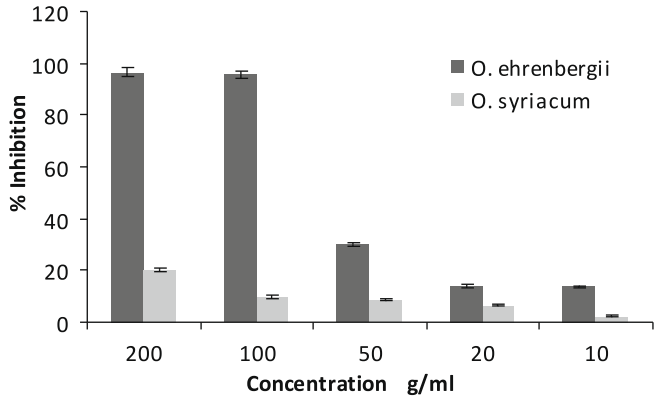

Fig. 2. Inhibition of NO production in LPS induced RAW 264.7 macrophages by *Origanum* essential oils. Data are given as means ± S.D. (n = 3).

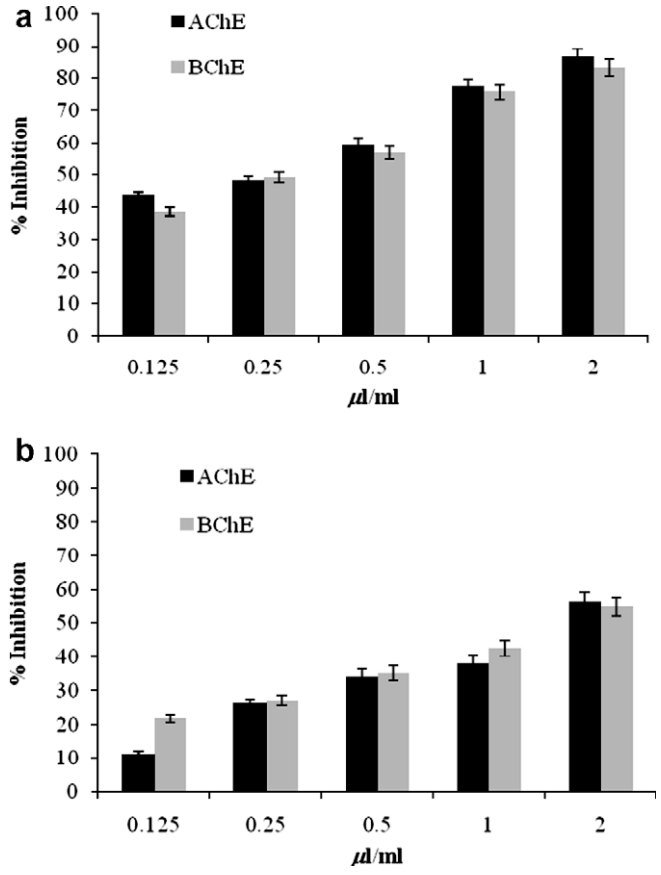

Fig. 3. Dose-dependent anticholinesterase inhibitory activity of *Origanum ehrenbergii* (a) and *O. syriacum* (b). Data are given as means ± S.D. (n = 3).

3.4. Anticholinesterase activity

AChE plays an important role in the central nervous system. It is one of the fastest known enzymes and catalyzes the cleavage of acetylcholine in the synaptic cleft after depolarisation. Inhibitors of AChE, such as galanthamine, are used frequently in the pharmacotherapy of AD. The less specific BChE has recently been a focus of research, because BChE concentration stays the same, or is even up-regulated, while AChE is dramatically down-regulated in the brains of patients suffering from AD (Giacobini, 2004). Since a large amount of evidence demonstrates that oxidative stress is intimately involved in age-related neurodegenerative diseases,

Table 4
Cholinesterase inhibitory activity of *O. ehrenbergii* and *O. syriacum* essential oil (IC_{50} μg/ml).

Essential oil	AChE	BChE	SI (BChE/AChE)
O. ehrenbergii	0.3 ± 0.02	0.3 ± 0.04	0.9
O. syriacum	1.7 ± 0.8	1.6 ± 0.05	1.0
Physostigmine	0.1 ± 0.003	0.2 ± 0.07	2.4

IC_{50} values are mean ± S.D. (n = 3).

there have been a great number of studies which have examined the positive benefits of antioxidants to reduce or to block neuronal death occurring in the pathophysiology of these disorders (Ramassamy, 2006). *O. ehrenbergii* exhibited an interesting activity against both cholinesterases in a dose-dependent manner (Figs. 1–3) with IC_{50} values of 0.3 μg/ml for both AChE and BChE, and a selectivity index of 0.9. On the contrary *O. syriacum* showed a lower activity (Table 4). Previously, Salah and Jäger (2005) reported that *O. syriacum* was recommended by herbalists for the treatment of AD and other neurological conditions. It was previously reported that essential oils rich in monoterpenes exhibit a strong inhibitory activity against both enzymes. Moreover, the AChE inhibitory property was recently demonstrated for thymol and carvacrol, the main components of both oils (Loizzo, Tundis, Menichini, & Menichini, 2008).

The significant activity on BChE is of a certain interest since in late stages of AD, levels of AChE decline by up to 85% and BChE represents the predominant cholinesterase in the brain. Such studies have targeted BChE as a new approach to intervene in the progression of AD (Ballard et al., 2005).

Beside being appreciated for their aromatic properties, *O. ehrenbergii* and *O. syriacum* essential oils provide interesting properties from a functional perspective in prevention of neurodegenerative disorders. In fact, both oils exhibited a good antioxidant and antiradical activity, with some properties relevant to an anti-inflammatory action. Moreover, even at very small concentrations, both *O. ehrenbergii* and *O. syriacum* oils demonstrated an interesting inhibitory activity on AChE and BChE, key enzymes in the pathogenesis of AD. The ability of these oils to act in a multi-factorial pattern leads to the suggestion that they may be candidates for valuable new flavours with functional properties in food, or for nutriceutical products with particular relevance in supplements for the elderly.

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Food Chemistry 117 (2009) 181–188

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Ascorbate, not urate, modulates the plasma antioxidant capacity after strawberry intake

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ARTICLE INFO

Article history:
Received 18 September 2008
Received in revised form 24 February 2009
Accepted 26 March 2009

Keywords:
Strawberry
Human acute consumption
Plasma antioxidant capacity
Ascorbate
Urate

ABSTRACT

The consumption of antioxidant-rich foods has already been associated with acute significant increases in plasma total antioxidant capacity (TAC) in humans, but there is no consensus among different literature reports. Furthermore, the acute rise in plasma TAC observed after consumption of several non-berry fruits seems not to be necessarily related to the absorption of dietary antioxidants, such as micronutrients and phenolic substances, but is attributed to the relevant increase of endogenous urate in serum. In this study, we first compared the nutritional quality of strawberry fruits from different genotypes, all cultivated in the same experimental field. Significant genotype-to-genotype differences were observed in the antioxidant capacity and in the vitamin C, folate and phenolic contents of the fruits. Second, in order to investigate the individual effects of the selected strawberry genotypes on the post-prandial plasma antioxidant status in humans, we assessed the acute effects of a single dose of strawberries in healthy subjects. The variation of plasma TAC through the FRAP and TEAC assays, and any eventual changes in ascorbate and urate levels in serum, were measured during the three hours following the strawberry intake. The acute intake of strawberries resulted in a significant increase in plasma FRAP values in all the subjects, independently of the individual TAC baseline levels. Interestingly, the lowest increases in plasma TAC and in serum ascorbate levels were associated with consumption of the nutritionally-poorest cultivars. In addition, significant increases in ascorbate, but not in urate, concentrations were observed in serum. These findings suggested that the fructose-dependent hyperuremic effect, observed after the intake of non-berry fruits, is not responsible for the plasma antioxidant changes following strawberry consumption.

1. Introduction

Increase in the consumption of foods rich in antioxidants, micronutrients and phytochemicals has long been proposed as a preventive measure to decrease the risk of several chronic diseases caused by oxidative stress. In particular, the improvement in plasma antioxidant status (and the increase in serum antioxidants) has been recognised as a potential tool to prevent the development of cardiovascular, proliferative, degenerative and other diseases (Kay & Holub, 2002).

Significant increases in the plasma total antioxidant capacity (TAC) have already been reported in human studies following both the acute and long-term consumption of antioxidant-rich foods and beverages, such as several fruits and vegetables, red wine and tea (Cao, Booth, Sadowski, & Prior, 1998; Cao, Russell, Lischner, & Prior, 1998; García-Alonso, Ros, Vidal-Guevara, & Periamo, 2006). However, there is no absolute consensus among the literature reports. For instance, other studies have not confirmed the same correlation between fruit consumption and plasma antioxidant status in humans (Bub et al., 2003; Duthie et al., 2006). In parallel, although relevant health-promoting effects have been associated *in vitro* with the polyphenol antioxidants contained in fruit, such as flavonoids (Hollmann, 2001; Yi-Fang, Jie, Xianzhong, & Rui, 2002), a clear *in vivo* demonstration of polyphenol absorption

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); FIA, flow injection analysis; FW, fresh weight; RDA, recommended daily allowance; TAC, total antioxidant capacity; TE, trolox equivalents; TEAC, trolox equivalent antioxidant capacity assay; FRAP, ferric reducing antioxidant power assay.

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doi:10.1016/j.foodchem.2009.03.096

and plasma circulation is still lacking, and their contribution to the plasma antioxidant status seems to be negligible. Firstly, this could be partly due to persistent analytical and methodological difficulties in recovering and identifying phenolic metabolites in complex biological fluids (Day & Morgan, 2003). Secondly, it is hardly likely that the rapid rise in plasma TAC, often observed after flavonoid-rich food intake, could be mainly ascribed to the direct contribution of these dietary compounds. For instance, the increase in plasma TAC after the consumption of antioxidant-rich berry fruits and juices has usually been attributed to the high levels of polyphenolic antioxidants provided by the berries. However, many recent short-term human intervention studies have reported that flavonoid compounds, such as anthocyanins, which are among the most represented phenols in berries, show a rapid but very poor absorption (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). These substances seem to reach only very low concentrations in human plasma and only immediately after the consumption of flavonoid-rich foods. Furthermore, the derivative chemical structures resulting from the extensive hepatic metabolism of the ingested flavonoids seem not to necessarily express the native antioxidant activities (Natsume et al., 2004). Finally, from a recent comparison of different analytical methods for assessing the TAC of human serum, it was found that the major contributors to TAC values were uric acid and vitamin C, followed by protein, bilirubin, and α-tocopherol (Cao & Prior, 1998). Other antioxidants, including plasma/serum phenols and polyphenolic compounds, have been thought to contribute about 5% of the FRAP values, so that the contribution of polyphenols to FRAP could be considered negligible (Cao & Prior, 1998).

On the other hand, several research groups have recently paid renewed attention to the putative role of other non-phenolic substances contained in fruit, both micronutrients and macronutrients, which could be directly or indirectly responsible for the postprandial variations in the plasma antioxidant pool (Lotito & Frei, 2006). Interestingly, a great number of investigators observed relevant increases of serum urate after consumption of several fruits, which otherwise did not contain urate or its precursors (Lotito & Frei, 2004). This endogenous metabolite expresses high reducing and free radical-scavenging activities, thus acting as an antioxidant, and alone may contribute to up to 60% of the plasma TAC, when evaluated by the FRAP assay. Thus, the rapid increase in the antioxidant status in humans after fruit consumption could not be ascribed to the absorption of dietary flavonoid or non-flavonoid antioxidants, but rather to an overproduction and circulation of endogenous antioxidant metabolites (Day & Stansbie, 1995; Natella, Nardini, Giannetti, Dattilo, & Scaccini, 2002). The mechanisms implicated in this hyperuremic response to fruit consumption have been partly explained and related to the absorption of fructose and related carbohydrates (Heuckenkamp & Zollner, 1971), responsible for accelerating the degradation of AMP to urate (Mayes, 1993).

Meanwhile, fructose-rich but vitamin-C-poor foods and beverages have often been tested in the *in vivo* studies carried out up to now, so that hyperuremic effects were observed while the contribution of vitamin C in the increase of plasma TAC was negligible and the role of serum urate resulted as a predominant factor.

In this study, strawberry was chosen as the fruit model to evaluate the possible acute plasma effects resulting from a high single dose intake. Compared to other non-berry fruits previously used in human studies (Lotito & Frei, 2004), strawberry is a relevant source of antioxidants and micronutrients, being among the richest natural food source of vitamin C and folate (Olsson et al., 2004), and it possesses a higher water content, a lower content of low molecular weight carbohydrates and a greater glucose/fructose ratio (Olsson et al., 2004; Vinson, Bose, Proch, Al Kharrat, & Samman, 2008). Among berries, the strawberry also possesses a more equilibrated phenolic composition (Aaby, Ekeberg, & Skrede, 2007) and is the most commonly consumed fruit, therefore having a major potential impact on human health (Hannum, 2004).

Six different strawberry genotypes were selected for the *in vivo* study. First, the antioxidant, micronutrient (vitamin C and folate) and phenolic contents of the selected fruits were analysed and compared, in order to calculate the individual intake corresponding to a single dose of strawberries consumed during the test, and to identify any relevant genotype-to-genotype differences in the nutritional quality of the fruits. Second, the eventual genotype-to-genotype differences in the plasma response to acute strawberry consumption were investigated, in order to test the influence of the nutritional quality (NQ) of the fruits on the changes in plasma TAC and in serum ascorbate and urate concentrations. Six parallel feeding tests were carried out involving healthy nonsmoking subjects; furthermore, for an internal control and a check of any genotype-dependent differences in plasma response to the acute intake, a subgroup of the subjects was asked to consume strawberries from two different genotypes with a washout period of at least one week between the two intakes.

2. Materials and methods

2.1. Test strawberries

In May 2006, six different strawberry genotypes (“Alba”, “Irma”, “Patty”, “Adria”, “Sveva”, “AN99.78.51”), cultivated in the experimental field for genetic improvement of the Azienda Agraria Didattico Sperimentale (Marche Polytechnic University), were selected for the *in vivo* acute test.

During three separate sessions, corresponding to the main harvest times of the selected clones, fruits were harvested and distributed to the subjects within one hour. Fruits from each studied genotype were also collected for composition analyses, immediately stored at −20 °C and at −80 °C (see vitamin C and folate measurement) and analysed within three months. Compounds extraction was carried out *via* homogenisation or *via* sonication, depending on the analysis to be performed. For spectrophotometric general assays, frozen strawberries were thawed for 60 min at 4 °C; then, 10 g aliquots of the fruits were added to 100 ml of the extraction solution (1:10 w/v), consisting of an 80% methanol aqueous solution, containing 0.1% formic acid (FA). Fruits were homogenised using an Ultraturrax T25 homogeniser (Janke & Kunkel, IKA Labortechnik) at medium-high speed for 2 min, and the extraction was maximised by stirring the suspension for 2 h in the dark at room temperature. Then tubes were centrifuged at 3500 rpm for 15 min (twice sequentially), to sediment solids, and supernatants were filtered through a 0.45 μm Minisart filter (PBI International), transferred to 5.0 ml amber glass vials and stored at −20 °C prior to analysis.

2.2. Standard colorimetric methods

The TAC of the hydroalcoholic extracts of the fruits was assessed by two methods, the trolox equivalent antioxidant capacity (TEAC) assay and the ferric reducing antioxidant power (FRAP) assay, as described below. Results are expressed as moles of trolox equivalents (TEq) per kg of fresh weight of strawberries (mmoles TEq/kg FW). The results represent the mean values ± SD of eight measurements. The total phenolic content of the hydroalcoholic extracts was determined by the Folin–Ciocalteu colorimetric method, as modified by ,Slinkard and Singleton (1977), quantifications were calculated through a calibration curve, daily prepared with known concentrations of gallic acid (GA) standards and results were expressed as grammes of GA equivalents per kg of fresh

weight of strawberries (g GAEq/kg FW, mean value ± SD of eight measurements). The total flavonoids was determined according to a colorimetric method previously described (Dewanto, Wu, Adom, & Liu, 2002) and catechin (C) was used as reference standard compound. Results were expressed as grammes of C equivalents per kg of fresh weight of strawberries (g CEq/kg FW, mean value ± SD of eight measurements). The total anthocyanins was measured using a modified pH differential method previously described (Giusti, Rodriguez-Saona, & Wrolstad, 1999) with slight modifications, and absorbance readings were converted to quantifications through a calibration curve obtained by known concentrations of pelargonidin-3-glucoside (Pg-glc) standards. Results are expressed as Pg-glc equivalents per kg of fresh weight of strawberries (g Pg-glcEq/kg FW, mean value ± SD of eight measurements).

2.3. Measurement of vitamin C content

For vitamin C and folate measurements, fruits stored at −80 °C were snap-frozen in liquid nitrogen, and ground to a fine powder by using a precooled coffee grinder (IKA A11 basic); the frozen powders were stored at −80 °C prior to analysis. Immediately before analysis, 2 ml of ice-cold extraction solution were added to 0.5 g FW of frozen powder weighed in 10 ml ice-cold glass tubes, and the mixture was sonicated in the dark at 4 °C for 15 min. For vitamin C quantification, the extraction solution consisted of MilliQ water containing 5% meta-phosphoric acid and 1 mM diethylenetriaminepentaacetic acid (DTPA), stirred well prior to use, sonicated until dissolved and stored in a refrigerator. After the ultrasound-assisted extraction, the cell walls and proteins were precipitated by centrifugation at 2500 rpm for 10 min at 4 °C; the surnatant was filtered through a 0.2 μm PTFE filter into 1.8 ml HPLC vials, and immediately analysed. Vitamin C in the fruits was analysed by reversed-phase HPLC, as previously described by our group (Tulipani et al., 2008). The HPLC system consisted of a Waters 600 controller, a Waters 996 Photodiode array (PDA) detector set at absorbances of 262 and 244 nm, and a column incubator at 30 °C. The HPLC column used was a YMC Pack Pro, 150 × 4.6 mm. The elution was isocratic with 50 mM potassium phosphate (KH_2PO_4) in MQ water, leading to pH 3.2 by adding orthophosphoric acid, and analysis consisted of a 10 min run, after which the column was cleaned with 50% acetonitrile. Vitamin C eluted at RT ≈ 5.3 min. Quantification of the vitamin C content was carried out through a calibration curve prepared by running standard concentrations of vitamin C prepared as were the extracts, and measured in duplicate at the beginning and at the end of the analysis. Results are expressed as g vitamin C (vit C) per kg of fresh weight of strawberries (g vit C/kg FW, mean value ± SD of three measurements).

2.4. Measurement of folate content

The total folate content in strawberries was quantified by a microbiological assay (MA), which used the growth response of a folate-dependent test micro-organism (generally *Lactobacillus rhamnosus* ATCC 7469) in extracts that were enzymatically treated to release the bound vitamin. For folate extraction, 0.15 g FW strawberry frozen powder was added to 1.5 ml of ice-cold extraction solution, consisting of 0.1 M sodium acetate (CH_3CO_2Na) buffer containing 0.1% ascorbic acid and 20 μm 1,4-dithiothreitol (DTT), pH 4.7. The mixture was vortexed, sonicated for 5 min, and kept for 10 min at 100 °C in a boiling water bath to denature any eventual enzyme before the deconjugation treatment. The deconjugation treatment involved incubating, for 4 h at 37 °C, 300 μl of strawberry extract with 450 μl of deconjugation working solution, consisting of a 1:24 (v/v) dilution of a 1.2% human plasma (w/v) and 0.7% 2-mercaptoethanol aqueous solution in 0.1 M sodium acetate solution, pH 4.7. Both the untreated (control) and the enzyme-treated strawberry extracts were then heated in a boiling water for 5 min, to denature enzymes, and filtered through 0.22 μm PTFE sterile filters (Acrodisc, GelmanSciences). The strawberry folate-dependent *Lactobacillus rhamnosus* growth was determined by measuring the increase in optical density at 580 nm, using a ninety-six-well microplate reader Emax (Molecular Devices Corporation, Sunnyvale, Calif.), as previously described (Sybesma, Starrenburg, Tijsseling, Hoefnagel, & Hugenholtz, 2003). Results were expressed as μg folate (Fol) per kg of fresh weight of strawberry fruit (μg Fol/kg FW, mean value ± SD of four measurements).

2.5. Subjects

Eight healthy nonsmoking subjects (3 men and 5 women) were recruited for the study. The subjects aged 26 to 45 years, with body mass indeces ranging from 22 to 27 kg/m^2 (Table 1). The main exclusion criteria were a case history of allergy to strawberries or other berries and the diagnosis of any chronic disease which could interfere with alignment to the guide lines of the test. Smokers and subjects with acute inflammatory disease or taking dietary supplements or medications were also excluded from the test. All participants were in good health, as confirmed by the results from clinical laboratory tests, and all biochemical parameters at the baseline time were within the normal reference ranges for adults (see Table 4 below). The human study was reviewed by the local ethics committee of the Marche Polytechnic University and Regional Hospital of Torrette-Umberto I (Ancona, Italy); the experimental design was carefully explained to the volunteers prior to the test, and their written informed consent was obtained.

2.6. Study design

The subjects were asked to abstain from eating fruits, vegetables and their juices, wine, tea or coffee for 12 h before the test to partially standardise the baseline points, and to maintain the usual intake of antioxidant compounds during the days before the experiment. After a night of fasting, they were invited to eat 1 kg of strawberries within a space of 10 min, and to refrain from consuming other foods or beverages during the test. Heparinized plasma was obtained immediately before (baseline timepoint) and up to 3 h after the consumption of the fruits, and stored at −80 °C until analysis.

2.7. Plasma and serum assays

2.7.1. Measurement of plasma total antioxidant capacity (TAC)

As for the fruits, plasma TAC was determined using (in parallel) the TEAC and FRAP assays. The TEAC assay was carried out as recently modified by a flow injection analysis (FIA) system (Bompadre, Leone, Politi, & Battino, 2004). The improved TEAC method, also called FIA–ABTS decolorization assay, is based on the ability of antioxidant compounds to quench the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\cdot+}$) and reduce the radical to the colourless neutral form. The undiluted plasma

Table 1
Physical characteristics of the subjects involved in the study (mean value ± standard deviation).

Subjects (*n*)	8
Gender (F/M)	5/3
Age (years ± standard deviation)	30 ± 6
Height (m ± standard deviation)	1.70 ± 0.1
Body mass (kg ± standard deviation)	70 ± 8
Body mass index (kg/m^2 ± standard deviation)	24 ± 2

sample (5 μl) is injected into a serpentine-knotted reaction coil and allowed to react with the $ABTS^{\cdot+}$ working solution pumped into the coil at a flow rate of 1.2 ml/min. The extent of decolorization, expressed as percentage of inhibition of absorbance, is then plotted as a function of concentrations of the antioxidants in the plasma sample. Each sample was analysed in four replicates and TEAC results were expressed as plasma millimolar concentration (mM) of trolox equivalents.

The FRAP assay was carried out as described by Benzie and Strain (1996), with minor modifications. Trolox aqueous dilutions were used for calibration. Each sample was analysed in eight replicates and FRAP results were expressed as plasma micromolar concentration (μm) of trolox equivalents.

2.7.2. Measurement of serum vitamin C and uric acid levels

The concentrations of ascorbic and uric acids in serum were measured by HPLC with electrochemical detection, as previously described by Lotito and Frei (2004). Briefly, 200 μl of plasma was mixed with an equal volume of ice-cold 5% (w/v) aqueous solution of metaphosphoric acid (MPA), vortexed and incubated at 4 °C for 10 min in the dark. The samples were centrifuged at 10,000 rpm for 5 min to remove the precipitated proteins. The supernatant was collected and diluted 1:6 in the mobile phase (see below). A 20 μl aliquot of this solution was immediately injected into the HPLC system, consisting of a Supercosil C-18 column (25 cm × 4.6 mm, 5 μm particle size) as stationary phase. The mobile phase consisted of 40 mM sodium acetate, 0.54 mM Na_2EDTA and of the ion pairing agent 3 mM hexadecyltrimethylammonium bromide (CTAB, Acros Organics), adjusted to pH 4.75 with glacial acetic acid. Ascorbic and uric acids were determined by amperometric electrochemical detection by using a model 5100A Coulochem II EC detector (ESA, Inc.) equipped with a dual analytical cell (model 5020).

For optimum detection of the compounds, the electrode potentials for the electrodes 1 (E1) and 2 (E2) were set at 200 and 350 mV, respectively. These potentials provided peak area responses with minimum background and allowed quantitative analysis. Peak area analysis was provided by Star WS software (Varian Inst. Inc.), based on calibration curves generated for each of the two compounds. Each sample was analysed in four replicates, and results were expressed as serum micromolar concentrations (μm).

2.8. Statistical analysis

Statistical analyses of both strawberry fruits and plasma data were performed using the STATISTICA software package (Statsoft Inc., Tulsa, OK, USA). For all the parameters evaluated in the strawberry fruits, results were subjected to the HSD Tukey' multiple range test. Plasma and serum values were subjected to the Wilcoxon paired samples test. Data are reported as means ± standard error (SEM) and as% changes from the baseline values (see Table 4 below). Differences at $p < 0.05$ were considered statistically significant.

3. Results and discussion

The strawberry fruits under study came from six different clones with a well-known genetic background, all previously selected for their different antioxidant and phenolic content (Battino & Mezzetti, 2006; Tulipani et al., 2008).

Table 2 shows the micronutrient and phytochemical contents of the strawberries analysed in this study, corresponding to the single dose consumed by the subjects (1 kg). Significant differences were observed among strawberry genotypes, confirming that the genetic background has an important role in influencing the nutritional value of the fruits. The results of the TEAC and FRAP assays for total antioxidant activity were similar and outlined the same cultival-to-cultivar differences, thus confirming that the two assays are almost comparable and inter-changeable for fruit analysis. FRAP values were, in each case, slightly lower than the corresponding TEAC values (Table 1), nevertheless a strongly significant correlation between the two methods was observed ($r = 0.95$, $p < 0.0001$). The variety Sveva showed the highest TAC values and the highest total phenolic and flavonoid contents, followed by the varieties Adria, Alba and the selection AN99.78.51, while the highest folate and anthocyanin contents were found in the selection AN99.78.51, together with the variety Alba. In keeping with previous observations (Prior et al., 1998), our data confirmed that the association between the TAC of the fruit and the proportion of phenolics present as anthocyanins was not very evident in strawberry. Even if individual anthocyanin compounds have strong antioxidant properties, several other phenolic compounds seem to give an important cumulative contribution to the TAC of the fruits, and the quantitative variation of the non-anthocyanin antioxidant phenolics among genotypes could explain why the total antioxidant capacity of the extracts is not strongly correlated with the anthocyanin content.

Similar vitamin C contents were observed among the TAC-richest genotypes Alba, Adria, Sveva and AN99.78.51 (Alba > Adria > Sveva > AN99.78.51). These clones also showed higher vitamin C values than the two remaining cultivars (Patty > Irma), confirming that the latter were the nutritionally-poorest fruits for all the parameters studied (Table 2).

The TAC of plasma samples was estimated, from their capacity to quench $ABTS^{\cdot+}$, reducing the radical to the colourless neutral form, using the TEAC assay, and by evaluating the plasma ability to reduce ferric iron by the FRAP assay. Table 3 shows the changes of plasma TAC (measured by both FRAP and TEAC assays) and of serum ascorbate and urate after the intake of strawberries. The data represent the mean values among all the strawberry genotypes consumed, and are reported both as absolute values

Table 2
Antioxidant and phenolic content of the selected strawberry genotypes corresponding to the single dose (1 kg) consumed by each subject. Means ± standard deviation in the same row with different superscript letters are significantly different at $p < 0.05$, using HSD Tukey' multiple range test.

Parameter	Cultivar/selection					
	Alba	Irma	Patty	Adria	Sveva	AN99.78.51
TAC (mmol TE/kg FW)						
FRAP	$9.04 \pm 1.3^{b,c}$	8.41 ± 0.5^{c}	8.34 ± 0.6^{c}	$9.83 \pm 0.3^{a,b}$	11.0 ± 0.5^{a}	$8.84 \pm 1.1^{b,c}$
TEAC	$12.5 \pm 1.6^{b,c}$	11.3 ± 2.2^{c}	$11.9 \pm 0.9^{b,c}$	13.5 ± 0.4^{b}	16.1 ± 0.6^{a}	$12.0 \pm 1.4^{b,c}$
Vit C (g/kg FW)	0.40 ± 0.03	0.28 ± 0.06	0.29 ± 0.00	0.35 ± 0.02	0.31 ± 0.03	0.31 ± 0.02
Fol (μg/kg FW)	237 ± 17^{b}	$172 \pm 15^{b,c}$	$171 \pm 24^{c,d}$	128 ± 11^{d}	187 ± 32^{c}	369 ± 26^{a}
TPC (g/kg FW)	2.15 ± 0.1^{b}	1.55 ± 0.2^{d}	1.85 ± 0.2^{c}	2.15 ± 0.1^{b}	2.62 ± 0.1^{a}	1.80 ± 0.3^{c}
FLAVO (g/kg FW)	$0.48 \pm 0.1^{a,b}$	0.36 ± 0.1^{e}	$0.39 \pm 0.0^{d,e}$	0.54 ± 0.0^{b}	0.71 ± 0.0^{a}	$0.44 \pm 0.1^{c,d}$
ACY (g/kg FW)	$0.49 \pm 0.0^{a,b}$	0.37 ± 0.1^{b}	$0.46 \pm 0.2^{a,b}$	0.41 ± 0.0^{b}	0.35 ± 0.0^{b}	0.56 ± 0.0^{a}

Table 3
Average changes in plasma TAC (measured by FRAP and TEAC assays) and in serum levels of ascorbic and uric acids among all the subjects, after consumption of a single dose (1 kg) of strawberry fruits. Data are presented as means ± SEM. Values in brackets indicate the % variation from the baselines.

	FRAP (μmol TE/l)	TEAC (mmol TE/l)	Ascorbic acid (μm)	Uric acid (μm)
Baseline	34.8 ± 3	3.2 ± 0.1	28.8 ± 1	124 ± 13
After 1 h	40.0 ± 2* (+18.1%)	3.2 ± 0.1 (+0.1%)	49.5 ± 1* (+73.7%)	125 ± 13 (+0.8%)
After 2 h	42.9 ± 3* (+22.8%)	3.2 ± 0.1 (+0.1%)	55.9 ± 2* (+96.0%)	125 ± 14 (0.0%)
After 3 h	42.0 ± 3* (+22.0%)	3.2 ± 0.1 (+0.6%)	55.8 ± 2* (+95.4%)	125 ± 14 (- 0.4%)

* $P < 0.05$ in respect to baseline values (0 h), using the Wilcoxon paired samples test.

Table 4
Biochemical parameters of the volunteers during the acute study, as measured by clinical laboratory tests. Results are expressed as mean values among all the subjects ± SEM ($n = 8$).

Parameter	Glucose (mg/dl)	Uric acid (mg/dl)	Cholesterol (C) (mg/dl)	HDL-C (mg/dl)	C/HDL-C ratio	LDL-C (mg/dl)	Triglycerides (mg/dl)	Albumin (mg/dl)
Reference value	60–110	2.5–7.0	<200	(M) > 40 (F) > 45	0.0–6.0	<130	<170	3.5–5.0
Baseline	90.0 ± 1.1	4.2 ± 0.4	158 ± 5.1	52.2 ± 2.0	3.03	95.2 ± 5.9	65.1 ± 9.9	4.8 ± 0.1
After 1 h	90.8 ± 3.4	4.2 ± 0.4	160 ± 5.2	53.8 ± 2.2	2.96	95.6 ± 5.9	57.8 ± 8.6*	4.9 ± 0.1
After 2 h	87.4 ± 1.3*	4.0 ± 0.4*	157 ± 4.8	53.3 ± 2.3	2.94	93.8 ± 5.2	48.7 ± 6.6*	4.8 ± 0.1
After 3 h	86.7 ± 1.1*	4.1 ± 0.4*	159 ± 4.5	53.8 ± 2.0	2.95	95.6 ± 5.3	55.5 ± 7.1	4.9 ± 0.0

* Significant difference in respect to baseline values ($p < 0.05$), using the Wilcoxon paired samples test.

(means ± SEM) and as percentage variations from the baseline level (values in brackets). In confirmation of findings previously reported by similar short-term studies (Cao & Prior, 1998; Netzel et al., 2002; Pedersen et al., 2000), a significant acute increase in plasma TAC was shown only by the FRAP assay (Table 3), already 1 h after fruit intake (18.1% increase) and this persisted during the following 2 h (22–23% increase); by contrast, no significant variation in plasma TAC was measured by the TEAC assay. The lack of correlation between the two assays has been previously reported (Cao & Prior, 1998) and traced back to the different technologies and specificities of the two methods, leading to different responses to the antioxidant compounds present in plasma. Particularly, the main difference between FRAP and TEAC specificities lies in the FRAP lack of sensitivity for SH group-containing antioxidants, so that plasma proteins such as albumin have a smaller influence on the FRAP data, while they make a greater relative contribution to the TEAC values (Benzie & Strain, 1996). This suggests that the absence of any evident changes in the TEAC values after strawberry consumption could be explained by the masking effects of SHgroup-containing plasma antioxidants, with respect to the finer variation of less representative compounds.

Together with the rise in FRAP values, the chromatographic quantification of the two main FRAP-responsive hydrophilic antioxidants in serum showed a highly significant increase in the serum concentration of ascorbate (74% increase after 1 h from the strawberry intake and up to 96% increase during the following 2 h) but, interestingly, no changes in the serum urate levels of the subjects throughout the test (Table 3).

These data indicated that vitamin C absorbed from strawberries probably did make a significant contribution to the change in plasma antioxidant capacity, observed in our study. The quantity of fruits consumed provided about 320 mg of vitamin C, a much higher dose compared to other fruits previously studied. For example, the 1 kg dose of apples recently used in a similar acute intake study (Lotito & Frei, 2004) furnished approximately 60 mg of vitamin C, and hence plasma ascorbate increased only slightly after apple consumption. Moreover, the lack of significant changes in the serum uric acid levels was surprising, even if a high interindividual variability was observed for this parameter, ranging from 7% decrease to 16% increase one hour after fruit intake. In addition, when urate levels were measured by routine clinical-chemical automatised laboratory tests (Analysis Laboratory of Torrette–Umberto I Regional Hospital, Ancona, Italy), a small but significant average reduction in the serum concentrations was revealed 2 and 3 h after fruit intake (Table 4).

This finding clearly conflicts with the hypothesis of an acute hyperuremic effect following strawberry intake, and with the data obtained by previous fruit consumption studies. For instance, fructose in consumed apples was recently shown to elevate plasma urate, and this correlated with the plasma antioxidant activity (FRAP) increase measured (Lotito & Frei, 2004). Moreover, the relevant differences between the apple study and our strawberry experiment included the lower fructose concentration, the lower total sugar content and the greater glucose/fructose ratio generally found in strawberries (Cordenunsi, Oliveira do Nascimento, Genovese, & Lajolo, 2002; Olsson et al., 2004) compared to apples (Lotito & Frei, 2004; Wrolstad & Shallenberger, 1981).

The absence of a relevant contribution of uric acid in the significant increase of plasma TAC after strawberry intake should be confirmed after a prolonged consumption of strawberries, and a check of whether the inter-individual variation persists over a medium-term period. Few studies have been published on the effects of berry consumption and urate metabolism and excretion, and results are controversial. The most interesting results come from the study of cherries. Clinical case reports of patients with gout showed that consumption of a daily serving of cherries and cherry products for up to three months reduced plasma urate to normal levels and alleviated arthritic pain and gout (Blau, 1950). Recent experimental studies, conducted to determine the extent of these effects on healthy subjects, have confirmed a decrease in plasma urate after cherry consumption, supporting the reputed anti-gout efficacy of cherries (Jacob et al., 2003). Anyway, we still do not know which compounds or which synergistic combination in cherries might be responsible for these actions. The findings suggest the need for further investigations to confirm the clinical data and to check for the potential effects of other berry fruits.

Finally, Figs. 1 and 2 illustrate the percentage variation, from baseline, of the analysed parameters according to the genotype of strawberries consumed by the subjects. The changes in plasma TAC measured by the FRAP test are represented in Fig. 1, while the trends in ascorbic and uric acid serum concentrations are in Fig. 2. No data referring to the statistical significance of the observed genotype-dependent changes are reported in Figs. 1 and 2, since only two subjects were tested for each strawberry

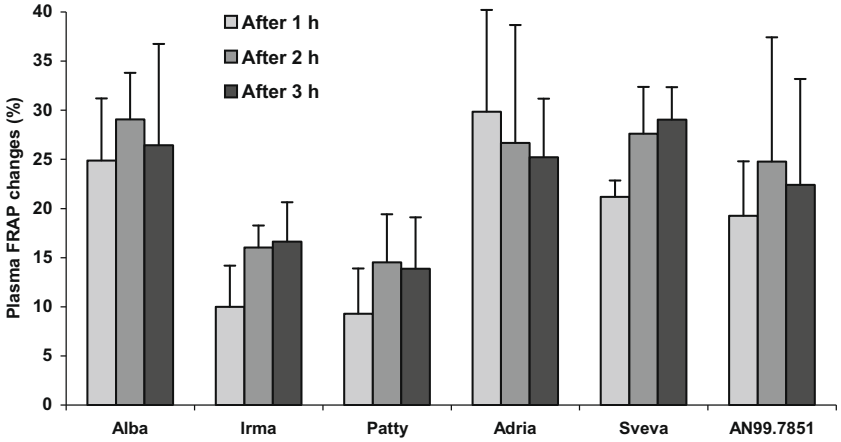

Fig. 1. Plasma% variation of total antioxidant capacity following acute intake of strawberries from different genotypes, when measured by FRAP assay. Data are presented as mean% changes from baseline value ± SEM.

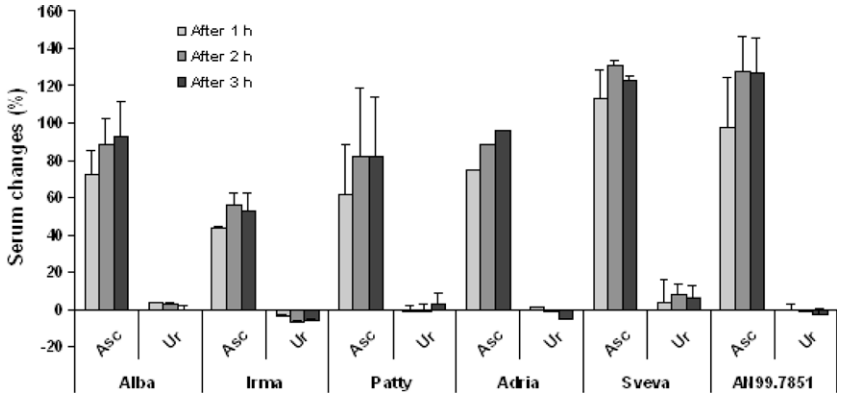

Fig. 2. Serum% variation in the levels of vitamin C ("Asc" columns) and uric acid ("Ur" columns) following acute intake of strawberries from different genoypes. Data are presented as mean% changes from baseline value ± SEM.

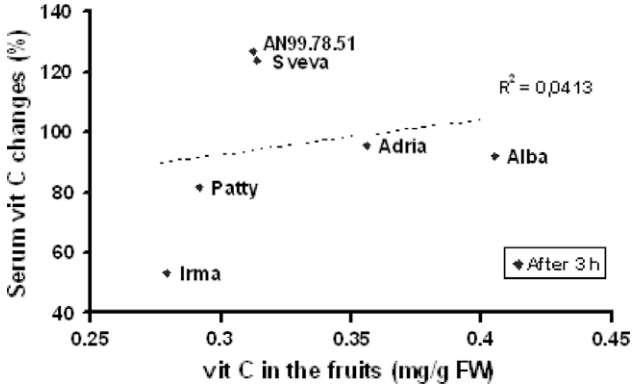

Fig. 3. The% increase of serum ascorbate 3 h after the strawberry consumption plotted against the original vitamin C content in the individual strawberry genotypes. The squared correlation coefficient (R^2) is shown.

clone. The highest increases in plasma TAC were clearly observed, in correspondence with the intake of Sveva > Adria > AN99.78.51 > Alba while, in keeping with their poorest antioxidant, micronutrient and phytochemical contents, the consumption of strawberry cultivars Irma and Patty was associated with the lowest acute increases in plasma TAC (Fig. 1). The intake of the vitamin C-poorest cultivar (Irma) was also correlated with the lowest serum increase of this vitamin (Figs. 2 and 3), while an over 2-fold higher serum ascorbate increase was observed after the consumption of Sveva and AN99.78.51 fruits. The intake of Adria and Alba fruits corresponded to an intermediate rise in serum ascorbate, thus no linear correlation between the vit C contents in the fruits and the human% absorption was observed (Fig. 3). Even when evaluating the data for each strawberry genotype consumed, no significant changes in serum uric acid concentration were observed after strawberry intake.

No correlation was found between the extent of increase in plasma FRAP and the genotype-dependent content of anthocyanins, which are the most represented class of antioxidant polyphenols in strawberries. This finding may confirm that dietary anthocyanins ingested with a strawberry meal do not crucially contribute to the acute increase in the plasma TAC observed.

Finally, together with the putative strawberry-dependent changes in urate levels in serum, Table 4 illustrates the main biochemical parameters analysed throughout the acute study. Results are expressed as means ± SEM, and reference values are indicated for all the parameters measured. Interestingly, a postprandial decrease in serum triglycerides was observed within the two hours following fruit consumption, together with a slight non-significant decrease in the cholesterol/HDL-cholesterol ratio. In a recent study, the effects of the fructose ingested, through a typical American diet, on plasma lipids were evaluated (Bantle, Raatz, Thomas, & Georgopoulos, 2000), and a diet providing 17% of energy as fructose produced significantly higher fasting, postprandial and

daylong plasma triacylglycerol concentrations than did a nearly fructose-devoid diet. Our data did not necessarily conflict with the mentioned study, due to the lower intake of fructose obtained through the strawberry consumption. However, our findings suggested a putative acute effect of strawberry intake on plasma lipids, and further investigations are strongly recommended for a deeper interpretation of the results.

4. Conclusion

Based on our results, it is reasonable to suggest that the increase in plasma TAC observed immediately after the ingestion of strawberries might not be caused by an increase in serum uric acid due to the rapid metabolism of fructose and/or other sugars in the fruits. A similar acute strawberry consumption study should be repeated in the future, and blood collected for up to several hours after the intake of the fruits, in order to evaluate whether 3 h is a sufficient time to allow the metabolism of strawberry fructose to uric acid, in the digestive tract of the average subject.

In the present study, significant genotype-to-genotype differences were observed in the antioxidant capacity and in the vitamin C, folate and phenolic contents of the selected fruits. The individual effects of the selected strawberry genotypes on the postprandial plasma antioxidant status were evaluated in healthy subjects and, interestingly, the lowest increases in plasma TAC and in serum ascorbate levels were associated with the consumption of the nutritionally-poorest cultivars. These findings underlined the importance of evaluating and distinguishing the NQ of fruits used for human consumption studies. Furthermore, the data confirmed the importance of the breeding approaches currently used to increase the content of specific bioactive compounds in strawberry fruits, since higher levels of micronutrients and phytochemicals in fruit may be an important tool to support a higher antioxidant intake, even in the case of low fruit consumption.

Further investigations should also be undertaken to assess the contribution of dietary flavonoids to the total antioxidant capacity of plasma in humans. For this reason, renewed attempts will be made to optimise analytical technologies and methodologies. Finally, the evaluation of medium- and long-term effects of strawberry consumption on the plasma and cellular antioxidant status should be considered.

Acknowledgements

COST Action 863 partially supported this study. The authors are indebted to Miss M. Glebocki for extensive editing of the manuscript.

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Food Chemistry 117 (2009) 189–195

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Differential synthesis of fermentative aroma compounds of two related commercial wine yeast strains

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ARTICLE INFO

Article history:
Received 28 January 2009
Received in revised form 18 March 2009
Accepted 30 March 2009

Keywords:
Wine yeast
Yeast strain
Wine aroma
Yeast effect
Temperature effect

ABSTRACT

The specific impact of the yeast strain on the wine flavour and aroma has not been well characterised yet because this effect is usually combined with other variables during the winemaking. In this study, the contribution to wine flavour of two *Saccharomyces cerevisiae* strains widely used in wine production, VIN13 and EC1118, was evaluated after fermentation at 15 °C. Chemical defined grape juice media fermented with the EC1118 strain showed higher solvent, fatty and pineapple aroma attributes, while that fermented with the VIN13 strain exhibited higher banana, fruity, yeasty and green attributes. Sensorial and chemical analyses evidenced that the production of flavour-active compounds is significantly affected by the yeast strain, as well as by the temperature of fermentation, as shown by comparing the former data with those from fermentations carried out at 28 °C under identical culture conditions.

1. Introduction

The sensory character of wines fermented with *Saccharomyces cerevisiae* starter cultures can differ significantly from those fermented with the indigenous grape flora (Domizio et al., 2007; Fleet, 2003; Lambrechts & Pretorius, 2000). Different strains of *S. cerevisiae* can produce significantly different flavour profiles when fermenting the same must. This is a consequence of both, the differential ability of wine yeast stains in releasing varietal volatile compounds from grape precursors, as well as the differential capacity to synthesise *de novo* yeast-derived volatile compounds (Swiegers, Francis, Herderich, & Pretorius, 2006; Ugliano, Bartowsky, McCarthy, Moio, & Henschke, 2006; Vilanova & Sieiro, 2006; Wondra & Boveric, 2001). Therefore, the selection of the proper yeast strain can be critical for the development of the desired wine style. At present, a wide range of wine yeast strains are commercially available giving winemakers the opportunity to explore different strains and include this selection as an additional variable in winemaking.

Among the hundreds of volatile compounds present in wine, only a subset is sensory detectable. Several fermentative aroma compounds show an olfactory impact despite being present in very low concentrations (micrograms or nanograms per litre) due to their low sensory detection thresholds (Lambrechts & Pretorius, 2000), or to the additive effect of compounds with similar chemical or aromatic properties (Ferreira, Escudero, Campo, & Cacho, 2008; Loscos, Hernandez-Orte, Cacho, & Ferreira, 2007). The odour activity value (OAV) – defined as the ratio between the concentration of the compound and its detection threshold – is usually used to evaluate the potential sensory contribution of a given compound (Guth, 1997), although it provides only a rough evaluation of the real contribution of each compound to the global aroma. In fact, the volatility and the perception of aroma compounds are significantly affected by the basic wine chemical composition, which can both mask the odour impact of certain compounds present in concentrations above their detection thresholds and favour the detection of other molecules present in concentrations below their detection thresholds (Ferreira et al., 2008). Thus, to detect differences in aroma perception between wines – and alcoholic solutions as well – discriminant sensory studies and quantitative descriptive analysis are frequently used (Campo, Ferreira, Escudero, & Cacho, 2005; Gómez-Míguez, Cacho, Ferreira, Vicario, & Heredia, 2007; Loscos et al., 2007). In addition, gas chromatography–olfactometry (GC–O) has become a fundamental tool in sensory characterisation of volatile compounds allowing both their separation from complex samples and the sensory detection and description of the corresponding aroma.

The aim of this work was to evaluate the fermentation performance and the formation of yeast-derived volatile compounds of

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doi:10.1016/j.foodchem.2009.03.116

two related commercial yeast strains widely employed by the wine industry: VIN13 strain, recommended for the elaboration of white wines with high thiol and ester content; and EC1118 strain, suggested for a wide range of fermentation temperatures and with relatively neutral flavour and aroma contribution. The statistical significance of the variables 'yeast strain' and 'fermentation temperature' on the production of each yeast-derived volatile compound were evaluated.

2. Materials and methods

2.1. Yeast strains and growth conditions

Two commercial wine yeasts, *S. cerevisiae* strain EC1118 (Lalvin, Canada) and *S. cerevisiae* strain VIN13 (Anchor Yeast, South Africa) were used in this study. The latter is a hybrid strain derived from *S. cerevisiae* N96 – equivalent to EC1118 – and *S. cerevisiae* 228 – also known as N76. The yeasts were grown separately in 3 ml of YPD medium (1% yeast extract, 2% peptone and 2% glucose) at 28 °C, then transferred to shake flasks containing 50 ml of modified MS300 culture medium (see below) with no anaerobic factors and with half the amount of sugar (60 g/l of glucose and fructose each), and grown overnight at 25 °C under aerobic conditions. This culture was used as inoculum for the wine fermentations carried out in 1-l Bioflo 110 bioreactors (New Brunswick Scientific, Edison, NJ, USA), in 800 ml of medium. Modified MS300 culture medium contained 120 g/l of glucose and 120 g/l of fructose, yeast assimilable nitrogen at a concentration of 300 mg/l (ammonium and amino acids), salts, vitamins and anaerobic factors (15 mg/l ergosterol, 5 mg/l sodium oleate and 0.5 ml/l Tween 80, dissolved in 5 ml of ethanol), as described elsewhere (Varela, Pizarro, & Agosin, 2004). This defined medium simulates standard grape juice, but does not contain grape varietal aromas or grape odourless precursors. Nitrogen was sparged to eliminate the oxygen from the medium before inoculation. The bioreactors were inoculated with 10^6 cells/ml of *S. cerevisiae* VIN13 or *S. cerevisiae* EC1118. Temperature was maintained at 15 °C, pH at 3.5, and agitation at 120 rpm. Fermentations were carried out in triplicate. Cell number, dry cell weight (DCW), optical density at 600 nm (OD_{600}), residual sugar, and the concentration of yeast-derived volatile products and organic acids were determined at different stages of the fermentation, as described below.

2.2. Analytical techniques

The concentrations of glucose, fructose, ethanol, glycerol, malic acid, citric acid and acetic acid were measured by high-performance liquid chromatography (HPLC) using a Bio-Rad HPX-87H column, as described in Molina, Swiegers, Varela, Pretorius, and Agosin (2007). Ammonia concentration and free α-amino nitrogen (FAN) concentration was determined as described in Molina et al. (2007).

2.3. HS-SPME-GC–MS using SIDA

Volatile compounds synthesised during fermentation were quantified using headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME-GCMS), with polydeuterated internal standards for stable isotope dilution analysis (SIDA), as described in Siebert et al. (2005). A gas chromatograph Agilent 6890 (Agilent Technologies) equipped with a Gerstel MPS2 multi-purpose sampler and coupled to a HP 5973 N mass selective detector was used. The control of the instrument and the data analysis were accomplished with Software ChemStation G1701CA (Agilent Technologies). A Phenomenex fused silica capillary column ZB-Wax ~60 m × 0.25 mm and 0.25 μm film thickness was used, and helium (Air Liquid or BOC, Ultra High Purity) as the carrier gas, with 36 cm/s of linear velocity and 2.0 ml/min of flow rate, in constant flow mode. The oven was started at 40 °C, held for 4 min, then increased to 220 °C at 5 °C/min and held at this temperature for 20 min. The inlet was fitted with a borosilicate glass SPME inlet liner (0.75 mm I.D., Supelco) and held at 200 °C. Sample preparation involved 1:10 and/or 1:100 dilution, injection of 100 μl of polydeuterated internal standard solution, salting out with NaCl and heating to 35 °C. A Carbowax/divinylbenzene (CW/DVB) 65 μm fibre (Supelco) was exposed to the sample headspace for 10 min. Then the fibre was desorbed in pulsed splitless mode, and after 30 s the splitter was opened (25:1). The fibre was baked in the inlet for 10 min. The mass spectrometer quadrupole temperature was set at 106 °C, the source at 230 °C, and the transfer line was held at 250 °C. Positive ion electron impact spectra at 70 eV were recorded in selective ion monitoring (SIM). The dwell times, the target and qualifier ions used for identification and quantitation, were the same than in Siebert et al. (2005), as well as the sample preparation. Thirty one volatile compounds were analysed, including ethyl- and acetate-esters, fatty acids and alcohols, but only 23 volatile compounds were identified and quantified in the collected samples. Principal Component Analysis (PCA) was performed using this data as detailed below. The odour activity value (OAV) of each compound was calculated by dividing the concentration of the compound by its detection threshold.

2.4. GC–Olfactometry analysis

Fermented musts were also analysed by GC-sniffing. Aroma extracts were obtained after liquid extraction with dichloromethane. The internal standard 4-nonanol was spiked into the samples prior to extraction to a final concentration of 3.5 μg/l. An Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), equipped with a polyethylene glycol DB-WAX column (Agilent Technologies), was employed. At the end of the column, the effluent was split evenly between a flame ionisation (FID) detector and a Sniffer 9000 port (Brechbühler Inc., Houston, TX, USA) connected by a Y shaped glass splitter. Helium, adjusted to a flow rate of 1.9 ml/min, was used as carrier gas. The injector was set at 180 °C and three microliters of extract were injected in splitless mode. The temperature program used was: 40 °C for 5 min, rise of 3° C/min up to 230 °C, 230 °C for 9 min, rise of 40 °C/min up to 240 °C, and 240 °C for 5.4 min. The temperature of the FID detector was 250 °C, the air flow 450 ml/min, and the hydrogen flow 49 ml/min. The sniffing port was kept at 190 °C with permanent humidification.

A panel of eight judges, five women and three men, sniffed all extracts in two sessions of 35 min each, one session a day. Half of the panel had an extensive experience with GC–O. The judges with no previous experience were subjected to an eight session training during which they became familiar with aroma standards, the description vocabulary, the detection of different intensities and the use of the evaluation scale system. An intensity scale of seven scores (0–3, including half values) was used as previously described (Gómez-Míguez et al., 2007). The aroma descriptor and intensity were recorded using the software Nose to Text® (Brechbühler Inc., Houston, TX, USA). The modified frequency (MF) was calculated based in the intensity (I) and frequency (F) of the aroma detection, according to the following formula (Campo et al., 2005): $MF(\%) = \sqrt{F(\%) \times I(\%)}$.

The MF values from attributes belonging to common aroma categories (floral, fatty, green-vegetal, etc.) were added to compare the total aromatic impact of each category for each sample. Few particular attributes (caramel, solvent, fruity and floral) were detected by the panel in the non-fermented control medium, at spe-

cific elution times. The MF values of the latter were subtracted from each corresponding category.

2.5. GC–MS

The aroma extracts employed for the GC-sniffing analysis were also analysed by GC–MS for identification of the volatile compounds. An Agilent 6890 gas chromatograph connected to an Agilent 5972 mass detector (Agilent Technologies, USA) was used. The same column, injection and temperature conditions than for the GC-sniffing analysis were applied in order to obtain comparable chromatograms. The data acquisition was carried out using Chemstation® software (Agilent Technologies), in full scan mode within the range 35–550 m/z, with 1.5 scans/s^{-1}. The data was analysed by Chemstation® and AMDIS (Stein, 1999) softwares.

2.6. Quantitative descriptive analysis and discriminant sensory evaluation

Quantitative descriptive analysis (QDA) using orthonasal evaluation was carried out by a panel of eight trained judges. Seven sensory terms were selected by the panel during preliminary sessions to describe and differentiate the samples. Standards for the selected descriptors were prepared, and a scale from 0 to 9 was used to score each attribute's intensity. At the end of the training period, the reproducibility and consistency of the panel evaluation was verified by ANOVA.

Discriminant sensory evaluation was performed through triangular tests to assess the significant differences between the fermented chemical media, in duplicate. The panel was the same than for QDA. An odourless colorant was added to the samples to remove the possible effect of colour differences in the evaluation.

2.7. Statistical analysis

Principal Component Analysis (PCA) and QDA data analysis were carried out employing the Senstools® software version 3.0.11 (OP&P Product Research, The Netherlands). For PCA, a correlation matrix was used in order to normalise the data. Multifactor Analysis of Variance (ANOVA) was performed using Statgraphics 4.0 in order to determine the statistically significant effect of the factors 'yeast strain' and/or 'fermentation temperature' on the final concentration of each volatile compound. Type III sums of squares was chosen so as the contribution of each factor is measured after removing the effects of all other factors.

3. Results

3.1. Fermentation parameters

The commercial wine yeast strains *S. cerevisiae* VIN13 and *S. cerevisiae* EC1118 were grown under wine fermentation conditions at 15 °C. For both strains, fermentation parameters were calculated and yeast-derived volatile compounds quantified. The EC1118 strain fermented faster than VIN13 and reached 4 g/l of residual sugar (wine dryness) approximately 400 h after inoculation. VIN13 needed 500 h to complete wine dryness.

Both strains showed a similar maximum growth rate (Table 1), although a significantly higher amount of glycerol was produced by VIN13. EC1118 showed a higher maximum rate of glucose and fructose consumption. No significant differences in the final ethanol content were observed between both fermentations.

3.2. Differential production of yeast-derived aroma compounds

Fermented musts were sensory evaluated by a trained panel. Triangular tests were used to determine if the global aroma of musts fermented with each strain was significantly different. The analysis revealed that these musts could be clearly differentiated with a 95% confidence (data not shown). Quantitative descriptive analysis showed that musts fermented with EC1118 had the highest score for the attribute 'pineapple', while musts fermented with VIN13 had the highest scores for the descriptors 'caramel', 'banana', 'raisins' and 'yeasty' (Fig. 1a).

Volatile products of the fermented musts were quantified by HS-SPME-GC–MS. The modified MS300 culture medium does not contain grape varietal aromas or precursors, allowing the exclusive determination of aroma compounds synthesised by wine yeast. Odour activity values (OAVs) for the most significant volatile compounds are summarised in Table 2. Thirteen compounds associated with the attributes 'nail polish remover', 'roses', 'banana', 'apple', 'pineapple', 'floral' and 'fruity' showed OAVs > 1 for both strains, suggesting that these compounds could significantly contribute to the perceived global aroma. Ethyl hexanoate and ethyl octanoate, associated to 'fruity', 'apple' and 'pineapple' descriptors, had the highest OAVs for both strains. In particular, the concentration of ethyl octanoate was higher in musts fermented with EC1118, coinciding with the higher 'pineapple' aroma obtained during the sensory descriptive analysis for these musts (Fig. 1a).

Sniffing analysis of the fermented musts using GC–Olfactometry permitted to further analyse the contribution of each single volatile compound to the aroma perception. The modified frequency (MF), indicating the aroma impact of each compound, was calculated based on the frequency and the intensity of detection of each volatile compound by the panel of judges, as detailed in the section Materials and Methods. Most of the compounds present in concentrations higher than their detection thresholds (OAV > 1) were detected by the panel, although at diverse intensities (Table 2). In addition, some volatile compounds present in concentrations below their detection threshold (OAV < 1) were sensed in the GC–O. An MF value higher than 70 – representing all the intermediate combinations from 'detection of high intensity aroma by half of the panel' to 'detection of medium intensity aroma by the entire panel' – was only observed for acetic acid, 2-methyl butanoic acid and 3-methyl butanoic acid, associated to the negative characters 'vinegar', 'rancid' and 'cheesy'. The rest of the volatile compounds

Table 1
Fermentation parameters of *S. cerevisiae* strains VIN13 and EC1118, in fermentations at 15 °C.

Strain	Max biomass (g DCW L^{-1})	Final ethanol conc. (%v/v)	Final glycerol conc. (g L^{-1})	μ max (h^{-1})	q_{Gluc} max (g L^{-1} h^{-1})	q_{Fruc} max (g L^{-1} h^{-1})
VIN13	5.4 ± 0.3	14.9 ± 0.2	7.6 ± 0.2	0.04	−0.64	−0.35
EC1118*	5.7 ± 0.3	14.8 ± 0.4	6.7 ± 0.1	0.04	−0.72	−0.44

DCW: Dry cell weight.
Conc.: concentration.
μ: Biomass growth rate.
q: Volumetric consumption rate.
* *(15).*

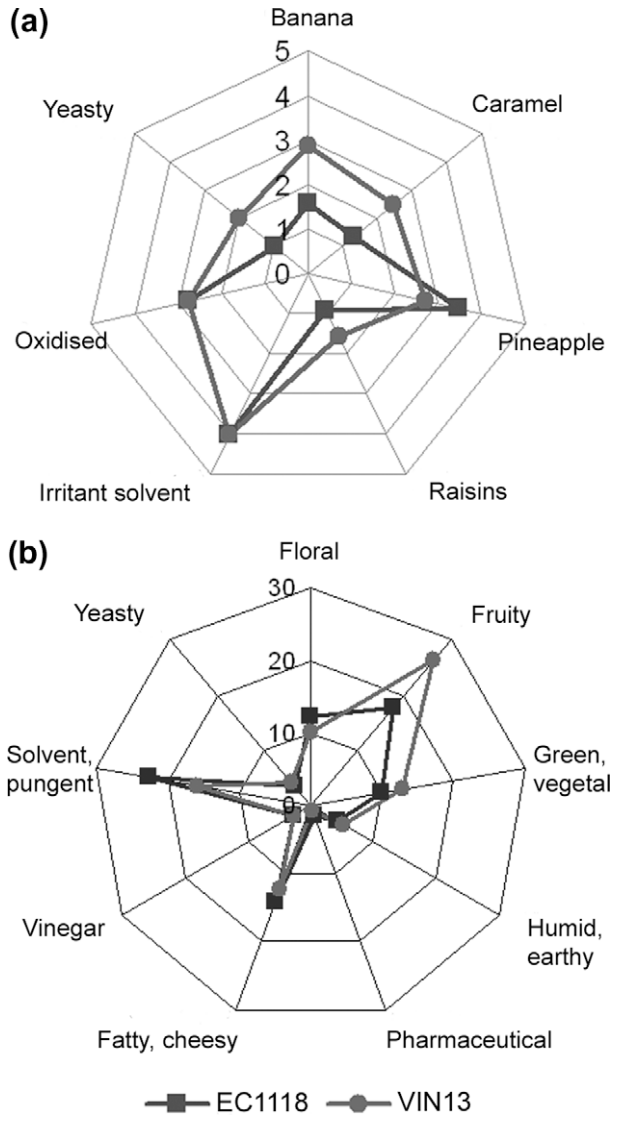

Fig. 1. Sensory evaluation of musts fermented with *S. cerevisiae* strains EC1118 and VIN13 at 15 °C. (a) Quantitative descriptive analysis. (b) Added MF values for descriptors of common aroma categories identified by a panel of judges by GC–O. Values are mean of two fermentations.

with OAV > 1 showed MF values between 50 and 70, meaning that they were detected in medium to low intensities or that they were not detected by the entire panel.

The major differences in volatile compounds among both strains were found for ethyl esters. Ethyl hexanoate and ethyl octanoate were strongly detected by the panel in fermentations carried out with EC1118, while ethyl decanoate was not detected in EC1118 fermentations and only barely detected in VIN13 fermentations. On the contrary, short chain ethyl esters were produced in higher concentrations by VIN13. Indeed, the calculated OAV for ethyl propanoate and ethyl butanoate agreed with the slightly higher MF values. Two compounds, ethyl 2-methyl propanoate and ethyl 2-methyl butanoate, had OAVs > 1 only in fermentations with VIN13, suggesting that the 'fruity' and 'strawberry' characters might characterise the musts fermented with this strain. Surprisingly, these two compounds were not detected by the judges in the GC–O analysis.

Among alcohols, the concentration of 2-phenyl ethanol was higher in musts fermented with EC1118, which agrees with a higher sensory perception of 'flowery' and 'roses' attributes detected by GC–O (Table 2). Conversely, 3-methyl butanol was produced in higher concentrations by strain VIN13, but it was sensed with higher MF values in musts fermented with strain EC1118. Acids other than acetic acid, 2-methyl butanoic acid and 3-methyl butanoic acid had OAV's < 1, but MF values ranging from 56 to 42, representing an intermediate detection of 'sweaty', 'onion', 'harsh', 'rancid' and 'cheesy' characters. A higher detection of the 'banana' attribute was found in musts fermented with strain VIN13 in the quantitative descriptive analysis (Fig. 1a), agreeing with the higher concentration of the associated compound 3-methyl butyl acetate (Table 2). Alternatively, 2-phenyl ethyl acetate was produced in concentrations slightly higher than its detection threshold by both strains, and was only weakly detected by the GC–O panel.

The aroma descriptors detected by GC–O were classified into eleven general aroma categories: fruity, banana, floral, fatty-cheesy, green-vegetal, solvent-pungent, yeasty, humid-earthy, pharmaceutical, vinegar (Fig. 1b). For each category, the MF values were added, including descriptors with unknown chemical identity. Both yeast strains produced musts with similar aroma composition (characterised by the "shape" of the spider webs). However, musts fermented with VIN13 showed higher values for the attributes 'fruity', 'green-vegetal', 'fatty-cheesy' and 'sweet-caramel' than musts fermented with EC1118 (Fig. 1b). Both strains had similar scores for the general categories 'floral' and 'solvent-pungent', while the attributes 'banana', 'yeasty', 'humid, earthy', 'vinegar' and 'pharmaceutical' resulted in modified frequencies below 200, and no significant differences between both strains.

3.3. Influence of the yeast strain and temperature on the production of volatile compounds

PCA was used to evaluate the statistical effect of the variables 'yeast strain' and 'temperature of fermentation' on the production of yeast-derived volatile compounds. For this purpose, data from the present study and from work previously published by our group (Molina et al., 2007) were used. Thus, three different fermentation conditions were analysed: VIN13 – 15 °C, EC1118 – 15 °C, and EC1118 – 28 °C. Strain VIN13 was not able to complete the fermentation at 28 °C (data not shown).

The first two Principal Components (PCs) explained 87% of the variance (PC1: 62%; PC2: 25%). Replicates of each 'strain-temperature' condition (VIN13 – 15 °C, EC1118 – 15 °C and EC1118 – 28 °C) were grouped and separated from the other conditions (Fig. 2). PC1 attributes with the highest weights (indicated in parenthesis) included hexanoic acid (1.00), 2-methyl butanoic acid (0.97), 2-methyl butanol (0.96), and ethyl propanoate (0.93). The concentrations of these compounds were higher in the VIN13 fermentations at 15 °C which grouped together on the right side of the graph. EC1118 Fermentations carried out at 28 °C had the lowest scores for both PC1 and PC2, and clustered in the lower left quadrant. Indeed, the highest negative weights in PC1 were assigned to 2-phenyl ethyl acetate (−0.97) and 2-methyl butyl acetate (−0.88), which were produced in higher concentrations in the EC1118 fermentations at 28 °C. Finally, EC1118 fermentations at 15 °C formed a separate group on the upper middle part of the graph and had the highest scores of PC2. This axis was characterised by decanoic acid (0.94) and ethyl decanoate (0.77) with the highest positive weights, and ethyl 2-methyl butanoate (−0.72) with the highest negative weight.

The significance of the effect of the yeast strain and the temperature of fermentation on the production of volatile compounds was assessed by Multifactor Analysis of Variance. The factor 'yeast strain' had a statistically significant effect at the 95% confidence level (p-value < 0.05) on 15 of the quantified volatile compounds, 12 of which had also a statistically significant effect from the factor 'temperature of fermentation' (Table 3). Additionally, the factor

Table 2
Odour activity values (OAV) and modified frequency (MF) of volatile compounds in fermentations of chemical defined grape juice media at 15 °C with *S. cerevisiae* strains EC1118 and VIN13.

Aroma compound	Threshold	OAV[a]		MF[b]		Aroma descriptor
	(mg/l)	EC1118[c]	VIN13	EC1118	VIN13	
Alcohols						
2-Methyl propanol	40[d]	**2.8**	**2.8**	**61.8**	37.8	Spirituous, fuel
2-Methyl butanol	30[e]	**1.5***	**2.2***	**71.8**	**58.6**	Harsh, nail polish remover
3-Methyl butanol	30[d]	**8.1***	**9.5***			
2-Phenyl ethanol	10[d]	**5.9***	**4.2***	**50.0**	20.4	Roses
Acids						
Acetic acid	200[d]	**4.3***	**3.5***	**70.9**	**87.2**	Vinegar
2-Methyl butanoic acid	3[d]	0.2*	0.3*	**82.9**	**91.3**	Cheese, rancid
3-Methyl butanoic acid	3[d]	0.1*	0.4*			
Hexanoic acid	3[d]	0.4*	0.6*	**50.0**	**53.2**	Cheese, sweaty
Octanoic acid	8.8[e]	0.4*	0.3*	48.4	42.7	Rancid, harsh
Decanoic acid	15[d]	0.3	0.1	**55.7**	**51.5**	Fatty
Acetates						
2-Methyl butyl acetate	0.005[f]	**28.8**	**26.1**	42.7	**67.7**	Banana
3-Methyl butyl acetate	0.03[d]	**61.4**	**65.3**			
2-Phenylethyl acetate	0.25[d]	**1.8***	**1.0***	25.0	32.3	Roses
Ethyl Esters						
Ethyl propanoate	1.84[e]	0.0*	0.1*	**50.0**	**56.1**	Fruity, pear, apple
Ethyl 2-methyl propanoate	0.015[d]	0.1*	**1.3***	**nd**	**nd**	Fruity
Ethyl butanoate	0.02[d]	**12.4**	**12.8**	**59.7**	**61.2**	Fruity, apple
Ethyl 2-methyl butanoate	0.001[d]	0.4*	**6.2***	**nd**	**nd**	Fruity, strawberry, pineapple
Ethyl hexanoate	0.005[d]	**89.0**	**82.6**	**62.4**	**55.9**	Fruity, apple
Ethyl octanoate	0.002[d]	**333.7**	**296.8**	**55.8**	48.0	Fruity, pineapple, pear
Ethyl decanoate	0.2[d]	**6.0***	**2.7***	**nd**	33.1	Floral
Ethyl acetate	7.5[d]	**6.1***	**6.8**	17.7	32.3	Nail polish remover, fruity

Fermentations were carried out in triplicate.
OAV > 1 and MF > 50 are in bold.
nd: not detected.
* Indicates OAV with significant differences between yeast strains (95% confidence level).
[a] OAV calculated as concentration/threshold.
[b] MF calculated based on the intensity and frequency of detection by GC–O, as detailed in Section 2.
[c] (*15*).
[d] (*11*).
[e] (*22*).
[f] (*23*).

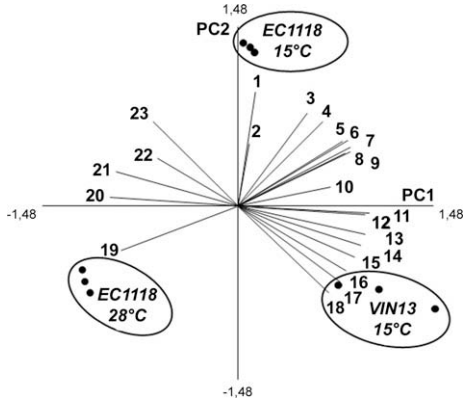

Fig. 2. Principal Component Analysis of fermentative volatile compounds produced during fermentation of a chemically defined must by *S. cerevisiae* strains VIN13 and EC1118 at 15 °C, and EC1118 at 28 °C. Each condition was tested in triplicate. 1: decanoic acid; 2: 2-methyl propanol; 3: ethyl decanoate; 4: octanoic acid; 5: ethyl butanoate; 6: ethyl acetate; 7: ethyl hexanoate; 8: ethyl octanoate; 9: 3-methyl butyl acetate; 10: 3-methyl butanol; 11: hexanoic acid; 12: 2-methyl butanol; 13: 2-methyl butanoic acid; 14: ethyl propanoate; 15: ethyl 2-methyl propanoate; 16: 2-methyl propanoic acid; 17: 3-methyl butanoic acid; 18: ethyl 2-methyl butanoate; 19: 2-methyl butyl acetate; 20: 2-phenylethyl acetate; 21: 2-phenylethanol; 22: 2-methyl propyl acetate; 23: acetic acid.

'temperature of fermentation' alone significantly affected the final concentration of six additional compounds. Neither of the two factors had a statistically significant effect on the final concentrations of 2-methyl propanol and 3-methyl butanol.

4. Discussion

In this study, the differential impact of two commercial *S. cerevisiae* wine strains on the aroma profiling and on the synthesis of volatile compounds was demonstrated. Strains EC1118 and VIN13 were selected because of their known differential contribution to wine aroma even though partially sharing their genetic background (EC1118 is a parental of the hybrid strain VIN13). Musts fermented by these two strains differed in sensory perception and descriptive analysis. The judges sensed slightly higher intensities of some descriptors in must fermented with VIN13, indicating a more complex aroma of these musts compared to musts fermented with EC1118. Twenty three volatile compounds were found to be produced by both strains, and most of them showed different final concentrations. VIN13 produced fifteen compounds in concentrations over the corresponding sensory thresholds, while EC1118 produced thirteen. The differential production of volatile compounds by VIN13 and EC1118 could result, at least in part, from the genetic contribution of the other parental strain, *S. cerevisiae* 228, to the genetic background of VIN13.

Musts fermented with VIN13 exhibited the highest concentration of short and branched-chain ethyl esters, and were characterised by sweet and fruity aroma characters. Both features constitute positive traits for VIN13 in the production of fresh and young white wines. This 'aroma producer' capacity is complemented by the good ability of VIN13 to release thiol precursors from grapes, thus enhancing the tropical flavours in Sauvignon Blanc wines (Swiegers et al., 2006).

Table 3
Analysis of variance (ANOVA) of the effect of the yeast strain and the temperature of fermentation on the final concentrations of yeast-derived volatile compounds.

Aroma compound	Temp		Strain	
	F-Ratio	*p*-Value	*F*-Ratio	*p*-Value
Alcohols				
2-Methyl propanol	1.62	0.250	2.21	0.187
2-Methyl butanol	6.20	0.047*	6.87	0.040*
3-Methyl butanol	2.02	0.205	0.08	0.791
2-Phenylethanol	35.64	0.001*	215.54	0.000*
Acids				
Acetic acid	6.75	0.041*	110.17	0.000*
2-Methyl propanoic acid	38.96	0.001*	327.50	0.000*
2-Methyl butanoic acid	7.94	0.030*	29.67	0.002*
3-Methyl butanoic acid	2.73	0.150	161.55	0.000*
Hexanoic acid	46.71	0.001*	46.55	0.001*
Octanoic acid	101.20	0.000*	9.87	0.020*
Decanoic acid	54.83	0.000*	40.44	0.001*
Acetates				
2-Methyl propyl acetate	0.01	0.935	8.25	0.028*
2-Methyl butyl acetate	37.61	0.001*	0.19	0.681
3-Methyl butyl acetate	17.38	0.006*	0.03	0.860
2-Phenylethyl acetate	188.14	0.000*	230.77	0.000*
Ethyl esters				
Ethyl propanoate	6.60	0.042*	21.21	0.004*
Ethyl 2-methyl propanoate	0.63	0.457	26.71	0.002*
Ethyl butanoate	103.66	0.000*	1.89	0.218
Ethyl 2-methyl butanoate	41.48	0.001*	698.18	0.000*
Ethyl hexanoate	809.79	0.000*	0.59	0.471
Ethyl octanoate	204.50	0.000*	0.08	0.791
Ethyl decanoate	56.16	0.000*	10.06	0.019*
Ethyl acetate	26.93	0.002*	0.95	0.368

* *p*-Value < 0.05 denotes a statistically significant effect of the factor on the final concentration of the volatile compound at the 95% confidence level. To determine the contribution of each factor, the effects of all other factors were removed.

VIN13, which is recommended for white wine fermentation between 12 °C and 16 °C, was not able to complete fermentation at 28 °C in the conditions used in this study. In contrast, EC1118, known for its tolerance to a wide range of fermentation temperatures, showed excellent performances both at 15 °C and 28 °C (Molina et al., 2007). When fermenting at 15 °C each strain presented different favourable winemaking properties. EC1118 strain had a higher rate of sugar consumption, and fermented faster than VIN13, while the latter produced more glycerol and less acetic acid, traits associated to wine quality.

The volatile compounds present in the wine matrix were extracted and quantified by HS-SPME using isotopically labelled internal standards. Compared with static head space gas chromatography, in which extraction of the compounds depends mostly on their volatility, in SPME the use of fiber involves selection of the extracted compounds depending on their partitioning among the liquid sample, the vial head space and the fiber. The method used in this study was specially optimised for accurate and precise quantification of key fermentative aroma compounds present in wines (Siebert et al., 2005). The use of labelled internal standards allowed overcoming the disadvantage of differential selectivity of the compounds, as well as matrix related effects. Equilibrium condition and affinity were assumed to be equal for each compound and its deuterated standard.

Based on their concentrations, the main volatile compounds allowing discrimination between both strains were 3-methyl butanoic acid, ethyl 2-methyl butanoate, 2-phenyl ethanol, 2-phenyl ethyl acetate, and acetic acid. Sniffing analysis pointed out further distinctive compounds. In addition to 3-methyl butanoic acid, 2-phenyl ethanol and acetic acid, compounds such as ethyl hexanoate, ethyl octanoate and 3-methyl butyl acetate showed different aroma contributions. Quantitative descriptive analysis matched these results, i.e. 'banana' attribute, associated to 3-methyl butyl acetate, characterised fermentations carried out with VIN13; while the 'pineapple' attribute, associated to ethyl octanoate, characterised fermentations with EC1118.

When using GC–O to quantify the impact of several odorant compounds, the number of judges in the panel is more decisive than the selection or experience of the judges (Ferreira, Pet'ka, Aznar & Cacho, 2003). In the present study, a panel of 8 judges was used to detect a number of volatile compounds. Since the detection threshold can vary from one person to the other, compounds present in concentrations near their reported detection thresholds were frequently sensed by part of the panel. However, ethyl 2-methyl propanoate and ethyl 2-methyl butanoate, two compounds produced in concentrations over their sensory thresholds only in fermentations with VIN13, were not detected by GC–O. These compounds probably co-eluted with other compounds that masked their aromas, and thus, further adjustments of the extraction method and the chromatographic run are necessary in order to detect these compounds by GC–O.

The factors 'yeast strain' and 'temperature of fermentation' have critical effects on the production of most yeast-derived volatile compounds. In a previous work, statistically significant effects of grape variety, yeast strain and fermentation temperature on the production of organic acids and higher alcohols were evidenced, and interactions between yeast type and temperature, and between temperature and grape variety were detected (Aragon, Atienza, & Climent, 1998). Our results agree with that previous report, and incorporate the statistical effect of yeast strain and fermentation temperature to additional fermentative aroma compounds as several fatty acids, ethyl esters and acetate esters.

In this study, the use of chemical defined grape juice media permitted to eliminate the effect of grape variety from yeast strain on the production of volatile compounds. However, the utilisation of natural grape must may change the effect that yeast strain and temperature can have on the level of volatile compounds, as complex interactions between grape variety, must nitrogen content and other variables may take place (Carrau et al., 2008; Delfini et al., 2001; Loscos et al., 2007; Ugliano et al., 2006). The results presented here demonstrate that both yeast strain and temperature of fermentation are critical variables influencing the aroma composition of a fermented must, and that the modulation of their effects could have a major impact on the final aroma and wine style.

Acknowledgments

We thank Robyn Kievit for her assistance with fermentations. This work was supported by FONDECYT grant 1050688; MECESUP grant PUC 9902/9903; Vinnova grant 05CTE01-06; 2005 Endeavour Australia Postgraduate and Postdoctoral Research Fellowships (DEST, Australian Government); and The Australian Wine Research Institute, through investment by the Grape and Wine Research Development Corporation.

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Food Chemistry 117 (2009) 196–203

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Quantification and characterisation of polyphenol oxidase from vanilla bean

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ARTICLE INFO

Article history:
Received 29 December 2008
Received in revised form 8 March 2009
Accepted 30 March 2009

Keywords:
Vanilla beans
Polyphenol oxidase
Quantification
Characterisation

ABSTRACT

Polyphenol oxidase (PPO) of *Vanilla planifolia* Andrews beans was extracted and purified through ammonium sulphate precipitation, dialysis, and gel filtration chromatography. PPO activity was measured by improved UV technique using 4-methylcatechol and catechol as substrates increasing substantial sensitivity of previous procedure. The optimum pH and temperature for PPO activity were found to be 3.0 and 3.4 and 37 °C, respectively. K_m and V_{max} values were found to be 10.6 mM/L and 13.9 OD_{300} min^{-1} for 4-methylcatechol and 85 mM/L and 107.2 OD_{300} min^{-1} for catechol. In an inhibition test, the most potent inhibitor was found to be 4-hexylresorcinol followed by ascorbic acid. The thermal inactivation curve was biphasic. Activation energy (E_a) and z values were calculated as 92.10 kJ mol^{-1} and 21 °C, respectively.

1. Introduction

Polyphenol oxidase (PPO) is a copper-containing oxidoreductase widely distributed in nature, which plays important roles in many plant metabolic processes. It is responsible for the enzymatic browning of fruits and vegetables. In the presence of oxygen, PPO catalyses two reactions: the hydroxylation of monophenols to diphenols (monophenolase EC 1.14.18.1) and oxidation of catechols to quinones (diphenolase EC 1.10.3.1), most of which are subsequently polymerised to dark pigments (Martinez & Whitaker, 1995; Matheis & Whitaker, 1984).

Enzymatic browning catalysed by PPO is generally considered detrimental to food quality from both sensory and nutritional points of view. Due to the importance of this reaction in the food industry, PPO has been intensively studied in several plants. Recent reports have shown that in some plants the protein is encoded by a multi gene family with a tissue specific expression pattern (Pereira, Donizeti, Murad, De Oliveira, & Evangekista, 2003; Yoruk & Marshall, 2003). The differences between PPOs may be identified by their physical, chemical or enzymatic properties such as electrophoretic mobility, optimal temperature and pH, substrate specificity and pI (Lu, Tong, Long, & Feng, 2006; Wang et al., 2007; Yoruk & Marshall, 2003).

It is well established that vanilla owes its flavour, aroma, and taste primarily to different enzymatic transformations that take place during the curing process. The traditional curing method is characterised by four phases: pod killing, sweating, drying and conditioning. From our point of view, the most important step of the curing process is pod killing. The objective of killing is to stop further vegetative development in the fresh pod and initiate wall disassembly by disrupting the cell membrane (Mariezcurrena, Zavaleta, Waliszewski, & Sanchez, 2008). Polyphenol oxidase in vanilla beans plays an important role in the colour qualities and commercial properties of cured beans. Importantly, vanilla bean browning appears to be driven by enzymatic activity. For example, prolonged or extreme heating during the curing process inhibits browning of the beans, probably due to enzyme denaturation. PPO driven browning in vanilla pods may result mostly from the oxidation of tyrosine, caffeic and chlorogenic acids, as well as other phenolic compounds (Havkin-Frenkel, French, Pak, & Frenkel, 2005).

Very little is known about PPO activity during growth, maturation and curing of vanilla beans. Wild-Altamirano (1969) studied dried acetone green vanilla powder and measured polyphenol oxidase activity in 14 substrates at pH 7 at an ambient temperature: however, these reaction conditions are not optimal for vanilla PPO, and the reaction products were measured for all substrates at a fixed 265 nm wavelength. Under these conditions, the highest substrate PPO affinities were to catechol and pyrogallol, but it is now well known that each substrate shows different reaction conditions with PPO (pH, temperature, substrate and enzyme concentration), and each substrate reaction product has different maximum absorbance wavelengths (Richard-Forget, Rouet-Mayer, Goupy, Philippon, & Nicolas, 1992). Debowska and Podstolski (2001) investigated PPO properties from vanilla shoot primordia culture revealing three isoenzymes shared the same optimum pH activity between pH 3 and 4.

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.03.118

Considering the importance of cured vanilla as a worldwide, it is important to understand the biochemical properties of vanilla bean PPO. The degree of enzymatic browning depends on the PPO activity, but also on the nature and amount of endogenous phenolic compounds, the presence of oxygen, reducing substances, pH, and temperature.

The aim of this paper was to extract, purify and characterise polyphenol oxidase from mature vanilla beans to determine kinetic parameters, optimum reaction conditions (pH and temperature), thermal stability, and inhibitor effects of various chemical compounds. In addition, the activation energy (E_a) and z values were calculated. In addition, an improved UV procedure for PPO quantification using 4-methylcatechol and catechol as substrates was developed.

2. Materials and methods

2.1. Plant material

Vanilla beans (*Vanilla planifolia*) with yellow apices were obtained from a recognised producer (Mr. Victor Vallejo, Papantla, Veracruz, México). Beans were washed with distilled water, cut into 1 cm pieces, lyophilised and stored in refrigeration in polypropylene vacuum bags.

2.2. Enzyme extraction and purification

Twenty-five grams of lyophilised beans were powdered using a domestic coffee grinder prior to homogenisation. The powder was homogenised for 5 min at 4 °C in an Ultra Turrax T 25 homogenizer (Ika Works Inc, Wilmington, NC) in the presence of 200 mL of 150 mM bis–tris-propane (BTP) buffer (pH 8.0) with 2 mM ethylenediamine–tetraacetic acid (EDTA), 3 mM of DL-dithiothreitol (DTT) and 1.25 g of polyvinylpolypyrolidone (PVPP). The homogenate was centrifuged at 37500×*g* for 20 min at 4 °C and filtered through a Whatman 1 paper filter (Dignum, Kerler, & Verpoorte, 2001). The enzyme extract was ultrafiltered in a Millipore stirred cell with a 10 kDa membrane and unfiltered solid was used as a crude enzymatic extract. The enzyme extract was submitted to protein precipitation with solid ammonium sulphate. Protein fractions at 30%, 30–40%, 40–50%, 50–60%, 60–70% and 70–80% of ammonium sulphate were separated by centrifugation at 10000×*g* for 20 min at 4 °C and the supernatant was removed. Each precipitate was dissolved in a small amount of 100 mM phosphate buffer (pH 7.0) and dialysed at 4 °C against the same buffer with four buffer changes every 6 h. For further purification, a dialysed enzyme aliquot was fractionated by gel filtration chromatography using a Sephacryl S-200 column with a 100 mL bed volume and equilibrated with 100 mM phosphate buffer (pH 7.0) and the elution rate was adjusted to 20 mL/h. The dialysed enzyme solution was applied to the column and 3 mL fractions were collected. The elution process continued until no absorbance at 280 nm was detected. The fraction with PPO activity was collected and the degree of purification was determined by measuring specific activity before and after purification. For determining PPO specific activity, a quantitative protein measurement was carried out. The protein concentration was determined using the Bradford (1976) method with Coomassie Plus Protein Assay Reagent (Pierce Chemical Co.) and bovine serum albumin was used as a protein standard.

2.3. PPO activity assay

The PPO assay was adopted and modified from Gauillard, Richard-Forget, and Nicolas (1993) technique. PPO activity was determined in a 2.0 mL assay mixture in a spectrophotometer (Beckman DU 7500) by measuring the increase in absorbance at 294 nm. Absorption spectrums collected during vanilla PPO reaction with catechol and 4-methylcatechol have shown the maximum wavelength of absorbance of the reaction product at 294 nm, and it is a major difference from the absorbance at 410–420 normally used to determine polyphenol oxidase activity with these substrates. The reaction mixture consisted of 0.9 mL of 0.1 M citrate buffer pH 3.0, 0.1 mL of enzyme, 0.5 mL of catechol or 4-methylcatechol in 0.1 M citrate buffer. The reaction was stopped by addition of 0.5 mL of 6% trichloroacetic acid. PPO activity measurements were performed over a 10 min reaction period and results were expressed as the mean. One unit of enzyme activity was defined as the amount of enzyme that caused 0.001 changes in absorbance at 294 nm per min. All runs were conducted in triplicate and the relative standard deviations were less than 1%.

2.4. SDS–PAGE electrophoresis

SDS–PAGE was performed according to Laemmli (1970), using a Bio-Rad Mini Gel system. The protein samples were fully denatured by boiling with β-mercaptoethanol and SDS and separated in a 12% polyacrylamide resolving gel and 4% stacking gel. Proteins were stained with Coomassie Blue and molecular weights were estimated by comparison with the molecular weight markers (Bio-Rad Precision Plus Protein Standard 161-0373).

2.5. Determination of isoelectric point

Preparative isoelectrofocusing was performed according to manufacturer's instructions for a Rotofor preparative cell (Bio-Rad) with a pH gradient from 3 to 10. Proteins were focused at 15 W constant electric power for 4 h at 4 °C. Twenty fractions, each of 2.5 mL were collected, their pH was measured and the samples were monitored at 280 nm for protein detection. This was followed by a Coomassie assay for protein and PPO activity to determine the location of PPO in collected fractions.

2.6. pH activity profile and pH stability

The PPO activity profile was analysed over a range of pH 3–7 using the following buffers: 0.05 M citrate buffer of pH 3–5 and 0.1 M phosphate buffer of pH 6–7. Catechol and 4-methylcatechol were used as substrate at 20 mM/L. PPO activity was assayed using the standard reaction mixture, but only changing the buffer. The optimum pH found for this enzyme was used in all other later studies. The purified enzyme was analysed for pH stability at the appropriate pH, ranging from 2.5 to 10 at 30 °C for 30 min prior to the assay for residual activity at a fixed pH of 3.4.

2.7. Temperature activity profile and heat inactivation

The optimal temperature was determined by measuring PPO activity under standard conditions using catechol as a substrate in a temperature range of 20–60 °C at pH 3.4. Thermal inactivation of purified PPO was studied at selected temperatures (60, 65, 70, 75, 80 and 85 °C) for various times in a glass tube. Tubes containing 900 μL of 0.1 M acetate buffer were heated to the selected temperature before addition of 100 μL of the enzyme solution than kept for isothermal inactivation. The time to reach the equilibrium temperature was measured, and this was taken into consideration in experiments and subtracted when calculating the inactivation time. At different time intervals, one tube was taken out of the water bath and rapidly cooled in an iced water bath to stop thermal inactivation for 30 min of the storage period, than transferred to a water bath at 37 °C. To conduct the enzymatic reaction for

10 min, 500 μL of 20 mM catechol in 0.1 M acetate buffer pH 4.8 at 37 °C was added. The experiments were performed in three repeated runs for each temperature. An enzyme sample that had not been heated was used as a blank. The percentage of residual activity was calculated by comparison with the unheated sample. The first-order inactivation constant (k) was calculated from the slope of the time course of denaturation using the equation: ln (A/A_0), where A_0 was the initial enzyme activity and A was the activity at time t. The slopes of these plots were determined by linear regression, and the rate constants were plotted. The half-life of the enzyme was calculated as $t_{1/2} = 0.693/k$. The decimal reduction time (D value) was estimated as $D = 2.303/k$. The z value, which is the temperature increase required for a $\log_{10}$ reduction (90% of decrease) of the D value, was determined from a plot of $\log_{10}D$ against temperature. The slope of the graph is equal to $1/z$ and the activation energy of denaturation (E_a) was calculated by multiplying the slope of the Arrhenius plot (ln (k) vs. $1/T$) by the universal gas constant R.

2.8. Enzyme kinetics

In order to determine the Michaelis-Menten constant (K_m) and maximum velocity (V_{max}), PPO activity was measured using catechol and 4-methylcatechol as substrates at various concentrations of 1–40, and 0–400 mM, respectively. K_m and V_{max} values of the enzyme were calculated from a plot of $1/V$ against $1/S$ using the method of Lineweaver and Burk method. Measurements were carried out in triplicate.

2.9. Effect of selected chemicals on enzyme activity

The purified PPO was incubated for 60 min at 4 °C in the presence of the following compounds (1 mM): L-ascorbic acid, dithiothreitol (DTT), β-mercaptoethanol, EDTA, metabisulfite, 4-hexylresorcinol, benzoic acid, cysteine, citric acid, borax, and sodium azide previously dissolved in 0.5 M citrate phosphate buffer (pH 3.8). SDS was used in this study but at a concentration of 1%. The presence of ethanol (0–90%) was also analysed after enzyme incubation for 48 h. The residual activity was measured by the standard assay technique (as described previously).

2.10. Effect of metal ions

The purified PPO was preincubated for 60 min at 4 °C in the presence of 1 mM of various metal ions (Mg^{+2}, Cu^{+2}, Ca^{+2}, K^{+1}, Mn^{+2}; Fe^{+2} and Hg^{+2}) previously dissolved in 0.5 M citrate phosphate buffer (pH 3.8). This was followed by measurement of the residual enzymatic activity.

2.11. Statistical analysis

Results are average of three determinations were analysed for variation (ANOVA) and statistical significance by t-test.

3. Results and discussion

3.1. Extraction and purification of PPO

The highest PPO activity of the precipitate was observed under conditions of 30–75% ammonium sulphate saturation, and this saturation point was used for all the extraction processes. Polyvinylpyrolidone was used during extraction to bind phenols that could inactivate the PPO. It is well documented that oxidation of phenolics by PPO produces quinones which can inhibit PPO (Matheis & Whitaker, 1984).

After ammonium sulphate precipitation, the PPO activity level in the dialysed extract decreased to 47.3%. This was probably due to loss of copper ions from the medium during dialysis (Matheis & Whitaker, 1984).

The results of PPO purification are shown in Table 1. The column was calibrated using a commercial protein kit containing five proteins of known molecular weights. The calibration protein graph had a linear regression equation of $y = 0.0135x + 3.3498$ ($R^2 = 0.9624$) and was used to calculate the molecular weight of PPO. The degree of purity was 9.5 after ammonium sulphate precipitation, and was 29.8 after gel filtration chromatography. The results of vanilla PPO purification by gel filtration chromatography are shown in Fig. 1. The active fractions of 30–41 eluates were collected to determine the degree of enzyme purity and for further characterisation studies. The molecular weight calculated by column chromatography was ~34 ± 2 kDa ($R^2 = 0.96$).

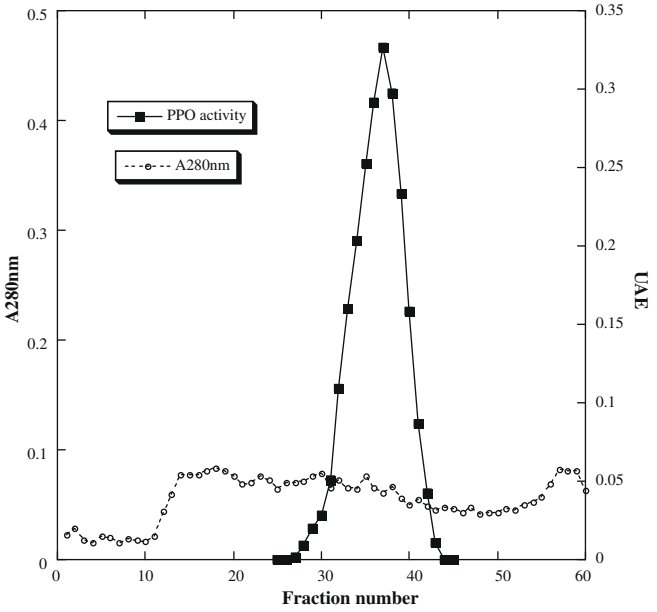

Fig. 1. Protein absorbance at 280 nm and polyphenol oxidase activity of fractions collected from Sephacryl S-200 column.

Table 1
Summary of results of PPO purification.

Sample	Total activity (units) 10^3	Activity recovery (%)	Total protein (mg)	Protein yield (%)	Specific activity (U/mg) 10^3	Fold purification
Crude	1482.8	100	109.6	100	13.5	1
UF*	1327.5	89.5	50.3	45.9	26.5	2
FG	701.8	47.3	5.5	5.0	128.6	9
$(NH_4)_2SO_4$	456.3	30.7	1.1	1.1	403.8	29.8

* Ultrafiltration on 10 KDa membrane.

3.2. Molecular weight and purity

After PPO isolation, the purity of the preparations and the molecular weight of the enzyme were examined by polyacrylamide slab gel electrophoresis, and the results after protein staining are shown in Fig. 2. In lane 2 of the Coomassie stained gel, the presence of several other proteins can be detected in the crude extract. Lane 3 represents the most active peak from the Sephacryl column, and the others bands have been eliminated leaving a pure PPO band. By comparing the molecular weight of PPO according to the gel with the known molecular weight of native PPO, we concluded that native PPO in vanilla bean is in a monomeric form of ~34 kDa. The molecular weight has previously been reported for coffee PPO (29 kDa) (Pereira et al., 2003), grape juice PPO (40 ± 2 kDa) (Okuda, Pue, Fujiyama, & Yokotsuka, 1999), marula PPO (71 kDa) (Mdluli, 2005), wheat PPO (40 kDa) (Kihara, Murata, Homma, Kaneko, & Komae, 2005) and apple PPO (45 kDa) (Ni Eidhin, Murphy, & O'Beirne, 2005).

3.3. Isoelectro focusing

Fig. 3 shows the PPO activity and pH profiles of the collected fractions resulting from the isolectro-focused preparations. The protein profile determined by absorbance at 280 nm showed only one peak. The peak obtained in fraction 5 corresponded to a pI of 4.0. Previous studies have shown that the pI of marula PPO was 5.4 (Mdluli, 2005) whilst, a pI of 3.6 was observed in palmito PPO (Robert, Rouch, Richard-Forget, Pabion, & Cadet, 1996).

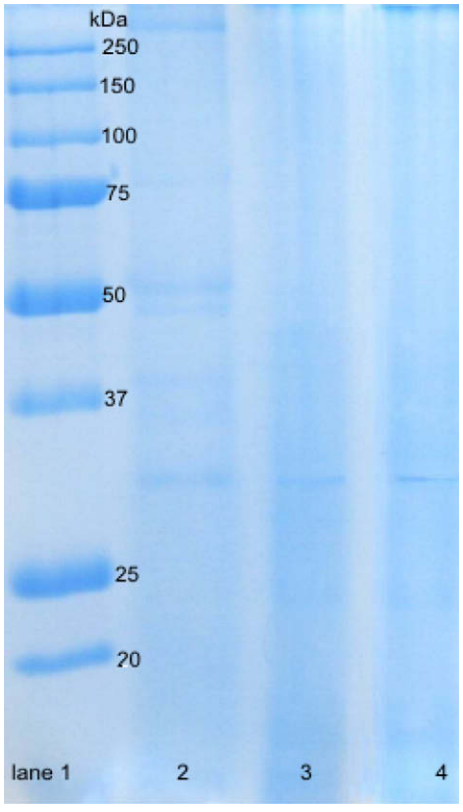

Fig. 2. SDS–PAGE of the purified PPO fractions. Lane 1, proteins standard; lane 2, crude enzyme extract; lane 3, PPO fraction after gel filtration; and lane 4, PPO fraction after preparative isoelectric focusing.

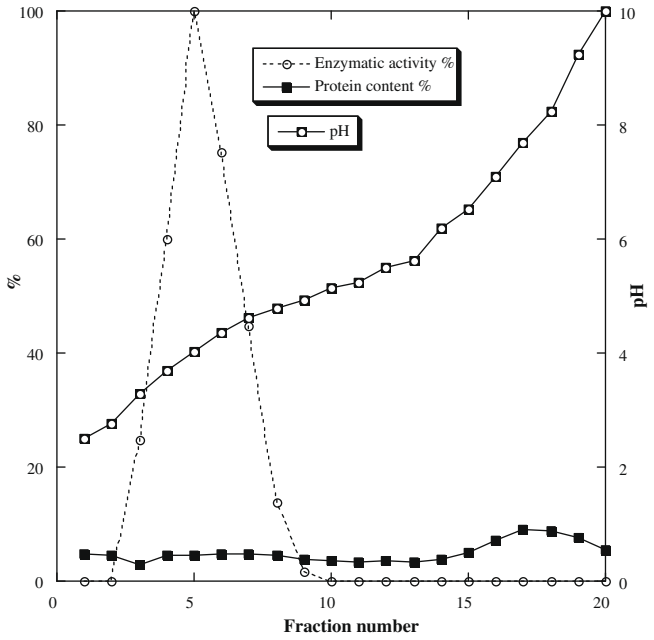

Fig. 3. Isoelectric focusing profile of PPO. Totally 20 fractions were collected at pH interval of 2–10.

3.4. PPO activity measurement

Many substrates were studied and compared for PPO activity, and showed that PPO has the best activity, the highest specificity and sensitivity toward catechol and 4-methylcatechol (Gauillard et al., 1991; Anil Kumar, Kishor Mohan, & Murugan, 2008; Dincer, Colak, Aydin, Kadioglu, & Guner, 2002; Gawlik-Dziki, Szymanowska, & Baraniak, 2007; Ngalani, Signoret, & Crouzet, 1993; Özen, Colak, Dincer, & Güner, 2004). Normally, PPO activity is measured by colorimetric method by estimation of oxidation products at very wide range of spectra at 390–430 nm. In fact, oxidation products of catechol and 4-M methylcatechol show two maxima absorbance, the highest and strong peak at 294 nm which clearly appeared after 10 min of reaction and the second weak and very wide peak indicating low sensitivity at 420 nm (Fig. 1), whilst the absorbance at 280 nm, characteristic for catechol and 4-methylcatechol slowly disappeared. To increase substantially assay sensitivity, we propose PPO assay activity with catechol and 4-methylcatechol at 294 nm after 10 min of reaction stopped by TCA. It offers advantage over colorimetric technique at 420 nm due to increase of measurement sensitivity as well as larger time of the linear period of the oxidation and large stability of oxidation products. Enzyme activity was measured for up to 10 min and stopped with TCA and the time of reaction of the linear part of absorbance vs incubation time. The initial rate was calculated from the slope of the absorbance–time curve. The increase in absorbance was linear with time for the first 15 min.

3.5. Optimal pH

Vanilla PPO had maximum activity at a pH approximately 3.4 for catechol and 3.0 for 4-methylcatechol (Fig. 4), which suggests that the enzyme may be found in acidic environments, such as the vacuole (Deepa & Arumughan, 2002). The optimum pH has previously been reported for Emir grape PPO 4.2 (Ünal & Sener, 2006). Erat, Sakiroglu, and Kufrevioglu (2006) found ferula leaf and stem samples optimum PPO activity for catechol (pH 7.0) and for 4-methylcatechol (pH 6.0), respectively. The optimum pH values for *Uapaca kirkiana* peel and pulp PPO were found to be 7.0 and

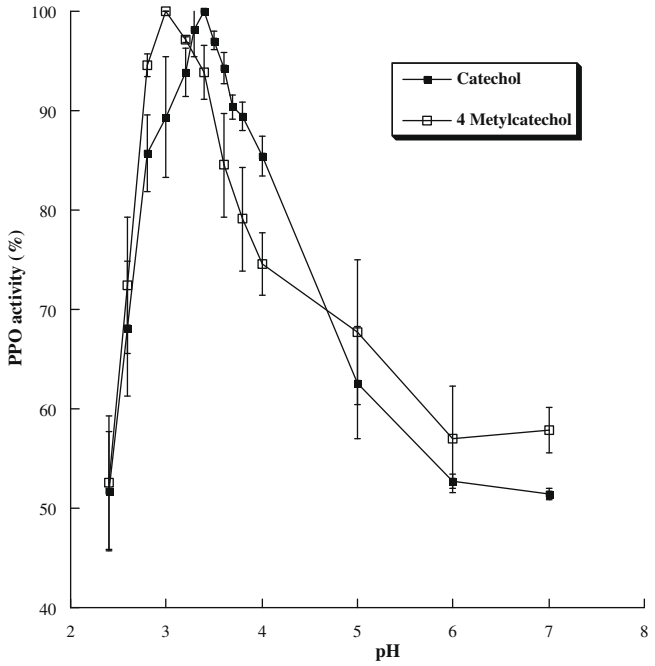

Fig. 4. pH optima of the purified vanilla bean PPO at 37 °C.

8.0, respectively (Muhuweti, Mupure, Ndhalala, & Kasiyamhuru, 2006). Chinese water chestnut PPO had an optimum pH of 6.5 with catechol (Lu et al., 2006) whilst marula fruit PPO showed optimum activity with catechol at pH 7.0 (Mdluli, 2005). In another study with catechol, pawpaw fruit (Fang, Zhang, Sun, & Sun, 2007) and mango pulp (Wang et al., 2007), PPO showed optimum pH of 7.0. These results do not coincide with the optimum pH of vanilla bean PPO, but a recent study reported that the optimum pH for PPO activity depends on the plant variety of the PPO, the nature of phenolic substrate and the extraction methods used (Ünal & Sener, 2006).

3.6. Effect of pH on PPO stability

The purified vanilla PPO showed the highest stability in an interval of pH 7–10 after 26 h of the enzyme incubation at 15 and 30 °C (Fig. 5). Whilst the enzymatic activity declined rapidly at pH below 5, the enzyme showed the least residual activity at pH 3. Erat et al. (2006) reported that *Ferula* PPO was more stable at pH 5.2 than at other pH values, whilst marula PPO was most stable at pH 6.0 (Mdluli, 2005). Grape juice PPO was stable at pH 6–8 (Okuda et al., 1999), whilst apple PPO was found to be most stable at pH 5.5–6.5 (Ni Eidhin et al., 2005). Chinese water chestnut PPO showed the highest stability at pH 6.5, which corresponded to the pH for optimal enzyme activity, but the relative activity at pH 3.0 was less than 10% (Lu et al., 2006). Enzyme instability at a low pH has also been reported for apple PPO (Ni Eidhin et al., 2005). This variation in PPO stability may be caused by differences in the binding ability of the substrate to the active site of the enzyme under ambient conditions (Martinez & Whitaker, 1995).

3.7. Optimal temperature

The effect of temperature on PPO activity was investigated in the range of 20–60 °C (Fig. 6). The optimum temperature for maximum vanilla PPO activity for catechol and 4-methylcatechol was found to be 37 °C, and the enzyme was active over relatively broad temperature range of 20–50 °C. The enzyme retained 80% or more activity between 30 and 45 °C. At temperatures above 50 °C the decline in enzymatic activity was more drastic, and only 10% of the activity was remained at 60 °C. Previous reports of thermal stability of PPO in other fruits have shown that, ferula PPO has an optimum temperature of 12 °C for catechol and 25 °C for 4-methylcatechol (Erat et al., 2006), *Uapaca kirkiana* PPO has an optimum temperature of 35 °C (Muhuweti et al., 2006), Emir grape PPO

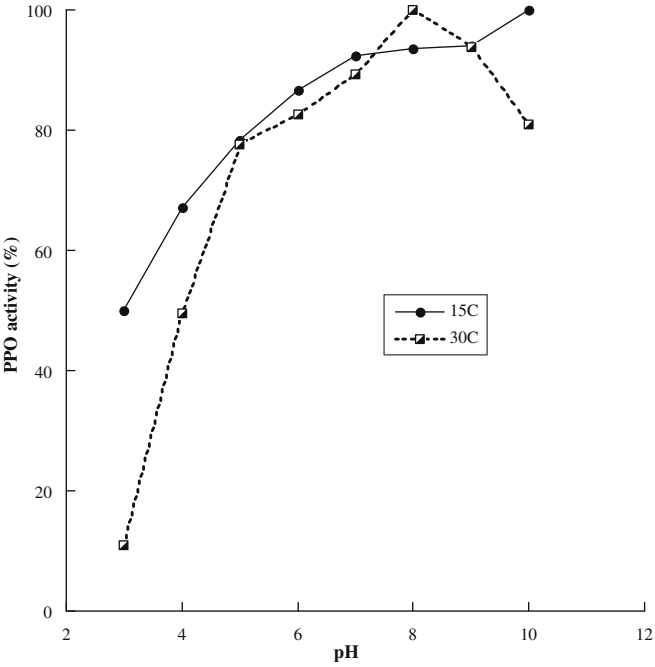

Fig. 5. Effect of pH on enzyme stability after the enzyme preparation incubation for 26 h at 15 and 30 °C in buffer solutions at various pH (3–10).

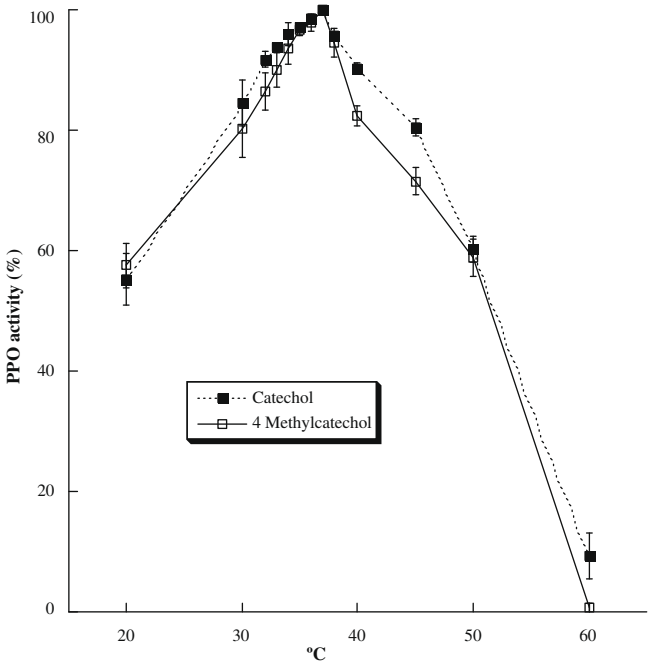

Fig. 6. Effect of temperature on PPO activity at pH 3.4 for catechol and pH 3.0 for methylcatechol. Results are means of duplicate + analyses of two replicates.

has optimum temperature of 25 °C (Ünal & Sener, 2006), the optimum temperature for grape juice PPO ranges 25 and 30 °C (Okuda et al., 1999), and apple PPO has an optimum temperature of 30 °C (Ni Eidhin et al., 2005).

3.8. Thermal inactivation

The thermal inactivation profile of vanilla PPO expressed as percentage of residual activity is shown in Fig. 7. The thermal inactivation profile is biphasic, which is more pronounced at 80 and 85 °C. The first phase represents thermolabile behaviour and the second phase thermoresistant behaviour. The biphasic behaviour may indicate the presence of two different isoenzymes with different thermal properties, which was not confirmed by gel electrophoresis. The enzyme was thermos table at 65 °C and retained up to 90% of activity after 20 min of heating. Biphasic thermal inactivation behaviour has been reported for Emir grape PPO (Ünal & Sener, 2006), Ferula PPO showed a decrease in activity of 40%, 65% and 100% at 30, 40 and 50 °C, respectively after 60 min of heat treatment and the enzyme was completely inactivated after 40 min at 60 °C (Erat et al., 2006). In comparison to other studies, marula fruit PPO treated at 60 °C was relatively thermostable retaining up to 60% of enzyme activity after 16 min of heating (Mdluli, 2005). Wheat PPO activity decreased below 30% after heat treatment at 70 °C for 10 min (Kihara et al., 2005).

Fig. 8 shows the Arrhenius plot of the kinetic expression for the thermal inactivation of PPO. Inactivation parameters were calculated from the initial part of the inactivation curve (Table 2). The half-life ($t_{1/2}$) value is an important parameter used in the characterisation of enzyme stability. The half-life values in the temperature range 60–85 °C varied between 2.6 and 0.2 min. In comparison with data from the literature, half-life values for marula fruit PPO at 60 and 70 °C were 1.4 and 0.8 min, respectively (Mdluli, 2005). Ünal and Sener (2006) reported half-life values for Emir grape PPO of 64.2 and 1.5 min at 65 and 80 °C, respectively. Since the half-life of inactivation obtained for vanilla PPO was low (0.8 min at 75 °C), and was less than the half-life of inactivation for Emir grape PPO (4.0 min at 75 °C), it is probable that vanilla PPO is less thermostable than other PPOs.

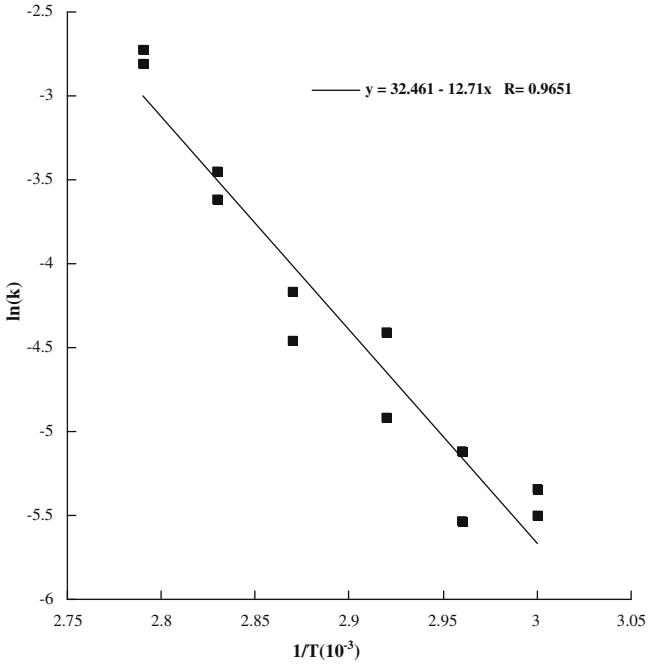

Fig. 8. The Arrhenius plot for heat inactivation of the purified PPO.

Table 2
Thermal inactivation parameters of purified PPO.

Temperature (°C)	$\Delta H^{\#}$ (J mol^{-1})	$\Delta G^{\#}$ (J mol^{-1})	$\Delta S^{\#}$ (J mol^{-1}K^{-1})	$t_{1/2}$ (min)	D_{value} (min)
60	89331.4	96848.8	−22.57	2.608	8.667
65	89289.9	98031.6	−25.86	2.425	8.056
70	89248.3	97612.5	−24.39	1.263	4.197
75	89206.7	98119.2	−25.61	0.871	2.893
80	89165.2	97305.2	−23.06	0.396	1.317

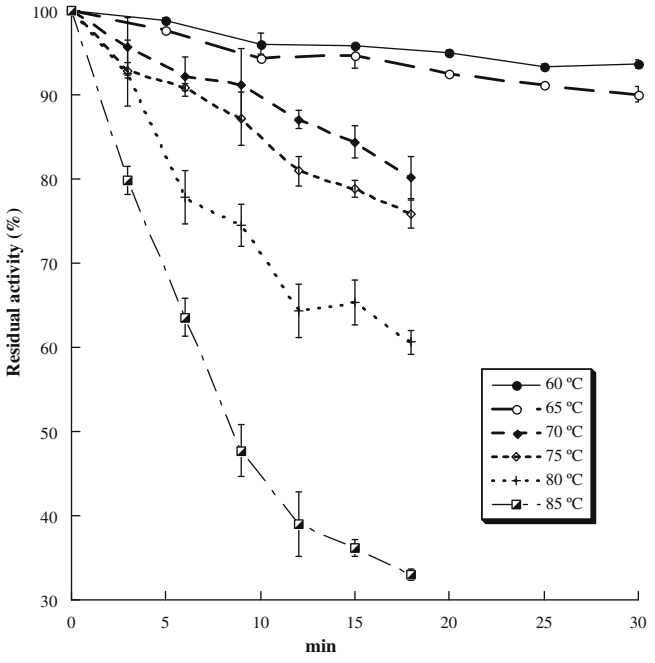

Fig. 7. Thermal inactivation profile for PPO. Values are means for duplicate analyses of two replicates.

The decimal reduction time (D value) is a parameter commonly used in the characterisation of enzyme stability and is defined as the time needed for a 90% reduction of the initial activity. D values for vanilla PPO ranged between 8.7 and 0.6 min at the temperatures studied (Table 2). D values previously reported for marula PPO were 4.6 and 2.5 min at 60 and 70 °C, respectively (Mdluli, 2005), whilst the D values of Emir grape PPO ranged between 213.2 and 4.9 min at 65 and 80 °C respectively (Ünal & Sener, 2006),

The z value is characterised by the temperature dependence of the decimal enzyme reduction activity time, which is the temperature increase needed for a $\log_{10}$ reduction (90% of decrease) in the D value. In general, low z values indicate greater enzyme sensitivity to heat, and high activation energy (E_a) values reflect a greater sensitivity of the enzyme to temperature change. Interestingly, z values may be influenced by the degree of ripeness and preparation method (Chutintrasri & Noomhorm, 2006) E_a and z values for vanilla PPO were calculated as 92.1 kJ mol^{-1} ($r^2 = 0.97$) and 21 °C ($r^2 = 0.94$), respectively. Some of the reported E_a values for PPO include 251 kJ mol^{-1} for Emir grape 25 °C (Ünal & Sener, 2006), and 37.9 kJ mol^{-1} for marula fruit (Mdluli, 2005). The E_a value for vanilla PPO is within the range of these values. Reported z values for PPO include 8.92 °C for Emir grape (Ünal & Sener, 2006) and 13 °C for an unspecified variety of grape (Weemaes, Ludikhuyze, Broeck, Hendricks, & Tobback, 1998). The z value found in this study is in agreement with these results.

Other transition state parameters, namely, $\Delta G^{\#}$, the activation Gibbs free energy change is considered as the energy barrier for enzyme inactivation, $\Delta H^{\#}$, enthalpy change, a measure of the number of covalent bonds broken, and $\Delta S^{\#}$, the entropy change, which is a measure of net enzyme and dissolvent disorder, were calculated using equations described by Forsyth, Owusu Apenten, and Robinson (1999). The $\Delta H^{\#}$ value found in this study is within the range of previously reported values of other PPO's. Mdluli (2005) reported values of $\Delta H^{\#}$ for marula PPO between 36168 and 34919 in a temperature interval of 60–90 °C. However, Forsyth et al. (1999) raised questions about the suitability of use of isolated $\Delta H^{\#}$ values as indicators of enzyme stability.

3.9. PPO kinetic parameters

The substrate specificity of PPOs varies widely depending on the source and purity of the enzyme. The affinity of a PPO for a particular substrate may even vary amongst multiple samples from of a particular preparation. It has often been suggested that the preferred substrate is the most abundant phenolic compound, which is not always the case. Catechins, cinnamic acid esters, 3,4-dihydroxyphenylalanine (DOPA) and tyrosine are the most important natural phenolic substrates of PPOs in fruits and vegetables (Matheis & Whitaker, 1984).

The kinetic parameters were measured at pH 3.4 and 3.0 for 4-methylcatechol and catechol, respectively at 37 °C. K_m and V_{max} were calculated using a Lineweaver–Burk plot. The K_m values for vanilla PPO were 10.6 mM and 85 mM using 4-methycatechol and catechol, respectively. K_m is a measure of the affinity of the enzyme for the substrate with smaller values representing a greater affinity. The affinity of vanilla PPO is low, as evidenced by its high K_m values. The affinity of plant PPOs for phenolic substrates is relatively low, usually around 1–10 mM. Ünal and Sener (2006), reported a K_m for Emir grape PPO of 25.1 mM for catechol, whilst ferula PPO had a K_m value of 2.34 mM for catechol and 6.58 for 4-methylcatechol (Erat et al., 2006). The K_m for marula PPO was 4.99 and 1.45 mM for catechol and 4-methylcatechol, respectively (Mdluli, 2005), whilst butter lettuce showed higher affinity to 4-methylcatechol than to catechol (Gawlik-Dziki, Zlotek, & Swieca, 2008) The Chinese water chestnut PPO K_m value for catechol was 10.32 mM (Lu et al., 2006) whilst mango PPO K_m was only 6.30 mM (Wang et al., 2007). The maximum reaction velocity (V_{max}) values for vanilla PPO were found to be 13.6 $OD_{300}\,min^{-1}$ and 107.2 $OD_{300}\,min^{-1}$ for 4-methylcatechol and catechol, respectively.

3.10. Effect of various chemicals on enzyme activity

The effects of inhibitors on vanilla bean PPO activity were studied at various concentrations using catechol as a substrate at pH 3.4. The results are given as a percentage of PPO inhibition in Table 3. It may be concluded that the most potent inhibitors were 4-hexylresorcinol and ascorbic acid. Ascorbic acid, cysteine and 4-hexylresorcinol when added to mango puree produced important PPO activity decreased (Guerrero-Beltran, Swanson, & Barbosa-Canovas, 2005). In addition, cysteine and ascorbate significantly inhibited cherry PPO activity (Anil Kumar et al., 2008). Ascorbic acid and metabisulfite are reducing agents that can reduce o-quinones to form stable colourless products (Ünal & Sener, 2006). There has been substantial interest in using ascorbic acid alone or with citric acid as a sulphite substitute. Due to safety concerns, sulphite was banned from use in fresh fruit and vegetables by the Food and Drug Administration (Martinez & Whitaker, 1995). In our study, citric acid was the least potent inhibitor and its inhibitory effect is normally attributed to its pH lowering effect and chelate formation with copper. In the case of vanilla PPO, citric acid produced two combined effects: it acted as a PPO activator by lowering pH (the optimum pH for catechol is 3.4), and as an inhibitor by chelate formation with copper. The inhibitor reaction mechanism varies depending on the inhibitor agent employed. Inhibition by thiol compounds is attributed to either the stable colourless products formed by an addition reaction with o-quinones, or by binding to the active centre of PPO (Muhuweti et al., 2006; Yoruk & Marshall, 2003).

Table 3
Effect of chemical compounds on PPO activity.

Compound (1 mM)	Residual activity (%)
Borax	110.52 ± 0.71
Citric acid	79.03 ± 7.20
Sodium azide	49.30 ± 1.98
Cysteine	22.54 ± 2.72
Benzoic acid	21.45 ± 1.91
EDTA	19.99 ± 0.11
Sodium dodecyl sulphate (1%)	18.30 ± 3.25
Sodium metabisulphite	6.46 ± 0.44
β-Mercaptoethanol	3.95 ± 0.46
Dithiothreitol	1.25 ± 0.17
Ascorbic acid	0.23 ± 0.03
4-Hexylresorcinol	0.15 ± 0.02

Values represent the mean of three replicates.

Table 4
Effect of metal ions on PPO activity.

Metal ion (1 mM)	Residual activity (%)
$FeCl_2$	165.7 ± 8.7
$CaCl_2$	80.4 ± 6.9
$MnCl_2$	81.6 ± 6.2
$MgCl_2$	79.7 ± 2.4
$HgCl_2$	48.8 ± 7.1
$CuCl_2$	89.2 ± 1.4

Values represent the mean of three replicates.

SDS is known to modify the tertiary and quaternary structures of proteins, resulting in 80% inactivation of vanilla PPO. In a previous study on PPO of *Vicia faba*, the presence of SDS in the reaction medium eliminates PPO activity at acid pH and greatly increases PPO activity at a neutral pH (Jimenez & Garcia-Carmona, 1996). EDTA, a metal chelator also resulted PPO inhibition of 80%. The enzyme was inhibited by thiol compounds such as cysteine by 78%.

PPO activity decreased as the ethanol concentration was increased. At an ethanol concentration of 10%, the enzyme lost 30% of its activity, at a 20% ethanol concentration, the loss in activity of PPO was almost 90% and at a concentration of 30% ethanol, the enzyme activity was completely inhibited. A decrease in enzymatic activity in the presence of ethanol has previously been reported for bromelain (total inhibition at 50% ethanol) (Hasan, Rasheedi, & Khatun, 2003).

3.11. Effect of metal ions on enzyme activity

Table 4 shows the effect of metals ions at a concentration of 1 mM on PPO activity. Some halides activated the enzyme, and this activation may be caused by the substitution between a halide ion and copper in the enzyme. This result indicates that metal ions are involved in PPO enzymatic activity, as has previously been described.

4. Conclusions

This study describes the quantification, isolation and characterisation of PPO from vanilla beans. To increase substantially assay

sensitivity, we propose PPO assay activity with catechol and 4-M methylcatechol at 294 nm after 10 min of reaction. The activity of vanilla PPO has some similarities with other plant PPO's. The enzyme has the greatest substrate specificity for 4-methylcatechol over catechol at an optimum temperature of 37 °C, and at pHs of 3.0 and 3.4 for each substrate, respectively. The most potent inhibitor was 4-hexylresorcinol, followed by ascorbic acid. PPO plays an important role in the colour qualities and commercial properties of cured beans and their final alcoholic extract. The data obtained in this study may help understand vanilla bean PPO activity mainly when heat treatment is used to fruit killing during curing process. Vanilla bean PPO utilises mono- and diphenols to catalyse the oxidation of various cellular substrates, probably including vanillin and this reaction may decrease vanillin content in the cured bean.

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Food Chemistry 117 (2009) 204–206

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Selenium concentration in St. John's wort (*Hypericum perforatum* L.) herb after foliar spraying of young plants under different UV-B radiation levels

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ARTICLE INFO

Article history:
Received 25 November 2008
Received in revised form 22 February 2009
Accepted 30 March 2009

Keywords:
Herb
Nutritional supplement
Selenium
UV-B radiation

ABSTRACT

St. John's wort (*Hypericum perforatum* L.) was grown under different levels of UV-B radiation, with selenium (10 mg l^{-1} Se applied by foliar spraying in the form of sodium selenate) or without foliar Se application. The different levels of UV-B radiation comprised an enhanced level simulating 17% ozone depletion, ambient level, and a reduced level of UV-B radiation. The concentration of Se in unsprayed plants was from 20 ng g^{-1} to 120 ng g^{-1}. The concentration of Se in the organs of plants foliarly sprayed with Se ranged from 1000 ng g^{-1} to 12,000 ng g^{-1}, the highest concentration being detected in plants grown under reduced levels of UV-B radiation. Foliar application of Se fertiliser is feasible and effective in St. John's wort and results in Se-enriched nutritional supplements.

1. Introduction

St. John's wort herb is traditionally used in Europe to relieve mild or moderate depression. Its extracts are also known for their *in vitro* antimicrobial and free radical-scavenging activity (Radulovic et al., 2007; Silva, Ferreres, Malva, & Dias, 2005; Silva, Malva, & Dias, 2008). Several new products were developed recently, which contain St. John's wort herb or its extracts as additives; several brands of food, beverages and yoghurts include St. John's wort herb (Ang et al., 2004; Silva et al., 2005). The consumption of its products has much increased, St. John's wort now being one of the most consumed of medicinal plants.

Seed soaking, or spraying of plants with a selenium (Se) solution may enrich the utilisable plant parts with Se compounds in concentrations of nutritional importance (Germ, Stibilj, Osvald, & Kreft, 2007; Ožbolt, Kreft, Kreft, Germ, & Stibilj, 2008; Smrkolj, Stibilj, Kreft, & Germ, 2006). In the case of plant products, which are part of food additives, a higher concentration of Se is desired to balance diets, otherwise deficient in Se. In fact, Se could be added to the preparation, or simply supplemented to the diet. However, among consumers, at least in Europe, is a growing interest for the original plant constituents and not for chemicals added to the preparations or foods.

Interactions between the effect of UV-B radiation and Se addition on its uptake in plants are known for buckwheat (Ožbolt et al., 2008). St. John's wort is a plant which thrives in direct sunshine, including natural UV-B radiation. However, as far as the present authors know, no studies have been carried out concerning the impact of UV-B radiation and/or supplementation with selenium (Se) by foliar spraying on the Se concentration in St. John's wort.

The studies of Gaberščik, Vončina, Trošt, Germ, and Björn (2002), Kreft, Štrukelj, Gaberščik, and Kreft (2002), Liang, Huang, Zhang, Zhang, and Zhou (2006), and Alonso-Amelot, Oliveros-Bastidas, and Calcagno-Pisarelli (2007) revealed that UV-B radiation affects the synthesis of secondary substances.

The aim of this work was to investigate the impact of Se spraying of young plants, and of UV-B radiation on the concentration of Se in the herb of *Hypericum perforatum*, in order to achieve concentrations of nutritional importance for food supplements or drugs.

2. Materials and methods

2.1. Plants and growth conditions

St. John's wort (*H. perforatum* L.) seedlings were obtained by cloning a sibling plant. Seedlings were planted in May 2005 in

Abbreviations: DM, dry mass; Se, selenium; Se0, without selenium; Se+, with selenium; UV−, reduced UV-B level; UV0, ambient UV-B level; UV+, enhanced UV-B level.

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.03.099

sandy soil in pots (15 × 15 × 15 cm), one plant per pot, on an outdoor research plot (Botanical Garden, University of Ljubljana: 320 m above sea level, 46°35′N, 14°55′E). The plants for each treatment were chosen randomly out of 100 specimens.

A stepwise UV-B supplement system was used. Three different treatments were used: a reduced level of UV-B radiation (UV-B(−)), using Mylar foil which cuts out wavelengths below about 320 nm (Gehrke et al., 1996); elevated UV-B radiation, simulating 17% ozone depletion (UV-B(+)), using Q-Panel UV-B 313 lamps with cellulose diacetate filters, which block the UV-C range (wavelengths lower than 280 nm); ambient radiation (UV-B(0)), produced with Q-Panel UV-B 313 lamps, filtered with Mylar foil to correct for the effects of UV-A radiation (control). Lamps were positioned at a distance of 25 cm from each other. Above the plants (80 cm) was placed an UV-B transmitting shield, to minimise the differences among treatments, due to wind, precipitation or temperature. The Mylar foil filter was positioned 80 cm above the plants. The systems were timer controlled. The biologically effective UV-B ($UV\text{-}B_{BE}$) doses were calculated and adjusted weekly using the programme of Björn and Murphy (1985), based on the generalised plant action spectrum of Caldwell (1968). During the experiment the methodology established during EU project UV-AQUTER (PL 970637) was strictly followed.

Three weeks after planting, plants were sprayed with a solution of detergent (Triton T-100, Sigma, 0.2 ml l^{-1}), with Se (Se+) (10 mg l^{-1} Se, in the form of sodium selenate) or without Se (Se0), to wet the whole plant. Detergent was added for uniform distribution of sprayed solutions on plants. There were six different groups: all combinations of 3 UV-B treatments and 2 Se treatments (with and without Se, respectively). In each UV-B and Se treatment group there were 10 plants, used randomly for analysis. At the time of Se foliar spraying, the young plants had no more than four leaves. These first four lower leaves (until the termination of the experiment they mainly developed into runners) were not included in the samples of leaves for analysis, to avoid possible contamination by Se not absorbed by the plants. Plants were harvested in week 14 after planting.

Samples were lyophilised, milled and stored in a refrigerator until analysed.

2.2. Determination of the Se content

Se content in stems, leaves and flower samples was determined by hydride generation atomic fluorescence spectrometry (HG–AFS) (Smrkolj & Stibilj, 2004). Digestion of samples was carried out in closed Teflon tubes under the following conditions: To 0.150–0.200 g of lyophilised sample of leaves, stems or flowers, 1.5 ml HNO_3 and 0.5 ml H_2SO_4 were added. Tubes were heated for 60 min at 130 °C in an aluminium block. Two millilitres of H_2O_2 were added and the samples were reheated at 115 °C for 10 min. Then, 0.1 ml 40% HF were added (only to leaves and stem samples) and the samples were heated at 115 °C for 10 min. Again 2 ml of H_2O_2 was added and the tube reheated at 115 °C for 10 min. After digestion, the solutions were cooled to room temperature and 0.1 ml of V_2O_5 in H_2SO_4 were added and the tubes were again heated at 115 °C for 20 min. Reduction of Se^{VI} to Se^{IV} with 2.5 ml of conc. HCl was carried out at 100 °C for 10 min. Samples were diluted and Se was determined by HG–AFS. Optimal measurement conditions are as follows: carrier flow rate 1 ml/min, argon flow rate 260 ml/min, nitrogen flow rate 3 ml/min, concentration of $NaBH_4$ 1.2%, concentration of HCl for hydride generation 2 M and concentration of HCl in the carrier 2 M. A schematic presentation of the procedure was published by Stibilj, Mazej, and Falnoga (2003). Each sample was analysed at least in duplicate.

The accuracy and precision of the method was checked by analysis of the certified reference material "Trace Elements in Spinach Leaves", NIST 1570a. Good agreement was found between the analysed and certified values of 119 ± 8 ng g^{-1} (four determinations) and 117 ± 9 ng g^{-1}, respectively.

All results are given on a dry mass basis (DM).

2.3. Statistical analyses

The data were evaluated by ANOVA (Statgraphics Version 4) and significance accepted at $p < 0.05$.

3. Results and discussion

The concentration of Se in unsprayed plants was from about 20 ng g^{-1} to 120 ng g^{-1} (Fig. 1). The concentration in foliarly sprayed plants was in the range from about 1000 ng g^{-1} to 12,000 ng g^{-1}, lowest in stem, higher in flowers and with the highest concentration in the leaves (Fig. 2). No visual symptoms of Se toxicity appeared on Se-sprayed plants, nor any symptoms due to UV-B irradiation of plants, or any other damage.

The present study revealed that a high amount of Se was efficiently transported from treated leaves to new, young leaves, emerging after the foliar treatment. It is known from previous studies that Se is easily transferred to other parts of the buckwheat plant when foliarly applied in low amounts (Germ & Stibilj, 2007; Smrkolj et al., 2006). Potato tubers and leaves of chicory, developed after Se spraying, contained significantly more Se, in comparison to Se-untreated plants (Germ, Kreft, Stibilj, & Urbanc-Berčič, 2007; Germ, Stibilj, Osvald, & Kreft, 2007).

Among the Se-sprayed plants the highest concentration of Se was found in plants exposed to reduced UV-B radiation (Fig. 2). The transformation of selenate into the plant's own Se-species and their translocation to the new, younger leaves depends on energy availability (Germ, Kreft, Stibilj, & Urbanc-Berčič, 2007; Germ & Osvald, 2005; Germ & Stibilj, 2007; Germ, Stibilj, & Kreft, 2007; Germ, Stibilj, Osvald, & Kreft, 2007). Plants not exposed to UV-B stress, had enough energy for efficient uptake of Se and its distribution in their tissue.

In contrast, among plants not sprayed with Se its concentration in leaves and stems was the highest in those plants exposed to enhanced UV-B radiation (Fig. 1), where the need for antioxidative effects was the highest.

The mass of the flowers was less than 200 mg per plant in the conditions of enhanced UV-B radiation with or without Se treatment, and therefore their analysis was not performed (Figs. 1 and 2).

No significant effects of UV-B or Se treatment were found on the length of the main shoot or the mass of shoots, except for the mass of shoots of plants treated both with Se+ and UV-B+, which was only 72% of that of untreated plants. In any case, Se-sprayed plants protected from UV-B radiation showed the highest concentration

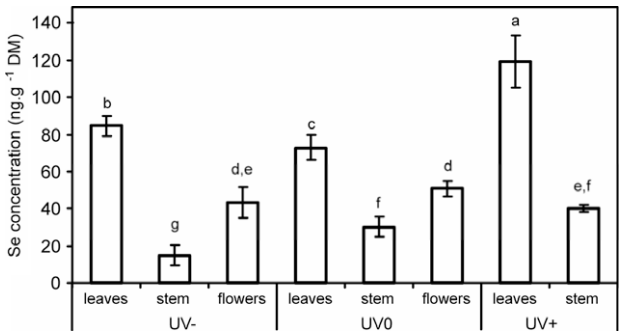

Fig. 1. Concentration of Se in plants not treated with Se (in ng g^{-1} DM ± SD, $n = 4$; UV−, reduced UV-B level; UV0, ambient UV-B level; UV+, enhanced UV-B level).

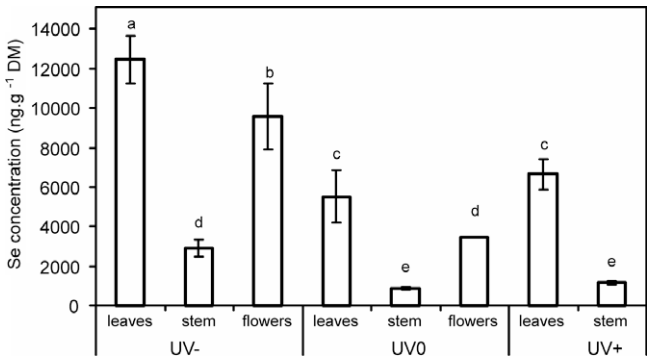

Fig. 2. Concentration of Se in plants treated with Se (in ng g^{-1} DM ± SD, $n = 4$; UV−, reduced UV-B level; UV0, ambient UV-B level; UV+, enhanced UV-B level).

of Se, and, hence, from the point of view of Se, this would be the most suitable way to grow St. John's wort for food supplements.

The recommended dietary intake of Se for adults is 55 micrograms per day (Food and Nutrition Board, 2000). Thus, a considerable part of this could be covered by products containing St. John's wort, subjected to foliar spraying with Se in the young plant phase. In conclusion, foliar application of Se fertiliser is feasible and effective in St. John's wort and results in Se-enriched products. This is in addition to its other important constituents, relieving mild or moderate depression, and exerting antimicrobial and free radical-scavenging activities.

Acknowledgements

The authors are grateful to Dr. A.R. Byrne for critical reading of the manuscript, and Neža Kenda for the assistance in Se analyses. This research was a part of the projects financed by the Slovenian Research Agency (ARRS), Ljubljana, Slovenia, entitled "The influence of UV-B radiation on antioxidant content and distribution in cultivated plants" (J7-9805-0106-06, and J4-9673-0481-06); and Programmes P1-0255, P1-0212 and P4-0085.

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Food Chemistry 117 (2009) 207–211

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Physicochemical characteristics, proximate analysis and mineral composition of ostrich meat as influenced by muscle

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ARTICLE INFO

Article history:
Received 31 October 2008
Received in revised form 25 February 2009
Accepted 30 March 2009

Keywords:
Ostrich meat
Muscle
Physicochemical characteristics
Proximate analysis
Mineral composition

ABSTRACT

The influence of muscle on the physicochemical characteristics, proximate analysis, and mineral composition of meat from 10 ostriches (10–12 months old), slaughtered according to commercial abattoir procedures, were evaluated. Muscle had no influence ($p > 0.05$) on L^*-values (32.5), a^*-values (11.9), water-holding capacity (11.9%), final pH (pH_{24}) values (6.07), and ash contents (1.12 g/100 g edible meat). However, intramuscular lipid contents varied ($p < 0.05$) from 0.88 (*M. fibularis longus*) to 1.44 (*M. flexor cruris lateralis*) g/100 g edible meat, at a mean value of 1.16 g/100 g edible meat for 10 different muscles. Sodium (34.7 mg/100 g edible meat) and iron (3.14 mg/100 g edible meat) contents, both influenced ($p < 0.05$) by muscle, possessed substantially lower and higher values, respectively, than values reported for beef and chicken.

1. Introduction

Despite domestication of the ostrich (*Struthio camelus*) in the middle of the 19th century due to a fashion for ostrich feathers, this animal remained unknown as a potential meat producer till the beginning of the 1990s. At this time the single channel marketing scheme was lifted by the Klein Karoo Agriculture Co-operative, Oudtshoorn, South Africa, and ostriches and ostrich products could be marketed freely worldwide (Drenowatz, Sales, Sarasqueta, & Weilbrenner, 1995). Surprisingly, notwithstanding little and fragmented research, several reviews (Balog & Almeida Paz, 2007; Hoffman, 2005, 2008; Paleari, Corsico, & Beretta, 1995; Sales, 1999; Sales & Horbańczuk, 1998; Sales & Oliver-Lyons, 1996) have been produced on ostrich meat. From the above it has been concluded that ostrich meat is characterised, relatively to meat from other species, by a

(1) high (>6.0) final pH (pH_f), which is beneficial for the colour and water-binding capacity of meat, but is undesirable for keeping quality and flavour,
(2) low intramuscular lipid content,
(3) low sodium (Na) content, and
(4) high iron (Fe) content.

Although several studies have evaluated the lipid and fatty acid composition of ostrich meat, a single study (Sales & Hayes, 1996) has determined the mineral profile of ostrich meat, with concentrations of iron (Fe) (Lombardi-Boccia, Lanzi, & Aguzzi, 2005; Lombardi-Boccia, Martinez-Dominguez, & Aguzzi, 2002), selenium (Se) (Duan & Åkesson, 2004), zinc (Zn) (Lombardi-Boccia et al., 2005), and copper (Cu) (Lombardi-Boccia et al., 2005) reported by some others. Furthermore, mineral concentrations have been evaluated in a limited number of different muscles, the unit used to sell ostrich meat. In addition to diet, hormones, age, gender and region, mineral concentrations vary among different muscles in the animal carcass, due to varying intensity of physical activity and the effects of fibre type (Doornenbal & Murray, 1981; Lin et al., 1989).

In order to evaluate the nutritional potential of ostrich meat, and to extend current knowledge, the present study aimed at evaluation of selected physicochemical characteristics, proximate analysis, and mineral composition of ostrich meat as influenced by muscle.

2. Materials and methods

2.1. Animals and sampling

Ostriches, predominantly from the subspecies *S. camelus australis* (Blue Neck), were reared on a commercial ostrich farm in Paprotno, Poland, on a commercial ostrich starter (16% crude

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doi:10.1016/j.foodchem.2009.03.100

protein, 9.6% fibre, 9.7 MJ/kg) feed mixture (pellets) till 3 months of age, whereafter a commercial (14.5% crude protein, 10.7% fibre, 9.5 MJ/kg) ostrich grower (pellets) was supplied. Ten ostriches (males) were slaughtered at the age of 10–12 months in a commercial abattoir for cattle and swine in Chruścina, Poland, with the slaughtering process adapted for ostriches. Ostriches were fasted for 24 h, and stunned with a captive bolt stunner. The unconscious birds were shackled by one leg, hoisted to a height and bled by severance of the heart, carotid artery and jugular vein through the thoracic inlet, anterior to the sternum plate. After feather and skin removal internal organs were removed. Carcasses were divided into two sides, and chilled for 24 h at 4 °C. Subsequently, legs and thighs were excised from the pelvic limbs.

The following 10 muscles were excised from the left side of each carcass: *M. gastrocnemius*, *M. fibularis longus*, *M. obturatorius medialis*, *M. flexor cruris lateralis*, *M. iliofibularis*, *M. femorotibialis medius*, *M. iliotibialis lateralis*, *M. iliofemoralis externus*, *M. ambiens*, and *M. iliotibialis cranialis*. External fat and epimysial connective tissue were removed and muscles were divided perpendicularly to their longitudinal axis into two equal parts. Whereas the caudal part of each muscle was stored at −18 °C till analysed for thawing and cooking losses, the cranial part was ground twice through a 4 mm sieve to ensure homogeneity of samples. The latter was portioned into two separate parts, with one of them being stored at −18 °C for proximate analysis and assaying mineral concentrations, and the second used fresh for immediate determination of meat colour, water-holding capacity (WHC), and final pH (pH_{24}).

2.2. Physicochemical characteristics

Six muscles (*M. gastrocnemius pars interna*, *M. fibularis longus*, *M. iliofibularis*, *M. femorotibials medius*, *M. iliotibialis lateralis*, and *M. iliotibialis cranialis*) were used for evaluation of physicochemical characteristics. To evaluate thawing and cooking losses of the thawed meat, the caudal part was weighed and then frozen at −18 °C for 2–3 months. Meat juice losses during thawing were calculated based on the difference of sample weight before freezing and after a 24-h thawing period at 4 °C (in a household refrigerator). These losses were expressed as a percentage in relation to the weight of the sample before freezing.

For determination of cooking losses, 100 g samples, 20 mm thick, were cut from the muscles after thawing, and placed in 500 ml glass jars and covered with 300 ml water. Glass jars with meat samples were closed and placed in a water bath until a temperature of 85 °C was obtained inside the muscles, according to Baryłko-Pikielna, Kossakowska, and Baldwin (1964). Thereafter, meat samples were removed from the glass jars and exposed to dripping and cooling in a household refrigerator at 4 °C for a period of 30 min. Cooking losses were calculated based on the difference in sample weights before and after cooking, expressed as a percentage of sample weight before cooking.

The following determinations were made on fresh ground meat tissue from the cranial part of each muscle.

2.2.1. Colour measurements

These were taken after keeping ground meat samples in measurement cells in a household refrigerator at 4 °C for 20 min, to enable myoglobin oxygenation on the surface layer of meat. Colour was measured using a HunterLab MiniScan XE Plus 45/0 apparatus with a measuring port diameter of 31.8 mm, adopted for measuring the colour of ground meat, applying the CIELAB $L^*a^*b^*$ scale (Commission Internationale de l'Eclairage (CIE), 1976) and a D65 illuminant and 10° standard observer. Standardisation of the apparatus was done in relation to black and white colour standard references with the following coordinates: $X = 78.5$, $Y = 83.3$ and $Z = 87.8$ (for D65 illuminant and 10° standard observer).

2.2.2. Water-holding capacity

This was based on the percentage of free water in meat, according to the method of Grau and Hamm (1953), as modified by Pohja and Niinivaara (1957). Ground meat samples, 0.3 g each (weighed accurately to 0.001 g), placed on Whatman No. 1 paper-filter, were exposed to 2 kg pressure between two glass plates for a period of 5 min. Thereafter, using a planimeter, the area of two spots created by extruded meat juice and meat, respectively, was determined (in cm^2). In order to determine the percentage of free water in meat, the infiltrate area expressed in cm^2 obtained from the difference in the areas of these two spots was divided by the weight of the sample.

2.2.3. pH_{24} measurement

This was done 24 h after slaughter with a combined glass electrode (ESAgP-306 W type) of a CyberScan 10 pH-meter (Eutech Cybernetics Pte Ltd., Singapore) in a water extract (distilled water), with a 1:1 meat to water ratio, after 1 h of extraction.

2.3. Proximate analysis

The following chemical constituents were determined on thawed ground samples of all muscles according to the official methods of analysis of the AOAC (2003): moisture content by oven drying a *ca.* 2-g test sample at 102 °C to a constant weight (950.46B, see p. 39.1.02); ash content by igniting a *ca.* 3–5-g test sample in a muffle furnace at 550 °C until light grey ash results (920.153, see p. 39.1.09); crude protein content by the classical macro-Kjeldahl method (981.10, see p. 39.1.19); and lipid (crude) content by petroleum ether extraction using a Soxhlet apparatus (960.39 (a), see p. 39.1.05).

2.4. Mineral composition

Chemical elements in the examined material were determined by inductively-coupled argon plasma optical emission spectrometry (ICP-OES), using a Perkin–Elmer Optima 2000 DV system, after prior mineralisation in an Anton Paar Multiwave microwave oven (Anton Paar Ltd., Hertford, UK). Meat samples, *ca.* 0.5 g each, were weighed exactly to four decimal places, and transferred to pressurised quartz vials, into which 5.0 ml 65% HNO_3 and 0.5 ml 35% HCl were added. Vials were sealed with Teflon plugs, secured in mineralisation bomb units, and placed in a microwave oven equipped with a permanent temperature and pressure control system in each quartz vessel. Mineralisation was conducted after selecting the "Meat" procedure proposed by the equipment manufacturer: 0–5 min – power gradient from 100 to 600 W; 6 to 10 min – 600 W (constant); 11 to 20 min – 1000 W or less after reaching critical values (75 MPa or 300 °C); 21 to 35 min – vial cooling. The cooled mineralisate was diluted with deionised water to a final volume of 10 ml. The following minor chemical elements were determined in the prepared solutions: boron (B), barium (Ba), cobalt (Co), chromium (Cr), Cu, Fe, manganese (Mn), nickel (Ni), Se, silicon (Si) and Zn. In order to obtain an optimum concentration range for the ICP method, calcium (Ca), potassium (K), magnesium (Mg), Na and phosphorus (P) concentrations were determined after a 100-fold dilution of the mineralisate. As a standard, the certified multi-element standard solution "ICP Multi-element Standard IV" (Merck, Darmstadt, Germany) was used.

2.5. Statistical analysis

Results were analysed using the ANOVA procedures of the software STATISTICA (data analysis software system, Version 7.1; StatSoft, Tulsa, OK). Individual animals were used as blocks to remove variation, due to differences among animals, from the error sum of

squares (Sales, 1996). Means for individual muscles were compared using Tukey's HSD tests.

3. Results and discussion

3.1. Physicochemical characteristics

Measured objective values for lightness (L^*), and the difference between red and green (a^*), did not differ ($p > 0.05$) among the six muscles evaluated for colour. However, the *M. iliotibialis cranialis*, compared to the *M. gastrocnemius pars interna*, *M. fibularis longus*, *M. femorotibialis medius*, and *M. iliotibialis lateralis* (Table 1), showed a higher ($p < 0.05$) intensity of yellow than blue (b^*).

Although present values for L^* correspond to values (27.4–34.4) reported for 10 different muscles from different subspecies (South African Black, Zimbabwean Blue, hybrids) of ostriches by Hoffman, Muller, Cloete, and Brand (2008), the latter study found significant differences among muscles, with the *M. fibularis longus* possessing the darkest muscle. Similarly, significant differences in a^*-values (10.7–17.1) have been indicated among muscles in the latter study. However, in agreement with present results, the highest (8.3–9.3) and lowest (6.0–6.7) b^*-values have been found in *M. iliotibialis cranialis* and *M. fibularis longus*, respectively. Results for lightness found in the present study are contradictory to subjective colour measurements presented by Morris et al. (1995), who found significant differences in lightness among 10 different muscles from the ostrich carcass, with the highest degree of lightness in the *M. iliotibialis cranialis*. The above differences among studies could partly result from the use of different *post-hoc* statistical tests, to identify differences among muscles. However, colour determination on ground meat in the present study *versus* whole muscles in other studies could be another contributing factor. Fragmentation of the muscle structure by grinding assists in the release of liquids from the interior of muscle cells, including soluble protein such as myoglobin, and destruction of some of the muscle's aerobic-reducing system, which could influence colour development in the final product (Fernández-López, Pérez-Alvarez, & Aranda-Catalá, 2000). Although L^*- and a^*-values were within ranges (27.4–38.0 and 5.48–22.8, respectively) reported for ostrich meat by several studies (summarised by Hoffman et al., 2008), b^*-values in the current study were considerably higher than 0.06–6.57, indicating that muscles showed a higher yellow intensity with less blue undertones. Fernández-López et al. (2000) reported a significant decrease in a^*- and increase in b^*-values with grinding of pork, partly attributed to higher metmyoglobin formation. Furthermore, the incorporation of air and consequent formation of oxymyglobin could contribute to higher b^*-values in ground meat than whole muscle.

Neither WHC nor pH_{24} differed ($p > 0.05$) among muscles (Table 1), in contrast to studies by Sales (1996) and Hoffman et al. (2008), which reported significant differences in pH_{24} among different ostrich muscles. However, the pH_{24} of around 6 found in the present study is typical for ostrich muscles (Sales & Mellett, 1996). Thawing losses differed ($p < 0.05$) among muscles (Table 1), and are comparable to losses of 1.28–4.28% reported for ostrich *M. femorotibialis medius* at different thawing rates by Hong et al. (2005). Cooking losses, with the highest ($p < 0.05$) value presented by the *M. femorotibialis medius*, were higher than values of 31.9–37.7 (Sales, 1996) and 28.6–38.7% (Hoffman et al., 2008) found with different ostrich muscles immersed in a water bath at 75 °C (50 min) and 80 °C (60 min), respectively.

3.2. Proximate analysis

Despite significant differences among muscles, dry matter contents varied with 1.66% units between the highest (*M. obturatorius medalis*) and lowest (*M. iliofibularis*) mean values (Table 2), and presented an overall mean moisture content (76.27 g/100 g edible meat) that are in the upper limit of that (69.5–76.5 g/100 g edible meat) reported for raw mammalian and poultry muscles (Forrest, Aberle, Hedrick, Judge, & Merkel, 1975). A similar tendency has been found for protein content, with an overall mean value of 21.16 g/100 g edible meat for all muscles.

However, intramuscular lipid content varied from a mean value of 0.88 g/100 g edible meat in the *M. fibularis longus* to 1.44 g/100 g

Table 1
Physicochemical characteristics of different ostrich muscles (mean ± SD).

Muscle	L^*	a^*	b^*	Water-holding capacity (%)	Thawing losses (%)	Cooking losses (%)	pH_{24}
M. gastrocnemius pars interna	31.8 ± 1.36	16.7 ± 1.14	11.3 ± 0.64^{b}	11.3 ± 2.33	3.11 ± 1.44^{abc}	41.7 ± 2.21^{abc}	6.07 ± 0.06
M. fibularis longus	31.5 ± 1.97	17.1 ± 2.25	11.3 ± 1.50^{b}	11.0 ± 4.08	2.53 ± 0.92^{bc}	41.1 ± 2.59^{bc}	5.99 ± 0.07
M. iliofibularis	33.5 ± 0.43	17.7 ± 2.21	12.5 ± 1.07^{ab}	13.2 ± 3.87	3.26 ± 1.19^{abc}	43.7 ± 2.34^{ab}	6.01 ± 0.04
M. femorotibials medius	32.6 ± 2.46	16.9 ± 1.84	11.4 ± 1.22^{b}	13.8 ± 3.89	3.88 ± 1.42^{ab}	45.1 ± 3.11^{a}	5.99 ± 0.02
M. iliotibialis lateralis	32.8 ± 4.83	18.5 ± 5.74	11.4 ± 2.41^{b}	11.9 ± 4.87	4.21 ± 1.08^{a}	38.2 ± 10.09^{c}	6.01 ± 0.04
M. iliotibialis cranialis	32.9 ± 0.55	19.1 ± 2.13	13.3 ± 1.20^{a}	10.3 ± 1.63	2.16 ± 0.26^{c}	40.0 ± 2.38^{bc}	6.04 ± 0.02
Mean	32.5 ± 2.36	17.7 ± 2.86	11.9 ± 1.54	11.9 ± 3.57	3.16 ± 1.28	41.7 ± 5.18	6.07 ± 0.01

$^{a–c}$Values with different superscript letters in the same column are different ($p < 0.05$).

Table 2
Proximate analysis (g/100 g edible meat) of ostrich muscles (mean ± SD).

Muscle	Dry matter	Protein	Intramuscular lipid	Ash
M. gastrocnemius pars interna	23.3 ± 1.20^{bcd}	21.3 ± 0.73^{abc}	0.90 ± 0.16^{b}	1.10 ± 0.05
M. fibularis longus	23.3 ± 1.12^{bcd}	21.5 ± 0.81^{ab}	0.88 ± 0.23^{b}	1.12 ± 0.05
M. obturatorius medalis	24.5 ± 0.69^{a}	21.7 ± 0.49^{a}	1.22 ± 0.27^{ab}	1.14 ± 0.08
M. flexor cruris lateralis	24.2 ± 1.39^{ab}	21.5 ± 0.92^{ab}	1.44 ± 0.31^{a}	1.14 ± 0.06
M. iliofibularis	22.8 ± 0.93^{d}	20.7 ± 0.52^{bc}	1.10 ± 0.25^{ab}	1.07 ± 0.07
M. femorotibialis medius	23.0 ± 1.14^{cd}	20.8 ± 0.67^{bc}	0.95 ± 0.11^{b}	1.15 ± 0.07
M. iliotibialis lateralis	23.8 ± 1.05^{abcd}	21.4 ± 0.58^{abc}	1.21 ± 0.22^{ab}	1.17 ± 0.08
M. iliofemoralis externus	23.8 ± 1.29^{abc}	20.7 ± 0.79^{bc}	1.22 ± 0.12^{ab}	1.14 ± 0.14
M. ambiens	24.1 ± 1.04^{ab}	21.3 ± 0.53^{abc}	1.34 ± 0.35^{a}	1.09 ± 0.06
M. iliotibialis cranialis	24.4 ± 1.49^{a}	20.6 ± 0.78^{c}	1.36 ± 0.24^{a}	1.13 ± 0.12
Mean	23.7 ± 1.24	21.2 ± 0.76	1.16 ± 0.29	1.12 ± 0.08

$^{a–d}$Values with different superscript letters in the same column are different ($p < 0.05$).

edible meat in the *M. flexor cruris lateralis* (Table 2). Studies of the influence of muscle on lipid content of ostrich meat are contradictory (summarised by Hoffman, 2005). Substantially lower values (mean of 0.48 g/100 g edible meat) have been reported for 10 different ostrich muscles and muscle groups by Sales (1996). However, similarly to present results, the *M. flexor cruris lateralis* (0.82 g/100 g edible meat) and *M. fibularis longus* (0.24 g/100 g edible meat) were the muscles with the highest and lowest lipid contents, respectively. It is important to emphasise that lipid contents will depend on the methodology of the lipid analysis, with different solvents used for extraction causing different results (Jensen, 2004). In accordance with findings by Sales (1996), ash content did not differ ($p > 0.05$) among muscles (Table 2), and were comparable to an overall mean value of 1.14 g/100 g edible meat presented by the former study.

3.3. Mineral composition

Although mean Ca values varied from 4.76 (*M. iliofemoralis externus*) to 6.03 (*M. ambiens*) mg/100 g edible meat, no significant differences were detected among muscles (Table 3). The *M. flexor cruris lateralis* was the muscle with the highest ($p < 0.05$) mean contents of Mg (25.4 mg/100 g edible meat) and P (228 mg/100 g edible meat), whereas the lowest ($p < 0.05$) contents of Mg (23.3 mg/100 g edible meat) and K (234 mg/100 g edible meat), and highest ($p < 0.05$) content of Na (39.0 mg/100 g edible meat), were found in the *M. ambiens*.

Values for Ca, a mineral receiving a great deal of attention in nutrition, are somewhat lower than 7.29–9.00 mg/100 g edible meat reported for ostrich *M. gastrocnemius pars interna*, *M. iliofibularis* and *M. femorotibialis medius* by Sales and Hayes (1996), with no significant differences due to muscle found in the latter study. However, higher contents than in the present study for Na (40.6–46.6 mg/100 g edible meat) and K (266–272 mg/100 g edible meat), with contents of Mg as 20.6–22.7 mg/100 g edible meat and P as 206–220 mg/100 g edible meat, were presented by Sales and Hayes (1996). Calcium showed a mean overall value of 5.43 mg/100 g edible meat, comparable to 7 mg/100 g reported for lean beef (Holland et al., 1991), but considerably lower than a value of 12 mg/100 g for chicken without skin (USDA, 1979). With the adverse effects of excessive Na consumption, ostrich meat, with an overall mean Na content of 34.7 mg/100 g edible meat found in the present study, would be beneficial compared to values of 61 (Holland et al., 1991) and 77 (USDA, 1979) mg/100 g reported for lean beef and chicken without skin, respectively.

Iron content varied from a mean value of 2.32 in the *M. iliofibularis* to 4.02 mg/100 g edible meat in the *M. flexor cruris lateralis* (Table 3), with an overall mean value of 3.14 mg/100 g edible meat, substantially higher than 2.1 mg/100 g found in lean beef (Holland et al., 1991), and 0.9 mg/100 g (USDA, 1979) in chicken without skin. Values in the present study for Fe are higher than 1.97–2.51 mg/100 g edible meat reported (Sales & Hayes, 1996) for ostrich *M. gastrocnemius pars interna*, *M. iliofibularis* and *M. femorotibialis medius*, and 2.34–2.57 mg/100 g edible meat found in raw ostrich fillet, sirloin and leg (Lombardi-Boccia et al., 2005). Furthermore, contradictory to present results, the highest Fe content (2.51 mg/100 g edible meat) in the former study has been presented by the *M. iliofibularis*.

Contents of Cu, Se, Si and Zn were variable among muscles (Table 3), with overall mean values of 0.14, 0.03, 1.33 and 3.13 mg/100 g edible meat, respectively. These were comparable to 0.08–0.10 mg/100 g edible meat for Cu content, and in the upper limit of values (1.06–3.10 mg/100 g edible meat) for Zn content, reported in earlier studies (Lombardi-Boccia et al., 2005; Sales & Hayes, 1996) on the mineral composition of ostrich meat. Substantially higher values have been found for Se content (0.11 mg/100 g)

Table 3
Mineral composition (mg/100 g edible meat) of different ostrich muscles (mean ± SD).

Mineral	*M. gastrocnemius pars interna*	*M. fibularis longus*	*M. obturatorius medialis*	*M. flexor cruris lateralis*	*M. iliofibularis*	*M. femorotibialis medius*	*M. iliotibialis lateralis*	*M. iliofemoralis externus*	*M. ambiens*	*M. iliotibialis cranialis*
Major elements										
Ca	5.45 ± 1.06	5.94 ± 1.52	5.15 ± 0.66	5.18 ± 0.86	5.62 ± 1.36	5.01 ± 0.98	5.40 ± 1.02	4.76 ± 1.24	6.03 ± 1.80	5.80 ± 0.86
K	243 ± 10.0^{abc}	245 ± 12.3^{abc}	244 ± 16.1^{abc}	238 ± 14.3^{bc}	240 ± 13.0^{abc}	256 ± 11.7^{a}	254 ± 21.3^{ab}	237 ± 7.32^{c}	234 ± 16.4^{c}	244 ± 11.3^{abc}
Mg	24.3 ± 1.29^{ab}	23.8 ± 1.29^{ab}	25.3 ± 1.77^{a}	25.4 ± 1.63^{a}	23.8 ± 1.09^{ab}	24.3 ± 1.10^{ab}	24.6 ± 1.76^{ab}	24.6 ± 1.14^{ab}	23.3 ± 1.63^{b}	25.1 ± 1.04^{ab}
Na	36.1 ± 5.19^{abc}	33.4 ± 2.24^{b}	38.7 ± 3.99^{ab}	33.9 ± 3.41^{abc}	32.6 ± 1.26^{c}	32.1 ± 2.11^{c}	32.0 ± 2.66^{c}	34.1 ± 3.39^{abc}	39.0 ± 5.21^{a}	35.4 ± 4.85^{abc}
P	216 ± 13.2^{ab}	220 ± 10.8^{ab}	224 ± 14.49^{ab}	228 ± 12.0^{a}	210 ± 6.48^{b}	222 ± 11.1^{ab}	218 ± 10.8^{ab}	218 ± 4.57^{ab}	211 ± 13.1^{b}	223 ± 11.0^{ab}
Minor elements										
B	0.031 ± 0.012^{abc}	0.026 ± 0.007^{abc}	0.041 ± 0.020^{a}	0.035 ± 0.016^{ab}	0.028 ± 0.012^{abc}	0.025 ± 0.008^{bc}	0.021 ± 0.011^{bc}	0.023 ± 0.006^{bc}	0.021 ± 0.005^{bc}	0.020 ± 0.004^{c}
Ba	0.007 ± 0.001	0.006 ± 0.001	0.006 ± 0.001	0.007 ± 0.001	0.006 ± 0.0004	0.006 ± 0.001	0.006 ± 0.001	0.006 ± 0.001	0.007 ± 0.001	0.007 ± 0.001
Co	0.008 ± 0.0005	0.009 ± 0.0009	0.009 ± 0.0008	0.009 ± 0.002	0.009 ± 0.001	0.009 ± 0.001	0.009 ± 0.001	0.009 ± 0.002	0.009 ± 0.002	0.009 ± 0.0008
Cr	0.006 ± 0.002^{b}	0.008 ± 0.002^{ab}	0.008 ± 0.002^{ab}	0.008 ± 0.001^{ab}	0.006 ± 0.002	0.008 ± 0.002^{ab}	0.008 ± 0.001^{ab}	0.012 ± 0.007^{b}	0.007 ± 0.002^{b}	0.007 ± 0.002^{b}
Cu	0.116 ± 0.023^{d}	0.112 ± 0.029^{d}	0.162 ± 0.030^{ab}	0.187 ± 0.032^{a}	0.108 ± 0.009^{d}	0.103 ± 0.006^{d}	0.127 ± 0.010^{cd}	0.178 ± 0.019^{a}	0.132 ± 0.007^{bcd}	0.156 ± 0.029^{abc}
Fe	2.88 ± 0.391^{cde}	3.14 ± 0.504^{bcd}	3.04 ± 0.366^{cd}	4.02 ± 0.522^{a}	2.32 ± 0.188^{e}	2.49 ± 0.140^{de}	3.22 ± 0.375^{bc}	3.45 ± 0.711^{ac}	3.76 ± 0.481^{a}	3.14 ± 0.599^{bc}
Mn	0.012 ± 0.001^{c}	0.012 ± 0.002^{c}	0.017 ± 0.002^{b}	0.022 ± 0.003^{a}	0.012 ± 0.001^{c}	0.012 ± 0.001^{c}	0.014 ± 0.001^{bc}	0.021 ± 0.005^{a}	0.012 ± 0.001^{c}	0.019 ± 0.003^{ab}
Ni	0.085 ± 0.005	0.090 ± 0.008	0.090 ± 0.010	0.090 ± 0.010	0.083 ± 0.009	0.090 ± 0.011	0.090 ± 0.002	0.091 ± 0.011	0.089 ± 0.011	0.086 ± 0.010
Se	0.020 ± 0.019^{b}	0.028 ± 0.019^{ab}	0.041 ± 0.007^{a}	0.038 ± 0.016^{a}	0.031 ± 0.011^{ab}	0.034 ± 0.016^{ab}	0.038 ± 0.011^{a}	0.034 ± 0.016^{ab}	0.033 ± 0.017^{ab}	0.032 ± 0.015^{ab}
Si	1.67 ± 0.619^{a}	1.31 ± 0.194^{ab}	1.57 ± 0.728^{ab}	1.37 ± 0.382^{ab}	1.25 ± 0.232^{ab}	1.10 ± 0.206^{b}	1.29 ± 0.270^{ab}	1.14 ± 0.172^{ab}	1.32 ± 0.273^{ab}	1.22 ± 0.144^{ab}
Zn	3.55 ± 0.471^{bc}	3.91 ± 0.468^{abc}	2.29 ± 0.648^{d}	2.02 ± 0.549^{d}	2.43 ± 0.502^{b}	3.74 ± 0.460^{ab}	3.13 ± 0.462^{c}	3.30 ± 1.101^{a}	4.30 ± 0.376^{a}	2.68 ± 0.339^{d}

$^{a-e}$Values with different superscript letters in the same column are different ($p < 0.05$).

of raw emu (*Dromaius novaehollandiae*) meat (Pegg, Amarowicz, & Code, 2006), and in three muscles (*M. obturatorius medalis*, *M. iliotibialis lateralis*, *M. iliofibularis*) from rhea (*Rhea americana*) (0.07–0.09 mg/100 g edible meat; Ramos, Cabrera, del Puerto, & Saadoun, 2009). However, Se content of meat is highly correlated to the Se contents of soil, grass, and feed ingredients (Ramos et al., 2009). No differences ($p > 0.05$) were found in contents of Co, Ni and Ba among muscles, whereas differences ($p < 0.05$) in contents of Mn and Cr are small, probably without any practical significance (Table 3).

Results from the present study are presented in the way that the product would be traded and is applicable to nutritionists and dieticians, namely a fresh tissue basis (Lin et al., 1989). It can be concluded that this study contributes to a description of the physicochemical and proximate composition of ostrich meat, obtained on several different muscles, which could be use to extend existing information. Furthermore, the mineral composition of a wide range of ostrich muscles are presented, some of the minor elements for the first time, in order to establish a database on the nutrient composition on ostrich meat for further use in research on human consumption of this relative unknown type of meat.

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Food Chemistry 117 (2009) 212–218

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Juice of New citrus hybrids (*Citrus clementina* Hort. ex Tan.×*C. sinensis* L. Osbeck) as a source of natural antioxidants

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ARTICLE INFO

Article history:
Received 2 December 2008
Received in revised form 16 March 2009
Accepted 30 March 2009

Keywords:
Citrus clementina Hort. ex Tan.× [*Citrus sinensis* (L.) Osbeck]
Anthocyanins
Flavanones
Ascorbic acid
ORAC
Tarocco orange
Moro orange

ABSTRACT

Fruit juices of pigmented and non-pigmented new citrus hybrids obtained by crossing clementine cv. Oroval with different cultivars of blood oranges were analysed to determine parameters related to fruit quality (total soluble solids titratable acidity, pH), and the content of ascorbic acid, total phenolics, flavanones, anthocyanins and phenolic acids. Antioxidant properties were evaluated by oxygen radical absorbance capacity (ORAC), and inhibition of induced linoleic acid peroxidation (InLAP) assays. The results of this study show that some hybrids with high antioxidant activity owing to considerable polyphenol content may be considered rich sources of phytochemicals. The OTA 9 hybrid was shown to be richest in polyphenols, suggesting that consumption of OTA 9 fruit or juice could be useful in health promotion and a disease-preventing diet. Moreover, the juice of this hybrid could be used as raw material to produce antioxidant ingredients for dietary, pharmaceutical, or cosmetic purposes.

1. Introduction

Citrus fruit contains vitamin C and other bioactive compounds, including flavonoids and hydroxycinnamic acids, with potential health-promoting properties (Widmer & Montanari, 1996). Flavanone glycosides are the most abundant phenolic compounds present in citrus fruit, but significant concentrations of other flavonoids such as methoxylated flavones, flavonols and anthocyanins (the last only in blood oranges) have also been found (Horowitz, 1961). Previous studies have shown that hesperidin, a member of the flavanone class, has antioxidant, anticancer and anti-inflammatory properties (Attaway, 1994). Hydroxycinnamic acids possess significant antioxidant activity and chemoprotective effects, as shown by *in vitro* and *in vivo* studies (Natella, Nardini, Di Felice, & Scaccini, 1999). Anthocyanins have also been associated with potentially beneficial effects against various diseases, such as capillary fragility, diabetic retinopathy and human platelet aggregation (Mazza & Miniati, 1993). In addition, anthocyanins are known to be potent antioxidants (Kähkönen & Heinonen, 2003; Wang, Cao, & Prior, 1997) and anthocyanin-rich fruit or juice has been associated with higher antioxidant capacity.

Blood (pigmented or red) oranges (Moro, Tarocco and Sanguinello) are the most cultivated varieties of sweet oranges [*Citrus sinensis* (L.) Osbeck] in Italy. The main characteristic that distinguishes these cultivars is the presence of anthocyanins in the flesh, as well as a higher content of vitamin C, flavanones and hydroxycinnamic acids compared to blond (or common) orange varieties (Rapisarda, Bellomo, & Intrigliolo, 2001). Previous studies have demonstrated that the major anthocyanins in blood orange juice are cyanidin 3-glucoside (Cy3G) and cyanidin 3-(6″malonyl)-glucoside (Cy3MG) (Maccarone, Rapisarda, Fanella, Arena, & Mondello, 1998), and their level in the fruit always varies according to the following order: Moro > Sanguinello > Tarocco. Moreover, marked differences in anthocyanin content among individual Tarocco clones have been observed (Rapisarda & Russo, 2000). The antioxidant activity of blood orange juices is higher than that of blond orange juices, and the antioxidant efficiency appears to be correlated with the anthocyanin level (Rapisarda, Tomaino, Lo Cascio, Bonina, & De Pasquale, 1999). Thus, a breeding programme that focuses on the bioactive phytochemical content of fruit, such as vitamin C, anthocyanins and other phenolics, represents a new and important strategy in the citrus industry. Since 1951, the CRA-Centro di Ricerche per l'Agrumicoltura e le Colture Mediterranee (CRA-ACM) has been working on a genetic improvement programme for extant blood orange cultivars and the development of new pigmented citrus hybrids with ease of peeling, increased size, new original sensory characteristics, and a high

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doi:10.1016/j.foodchem.2009.03.101

anthocyanin content. New pigmented hybrids were obtained by crossing some clones of clementine (*C. clementina* Hort. ex Tan.) with different blood orange [*C. sinensis* (L.) Osbeck] varieties.

In order to investigate the heritability of traits, such as phenolic content and antioxidant activity, the fruit juices of pigmented and non-pigmented new citrus hybrids belonging to the OTA (Oroval clementine × Tarocco orange) and OMO (Oroval clementine × Moro orange) groups were analysed, to evaluate parameters related to fruit quality, as well as the content of health-promoting components such as ascorbic acid, total phenolics, flavanones, anthocyanins and phenolic acids. In addition, the total antioxidant capacity of the juices was measured using two different *in vitro* tests, oxygen radical absorbance capacity (ORAC) and inhibition of induced linoleic acid peroxidation (InLAP).

2. Materials and methods

2.1. Crosses

OTA and OMO hybrids were obtained by conventional citrus breeding methods. Controlled pollination between Oroval clementine (*C. clementina* Hort. ex Tan.), used as the female parent, and Tarocco 57-1E-1 or Moro NL 58-8D-1 [*C. sinensis* (L.) Osbeck], used as the male parent, was carried out. Seeds obtained from the fruit of these crosses were placed in pots, and the hybrid seedlings were grown in a greenhouse. After two years, scions from the apical part of the seedlings were cut and grafted onto *Poncirus trifoliate* L. Raf. rootstocks. One year later, the grafted plants were transferred to the Palazzelli experimental farm of CRA-ACM, where fruiting occurred after four years. For this study, the OTA 8, OTA 9, OTA 20, OTA 31, OTA 43, OMO 24 and OMO 30 hybrids were selected from the F1 progeny.

2.2. Chemicals

Ascorbic acid, hydroxycinnamic acids (*p*-coumaric, ferulic, caffeic and sinapic acids), Folin–Ciocalteu's phenol reagent, AAPH (2,2́-azobis(2-methylpropionamidine)dihydrochloride), Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), fluorescein, linoleic acid and lipoxygenase (E.C.1.13.11.12) were purchased from Sigma–Aldrich (Milan, Italy). Hesperidin, cyanidin 3-glucoside and (±)-catechin were from Extrasynthèse (Geney, France). All other chemicals were of analytical grade and the solvents used for chromatography were HPLC grade (Merck, Darmstadt, Germany).

2.3. Plant material

Fruits were harvested from three trees for each genotype: in December 2006, the Oroval clementine; in February 2007, the Tarocco 57–1E-1 orange; in March 2007, the Moro NL 58–8D-1 orange and the different hybrids (OTA 8, OTA 9, OTA 20, OTA 31, OTA 43, OMO 24 and OMO 30). Juice was extracted by a domestic squeezer and used for chemical analyses and antioxidant activity determination.

2.4. Physicochemical analysis

Total soluble solids (TSS), titratable acidity (TA) and pH were determined according to conventional methods. The ascorbic acid concentration was evaluated by liquid chromatography using a Waters Alliance 2695 HPLC (Waters Corporation, Milford, MA) equipped with a Waters 996 photodiode array detector and Empower software (Rapisarda & Intelisano, 1996). Briefly, 5 ml of centrifuged juice was poured into a flask and made up to 50 ml with 3% metaphosphoric acid solution. An aliquot of the solution was 0.45 μm filtered, and 20 μl injected into the HPLC. The mobile phase was 0.02 M H_3PO_4 and the detector was set at 260 nm.

The orange juices were analysed for total phenolics by the Folin–Ciocalteu (FC) colorimetric method (Singleton, Orthofer, & Lamuela-Raventos, 1999). Appropriately diluted samples (1 ml) were mixed with 5 ml of FC commercial reagent (previously diluted with water, 1:10 v/v) and 4 ml of a 7.5% sodium carbonate solution. The mixture was stirred for 2 h at room temperature away from strong light. The absorbance of the resulting blue solution was measured spectrophotometrically at 740 nm and the concentration of total phenolics was expressed as (±)-catechin equivalents (mg/l). Total anthocyanin content was determined spectrophotometrically (Varian UV–vis spectrophotometer, model Cary 100 Scan; Varian Inc., Palo Alto, CA) by the pH differential method (Rapisarda, Fanella, & Maccarone, 2000) and expressed as cyanidin 3-glucoside equivalents (mg/l).

Individual anthocyanin analysis was carried out by HPLC. A sample of 5 ml of centrifuged juice was loaded onto a C_{18} Sep-Pak cartridge, previously conditioned with 5 ml of methanol and 5 ml of pure water. The cartridge was eluted with acidified methanol (0.1% HCl). One millilitre of the extract was filtered through a 0.45 μm cartridge and injected into a Waters 600-E liquid chromatograph equipped with a Waters 996 photodiode array detector (PDA); the data were processed by Millennium 3.2 software. The column was a 250 mm × 4.6 mm i.d., 5 μm, Hypersil ODS (Phenomenex, Torrance, CA) and the solvent system was **A**, water:formic acid (85:15) and **B**, water:formic acid:acetonitrile (35:15:50). The percentage of **B** was increased linearly from 10% to 30% in 30 min at a flow rate of 1 ml/min. Elution was monitored at 520 nm, and the column temperature was maintained at 35 °C.

Flavanone glycosides, expressed as hesperidin equivalents (mg/l), were determined by HPLC (Rouseff, Martin, & Yotsey, 1987) using the HPLC-PDA equipment described above. A sample of centrifuged juice was diluted at a ratio of 1:5 with the mobile phase, 0.45-μm filtered, and injected directly into the column. The eluent used was water:acetonitrile:acetic acid (79.5:20:0.5), and the flow rate was 0.8 ml/min. The column effluent was monitored at 280 nm.

Hydroxycinnamic acids (*p*-coumaric, ferulic, caffeic and sinapic acids) were extracted from the juice by solid-phase extraction after alkaline hydrolysis of hydroxycinnamic esters (Rapisarda, Carollo, Fallico, Tomaselli, & Maccarone, 1998). A 10-ml sample of centrifuged juice was added to 10 ml of 2 M sodium hydroxide and stored at room temperature in the dark. Complete hydrolysis of bound hydroxycinnamic acids occurred in 4 h. The solution was then acidified with 2 M HCl to pH 2.5 and passed through a C_{18} SPE cartridge (Waters). Hydroxycinnamic acids were eluted with 0.1% HCl in methanol. The alcoholic solution was 0.45-μm filtered, and 20 μl of the solution was analysed by HPLC as above. The eluents used were water:acetic acid (98:2) (solvent **A**) and methanol (solvent **B**) with a gradient transition from 95% to 70% of solvent **A** over 35 min. The flow rate was 1 ml/min, and detection was at 300 nm.

2.5. Antioxidant activity

Antioxidant activity was determined using the ORAC assay, as described by Ou, Hampsch-Woodill, and Prior (2001), with some modifications. Briefly, the measurements were carried out on a Wallac 1420 Victor III 96-well plate reader (EG & Wallac, Turku, Finland) with a fluorescence filter (excitation 485 nm, emission 535 nm). Fluorescein (116 nM) was the target molecule for free radical attack from AAPH (153 mM) as the peroxyl radical generator. The reaction was conducted at 37 °C and pH 7.0, with Trolox (10 μM) as the control standard and 75 mM phosphate buffer

(pH 7.0) as the blank. All solutions were freshly prepared prior to analysis. All sample were diluted with phosphate buffer (1:25–100, v/v) prior to analysis and results were reported as micromoles of Trolox equivalents per 100 ml of juice.

Antioxidant activity was also determined through the inhibition of induced linoleic acid peroxidation (InLAP) assay. The enzymatic oxidation of linoleic acid was obtained by adding lipoxygenase from soybean (E.C.1.13.11.12) (Lo Scalzo, Iannoccari, Summa, Morelli, & Rapisarda, 2004), and monitored in the absence (blank test) and presence (sample test) of orange juice by recording the linear increase in absorbance at 234 nm (A_{max} of conjugated diene peroxides from linoleic acid oxidation). In the sample test, InLAP was measured in a solution containing 2.65 ml of 0.1 M phosphate buffer (pH 7.0), 0.3 ml of 2.28 mM linoleic acid–water emulsion, 0.05 ml of orange juice, and 0.025 ml of lipoxygenase solution (15 mg of lipoxygenase standard in 25 ml of 0.1 M phosphate buffer at pH 7.0). In the blank test, orange juice was substituted with 0.05 ml of 0.1 M phosphate buffer (pH 7.0). Antioxidant activity was expressed as the protection percentage of linoleic acid enzymatic degradation, where the blank represents 100% degradation of linoleic acid.

2.6. Statistical analysis

One-way ANOVA was performed for each genotype to obtain a statistical assessment of the physicochemical parameters and antioxidant activity. Means were separated by the Tukey's test at $p \leqslant 0.05$, $p \leqslant 0.01$ and $p \leqslant 0.001$ significance. A correlation analysis of antioxidant activity values *versus* ascorbic acid or phenolic components (anthocyanins, flavanones, hydroxycinnamic acids and total phenolics) was performed using MSTAT WIN 10.

3. Results

3.1. Fruit quality parameters

TSS, TA, TSS/TA and pH of the different genotypes studied are shown in Table 1. The TSS and TA values of clementine, Tarocco and Moro juices ranged within known values for mature fruit. As expected, the TSS/TA was higher in clementine (12.35) than in Tarocco (10.84) and Moro oranges (10.04), mainly due to the higher acidity of the blood orange varieties. Since the TSS/TA is related to maturity in citrus fruit, it is possible that OTA 9, OTA 31, OMO 24 and OMO 30 matured in March, while OTA 43 reached fruit maturity early. The high acidity found in OTA 8 and OTA 20 suggests that this is a persistent trait also in over-ripened fruits, since fruit senescence and drop were observed after March. The pH values of the hybrids and their parents reflect the acid content of these genotypes.

Table 1
Fruit quality parameters of OTA and OMO hybrids and their parents.[a]

	Genotype	pH		TA (%)		TSS (%)		TSS/TA	
OTA group	Clementine	3.86	A	1.02	E	12.64	D	12.35	B
	Tarocco	3.52	CD	1.12	D	12.18	E	10.84	C
	OTA 8	3.33	E	1.45	B	10.85	F	7.47	D
	OTA 9	3.45	D	1.22	C	13.09	C	10.69	C
	OTA 20	3.32	E	2.25	A	13.52	B	6.01	E
	OTA 31	3.54	CD	1.05	E	13.69	B	13.01	B
	OTA 43	3.75	B	0.87	F	15.10	A	17.29	A
OMO group	Clementine	3.86	A	1.02	C	12.64	A	12.35	A
	Moro	3.37	B	1.18	C	11.90	C	10.04	C
	OMO 24	3.33	C	1.15	B	12.61	A	10.85	B
	OMO 30	3.35	C	1.23	A	11.47	B	9.29	D

[a] Values in the same column for each genotype having different letters are significantly different ($p \leqslant 0.01$).

3.2. Antioxidant components

Similar values for ascorbic acid were observed in OTA 8 and clementine, while the other hybrids showed higher levels than the female parent (Table 2). In addition, OTA 20 and OTA 31 juices exhibited higher ascorbic acid contents than the Tarocco orange, which is known as the richest source of vitamin C among orange varieties. The ascorbic acid level in OMO 24 was intermediate between those of the clementine and Moro orange, while the value obtained for the OMO 30 hybrid was similar to that of the male parent.

Total phenolics measured by the Folin–Ciocalteu (FC) assay were different depending on the genotype studied. The OTA 9 hybrid had a much higher content of total phenolics than the other citrus genotypes (Table 2). The mean content of total phenolics found in the other hybrids was intermediate between their respective parents, with higher values found in those hybrids that were richer in ascorbic acid or flavanones. Folin–Ciocalteu's phenol reagent is nonspecific for phenolic compounds since it measures sample reducing capacity through electron transfer-based antioxidant capacity and, thus, it can be reduced by many non-phenolic compounds, such as vitamin C (Huang, Boxin, & Prior, 2005). Therefore, due to the contribution of ascorbic acid, the response of some genotypes, such as OTA 20, OTA 31 and OTA 43, to the FC assay was high.

Crossbreeding between clementine and the two blood orange varieties produced pigmented and non-pigmented hybrids in both the OTA and OMO groups. In the former, OTA 9 and OTA 20 were pigmented, while in the latter, only OMO 30 contained anthocyanins. The anthocyanin contents in OTA 20 and OMO 30 were lower than those observed for the Tarocco and Moro juices, respectively, whereas the pigment concentration reached about 500 mg/l in the OTA 9 hybrid, an almost 5-fold higher value with respect to its male parent (Table 3). The anthocyanin profiles of pigmented hybrids, Moro and Tarocco orange juices are shown in Figs. 1 and 2. Peak assignments were made on the basis of their retention time, UV–vis spectra, and co-chromatography with authentic standards, as discussed in previous works (Maccarone et al., 1998; Rapisarda, Pannuzzo, Romano, & Russo, 2000). Even though the pattern of anthocyanins present both in male parents and hybrids was similar, a different distribution of individual anthocyanins was noted. In all genotypes studied, cyanidin 3-glucoside (peak 2) was the major anthocyanin, with delphinidin 3-glucoside (peak 1), delphinidin 3-(6″malonyl)-glucoside (peak 3), and cyanidin 3-(6″malonyl)-glucoside (peak 4), representing the other predominant anthocyanins.

Table 2
Ascorbic acid content and total phenolics[a] in OTA and OMO hybrids and their parents.[b]

	Genotype	Ascorbic acid (mg/100 ml)		Total phenolics (mg/l)	
OTA group	Clementine	51.64	E	529.13	G
	Tarocco	68.29	C	890.52	B
	OTA 8	53.80	E	571.99	FG
	OTA 9	61.90	D	1554.67	A
	OTA 20	81.07	B	693.10	D
	OTA 31	91.60	A	796.15	C
	OTA 43	70.41	C	608.33	EF
OMO group	Clementine	51.64	C	529.13	C
	Moro	56.92	A	1065.10	A
	OMO 24	54.22	B	634.23	B
	OMO 30	56.75	A	595.43	B

[a] Expressed as cathechin equivalents.
[b] Values in the same column for each genotype having different letters are significantly different ($p \leqslant 0.01$).

Table 3
Anthocyanins content of pigmented hybrids and their parents[a] and relative percentage of main individual anthocyanins.

Genotype	Total anthocyanins (mg/l)		D3G[b] (%) [c]	Cy3G[b] (%)[c]	D3MG[b] (%) [c]	Cy3MG[b] (%)[c]
Tarocco	116.79	B	2.9	41.2	16.1	18.5
OTA 9	497.45	A	23.0	31.7	10.9	10.4
OTA 20	28.99	C	9.8	21.1	7.8	13.8
Moro	278.90	A	2.2	41.2	11.7	21.2
OMO 30	62.86	B	14.7	16.1	11.9	11.4

[a] Values in the same column for each sample having different letters are significantly ($p \leqslant 0.01$) different.
[b] D3G, delphinidin 3-glucoside; Cy3G, cyanidin 3-glucoside; D3MG, delphinidin 3-(6″malonyl)-glucoside; Cy3MG, cyanidin 3-(6″malonyl)-glucoside.
[c] Relative percentage of anthocyanins was based on HPLC peak areas.

Oranges and clementines mainly accumulate the tasteless flavanones rutinoside, narirutin, and hesperidin (Horowitz, 1961), but other flavonoids such as polymethoxylated flavones, flavonol glucosides, and flavone C-glycosides also occur in these species (Caristi, Bellocco, Gargiulli, Toscano, & Leuzzi, 2006; Leuzzi, Caristi, Panzera, & Licandro, 2000). The major flavanone glycoside found in the parental and all hybrid fruit juices was hesperidin, representing about 50–80% of the total flavonoids (Table 4). Narirutin and dydimin were also present, confirming that hybrids resulting from crosses between oranges and clementines principally contain patterns of rutinosyl glycosides. The hybrid representing the richest source of flavonoid glycosides (not including anthocyanins) was OTA 9, with 1018.34 mg/l, followed by OMO 30, with 307.66 mg/l. A study is currently in progress to identify the minor anthocyanins and other flavonoids present in these hybrids using HPLC-ESI-MS/MS.

The particular trend observed for OTA 9 was also reflected in the concentration of hydroxycinnamic acids, the values of which made OTA 9 the richest hybrid in polyphenols content. Total hydroxycinnamic acid (HCA) content in most non-pigmented hybrids (OTA 31, OTA 43 and OMO 24) was similar to that of the clementine, whereas higher values than in the female parent were observed in pigmented hybrids (OTA 20, OMO 30) (Table 5). The HCA content in the OTA 9 was about 1.5 times higher than in the Tarocco orange, its male parent. The distribution of individual phenolic acids in the hybrids with respect to their parents showed higher values of *p*-coumaric and ferulic acids in the OTA 9 and *p*-coumaric acid in the OMO 30 hybrids, while caffeic acid was higher in the OTA 20 and OTA 43 hybrids. In the other hybrids, the amount of individual hydroxycinnamic acids was lower than, or comparable to, that of the two parents.

3.3. Antioxidant activity

All juices tested showed an apparent antioxidant effect that may be mostly attributed to their high content of polyphenols (Table 6). The OTA 9 hybrid exhibited higher ORAC activity than its parents, whereas that of the female parent clementine was the lowest. Statistically significant differences were observed between pigmented and non-pigmented hybrids in both OTA and OMO groups, with higher values found in anthocyanin-containing hybrids. The correlation between anthocyanins content and ORAC

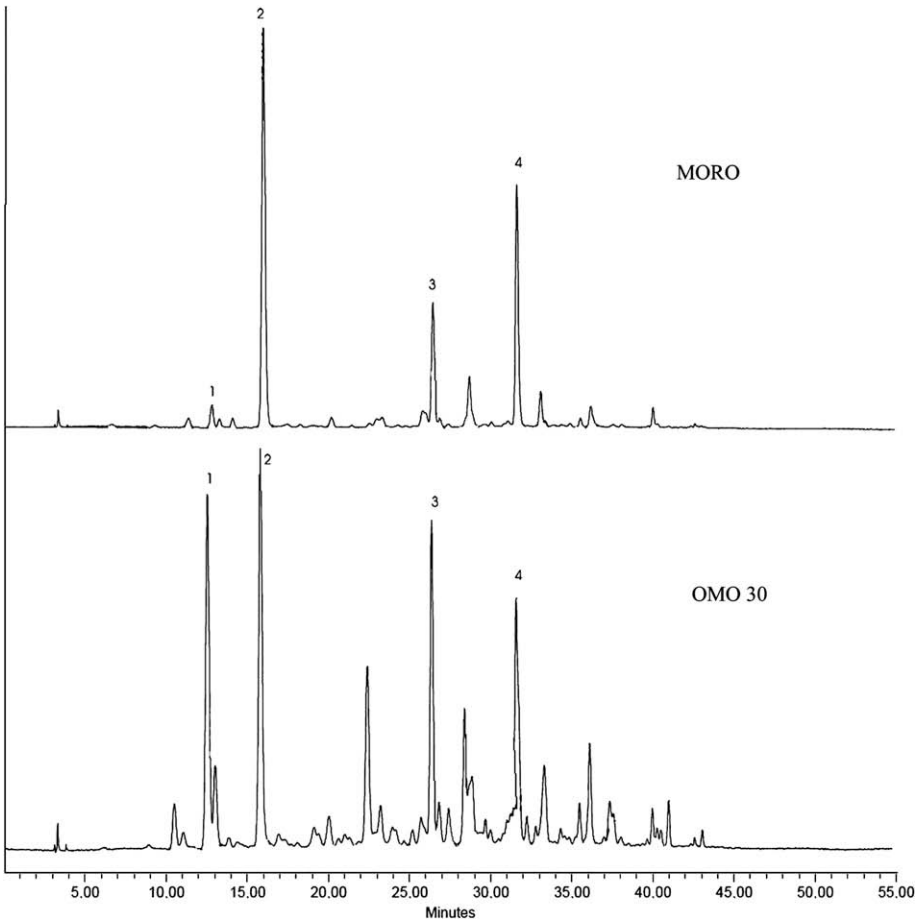

Fig. 1. HPLC chromatograms detected at 520 nm showing the anthocyanins present in Moro and OMO fruit juices. Peaks: 1, delphinidin 3-glucoside; 2, cyanidin 3-glucoside; 3, delphinidin 3-(6″malonyl)-glucoside; 4, cyanidin 3-(6″malonyl)-glucoside. Detector was set at 520 nm.

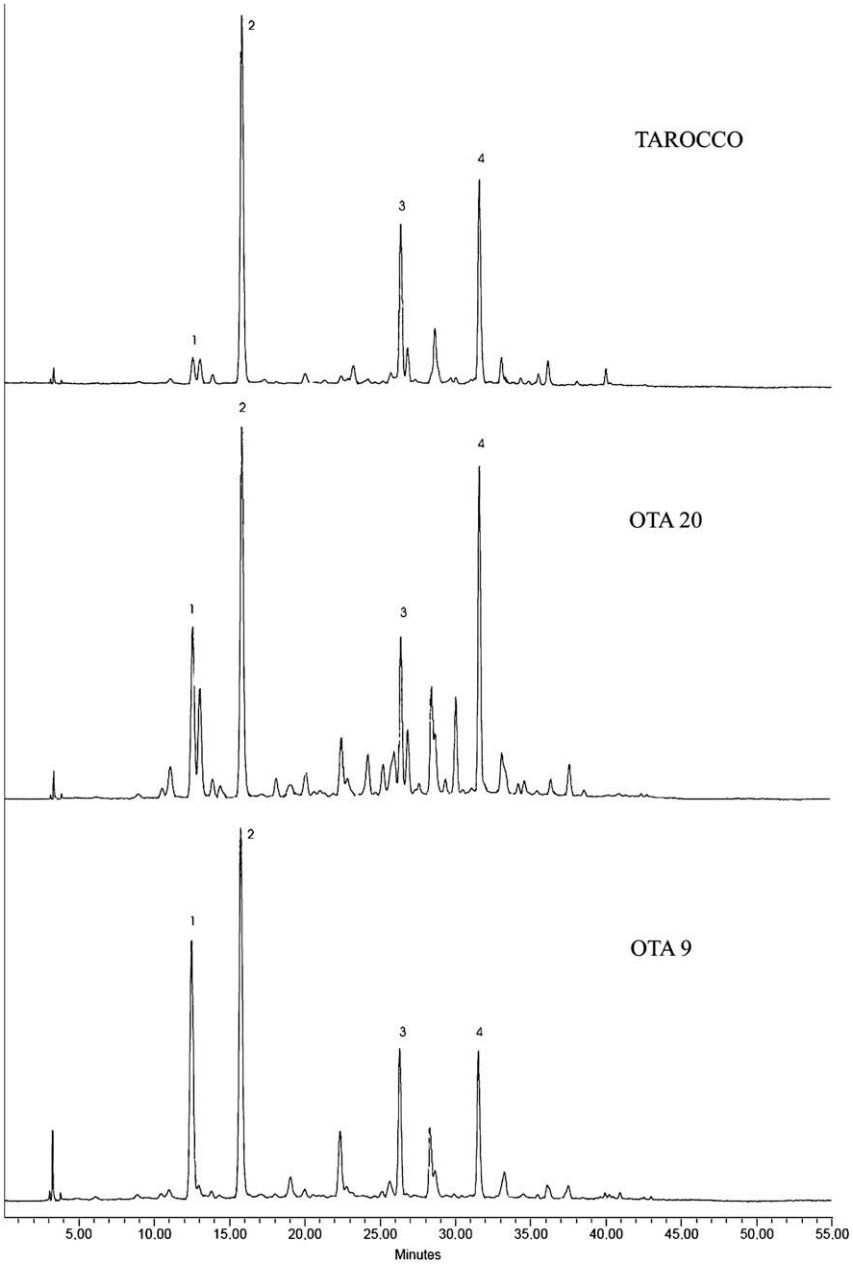

Fig. 2. HPLC chromatograms detected at 520 nm showing the anthocyanins present in Tarocco and OTA fruit juices. Peaks: 1, delphinidin 3-glucoside; 2, cyanidin 3-glucoside; 3, delphinidin 3-(6″malonyl)-glucoside; 4, cyanidin 3-(6″malonyl)-glucoside. Detector was set at 520 nm.

activity for the pigmented hybrid group was highly significant ($r^2 = 0.99$, $p \leqslant 0.001$ and $n = 9$). A high positive correlation was also found between ORAC and total phenolics ($r^2 = 0.98$, $p \leqslant 0.001$ and $n = 9$), flavanones ($r^2 = 0.96$, $p \leqslant 0.001$ and $n = 9$) and hydroxycinnamic acids ($r^2 = 0.85$, $p \leqslant 0.001$ and $n = 9$) in the pigmented hybrid group. Only a weak correlation was observed between flavanones and ORAC in the non-pigmented hybrids group ($r^2 = 0.42$, $p \leqslant 0.05$ and $n = 12$).

The OTA 9 and OTA 20 hybrids demonstrated significant activity in the InLAP assay, with respect to clementine and Tarocco parents. The formation of a conjugated diene at 234 nm was inhibited approximately 88% and 81% by the OTA 9 and OTA 20 juices. The InLAP values obtained for the other hybrids within the OTA group, ranged from 66% to 71%. No relevant differences were noted for OMO hybrids with respect to the Moro orange, although their antioxidant activity was higher than that of the female parent. Moreover, significant correlations between InLAP and polyphenols, such as anthocyanins ($r^2 = 0.81$, $p \leqslant 0.01$ and $n = 9$), flavanones ($r^2 = 0.72$, $p \leqslant 0.05$ and $n = 9$) and total phenolics ($r^2 = 0.88$, $p \leqslant 0.001$ and $n = 9$) were observed in pigmented hybrids, whereas only a high correlation between InLAP and flavanones ($r^2 = 0.81$, $p \leqslant 0.001$ and $n = 12$) was found in non-pigmented fruit.

4. Discussion

Improvement of fruit quality and agronomic performance has been a major breeding objective in the citrus industry. Due to the suggestion by certain epidemiological studies that antioxidant consumption can positively influence health, breeders have recently shown greater interest in developing citrus fruit with higher antioxidant activity. For this goal, it is important to study the heritability of certain traits, such as the chemicals level that contrib-

Table 4
Concentration of individual flavanones [b], unidentified flavonoids [b] and total flavonoids [b,c] in OTA and OMO hybrids and their parents.[a]

	Genotype	Narirutin (mg/l)		Hesperidin (mg/l)		Dydimin (mg/l)		Unidentified flavonoids (mg/l)		Total flavonoids (mg/l)	
OTA group	Clementine	14.10	G	66.92	D	1.56	D	17.22	CD	99.88	E
	Tarocco	42.16	B	164.29	B	17.12	B	90.01	B	313.58	B
	OTA 8	40.02	D	115.64	C	7.71	C	15.14	CD	178.51	C
	OTA 9	151.14	A	508.29	A	37.68	A	321.23	A	1018.34	A
	OTA 20	32.14	E	56.93	E	1.53	D	28.81	C	119.41	D
	OTA 31	41.02	C	63.23	D	7.88	C	19.74	CD	131.87	D
	OTA 43	18.80	F	45.41	F	1.54	D	10.63	D	76.38	F
OMO group	Clementine	14.10	C	66.92	D	1.56	D	17.30	C	99.88	D
	Moro	52.69	A	375.95	A	27.89	A	181.81	A	638.34	A
	OMO 24	29.93	B	212.71	B	4.12	C	13.77	C	260.53	C
	OMO 30	36.37	B	206.40	C	6.36	B	58.53	B	307.66	B

[a] Values in the same column for each genotype having different letters are significantly different ($p \leqslant 0.01$).
[b] Flavanones are expressed as hesperidin equivalents (mg/l).
[c] Total flavonoid content does not include the anthocyanin concentration.

Table 5
Hydroxycinnamic acids content in OTA and OMO hybrids and their parents.[a]

	Genotype	Caffeic acid (mg/l)		*p*-Coumaric acid (mg/l)		Ferulic acid (mg/l)		Sinapic acid (mg/l)		HCA[b] (mg/l)	
OTA group	Clementine	3.30	CD	5.47	DE	17.04	C	4.78	B	30.59	DE
	Tarocco	7.51	B	23.66	B	31.12	B	8.21	A	70.50	B
	OTA 8	1.99	D	2.37	E	12.67	CD	2.03	E	19.06	E
	OTA 9	4.06	C	48.56	A	41.89	A	9.41	A	103.92	A
	OTA 20	11.27	A	17.36	C	10.72	D	4.19	BC	43.54	C
	OTA 31	6.28	B	8.68	D	13.61	CD	3.48	CD	32.05	CD
	OTA 43	10.51	A	4.98	DE	14.76	CD	2.45	DE	32.69	CD
OMO group	Clementine	3.30	A	5.47	D	17.04	b	4.78	B	30.59	B
	Moro	3.91	A	29.92	B	22.42	a	7.32	A	63.57	A
	OMO 24	1.66	B	11.46	C	19.44	ab	3.72	B	36.28	B
	OMO 30	1.53	B	38.27	A	21.41	ab	5.64	AB	66.85	A

[a] Values in the same column for each genotype having different letters are significantly different ($p \leqslant 0.01$ capital letters; $p \leqslant 0.05$ small letters).
[b] Total hydroxycinnamic acids (HCA).

utes to antioxidant activity. Therefore, the evaluation of vitamin C, phenolics and antioxidant activity are useful tools when the aim is to produce citrus fruit with high antioxidant potential.

In this study, we revealed that the progeny of clementine × blood oranges produced fruit with different sugar-acid ratios. The acid levels in OTA 8 and OTA 20 were very high and, thus, neither of these two hybrids was fit to eat as a dessert fruit.

The ascorbic acid content of most hybrids was higher than that of the female parent. Therefore, the introduction of orange parentage enhanced the vitamin C level in these hybrids. High contents of ascorbic acid were found in OTA 20 and OTA 31, with values that were about 16% and 25%, respectively, higher than the Tarocco orange. The balanced level of sugar and acid and the observed highest content of ascorbic acid, mark the OTA 31 fruit as both excellent for eating and a significant source of dietary vitamin C.

Blood orange anthocyanins have been associated with potentially beneficial effects on various diseases, attributed in part to their antioxidant activity (Maccarone et al., 1998). The anthocyanin content of the pigmented hybrids was very low with respect to that of their parents, with the exception of OTA 9, which demonstrated the highest total anthocyanin concentration observed. This result demonstrates that OTA 9 hybrid may be selected on the basis of its superiority in total anthocyanin content in the fruit and/or juice. In addition, since cyanidin 3-glucoside, the major anthocyanin present in the OTA 9 hybrid, has a stronger antioxidant activity than other common anthocyanins (Wang et al., 1997), consumption of OTA 9 fruit or juice might be useful for disease prevention and health promotion. Moreover, the isolated and purified anthocyanin extract from this hybrid could be used in the pharmaceutical industry.

The OTA 9 fruit also contains higher concentrations of flavanones and hydroxycinnamic acids than other hybrids and their parents. These components are of great interest as pharmaceutical agents in human health and nutrition (Benavente-Garcia, Castillo, Marin, Ortuño, & Del Rio, 1997; Silva, Borges, Guimarães, Lima, & Matos et al., 2000). As a result, their recovery from a plentiful source might be economically important.

Table 6
Antioxidant activity of OTA and OMO juices determined by ORAC and InLAP assays [a].

	Genotype	ORAC units[b]		InLAP (% In.)[c]	
OTA group	Clementine	1592.34	E	67.41	D
	Tarocco	3369.63	B	73.32	C
	OTA 8	1969.74	D	71.39	CD
	OTA 9	6688.65	A	88.51	A
	OTA 20	2953.08	C	81.56	B
	OTA 31	2016.03	D	71.25	CD
	OTA 43	1766.02	DE	66.28	D
OMO group	Clementine	1592.34	D	67.41	B
	Moro	4535.43	A	75.30	A
	OMO 24	2027.94	C	78.30	A
	OMO 30	2964.22	B	79.05	A

[a] Values in the same column for each genotype having different letters are significantly different ($p \leqslant 0.01$).
[b] ORAC units are expressed as μmol Trolox equivalents/100 ml.
[c] Percentage of linoleic acid enzymatic-degradation inhibition.

Recent research has demonstrated the possibility of recovering anthocyanins, flavanones and hydroxycinnamic acids from blood orange juice or citrus byproducts (Rapisarda et al., 2001) and using this extract as an antioxidant ingredient for dietary supplements. A standardised red (blood) orange extract (ROE) containing 3.1% anthocyanins, 2.07% hydroxycinnamic acids, 8.1% flavanones and 5.0% ascorbic acid has shown strong antioxidant activity *in vitro* and a photoprotective effect against UVB-induced skin erythema

in vivo (Bonina, Saija, Tomaino, Lo Cascio, & Rapisarda et al., 1998). In addition, ROE has been demonstrated to be capable of protecting DNA from UV-induced damage (Russo et al., 2002) and decreasing oxidative stress in diabetic patients (Bonina et al., 2002). Having the highest concentration of polyphenols, the OTA 9 juice represents a richer source of antioxidant compounds than blood orange juice; it could potentially be used as raw material to make functional food or pharmaceutical and cosmetic products.

In order to measure the antioxidant activity of different citrus genotypes, two methods were used: the ORAC and the InLAP assays. The former measures the antioxidant scavenging activity against peroxyl radical induced by AAPH, and the latter evaluates the ability to inhibit lipid peroxidation by antioxidants. ORAC assay represent a hydrogen atom transfer (HAT) reaction mechanism, which is most relevant to human biology (Prior et al., 2003). Induced linoleic acid peroxidation (InLAP) is an assay in which the hydrophilicity or lipophilicity of antioxidants plays an important role in the evaluation of antioxidant capacity. To comprehensively study different aspects of antioxidants in orange and hybrid juice samples, both kind of assays have been performed.

The radical-scavenging activities of flavonoids and phenolic acids have been extensively reviewed (Cao, Sofic, & Prior, 1997; Rice-Evans, Miller, & Paganda, 1996). It has been demonstrated that anthocyanins and other flavonoids have 2–6 times the antioxidant activity of common antioxidants, such as ascorbic acid, glutathione, etc. (Prior et al., 1998). Therefore, the hybrid juices containing anthocyanins and/or high levels of flavanones or hydroxycinnamic acids, such as OTA 9, OTA 20, and OMO 30, showed the highest antioxidant activity. The absence of correlation between ORAC and ascorbic acid values demonstrated that the contribution of vitamin C to the ORAC activity was relatively low. The same results were obtained by Wang et al. (1997) for orange juice and Prior et al. (1998) for *Vaccinium* species.

Regarding InLAP assay, data showed significant correlations between InLAP and flavonoids, such as flavanones and anthocyanins, probably due to the lipophilic characteristics of hesperidin, narirutin, and dydimin and to the ability of anthocyanins to accumulate within the water phase of the emulsion and, therefore, to maintain their antioxidant efficiency (Kähkönen & Heinonen, 2003). The results of this study demonstrate an increasing trend in the oxygen radical absorption capacity (ORAC) with increased total phenolic content of the different genotypes. The InLAP assay showed a similar trend, suggesting that the phenolic compounds were likely to be significant contributors to the antioxidant activity.

In conclusion, we have identified two new citrus hybrids, OTA 9 and OTA 31, as novel sources of polyphenolic complex and vitamin C, respectively. In addition, these hybrids have shown a high antioxidant activity, suggesting that their consumption could be useful in the prevention of diseases in which free radicals are involved. Finally, extracts of new citrus hybrids, such as OTA 9, might have potential application as an antioxidant ingredient in nutraceutical, pharmaceutical, or cosmetic products.

Acknoweledgements

This work was supported by European Commission – sixth Framework Programme (Food-CT-2005, FLORA Project, Contract No. 007130).

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Food Chemistry 117 (2009) 219–225

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Effects of genotype and sowing date on phytostanol–phytosterol content and agronomic traits in wheat under organic agriculture

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ARTICLE INFO

Article history:
Received 19 December 2008
Received in revised form 5 February 2009
Accepted 30 March 2009

Keywords:
Bread wheat
Phytosterols
Phytostanols
Genetic variability
Crop management
Organic conditions
Bioaccumulation

ABSTRACT

Cereals are an important source of sterols and stanols in the human diet. The present study underlines the effect of genotype and weather conditions in bread wheat, on total sterol and stanol content (TSS), agronomic traits, proteins and ash content under organic conditions. Variations in TSS as well as other characters between two sowing dates were observed. A broad genotypic variability was also reported since extreme genotypes differed by more than 30 mg 100 g^{-1} DW for TSS, with total stanol content varying twofold. Moreover, two groups of genotypes that differed in agronomic production, ash and protein content were depicted, based on their response to an increase in temperature. This result suggests that the genotypic factor prevails over the sowing date factor for determining sterol and stanol traits in wheat cultivated under organic conditions. Nevertheless, a strong interaction exists between the two factors, which can be used to drive bioaccumulation of these molecules.

1. Introduction

Cereal grains contain a wide variety of nutrients and bioactive molecules that play an important role in disease prevention (Halvorsen et al., 2002). Among these molecules, much attention has been focused on phytosterols in the last few years. As minor constituents of lipids, they constitute a group of cholesterol-like molecules and are characterised by the presence of a methyl or ethyl group at carbon 24 of the basic cholesterol chemical structure (Moreau, Whitaker, & Hicks, 2002). Phytosterols occur in plants as free sterols and conjugated forms (Piironen, Lindsay, Miettinen, Toivo, & Lampi, 2000). They are key compounds for the formation of plant plasma membrane microdomains (Roche et al., 2008). These molecules play an essential role in the regulation of membrane fluidity and permeability by affecting the proteins associated to membranes. Moreover, they are precursors of brassinosteroids and are involved in embryogenesis (Schaller, 2003).

Phytosterols have been described as cancer-preventive bioactive molecules and have an immuno-modulating activity (Bouic, 2001). However, the most important property of phytosterols is their beneficial effect in reducing the low density lipoprotein (LDL) cholesterol level (Calpe-Berdiel, Escolà-Gil, & Blanco-Vaca, 2009). According to Valsta et al. (2004), even if the sterol level in cereals is lower than that of oilseeds, cereals remain the major source of natural sterols in the human diet due to the large amounts consumed daily. A comparison between sterol content in cereals was performed by Piironen, Toivo, and Lampi (2002). It appears clearly that rye, wheat and barley grains exhibit the highest sterol–stanol content whereas oat presented the lowest level of these same compounds. Moreover, wheat is one of the most important food crops and is grown worldwide.

Phytosterols in cereals are present in two forms: sterols and their completely saturated forms, stanols. This fact has oriented clinical experiments to focus on stanol versus sterol efficiency in reducing LDL cholesterol, and it seems that stanols are more efficient (Santos et al., 2007).

Several authors have studied the total phytosterol content in grains of different cereals (Moreau, Singh, Nunez, & Hicks, 2000) and in cereal by-products (Hakala et al., 2002; Jiang & Wang, 2005; Nyström, Paasonen, Lampi, & Piironen, 2007). However, in these studies, sterol content was measured on commercial samples, which considerably limits source traceability. Other experiments have been carried out to study sterol and stanol levels in

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doi:10.1016/j.foodchem.2009.03.102

oat (Määttä et al., 1999), rye (Piironen et al., 2002; Zangenberg, Boskov Hansen, Jorgensen, & Hellgren, 2004), corn (Harrabi et al., 2008) and bamboo (Lu, Ren, Zhang, & Gong, 2009) across locations as influenced by genotype under conventional conditions. However, information on the effects of genotype and environmental conditions on the stanol and sterol composition of wheat is lacking. Moreover, the effect of water deficit or high temperatures on technological traits as sterols content and protein content was studied under conventional conditions where pests and diseases were managed by chemicals (Dupont et al., 2006; Määttä et al., 1999; Zangenberg et al., 2004). In contrast, organic culture, conducted without chemicals, can be considered as stress for plants. Thus, this agricultural method may influence molecules composition in wheat grain.

Phytosterols used in industrial preparations are chemically extracted from oil plants, and phytostanols are obtained by hydrogenation of sterols (Mouloungui, Roche, & Bouniols, 2006), often by using harsh chemical methods that are not always in accordance with European Union recommendations and REACH legislation. It has in fact been recommended to decrease different inputs, particularly chemicals, in agriculture, and to develop environmentally-friendly production and extraction methods. This latter goal could be easily reached by the improvement of sterol thresholds in plant organs.

The objectives of this work were therefore to study the genotypic variability of phytosterols and phytostanols in bread wheat cultivated under organic conditions and to examine the effect of weather conditions during grain filling on target compound content in the kernel. Relationships between sterols–stanols and protein and ash content, which are the most important quality traits, are also examined.

2. Materials and methods

2.1. Plant material

Twenty bread wheat (*Triticum aestivum* L.) cultivars varying in yield potential and technological traits were used in this study, as well as three other synthetic populations currently used in organic culture in France. The details of genotype characteristics are presented in Table 1.

Table 1
Cultivars of bread wheat used in this study.

Cultivar	Origin	Type of genotype
Apache	France	Improved variety
Ataro	Belgium	Improved variety
Attlas	France	Improved variety
Aubusson	France	Improved variety
Caphorn	France	Improved variety
Esperia	Italy	Improved variety
Fiorenzo	Italy	Improved variety
Kalango	France	Improved variety
Lona	France	Improved variety
Mercato	France	Improved variety
Orpic	France	Improved variety
Pactole	France	Improved variety
Palladio	Italy	Improved variety
Pollux	Belgium	Improved variety
PR22R58	France	Improved variety
Quebon	France	Improved variety
Renan	France	Improved variety
Saturnus	France	Improved variety
Soissana	France	Improved variety
Stefanus	Belgium	Improved variety
Bulk 1[a]	France	Synthetic population
Bulk 2[a]	France	Synthetic population
Bulk 3[a]	France	Synthetic population

[a] Bulks 1, 2 and 3 are synthetic populations currently used in organic agriculture in south-west France.

2.2. Crop management and experimental conditions

Trials were carried out at the Regional Organic Agriculture Experimental Station in Auch (near Toulouse, south-west France, 43°38′47″ N, 0°35′08″ E). Two sowing dates were chosen. The first sowing was done on 24 November 2005. The second sowing was performed on 15 January 2006.

The crops were completely managed under organic and rainfed conditions without any chemical supply. Crushed feathers were applied as an organic fertiliser at a rate of 60 units ha^{-1} in April and May for the conventional (SD1) and later (SD2) sowings, respectively. Weeds were mechanically eliminated.

The soil was a clay-loam (organic matter content: 3.2%; pH 8.1) with a depth of about 1.2 m. Table 2 shows the temperature and rainfall during the plant cycle. The growing season was characterised by a total rainfall of 405 and 385 mm for SD1 and SD2, respectively. These amounts are 110 and 50 mm lower than the half-century average rainfall observed for the same period in our area. In contrast, temperature during the grain filling period was 18.4 and 22.4 °C in SD1 and SD2, respectively. These temperatures were at least 2 °C higher than the observed average temperature during the 50 past years in our region. It is clear that the grain filling period in SD2 coincided with higher temperatures and lower water availability.

The field experiments were conducted as a randomised complete block design with three replicates. Seeds were sown in 22.5 m^2 plots with seven rows per plot (20 cm between rows and 3 cm between plants). Anthesis occurred from beginning to mid-May and from mid-May to end-May for SD1 and SD2, respectively. Maturity took place at the end of June for SD1 and the first week of July for SD2.

2.3. Trait measurements

2.3.1. Agronomic measurements

The number of grains per ear was noted at maturity on ten ears (randomly selected) per plot. Plants were therefore separately harvested by plot in each block. The grain yield was determined by weighing and expressed in tonnes ha^{-1}. Two samples of 1000 grains were taken and then weighed in order to determine the 1000 grains weight of each genotype in each plot. Two aliquots, each of 10 g, were sampled in order to analyse protein and sterol–stanol content separately.

2.3.2. Sterol–stanol, protein, ash and moisture determination

The method of sterol determination was adapted from the one performed by Toivo, Lampi, Aalto, and Piironen (2000). Wheat samples (1.3 g) were added to each tube containing 100 μg of cholestanol (dihydrocholesterol; Aldrich Chemicals Co.), used as an internal standard. Saponification was performed by adding ethanolic KOH (1 M) (TITRINORM, Prolabo) for 60 min at 75 °C. One millilitre of distilled water was added to the samples and the unsaponifiable fraction was extracted from the saponified lipids with 6 ml of *iso*-hexane (Merck). Sterols and stanols were silylated by *N*-methyl-*N*-trimethylsilyl-heptafluorobutyramide (MSHFBA; Macherey-Nagel) mixed with 1-methyl imidazole (Sigma). One microlitre of sterol and stanol trimethylsilyl ether derivatives were injected into a Perkin–Elmer GC equipped with a CPSIL 5CB 30 m column (i.d.: 0.25 mm), and an on-column injector. Detection was performed with a flame ionisation detector. The thermal regime was the following: 160 °C (0.5 min), 10 °C min^{-1} to 260 °C,

Table 2
Prevailing weather conditions during the plant cycle at two different sowing dates (conventional (SD1) and late (SD2) sowings) in Auch (south-west France, near Toulouse). Comparisons are made with the half-century average values of precipitation and temperatures in the same location.

Phenological period	Weather parameter	2005–2006		Last 50 years	
		SD1	SD2	SD1	SD2
Grain filling	Rainfall (mm)	95.8	78.0	112.9	81.0
	Mean temperature (°C)	18.4	22.4	16.7	19.7
	Temperature sum (°C/day)	1120.2	1369.7	1014.7	1203.8
Plant cycle	Rainfall (mm)	405.0	385.4	515.0	434.1
	Mean temperature (°C)	9.9	13.3	11.3	12.4
	Temperature sum (°C/day)	2398.7	2922.4	2452.9	2839.7

2.5 °C min^{-1} to 300 °C, 25 °C min^{-1} to 350 °C, and 350 °C (1.5 min) for the oven temperature; 55 °C (0.5 min), 200 °C min^{-1} to 320 °C, 30 °C min^{-1} to 350 °C, and 350 °C (2.5 min) for the injector temperature; and 365 °C for the detector temperature. Total phytosterols and phytostanols detected included desmethylsterols (β-sitosterol, stigmasterol, campesterol, Δ-7 stigmastenol, Δ-7 avenasterol), campestanol and sitostanol. A typical GC chromatogram of the compounds analysed is shown in Fig. 1.

Grain protein content was determined by NIRS (AACC method 39-11, American Association of Cereal Chemists, 2000).

The moisture content of the samples was determined by drying them (5.0 g) at 105 ± 2 °C overnight. Ash content (expressed in mg g^{-1} dry weight) was determined after complete combustion of the powder aliquot in a muffle furnace at 900 °C and subsequent weighing of the residue. Each sample was analysed at least three times.

2.3.3. Statistical analyses

All the data were subjected to variance analysis using the GLM procedure of SAS (SAS Institute, 1987, Cary, NC, USA). Since genotypes were different in terms of flowering, there were differences for the number of days from sowing to heading. This trait was therefore taken as a covariate in an ANOVA and adjusted means were generated to overcome the effect of flowering date on other traits. The mean pairwise comparisons were based on the means test at 0.5% probability level. Correlation analysis was performed to determine the relationships between the traits using the SAS CORR procedure.

3. Results

3.1. Effect of genotype on sterol–stanol, protein and ash content in grain

In the collection studied, total sterols consisted of 21.3% stanols and 78.7% sterols, where the mean value of sterols + stanols was 65.6 mg 100 g^{-1} DW. The most abundant sterol was β-sitosterol, which represented nearly 70% of the total sterols and more than half of the sterols + stanols. Campestanol and sitostanol represented nearly one-third and two-third of the total stanol content, respectively (Table 3).

Significant differences were observed among the studied genotypes for sterol–stanol level and for both protein and ash content (Table 3). Total sterol (sterols and stanols, TSS) content varied between 49.4 (Bulk 3) and 79.6 mg 100 g^{-1} DW (Caphorn). The largest genotypic difference was observed for stanols since values varied twofold between Orpic (highest value, richer one) and Palladio (lowest one, poorer one). From the point of view of relative value, the latter genotype was the poorest genotype in terms of stanols compared to TSS, whereas stanols represented more than a quarter of TSS in the case of Kalongo. There were significant differences among bread wheat genotypes in our collection for each sterol and stanol. Caphorn, which showed the highest value of TSS, was the richest genotype in sterols, whereas Apache and ORP, the two other richest genotypes in TSS, exhibited the highest values in stanols. In contrast, PR22, the poorest cultivar in TSS, presented the lowest value of sterols.

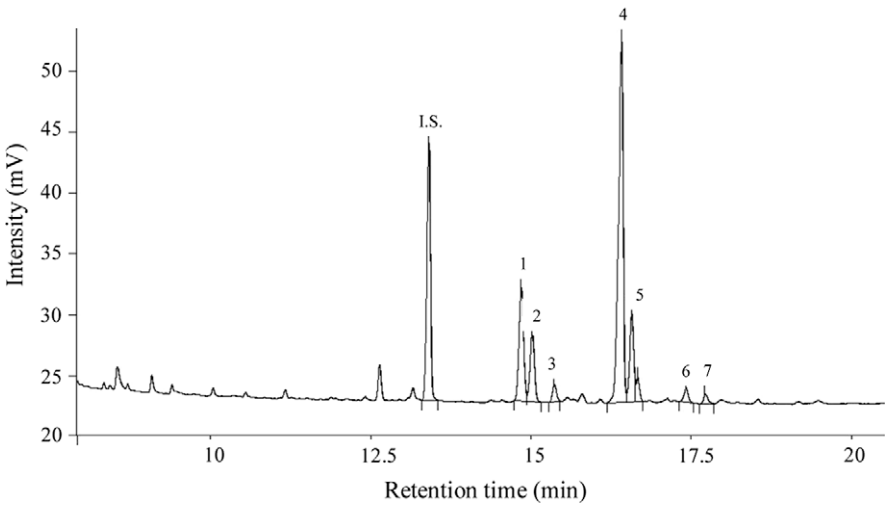

Fig. 1. A typical GC chromatogram of sterols and stanols found in bread wheat kernel. I.S. (internal standard) cholestanol, (1) campesterol, (2) campestanol, (3) stigmasterol, (4) β-sitosterol, (5) stigmastanol, (6) Δ-7 stigmastenol, (7) Δ-7 avenasterol.

Table 3
Sterol and stanol content (mg 100 g^{-1} DW), grain protein content (%), ash content (mg g^{-1} DW) grain yield (tonnes ha^{-1}), number of grains per spike (NGS), and 1000 grains weight (TGW, g) of 23 genotypes of bread wheat grown under rainfed conditions in Auch (south-west France) at two sowing dates (conventional (SD1) and late (SD2)).

Trait	SD1		SD2		Overall		Genotype (G) df = 22	Sowing date (SD) df = 1	G × SD	DH	LSD
	Mean	SE	Mean	SE	Mean	Range					
Campesterol	10.2^{B}	1.6	10.8^{A}	2.0	10.5	6.8–14.3	14.9***	15.9***	4.4***	1.2ns	0.3
Stigmasterol	1.8^{A}	0.3	1.8^{A}	0.4	1.8	1.3–2.8	0.5***	0.1ns	0.2***	0.1ns	0.1
β-Sitosterol	35.7^{A}	3.9	36.0^{A}	4.8	35.9	26.4–3.5	65.7***	2.9ns	49.4***	4.4ns	0.9
C/SSr	0.27^{B}	0.02	0.29^{A}	0.03	0.28	0.22–.33	0.00***	0.01***	0.00***	0.0ns	0.0
Desmethysterols	50.8^{A}	5.7	51.6^{A}	6.4	51.3	39.6–2.1	121.8***	0.2ns	96.7***	10.9ns	1.3
Campestanol	5.0^{A}	1.1	5.2^{A}	1.4	5.2	2.4–7.9	7.3***	0.8ns	1.7**	0.1ns	0.3
Sitostanol	10.0^{A}	2.0	8.4^{B}	2.3	9.2	4.2–13.0	20.2***	83.8***	6.2***	1.1ns	0.4
C/Sn	0.52^{B}	0.14	0.64^{A}	0.15	0.58	0.29–.89	0.1***	0.49***	0.04***	0.0ns	0.0
Total stanols	15.0^{A}	2.5	13.6^{B}	3.2	14.4	7.8–19.9	39.3***	68.5***	8.9**	2.0ns	0.7
Total sterols + stanols	65.8^{A}	6.6	65.4^{A}	8.9	65.6	49.4–9.6	206.6***	19.2ns	156.9***	18.4ns	1.8
Protein content	12.1^{B}	0.7	12.5^{A}	0.6	12.3	10.4–3.2	2.1***	6.9***	2.0**	0.4ns	0.2
Ash content	32.9^{B}	7.9	45.7^{A}	6.3	39.3	19.7–60.0	118.5***	5609.0***	82.4**	1.0ns	2.3
NGS	54.2^{A}	13.6	37.9^{B}	10.6	45.9	23.3–6.7	856.3***	9071.9***	3600.3***	8860.0***	5.7
TGW	34.0^{A}	3.5	28.3^{B}	3.1	31.0	23.3–9.5	72.6***	1145.3***	815.9**	1162.7***	0.9
Grain yield	2.6^{A}	0.5	2.2^{B}	0.4	2.4	1.5–3.6	94.1***	668.1***	634.9***	638.0***	1.1

Values presented are mean (across genotypes) and standard error (SE) values. Means indicated by a different letter are significantly different ($P \leqslant 0.05$). LSD: least significant difference. For each trait, *F*-value and degrees of freedom (df) of the genotype, sowing date and their interactions (G × SD), as well as the covariate effect of the number of days from sowing to heading (DH), are also displayed. ** and *** significance at 0.01 and 0.001 probability levels, respectively. ns: not significant. C/SSr: ratio of campesterol to stigmasterol + β-sitosterol; C/Sn: ratio of campestanol to sitostanol.

A wide variability was observed among the studied genotype for ash content. This trait varied from 19.7 (Pactole) to 60.0 mg g^{-1} DW (Quebon), with a mean value of 39.3 mg g^{-1} DW. Protein content varied less than sterol and ash content. Pollux contained nearly 3% more proteins than Aubusson, while Caphorn and Bulk 3, extreme genotypes for TSS, showed intermediate values for protein content.

3.2. Effect of sowing date on sterol–stanol, protein and ash content in grain

The objective of different sowing dates was to study the effect of the contrasting temperatures that occurred during grain filling. In this region, late sowing delays grain filling, which in turn coincides with high temperatures. Higher protein and ash content values were observed for later sowing.

Sowing date has shown a significant impact on sterol and stanol content in grain, except on stigmasterol, β-sitosterol, d_7-stigmasterol content and TSS. Two response patterns were depicted. The increase in temperature during grain filling (by sowing at the later date rather than the conventional date) improved the content for several sterols but did not significantly increase the most abundant one (i.e., β-sitosterol) and the total sterol level (Table 3). In contrast, and surprisingly, sitostanol, campestanol and total stanol content decreased when temperatures increased. Despite this, a strong increase of the campestanol/sitostanol ratio was observed for delayed sowing (Table 3). A significant increase of the ratio of campesterol to β-sitosterol + stigmasterol was observed in grain for late sowing (Table 3). The genotype by sowing date interaction was also significant for all traits (Table 3).

Two genotypic groups were different in response to high temperatures. A group made up of 18 genotypes reduced its TSS from the conventional (SD1) to the late sowing date (SD2) by more than 4.0 mg 100 g^{-1} DW, mainly due to the decrease in stanol content (Table 4). This group included Aubusson, Lona, Palladio, Quebon and the three bulk genotypes. In contrast, the second group composed of five genotypes (Fiorenzo, Kalongo, Orpic, Renan and Stefanus) increased TSS by nearly 13.0 mg 100 g^{-1} DW (2.1 mg 100 g^{-1} DW of stanols and 10.4 mg 100 g^{-1} DW of desmethylsterols) from SD1 to SD2 (Table 4). Moreover, the difference between SD1 and

Table 4
Mean values of sterol content, stanol content, grain protein content, ash content and agronomic traits of two groups contrasted for their response to delayed sowing. Group 1 consisted of five genotypes and Group 2 of 18 genotypes. The 23 genotypes of bread wheat were grown under rainfed conditions in Auch (south-west France) at two sowing dates (conventional (SD1) and late (SD2)).

Trait	Group 1			Group 2		
	SD1	SD2	LSD	SD1	SD2	LSD
Campesterol (mg 100 g^{-1} DW)	9.8^{B}	12.1^{A}	1.3	10.3^{A}	10.5^{A}	1.3
Stigmasterol (mg 100 g^{-1} DW)	1.8^{A}	2.2^{A}	0.6	1.9^{A}	1.7^{A}	0.3
β-Sitosterol (mg 100 g^{-1} DW)	33.7^{B}	41.0^{A}	4.5	36.3^{A}	34.7^{A}	2.9
C/SSr	0.28^{A}	0.28^{A}	0.0	0.27	0.29	0.0
Desmethylsterols (mg 100 g^{-1} DW)	47.5^{B}	57.9^{A}	6.0	51.8^{A}	50.0^{A}	4.0
Campestanol (mg 100 g^{-1} DW)	5.2^{A}	6.1^{A}	1.9	5.0^{A}	5.0^{A}	0.8
Sitostanol (mg 100 g^{-1} DW)	10.2^{A}	11.4^{A}	2.1	9.9^{A}	7.7^{B}	1.3
C/Sn	0.52^{A}	0.54^{A}	0.2	0.52^{B}	0.67^{A}	0.1
Total stanols (mg 100 g^{-1} DW)	15.4^{A}	17.5^{A}	3.0	14.9^{A}	12.7^{B}	1.8
Total sterols + stanols (mg 100 g^{-1} DW)	64.7^{B}	76.8^{A}	8.1	68.7^{A}	64.2^{A}	5.0
Protein content (%)	12.4^{A}	12.8^{A}	0.5	12.0^{B}	12.5^{A}	0.5
Ash content (mg g^{-1} DW)	40.1	46.7	7.5	33.5^{B}	45.5^{A}	8.3
Number of grains per spike	53.9^{A}	35.7^{B}	11.9	54.2^{A}	38.8^{B}	9.0
1000 grains weight (g)	34.4^{A}	29.9^{B}	6.5	34.0^{A}	28.1^{B}	2.0
Grain yield (tonnes ha^{-1})	2.6^{A}	2.3^{A}	0.5	2.6^{A}	2.2^{B}	0.3

C/SSr: ratio of campesterol to stigmasterol + sitostanol; C/Sn: ratio of campestanol to β-sitosterol.

SD2 was more pronounced for the genotypes of the former group than for the group of the five genotypes for the agronomic traits (Table 4).

3.3. Effects of sowing date and genotypes on agronomic traits

Both sowing date and genotype effects significantly influenced agronomic traits. Interaction between the two factors was also significant for all traits (Table 3).

Plants sowed at the conventional date produced more grains by spike and filled those more than grains sowed later. Consequently, grain yield was higher for conventional compared to late sowing. Large and significant genotypic differences were observed within the collection studied for agronomic traits. Saturius produced three times more grain than Esperia. Grains of Renan were more than two times heavier than those of Orpic. PR22 yielded two times more than Pollux.

4. Discussion

4.1. Effects of genotype and sowing date on agronomic traits, protein and ash content

Differences were observed in agronomic traits, ash and protein content within the collection of genotypes studied (Table 3). Grain yield varied twofold between extreme genotypes. These differences resulted from the variability in yield components (Table 3). These results were expected since genotypic variability in agronomic traits had been widely reported in cereals (Asseng & Milroy, 2006; Merah, Deléens, Souyris, & Monneveux, 2001; Xu, Yuan, Li, & Monneveux, 2007). Similarly, protein and ash content varied considerably among the studied genotypes (Table 3), confirming results of previous reports in several cereals (Ma et al., 2007; Merah et al., 2001; Roussel, Branlard, Vezine, Bertrand, & Balfourier, 2005; Xu et al., 2007).

Significant differences were observed for all traits between the two sowing dates (Table 3). Late sowing induced a decrease in agronomic characteristics. This decrease is probably related to the difference in weather conditions between the two sowing dates, primarily during the grain filling period (Merah, Deléens, & Monneveux, 1999; Xu et al., 2007). It clearly appears that terminal drought and higher temperatures were more pronounced at the late sowing date (Table 2). This may explain the differences in grain yield between SD1 and SD2, resulting from the decrease in the number of grains per ear and the grain weight due to increasing stress (Asseng & Milroy, 2006; Spiertz et al., 2006).

The negative correlation between ash content and grain yield ($r = 0.50$, $P > 0.01$, df = 44) found in our study is in agreement with reports of Xu et al. (2007) and Merah et al. (2001, 1999). These reports suggest that minerals accumulate in grains by phloem transport rather than passively from the transpiration stream. Therefore, mineral accumulation in the grain primarily depends on translocation from the leaves and on minerals removed from the lower parts of the plant after the onset of senescence. As a consequence, genotypes that cannot maintain high rates of stomatal conductance and photosynthesis during grain filling would fill their grains through retranslocation of photoassimilates from preanthesis reserves, and of minerals from early senescent vegetative tissues (Merah et al., 2001). The ash concentration in grain could be an indication of the importance of the retranslocation processes during grain filling (Merah et al., 1999). These results suggest that grain ash content is higher in genotypes more affected by drought and higher temperatures during grain filling. Moreover, Xu et al. (2007) reported that higher temperatures, drought or both induced an increase of grain ash content, regardless of genotype tolerance.

Differences between SD1 and SD2 in grain protein content were also observed. This result means that variation in protein content was a consequence of agronomic practices (late sowing in this case) due to differences in weather conditions, for the same location and the same genotype. Previous studies have emphasised the increase of protein content in grain or in flour as a result of moisture stress (Saint-Pierre et al., 2008) and high temperatures (Asseng & Milroy, 2006; Dupont et al., 2006; Spiertz et al., 2006). It has been reported that higher temperatures as well as water stress during the grain filling period induces more of an increase in nitrogen accumulation than in dry weight accumulation, leading to higher protein content in grain (Asseng & Milroy, 2006; Dupont et al., 2006; Spiertz et al., 2006). In our study, the average temperature during grain filling was 4 °C higher for plants that were grown later than the conventional sowing date, in conjunction with low water availability (Table 2). As a consequence, plants sowed later accumulate less dry matter per grain but increase their protein content (Table 3). A 1000 grains weight was in fact 6 g lower in SD2 than in SD1, whereas the grain accumulated more proteins in late sowing than in conventional sowing (Table 3), thereby confirming previous reports. This is also supported by the negative correlation found between protein content and grain yield ($r = -0.71$, $P < 0.001$, df = 44).

4.2. Effects of genotype and sowing date on phytosterol and phytostanol content

In our study, the range of genotypic variation was from 49.4 to 79.6 mg 100 g^{-1} DW. This wide range of variation was similar to those reported for wheat grain in other studies. Piironen et al. (2002) reported a mean value of two wheat genotypes of 69.0 mg 100 g^{-1}. Analysis of whole grains of commercial samples revealed 78.3 mg 100 g^{-1} DW (Nyström et al., 2007). It is clear that the range of variation in total sterol level in bread wheat grain observed within our collection is similar to those reported in the few studies done on wheat grain. Nevertheless, our results cannot be compared to those observed elsewhere. There are several reasons for this discrepancy. The main studies dealing with sterol content in grain or other plant organs were done on commercial samples. These samples may come from bulk grains of several genotypes obtained from crops in different locations and under different climates, and information on growing conditions in these studies was inadequate. Moreover, our study was done under organic conditions that are considered to be stressed growing conditions and that probably influence the potential expression of the genotypes.

The second reason is the difference in analytical methodology for sterol analysis. In our study, we focused on sterols and steryl fatty acid esters, whereas Nyström et al. (2007) and Piironen et al. (2002) also assessed conjugated forms. In spite of this difference, our values were similar to those of Piironen et al. (2002).

The third reason was that results reported in the different studies were expressed in lot of units and the comparison was therefore not possible. This fact has already been reported by Zangenberg et al. (2004). These authors, as well as Määttä et al. (1999), highlighted the influence of genotypes on sterol content in cereal grains, which could also partially explain the different values observed between our study and the other studies mentioned above.

The fourth reason was that the studies differed by the number and the genotypes used for the evaluation of sterol and stanol content. A large variation was observed for sterol and stanol content within the collection of bread wheat genotypes studied. Genotypic variation was reported for sterol content among seven oat cultivars (Määttä et al., 1999) and three rye cultivars (Zangenberg et al., 2004). Piironen et al. (2002) also reported differences in four cereals. In the latter study, the difference reported between the two

genotypes used was 5.0 mg 100 g^{-1} DW. This difference represents nearly a quarter of the observed genotypic range within our collection. For the other cereal species, genotypic ranges for TSS were quite lower than those observed in our study (Määttä et al., 1999; Piironen et al., 2002; Zangenberg et al., 2004). Even if the range of genotypic variation for stanol content was wider in our study, the mean reported value was quite equivalent to the values reported by Piironen et al. (2002) in wheat.

A difference was observed between the two sowing dates for campesterol. Surprisingly, sitostanol strongly decreased in later sowing, which in turn induced a reduction of stanol content in the grain. Consequently, TSS was lower in later sowing. In rye, a decrease of sitostanol was observed for three genotypes across years (Zangenberg et al., 2004). Unfortunately, climatic conditions were not recorded in this study and we can therefore not effectively compare our results to those obtained by Zangenberg et al. (2004).

In sunflower, high temperatures (late sowing) induce an increase of sterol content in the kernels (Roche, Bouniols, Mouloungui, Barranco, & Cerny, 2006; Valsta et al., 2004). Other studies have reported that variations in temperature may cause a difference in sterolic profiles, mostly for campesterol vs. stigmasterol + β-sitosterol (Hartmann, 1998; Schaller, 2003). In our study, we observed that the ratio of campesterol to stigmasterol + β-sitosterol (hereafter C/SSr) was not significantly different between SD1 and SD2. In contrast, the ratio of campestanol to sitostanol (hereafter referred to as C/Sn) strongly increased at the later sowing date (Table 3). This was due to the concomitant decrease of sitostanol with the slight reduction of campestanol in the grain from later sowing.

However, this is a general tendency and the two groups were differentiated on the basis of their response to higher temperatures. The first one (GRP1), made up of five genotypes, increased its TSS as a result of the increase in the stanol level and of desmethylsterols, in particular. In contrast, the second group (GRP2), composed of 18 genotypes, high temperatures led to a decrease of sterol and particularly stanol content (Table 4). Moreover, the genotypes of the former group had increased levels of mainly β-sitosterol and sitostanol from the conventional to the delayed sowing. These two components were the main molecules that were reduced by high temperatures in the second group. Sterols and stanols play an important role in membrane fluidity and permeability (Moreau et al., 2002; Schaller, 2003). Changes in the physical properties of membranes reflect variations in their composition resulting from alterations in metabolic processes. These processes are accompanied by an increase in the sterol-to-phospholipid ratio (Borochov & Woodson, 1989; Itzhaki, Borochov, & Mayak, 1990). Moreover, membrane fluidity is not only influenced by the level of sterols but also by the change in their composition (Moreau et al., 2002; Schaller, 2003). It seems that the increase in β-sitosterol content increases membrane fluidity, which in turn changes metabolic cell functioning. In contrast, the increase of campesterol content reduces the physical properties of the membrane, thus leading to increased fluidity. Variation in desmethylsterols (represented in our study by C/SSr) may result from the activity of sterol-methyl-transferase 2 (SMT2) (Schaeffer, Bronner, Benveniste, & Schaller, 2001). Interestingly, Schaller (2003) reported the importance of genotypic factor on this ratio, which is in accordance with our results (Tables 3 and 4). Moreover, our study emphasised the effect of temperature on C/SSr in grain, confirming previous reports on *Arabidopsis* (Schaeffer et al., 2001). Delayed sowing coincided with high temperatures during grain filling (Table 2). These conditions induced an increase in β-sitosterol and sitostanol from conventional to delayed sowing in GRP1. A decrease of these sterols and stanols was observed for GRP2 with high temperatures (Table 4). In addition, the C/SSr ratio as well as the C/Sn ratio remained fairly constant in the former group, whereas these ratios increased for GRP2 due to the decrease of β-sitosterol and sitostanol (Table 4).

In our study, different responses to high temperatures of the two groups of genotypes for agronomic traits were found. Grain yield and its component decreased with high temperature. This decrease was more pronounced in GRP2 than in GRP1 (Table 4). Similarly, genotypes of GRP2 accumulated 1.5 times more minerals in their grains in SD2 than in SD1, whereas genotypes belonging to GRP1 maintained a constant ash content (Table 4). We hypothesised that the difference in tolerance of high temperature and water shortage exists between the two groups of genotypes. Among the numerous mechanisms involved in this expressed genotypic difference, there is a difference in the maintenance of membrane fluidity. This could be the result of an increase in sterol content, which induces the changes in the physical properties of membranes and may therefore cause alterations in cell functioning and plant development, depending on the timing of the alterations during the plant cycle (Borochov & Woodson, 1989; Itzhaki et al., 1990). Our results also suggest that the genotype effect was the main factor influencing sterol content.

No significant correlation was found between sterols and agronomic traits (data not shown). Sterol content was not related to protein content in our study ($r = 0.05$, df = 44). The relationship between sterol and protein content has not been studied in cereals. However, phytosterols are distributed in endosperm, germ and pericarp (Harrabi et al., 2008), while proteins are localised in the aleurone cell layer. This fact could explain the lack of correlation in the accumulation of the two compounds. In sunflower cultivated under different water availability conditions, no significant correlation was found between protein and sterol content in the grain (Roche, 2005). This result is very interesting. If confirmed, it would mean that improvement of both sterol and protein content is possible and may be easily feasible within a selection programme.

5. Conclusion

Wheat is among the major sources of sterols and stanols. A wide variability of sterol and stanol content exists in bread wheat cultivated under organic conditions. Although the main factor remained the genotype, our data revealed a strong interaction with weather conditions that could easily be managed to promote the accumulation of targeted sterols or stanols and to provide added value to the wheat grain. Sterol and stanol content seems to be independent from other technological traits and, therefore, may be easily improved without any negative interaction with other characters of interest. These results are interesting and should be studied under other climatic conditions and with other genotypes. Much attention is being focused on functional foods at this time. The accumulation of compounds of interest such as sterols in plants should facilitate their extraction by soft methods, ultimately benefiting the consumer.

Acknowledgements

We would like to thank the European Union for financially supporting this work (FEDER 7440 and 12688). Thanks are due to Loïc Prieur and Laurent Laffont from CREAB for help in experiments and for providing grains. The participation of Dr. O. Merah was supported by IUT Paul Sabatier, Toulouse (University of Toulouse III), France.

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Food Chemistry 117 (2009) 226–231

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Effects of extraction temperature on the phenolic and parthenolide contents, and colour of aqueous feverfew (*Tanacetum parthenium*) extracts

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ARTICLE INFO

Article history:
Received 24 November 2008
Received in revised form 5 March 2009
Accepted 30 March 2009

Keywords:
Feverfew
Tanacetum parthenium
Total phenols
Parthenolide
Colour

ABSTRACT

The aim of this study was to determine the effects of extraction temperature on the colour as well as on the phenolic and parthenolide content of feverfew (*Tanacetum parthenium*) aqueous extracts, with a view to incorporating the extracts into a beverage. Results showed that extraction temperatures of 20–70 °C yielded dark-coloured extracts with low total phenol and a relatively high total tannin concentration. However, increasing the temperature to 75 °C produced lighter-coloured extracts with a significantly higher concentration of total phenols, lower total tannin fraction and maximum extraction of parthenolide. Higher extraction temperatures (80–100 °C) yielded suitably pale extracts rich in total phenols, but with progressively higher total tannin to non-tannin ratio and lower parthenolide content. The compromise temperature of 80 °C was found to provide extracts rich in parthenolide content, phenolic content and with a desirable colour, suitable for incorporation into a functional beverage.

1. Introduction

The utilisation of naturally-occurring bioactives as potential sources of functional food ingredients and, in particular, phytochemicals has increased in the recent years, because of their beneficial health effects. Consumer demand for health-related products, providing immune support, energy enhancement, healthy joint function and promoting overall well-being has led to the development of functional foods and beverages. One major success story in this regard is the use of beverages as dietary vehicles for phytosterols (Devaraj, Autret, & Jialal, 2006; Goncalves, Maria, Silva-Herdade, Silva, & Saldanha, 2007). The inclusion of these plant extracts in food products requires not only an optimum concentration of bioactives, but also a minimisation of the potential detrimental effect this inclusion could have on the taste and appearance of the food product. For example, the colour of the plant extract can impart an unfavourable green or brown hue to the final beverage, whilst the presence of large amounts of tannins could impart significant bitterness.

Phytochemical-rich plants have played a significant role in diet-based therapies to prevent and cure various ailments (Butt, Nazir, Sultan, & Schroen, 2008). Feverfew (*Tanacetum parthenium*), a medicinal herb commonly found throughout Europe, has a potential for use in functional foods. It has been used for years for the treatment of arthritis and migraine, due to its anti-inflammatory activity (Johnson, Kadam, Hylands, & Hylands, 1985; Williams, Harborne, Geiger, & Hoult, 1999). Feverfew has also been used for treating conditions like epilepsy, fever, stomach ache, toothache and insect bites (Jager, Gauguin, Adsersen, & Gudiksen, 2006; Jain & Kulkarni, 1999). Traditionally, feverfew leaves were used fresh, but preparations made from dried feverfew are more common today in the form of teas, tablets and capsules (Piela-Smith & Liu, 2001).

Pharmacological studies have shown that parthenolide is the main bioactive ingredient of feverfew, probably due to the presence of the α-methylene-γ-lactone ring and the epoxide group in this molecule (Bejar, 1996; Jain & Kulkarni, 1999; Kwok, Koh, Ndubuisi, Elofsson, & Crews, 2001; Macias, Galindo, Castelleno, & Valesco, 1999; Miglietta, Bozzo, Gabriel, & Bocca, 2004; Piela-Smith & Liu, 2001). The biological activity of feverfew is also due to its content of phenolic compounds, including apigenin, luteolin and tanetin (McVean, Xiao, Isobe, & Pelling, 2000; Pettit et al., 1996; Williams et al., 1999; Wu et al., 2006).

Several methods have been used to extract and quantify the bioactive compounds in feverfew. Solvent extraction and supercritical fluid extraction have been used for the extraction of sesquiterpene lactones (Kaplan, Simmonds, & Davidson, 2002) and bottle stirring, sonication and Soxhlet extraction have been used for the extraction of parthenolide (Fonseca, Rushing, Thomas, Riley, & Rajapakse, 2006; Zhou, Kou, & Stevenson, 1999). Wu et al. (2006) extracted parthenolide, luteolin, apigenin from feverfew in 80% alcohol.

Whilst these extraction methods have been carried out with the objective to obtain the maximum concentration of active

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doi:10.1016/j.foodchem.2009.03.103

constituents, the use of organic solvents prohibits the subsequent use of these extracts in beverages. Moreover, there is little or no information in the literature on the aqueous extraction of feverfew with a view to obtaining extracts suitable for the manufacture of functional foods and beverages. Therefore, the objective of this study was to determine the effect of extraction temperature on the total phenol and parthenolide contents, and the colour of aqueous feverfew extracts, for incorporation into a beverage with anti-inflammatory properties.

2. Materials and methods

2.1. Plant material

Feverfew was organically grown and harvested in Roscommon, Ireland. On the day of harvest the aerial parts of feverfew were transferred to the laboratory and frozen at −20 °C. The frozen plant material was subsequently dried for 72 h using a freeze dryer (Edwards Super Modulyo, Davidson & Hardy Ltd., Dublin, Ireland). The dried samples were then ground into a moderately fine powder (180–355 μm) (WHO (World Health Organization), 1998) using a lab mill (Christy and Norris Ltd., Christy Turner, Ipswich, UK). The dried samples were placed in brown Kraft envelope bags (Paper Nature's Packaging, Ireland) and stored in polypropylene buckets containing silica gel. The buckets were then sealed and stored at ambient temperature. Previous studies in our laboratory have shown that these storage conditions resulted in no appreciable loss of phenolics or parthenolide during the duration of the present study (unpublished data).

2.2. Extraction

To prepare aqueous extracts, the dried feverfew powder (2.5% w/w) was put into distilled water, and heated at 20, 60, 70, 75, 80, 90 and 100 °C for 10 min using a stirring hot plate (Ika® Werke GmbH and Co., Staufen, Germany). The extracts were cooled in ice to <10 °C and filtered under vacuum using Whatman No. 1 filter paper with a micro pore size of 11 μm. All the filtrates were stored in sterile containers (Sarstedt, Nümbrecht, Germany) at −20 °C prior to analysis.

Extraction under vacuum at 20 °C was also performed. The dried plant material (2.5 g) was weighed into a conical flask covered with a nylon cloth and placed in a vacuum desiccator connected to a vacuum pump for 40 min. Extraction was carried out in 100 ml distilled and degassed water. The water was degassed for two hours using a sonication bath (Ultrawave Ltd., Cardiff, UK).

Additional filtration using Acrodisc® premium 25 mm syringe with 0.2 μm GHP (hydrophilic polypropylene) membrane (Pall Life Sciences, Portsmouth, UK) was used to prepare filtrates for HPLC analysis.

2.3. Colour determination of feverfew extracts

The colour of feverfew extracts was determined using a Chroma metre CR-300 (Minolta Ltd, Milton Keynes, UK). The Hunter Lab scale was used with L^*, a^* and b^* axes expressing the lightness, redness–greenness and blueness–yellowness, respectively. To determine the effects of extraction temperature on the colour of the extracts, the hue angle (h°) and chroma (C^*) were calculated according to McGuire (1992). Hue angle is defined as a colour wheel with red–purple at an angle of 0°, yellow at 90°, bluish-green at 180° and blue at 270°.

The absorbance of the extracts was also measured at 430 nm using a UV–Vis spectrophotometer (UV-1240, Shimadzu, Kyoto, Japan).

2.4. Quantification of the total phenolic content

The total phenolic content was analysed according to the Folin–Ciocalteu method (Singleton & Rossi, 1965). A standard curve was prepared using gallic acid (Reagent grade; Sigma–Aldrich, St Louis, MO) of known concentrations (0–350 mg/l). The standards (0.2 ml) or feverfew extracts were mixed with 0.5 ml Folin–Ciocalteu reagent (Reagent grade, Merck, Darmstadt, Germany), 1.5 ml of 20% sodium carbonate (Reagent grade, Merck) and 7.8 ml of distilled water and allowed to stand for 2 h. Absorbance was measured at 760 nm using a UV–Vis spectrophotometer (UV-1240, Shimadzu). The total phenolic content of the extract was calculated as mg of gallic acid equivalents (GAE)/g of dry weight (d.b.) of the feverfew powder.

2.5. Separation of total tannin and non-tannin fraction

The total tannin (condensed and hydrolysable tannins) and non-tannin (flavonoids and simple phenols) fraction were separated according to the cinchonine precipitation method of Peri and Pompei (1971). The extract (1 ml) was mixed with 1 ml of 0.5% cinchonine hemisulfate (Reagent grade, Sigma–Aldrich) and placed in a 2 ml micro-centrifuge tube. The tubes were shaken well and centrifuged in a micro-centrifuge (Davidson & Hardy Ltd.) at 5668*g* for 5 min. The supernatant representing the non-tannin fraction was analysed using the Folin–Ciocalteu procedure. The total tannin fraction was determined by difference.

2.6. Separation and quantification of parthenolide

The extracts were analysed using a system comprising a P4000 pump, an AS3000 autosampler and a UV2000 ultraviolet detector (Thermo-Separation products, San Jose, CA) as described by Zhou et al. (1999) by HPLC. Reversed-phase chromatography was performed using a Phenomenex Luna C_{18} column (150 × 4.6 mm i.d., 5 μm), mobile phase of acetonitrile (HPLC grade, Sigma–Aldrich): water (55:45 v/v), flow rate of 2 ml/min and UV detection at 210 nm. The injection volume was 20 μl.

2.7. Coomassie protein assay

Coomassie dye was prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 (Reagent grade, Sigma–Aldrich) in 50 ml of 95% ethanol (Reagent grade, Sigma–Aldrich). The solution was added to 100 ml of phosphoric acid (85%) (Reagent grade, Sigma–Aldrich) and diluted to 200 ml with distilled water. This stock solution was stored in a dark bottle at 4 °C. The assay reagent was prepared with 25% (v/v) of dye stock in distilled water.

The protein assay was carried out according to the dye-binding method of Boyes, Strubi, and Dawes (1997).

2.8. Determination of polyphenol oxidase (PPO) activity

An aliquot of feverfew powder (2.5 g) was extracted in 100 ml of 0.02 M phosphate buffer (pH 6.8) at ambient temperature for 10 min. The extract was filtered through a Whatman filter paper No. 1 and incubated in a water bath at different temperatures (30–100 °C) for 10 min each. The extracts were cooled on ice prior to analysis. PPO activity was determined according to the method of Wakayama (1995). Absorbance at 420 nm was measured with time using a UV–Vis spectrophotometer (UV 4, ATI Unicam Ltd., Cambridge, England). One unit of enzyme activity was defined as the amount of enzyme that caused an initial change in absorbance of 0.001 per minute.

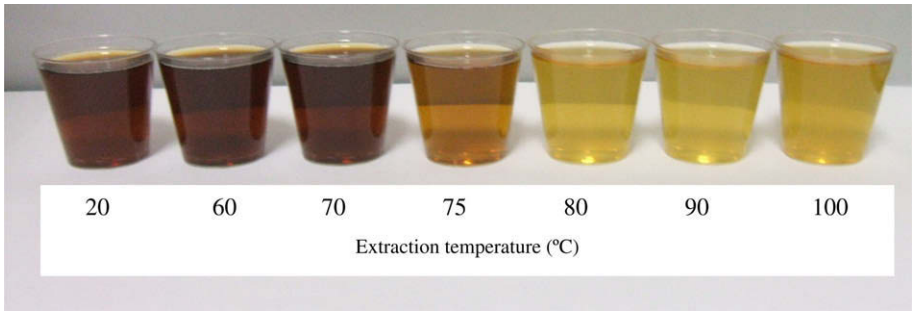

Fig. 1. Image of feverfew samples extracted in water at different temperatures.

2.9. Statistical analysis

Extraction at each temperature was carried out in triplicate and one measurement was carried out on each extract. Measurement of the PPO activity was carried out in triplicate. Statistical analysis was performed using SAS software (SAS Institute, Cary, NC) Version 9.1.3. Tukey pairwise comparison test was used for comparison of means. Mean values represented by the same letters were considered not significantly different at $p \geqslant 0.05$.

3. Results and discussion

3.1. Colour of feverfew extracts

Extraction of feverfew samples in distilled water at different temperatures yielded extracts with different colour intensities (Fig. 1). Generally, extraction temperature had a substantial effect on the lightness, chroma and hue angle of the extracts. The samples extracted at 20 to 70 °C had a brownish appearance with a hue angle of 348 ± 4°, chroma of 1 ± 0.1 and lightness of 49 ± 0.4, whereas samples extracted at higher temperatures of 80–100 °C were yellow in appearance with a hue angle of 55 ± 5°, chroma of 11 ± 3 and lightness of 57 ± 2 (Table 1). This dramatic change in colour with extraction temperature was unexpected, as herbal infusions extracted at higher temperatures usually contain more phenolic bioactives and are darker than those extracted at low temperatures. In order to determine the general chemical composition of the feverfew extracts, the concentration of total phenols, total tannin and non-tannin fractions and parthenolide were analysed.

Table 1
Hue angle, chroma and lightness of feverfew samples extracted at different temperatures in distilled water.

Extraction temperature (°C)	Hue angle ($h°$)	Chroma (C^*)	Lightness (L^*)
20	346 ± 3^{a}	1.0 ± 0.1^{c}	49.3 ± 0.3^{d}
60	351 ± 3^{a}	1.1 ± 0.1^{c}	49.1 ± 0.5^{d}
70	347 ± 3^{a}	1.2 ± 0.1^{c}	49.0 ± 0.6^{d}
75	20 ± 2^{c}	5 ± 2^{b}	51 ± 2^{cd}
80	56 ± 3^{b}	13 ± 2^{a}	59 ± 1^{a}
90	59 ± 6^{b}	13 ± 2^{a}	58 ± 3^{ab}
100	49 ± 3^{b}	8 ± 1^{b}	55 ± 1^{bc}

[a–d]Mean values ± standard deviations with the same letters within the same column are not significantly different at $p \geqslant 0.05$.

3.2. Total phenols, total tannins and non-tannins in feverfew extracts

The effect of extraction temperature on the total phenols is shown in Fig. 2. Results showed that extraction temperatures of 20 to 70 °C did not affect the total phenol content significantly ($p \geqslant 0.05$). An increase in temperature to 75 °C led to a significant increase in total phenols and further increasing the extraction temperature increased the total phenol concentration significantly, with the highest amount of 31 ± 1 mg/g GAE d.b. extracted at 100 °C. The effect of extraction temperature on the total tannin and non-tannin fractions is shown in Fig. 3. The total tannin fraction was not significantly affected by the extraction temperature, except for an increase at 100 °C. Extraction temperatures of 20–70 °C led to low non-tannin fractions, although increasing the temperature to 75 °C resulted in a significant increase. However, a further increase in temperature from 75 to 100 °C did not affect the non-tannin content. From these results it appears that the

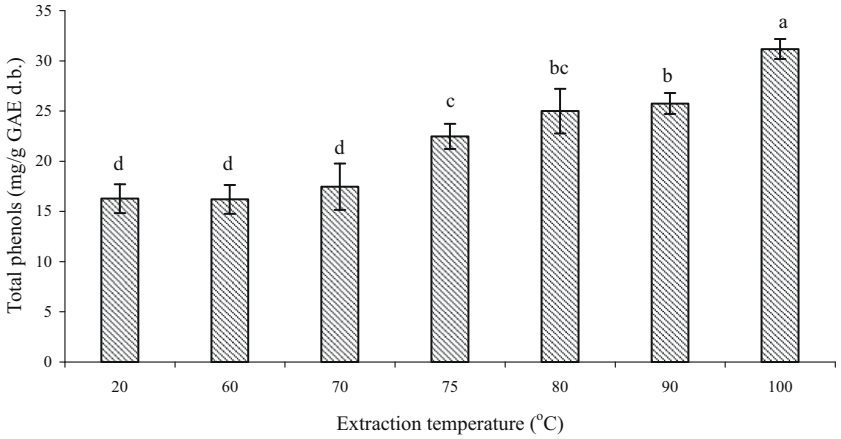

Fig. 2. Effect of extraction temperature on the total phenolic content in aqueous feverfew extracts. Bars with the same letters (a–d) are not significantly different at $p \geqslant 0.05$.

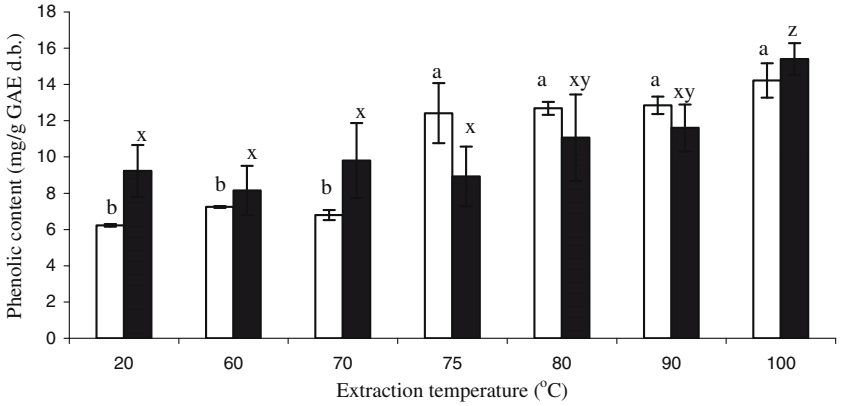

Fig. 3. Effect of extraction temperature on the total tannin (■) and non-tannin (□) fractions of aqueous feverfew extracts. Bars with the same letters (a–b, x–z) are not significantly different at $p \geqslant 0.05$.

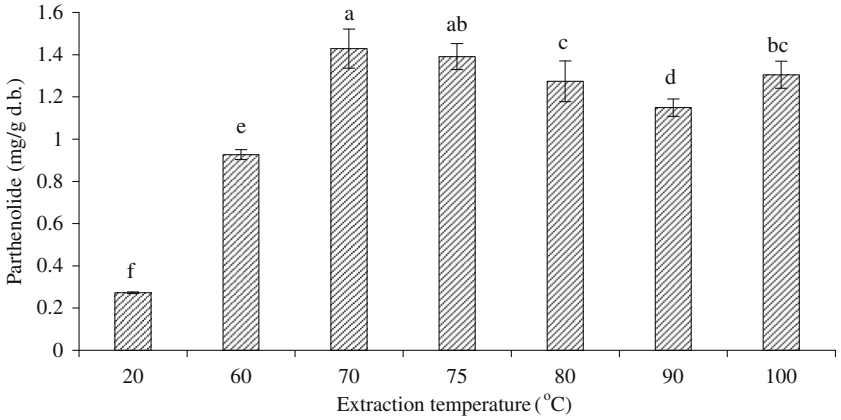

Fig. 4. Effect of extraction temperature on the parthenolide concentration in aqueous feverfew extracts. Bars with the same letters (a–f) are not significantly different at $p \geqslant 0.05$.

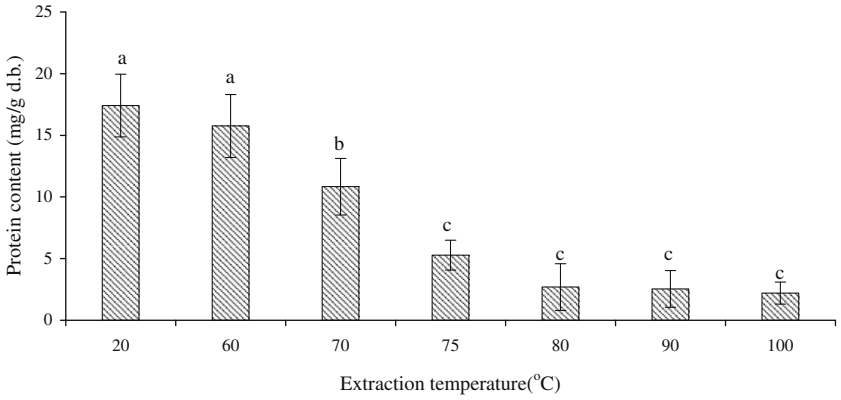

Fig. 5. Effect of extraction temperature on the protein content in aqueous feverfew extracts. Bars with the same letters (a–c) are not significantly different at $p \geqslant 0.05$.

significant increase in the total phenols content noticed at temperatures above 75 °C is due solely to an increase in the non-tannin fraction. But this significant increase in non-tannins at high temperatures could not explain the change in extract colour.

3.3. Parthenolide content in feverfew extracts

The effect of extraction temperature on the parthenolide content in aqueous feverfew extracts is shown in Fig. 4. Partheno-

Table 2
Evolution of absorbance at 430 nm on exposure to air for samples extracted *in vacuo* at 20 °C.

Time after extraction (min)	Abs at 430 nm
5	1.03
25	1.08
165	1.20
1440	1.52

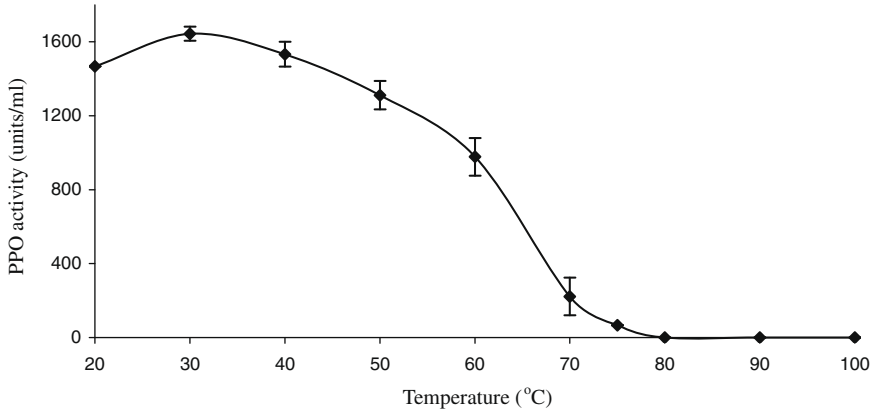

Fig. 6. Effect of temperature on the polyphenol oxidase activity in feverfew extracts.

lide concentration increased significantly with increasing extraction temperature from 20 to 70 °C. There was no significant difference ($p \geqslant 0.05$) in the amount of parthenolide extracted at 70 or 75 °C, but a slight decrease in the parthenolide content was observed with increasing extraction temperature above 75 °C. These results indicate that temperature had a significant effect on the recovery of parthenolide from the plant material. As reported by Smith and Burford (1992) parthenolide is present in the plant matrix at different sites, some of which can be readily extracted whilst others are tightly bound. Therefore, the increase in temperature may have influenced the release of the tightly bound parthenolide. However, the decrease in parthenolide content above 75 °C could be due to slight thermal degradation. This is in agreement with the results of Cretnik, Skerget, and Knez (2005) on the degradation of parthenolide at 80 °C.

3.4. Protein content in feverfew extracts

As some proteins are known to form complexes with phenolic compounds, the amount of protein in the extracts was measured using the Coomassie Blue assay. The effect of extraction temperature on the protein content in the feverfew extracts is shown in Fig. 5. At low extraction temperatures (below 70 °C), the amount of protein was found to be high (17.4 ± 3.0 mg/g d.b.) and four times higher than that found in the high temperature extracts (75–100 °C). These results showed that there is a relationship between the protein content and the colour of the extracts. At extraction temperatures of 20–70 °C when the extracts are brownish, the protein content is relatively high. Temperatures of and above 75 °C produce yellowish extracts with low amounts of protein. Quinones either extracted from the plant material or formed through oxidation of polyphenols are known to bind to proteins and may give brown pigments, which are observed at lower extraction temperatures. At higher temperatures, when the proteins and oxidising enzymes are denatured, the extracts are light-coloured. In order to examine this last hypothesis, the polyphenol oxidase activity of the extract prepared was measured. An extraction *in vacuo* at room temperature was performed first to determine if the quinones are present in the extract ab initio or are formed by the enzymatic oxidation of some phenols.

3.5. PPO activity

The feverfew sample extracted under vacuum at 20 °C yielded a significantly ($p \leqslant 0.05$) higher amount of phenols (23.5 ± 0.4 mg/g GAE d.b.), compared to the samples extracted in the presence of air. This extract was also significantly lighter in colour than the low temperature extracts prepared in presence of air, indicating that the quinones more than likely arise from enzymatic oxidation of phenols. Browning of this extract was measured spectrometrically at 430 nm (Table 2). Absorbance at 5 min (1.03) was low and the same as the absorbance of the samples extracted at 90–100 °C. The extract then progressively browns over time, increasing in absorbance to 1.52 after 24 h, comparable to that of samples extracted at 20 °C in the presence of air. The slow browning of this extract could be due to the slow oxidation of phenols to quinones, which then react irreversibly with protein, forming brown pigments (Harborne, 1984). In order to validate this hypothesis of enzymatic browning, the samples extracted at 20 °C in 0.02 M phosphate buffer (pH 6.8) were incubated at different temperatures (30–100 °C) and polyphenol oxidase (PPO) activity measured using catechol as the substrate. The effect of temperature on the PPO activity is shown in Fig. 6. The highest activity of PPO (1644 ± 38 units/ml) was exhibited at 30 °C. Increasing the temperature from 40 °C to 75 °C decreased the PPO activity from 1533 to 67 units/ml. No activity was detected in the samples extracted at 80–100 °C, probably due to temperature denaturation and inactivation of the enzyme. This agrees with the denaturation temperature (near 70 °C) of PPO in litchi pericarp reported by Liu et al. (2007).

4. Conclusion

The results obtained in this study demonstrate a strong relationship between the amount of total phenols, total tannins, non-tannins and the colour of the extracts at different extraction temperature as a result of PPO activity. At temperatures of 70 °C or below, when the PPO is enzymatically active, the oxidation of some non-tannin phenolics resulted in brown extracts with significantly lower amounts of total phenols. Inactivating the PPO at temperatures above 70 °C resulted in lightly coloured extracts with a higher total phenol content. The highest concentration of parthenolide was extracted at 70–75 °C. Feverfew extracts prepared at 80 °C were rich in parthenolide and phenolics, and had a desirable colour. These extracts may therefore be promising sources of natural ingredients for incorporation into a beverage with anti-inflammatory properties.

Acknowledgements

This work was funded by the Food Institutional Research Measure (FIRM) administered by the Department of Agriculture, Fisheries and Food, Republic of Ireland.

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Food Chemistry 117 (2009) 232–240

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Free radical scavenging activity and comparative proteomic analysis of antioxidative protein against H_2O_2-induced oxidative stress in neuronal cells

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ARTICLE INFO

Article history:
Received 13 November 2008
Received in revised form 13 March 2009
Accepted 30 March 2009

Keywords:
Artemisia annua
Enzymatic extracts
Free radical
H_2O_2
Proteomic analysis

ABSTRACT

Artemisia annua was enzymatically hydrolyzed by five proteases and seven carbohydrases. All enzymatic extracts scavenged DPPH, hydroxyl and alkyl radicals. Especially, the Protamex among the various proteases and Maltogenase among the various carbohydrases extracts exhibited the highest scavenging activity on hydroxyl radical. The extracts of *A. annua* clearly reduced neuronal cell death from H_2O_2-induced damage. In addition, a proteomic analysis, two-dimensional electrophoresis (2-DE) and matrix assisted laser desorption ionisation-time of flight/time of flight (MALDI-TOF/TOF) was used to identify the proteins of the neuronal cells whose expressions were or were not altered by the treatment of the Maltogenase extracts which showed the highest hydroxyl radical scavenging activity among all enzymatic extracts for 24 h. The protein characterisation revealed that translation elongation factor Tu (EF-Tu), Immunoglobulin E (IgE) and voltage-dependent anion channel 1 (VDAC-1) were involved in the cell survival effects against H_2O_2-induced apoptosis. These results suggest that EF-Tu, IgE and VDAC-1 have an important role in the reduction of neuronal apoptosis by oxidative stress, and the enzymatic extracts of *A. annua* shows potent antioxidative activities by regulating EF-Tu, IgE and VDAC-1.

1. Introduction

It has been reported that there is an inverse relationship between the antioxidative status and the occurrence of human diseases (Delanty & Dichter, 2000; Je, Park, & Kim, 2004). In addition, antioxidant compounds can be isolated and then used for the prevention and treatment of free radical-related disorders (Middleton, Kandaswami, & Theoharides, 2000; Packer, Rimbach, & Virgili, 1999). Therefore, research for identifying antioxidant compounds is an important issue.

Proteomic analysis using 2-DE and MALDI-TOF is a very useful tool for examining protein changes comprehensively before and after drug treatments. Recently, some researches examined the cytotoxic effect of xanthoangelol on drug-resistant neuroblastoma, and induction of apoptosis of the tumour cells, and also investigated the mechanism of this xanthoangelol-induced apoptosis in neuroblastoma using proteomic analysis (Motani et al., 2008).

Artemisia annua (sweet or annual wormwood, family Asteraceae) is an annual herb endemic to the northern parts of Chahar and Suiyuan provinces in China where it is known as 'quinghao' (green herb), and has been used to treat chills and fever for more than 2000 years (Klayman, 1985). During the past 30 years or so after its discovery in China in the early 1970s, artemisinin, which is a derivation of *A. annua,* has been widely promoted as a component of artemisinin combination therapies for treating drug-resistant malaria (Duffy & Mutabingwa, 2004; Woodrow, Haynes, & Krishna, 2005). Furthermore, artemisinin produces rapid parasite clearance, which is significantly faster than that of any other antimalarial drugs (Sriram, Rao, Chandrasekhara, & Yogeeswari, 2004).

However, while literature on the chemistry of *A. annua* and clinical trials reporting its effects are vast, research into the antioxidant effects of *A. annua* is scarce, or even non-existent. Thus, the present study aimed to investigate the free radical scavenging activities of enzymatic extracts from *A. annua* by ESR spectroscopy and their possible protective effects on cells against oxidative stress.

2. Materials and methods

2.1. Materials

Propidium iodide (PI), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), 2,2-azobis(2-amidinopropane) hydrochloride (AAPH),

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doi:10.1016/j.foodchem.2009.03.115

2,2-diphenyl-1-picrylhydrazyl (DPPH) and (4-pyridyl-1-oxide)-*N*-tert-butylnitrone (4-POBN) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RNase A, pBR 322 DNA and Tween-20 were purchased from Novagen Inc. (Darmstadt, Germany), Takara Biomedicals (Tokyo, Japan) and USB Co. (Cleveland, OH, USA), respectively. DMEM (Dulbecco's Modified Eagle Medium), Trypsin–EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin and certified foetal bovine serum were supplied by Invitrogen Co. (Carlsbad, CA, USA). The seven carbohydrases including Promozyme, Celluclast 1.5L FG, Maltogenase L, Viscozyme L, Termamyl SC, Dextrozyme E and AMG 300L, and the five proteases including Protamex, Flavourzyme 500 MG, Neutrase 0.8L, Pancreatic Trypsin Novo, Alcalase 2.4L FG were obtained from Novozyme (Novozyme Nordisk, Bagsvaerd, Denmark). Triton X-100 and MW electrophoresis calibration kits were purchased from Amersham-Pharmacia (Piscataway, NJ, USA). All of the chemicals used for 2-DE and MS were purchased from either BioRad (Hercules, CA, USA), or Applied Biosystems (Foster City, CA, USA). In addition, *A. annua* was obtained at a local oriental medicine market (Busan, Korea). All other reagents were of the highest grade available commercially.

2.2. Preparation of enzymatic extract from Artemisia annua

The samples were pulverised into powder using a grinder. The optimum pH, temperature and characterisation of the various enzymes used are summarised in Table 1. The enzymatic extracts were obtained according to the method described by Park et al. (2005). One hundred millilitres of buffer solution were added to 2 g of the dried sample, and then 40 μl (or mg) of each enzyme were added after pre-incubation for 30 min. The enzymatic hydrolysis reactions were performed for 8 h to achieve an optimum hydrolytic level and immediately heated at 100 °C for 10 min. Finally, the enzymatic extracts were obtained after filtering the supernatants, lyophilising, and were then stored at −20 °C until use (within 3 days).

2.3. Determination of total phenolic contents

The phenolic contents of the various enzymatic extracts were determined using a protocol similar to that of Chandler and Dodds (1983) described by Shetty, Curtis, Levin, Witkowsky, and Ang (1995). Briefly, 20 μl of the enzymatic extracts from *A. annua*, 20 μl of 95% EtOH, 100 μl of distilled water and 10 μl of 50% Folin–ciocalteu reagent were mixed. The mixtures were allowed to react for 5 min, followed by the addition of 20 μl of 5% Na_2CO_3, and then mixed thoroughly and placed in the dark for 1 h. Their absorbance was measured at 650 nm by an ELISA reader (Tecan, Grodig, Austria).

2.4. DPPH radical scavenging activity

DPPH radical scavenging activity was measured using the method described by Nanjo et al. (1996). A sample solution of 60 μl of each enzymatic extract was added to 60 μl of DPPH (60 μM) in methanol solution. After mixing vigorously for 10 s, the solution was then transferred into a 100 μl Teflon capillary tube, and the scavenging activity of each enzymatic extract on DPPH radical was measured using a JES-FA ESR spectrometer (JEOL Ltd., Tokyo, Japan). The spin adduct were measured on an ESR spectrometer exactly 2 min later. The experimental conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain, 6.3×10^5; and temperature, 298 K.

2.5. Hydroxyl radical scavenging activity

Hydroxyl radicals were generated by the iron-catalysed Haber-Weiss reaction (Fenton-driven Haber-Weiss reaction); these generated hydroxyl radicals can be rapidly reacted with nitrone spin-trap DMPO (Rosen & Rauckman, 1984). The resultant DMPO-OH adducts were then detected using an ESR spectrometer. Briefly, 0.2 ml of each enzymatic extract with various concentrations was mixed with 0.2 ml of DMPO (0.3 M), 0.2 ml of $FeSO_4$ (10 mM) and 0.2 ml of H_2O_2 (10 mM) in a phosphate buffer solution (pH 7.2) and then transferred into a 100 μl Teflon capillary tube. After 2.5 min, ESR spectrum was recorded using an ESR spectrometer. The experimental conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5; and temperature, 298 K.

2.6. Alkyl radical scavenging activity

Alkyl radicals were generated by AAPH. The PBS (pH 7.4) reaction mixtures containing 10 mM AAPH, 10 mM 4-POBN and indicated concentrations of tested samples were incubated at 37 °C in a water bath for 30 min and then transferred to 100 μl Teflon capillary tube. The spin adduct was recorded on an ESR spectrometer. The measurement conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude,

Table 1
Optimum hydrolysation conditions of particular enzymes.

Enzyme	Optimum conditions		Buffer used[a]	Enzyme composition
	pH	Temperature (°C)		
Protamex	7.0	50	0.1 M PB[b]	Hydrolysis of food proteins
Flavourzyme	7.0	50	0.1 M PB	Containing both endoprotease and exopeptidase activities
Neutrase	7.0	50	0.1 M PB	An endoprotease
Pancreatic trypsin	7.0	37	0.1 M PB	A serine protease
Alcalase	7.0	50	0.1 M PB	A endoprotease
Promozyme	5.0	60	0.1 M SB[c]	Debranching enzymes known as pullulanases
Celluclast	4.5	50	0.1 M SB	Catalysing the breakdown of cellulose into glucose, cellobiose and higher glucose polymer
Maltogenase	5.0	60	0.1 N SB	An α-amylase
Viscozyme	4.5	50	0.1 M SB	Arabanase, celluiase, β-glucanase, hemi-cellulase and xyianase
Termamyl	6.0	60	0.1 M PB	A heat-stable α-amylase
Dextrozyme	4.5	60	0.1 M SB	A glucoamylase and pullulanase
AMG	4.5	60	0.1 M SB	An exo-1,4-α-d-glucosidase

[a] In enzymatic hydrolysis.
[b] Sodium acetate–acetic acid buffer.
[c] Phosphate buffer.

2 G; microwave power, 1 mW; gain, 6.3×10^5; and temperature, 298 K.

2.7. Protective effect of the enzymatic extracts from Artemisia annua against H_2O_2-induced DNA damage

To study the protective effects of the enzymatic extracts against DNA damage by H_2O_2, the reaction was conducted in an eppendorf tube at a total volume of 13 μl containing 0.5 μg of pBR 322 DNA, 3 μl of 50 mM phosphate buffer (pH 7.4), 3 μl of 2 mM $FeSO_4$ and 2 μl of the enzymatic extracts at various concentrations. Then 4 μl of 30% H_2O_2 were added, and the mixture was incubated at 37 °C for 1 h (Rosen & Rauckman, 1984). Finally, the mixture was subjected to 0.8% agarose gel electrophoresis. The DNA bands (supercoiled, linear and open circular) were stained with ethidium bromide.

2.8. Flow cytometer analysis

For sub-G1 and cell cycle analysis, PC-12 cells were suspended in ethanol with 0.5% Tween-20 and left for 24 h at 4 °C. The cells were harvested by centrifugation and resuspended in 1 ml PBS with 0.05 mg/ml propidium iodide and 10 μl/ml. RNase A, and incubated at 37 °C for 30 min. Apoptotic cell death was assessed by measuring the hypodiploid DNA contents of the cells using a flow cytometer (FACS-caliber, Becton Dickinson, Franklin Lakes, NJ, USA). The cells belonging to the sub-G1 population were considered apoptotic cells and the percentage of cells in each phase of the cell cycle was determined.

2.9. 2-DE

Neuronal cells strips untreated or treated enzymatic extracts from *A. annua* prior to treatment with 1 mM H_2O_2 for 24 h were homogenised in 2-DE buffer containing 8 M urea, 2 M thiourea, 65 mM DTT, 2% CHAPS and 16 complete protease inhibitor cocktail (Roche Applied Science, Wiesbaden, Germany). The homogenates were centrifuged at 12,000*g* for 10 min at 10 °C and the supernatants were collected. The protein concentrations were determined using BioRad (Hercules, CA, USA) protein assay reagents with BSA as standard. The protein homogenates were diluted with rehydration buffer containing 8 M urea, 0.28% DTT, 0.5% CHAPS, 10% glycerol, 0.5% appropriate ampholyte and 0.002% bromophenol blue. The IPG strips (pH 3–10 nonlinear) were rehydrated at 50 V for 12 h at 20 °C in 80 μg of whole cell protein extract. IEF was carried out sequentially with a BioRad Protein IEF Cell system at 100 V for 2 h, 250 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 3000 V for 1 h, 5000 V for 1 h and 8000 V for 9 h at 20 °C. After IEF, the individual strips were equilibrated for 20 min in 10 mL of equilibration solution containing 50 mM Tris–HCl (pH 6.8), 6 M urea, 20% glycerol, 2% SDS, 0.01% bromophenol blue and 5 mM tributylphosphine. The equilibrated strips were transferred onto 12% acrylamide SDS gels, and a potential of 30 V was applied for 1 h followed by a potential of 80 V until the bromophenol blue marker reached the bottom of the gel. To visualise the proteins, the gels were incubated for 1 h in fixing solution containing 50% methanol, 12% acetic acid and 0.05% formaldehyde, and then washed twice for 20 min, each time in 50% ethanol. Sensitisation was carried out for 1 min in 0.02% $Na_2S_2O_3$. After three consecutive washes with distilled water, each for 20 s, the gels were impregnated with 0.1% silver nitrate solution containing 0.075% formaldehyde for 20 min. Excess silver nitrate was washed with distilled water for a few seconds and the gels were developed with a solution of 0.0002% $Na_2S_2O_3$, 0.025% formaldehyde and 3% Na_2CO_3 for 10 min. The development was stopped by the addition of 1.5% EDTA. The densities of silver-stained spots from three different experiment sets were detected and counted by both automation and manual spot detection, and statistically analysed with PDQuest software (Version 7.1.1, BioRad).

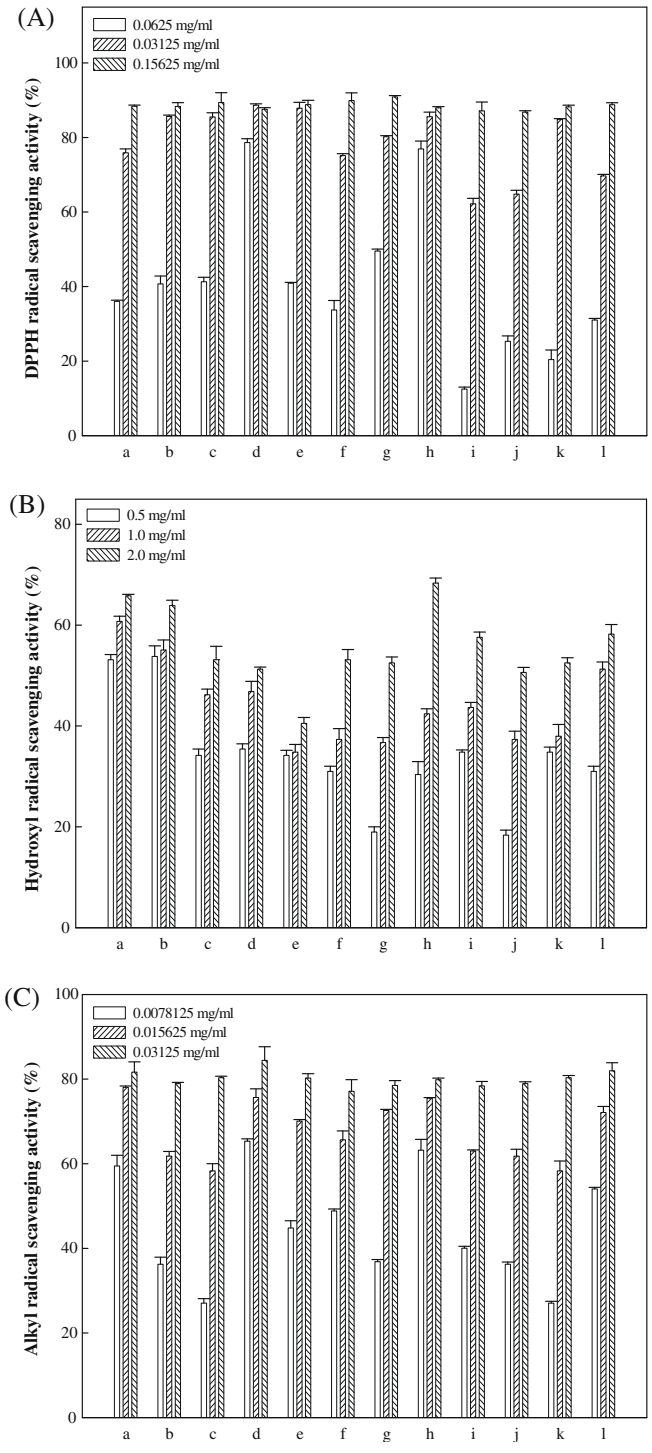

Fig. 1. DPPH (A), hydroxyl (B) and alkyl (C) radical scavenging activity of various enzymatic extracts from *Artemisia annua* obtained in various system at various concentrations. a, Protamex; b, Flavourzyme; c, Neutrase; d, Pancreatic trypsin; e, Alcalase; f, Promozyme; g, Celluclast; h, Maltogenase; I, Viscozyme; j, Termamyl; k, Dextrozyme; l, AMG. Means ± SD of determinations were made in triplicate experiments.

2.10. In-gel digestion and MS of protein spots

The silver-stained protein spots were excised from the stained gel and destained with freshly prepared 15 mM $K_3[Fe(CN)_6]$/ 50 mM $Na_2S_2O_3$ for 10 min. The gel pieces were washed with 50% ACN and 100 mM NH_4HCO_3 three times for 15 min. After dehydrating the spots with ACN for 15 min, each spot was dried in a SpeedVac centrifuge for 10 min. The samples were then reduced with DTT, and subsequently, alkylated with iodoacetamide. Finally, the samples were digested in 20 mL of digestion buffer (12.5 ng/mL trypsin in 20 mM NH_4HCO_3) at 37 °C for at least 16 h. The peptide samples were extracted with 50 mL of 50% ACN/0.1% formic acid and dried in a SpeedVac centrifuge. The extracts were resuspended in 10 mL of 0.1% TFA, and desalted with ZipTip C18 columns (Millipore) according to the manufacturer's instructions. The peptide samples were then mixed (1:1) with a matrix consisting of a saturated solution of CHCA prepared in 50% ACN/0.5% TFA. Aliquots of the samples (1 ml) were spotted onto stainless-steel sample target plates. Peptide mass spectra were obtained by a MALDI-TOF/TOF mass spectrometer (AB4700, Applied Biosystems) in the positive ion reflector mode. For the precursor ion selection, all fractions were measured in single MS before MS/MS was performed. For MS/MS spectra, the peaks were calibrated by default. The 10 most abundant precursor ions per sample were selected for subsequent fragmentation by high-energy CID. The collision energy was set to 1 keV and air was used as the collision gas. The criterion for precursor selection was a minimum S/N of 20. The mass accuracy was within 100 ppm for the mass measurement and within 0.2 Da for the CID experiments. The other parameters for searching were of trypsin, 1 missed cleavage, variable modification of carbamidomethyl, oxidation, propionamide and pyro-glu (N-term), peptide charge of 1+, and monoisotopic. For database searches, known contamination peaks such as keratin and autoproteolysis peaks were removed before searching. The spectra were processed and analysed by the Global Protein Server Explorer 3.0 software (Applied Biosystems). This uses an internal MASCOT (Matrix Science, UK) program for matching MS and MS/MS data against database information. The data

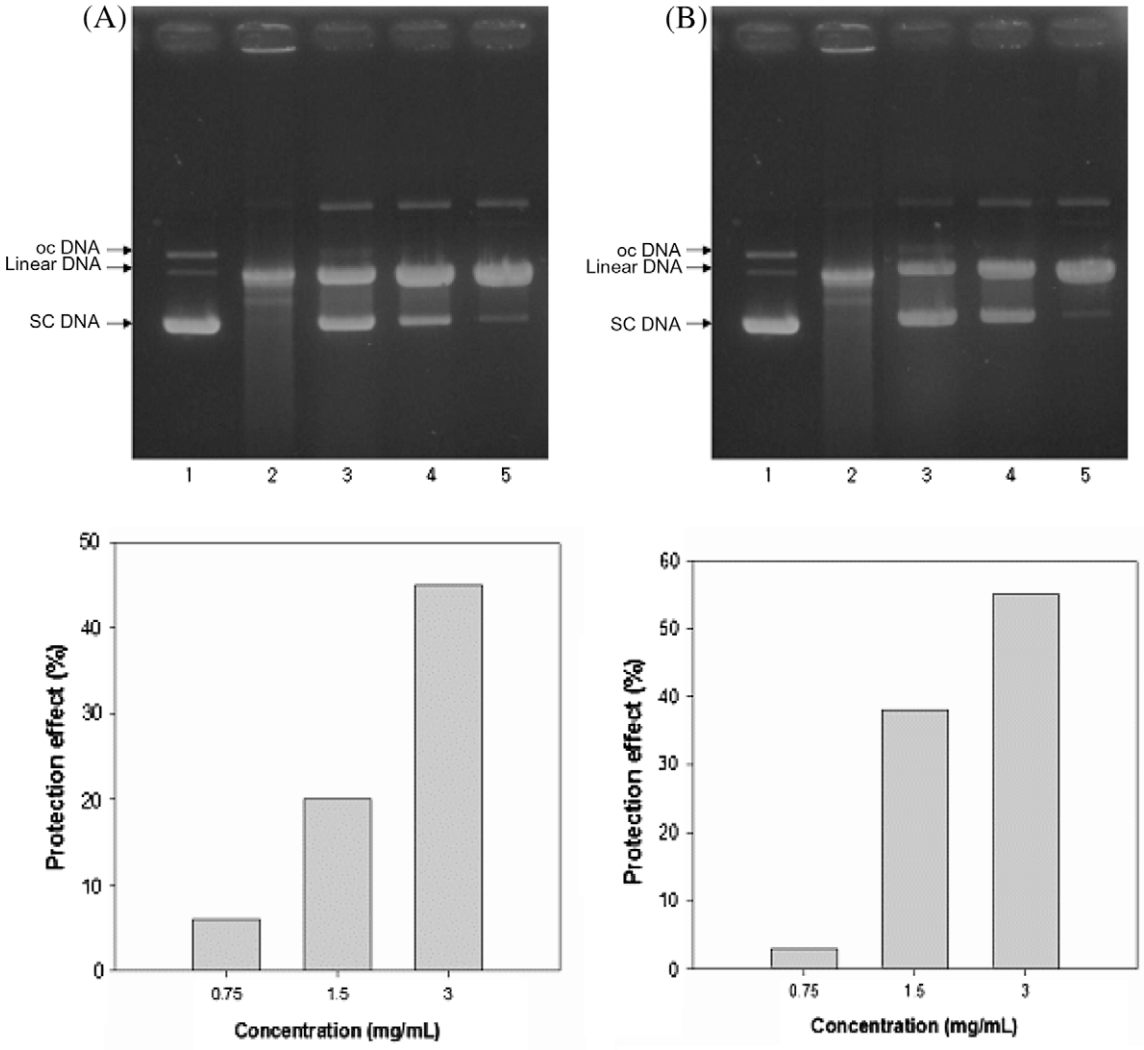

Fig. 2. Agarose gel electrophoretic patterns of plasmid DNA breaks by OH generated from a Fenton reaction in the presence of Protamex (A) and Maltogenase (B) extracts of *Artemisia annua*. An amount of 0.5 μg of pBR 322 DNA was incubated at 37 °C for 1 h in 2 mM $FeSO_4$ and 30% H_2O_2 with the following additive combinations: Lane 1, no addition (plasmid DNA control); Lane 2, $FeSO_4$ and H_2O_2 (DNA damage control); Lanes 3–5, $FeSO_4$ and H_2O_2 in the presence of Protamex and Maltogenase extracts from *Artemisia annua* with concentrations of 3, 1.5 and 0.75 mg/ml, respectively.

obtained were screened against rat databases downloaded from both NCBI (http://www.ncbi.nlm.nih.gov) and the Swiss-Prot/TrEMBL homepage (http://www.expasy.ch/sprot). Further confirmations of the protein identifications were obtained by using the MS-Fit (http://prospector.ucsf.edu) and ProFound (http://www.prowl.rockefeller.edu) programs.

2.11. Statistics

The experimental results were recorded as the mean ± SD of three parallel measurements. A variance analysis using the Duncan's multiple range test was performed to determine differences between the means of the treatments at $p < 0.05$.

3. Results and discussion

3.1. Total phenol contents of enzymatic extracts from Artemisia annua

Phenolic compounds, such as flavonoids, phenolic acids and tannins are considered to be major contributors to the antioxidative activity of medicinal plants. These antioxidants also possess diverse biological activities, such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic activities. These activities may be related to their antioxidative activity (Chung, Wong, Huang, & Lin, 1998).

The total contents of the proteolytic extracts by Pancreatic trypsin, Alcalase, Protamex, Neutrase and Flavourzyme from *A. annua* were 41.85, 35.94, 35.63, 32.43 and 27.67 μg/mg, respectively, (data not shown). The total contents of the extracts by carbohydrase such as Celluclast, Viscozyme, AMG, Maltogenase, Termamyl, Dextrozyme and Promozyme from *A. annua* were 53.74, 52.26, 45.67, 41.21, 31.84, 26.92 and 23.98 μg/mg, respectively (data not shown). It was observed that the total phenol contents of all the various protease and carbohydrase extracts fell within the range of 23.98–53.74 μg/mg of extract. Meanwhile, the enzymatic extracts with higher phenolic contents did not have higher flavonoid contents and antioxidative capacity. There was no significant relationship between the total phenol content and antioxidant capacity as similar to Maisuthisakul, Suttajit, and Pongsawatmanit (2005).

3.2. DPPH radical scavenging activity

DPPH is a stable free radical, which has been used to evaluate the free radical scavenging activity of natural antioxidants. In this study, the DPPH radical scavenging activities of the various protease and carbohydrase extracts are shown in Fig. 1A. The proteolytic extracts by Neutrase, Alcalase, Protamex, Flavourzyme and Pancreatic trypsin from *A. annua* had DPPH radical scavenging activities of 89.38%, 88.82%, 88.40%, 88.34% and 87.58% at 0.0625 mg/ml, respectively. The radical scavenging activity occurred in a concentration dependent manner. It was observed that the extracts by carbohydrase such as Celluclast, Promozyme, AMG, Maltogenase, Dextrozyme, Viscozyme and Termamyl had DPPH radical scavenging activities of 90.91%, 89.92%, 88.79%, 87.99%, 88.24%, 87.15% and 86.81% at 0.0625 mg/ml, respectively. In addition, the Neutrase and Celluclast extracts exhibited the strongest scavenging activities, among five proteases and seven carbohydrases extracts and their IC_{50} values were 18.71 and 15.83 μg/mg, respectively. These results indicate that all the enzymatic extracts possessed a scavenging activities against DPPH radicals.

3.3. Hydroxyl radical scavenging activity

Hydroxyl radicals generated in the Fe^{2+}/H_2O_2 system were trapped by DMPO, forming spin adducts detected by an ESR spectrometer, which may be due to the paramagnetic impurities contained in unpurified commercial DMPO (Bindoli, Rigobello, & Deeble, 1992). As shown in Fig. 1B, it was observed that the hydroxyl radical scavenging activities of the Protamez, Flavourzyme, Neutrase, Pancreatic trypsin and Alcalase extracts from *A. annua* were 65.82%, 63.92%, 53.16%, 51.27% and 40.51% at 2 mg/ml, respectively, which indicated that their scavenging capabilities followed the order: Protamex > Flavourzyme > Neutrase > Pancreatic trypsin > Alcalse. As shown in Fig. 1B, it was observed that 2 mg/ml of the extracts prepared with the seven types of carbohydrases such as Promozyme, Celluclast, Maltogenase, Viscozyme, Termamyl, Dextrozyme and AMG had hydroxyl radical scavenging activities of 53.16%, 52.53%, 68.35%, 57.59%, 50.63%, 52.53% and 58.23%, respectively, which indicated that their scavenging capabilities followed the order: Maltogenase > AMG > Viscozyme > Promozyme > Celluclast, Dextrozyme > Termamyl, and the radical scavenging activities were concentration-dependent. In addition, the Protamex and Maltogenase extracts had the highest scavenging activities among the various proteases and carbohydrases extracts and their IC_{50} values were 1.54 and 1.29 mg/ml, respectively.

3.4. Alkyl radical scavenging activity

The alkyl radical spin adduct of 4-POBN/free radicals were generated from AAPH at 37 °C for 30 min, and the decrease in the ESR

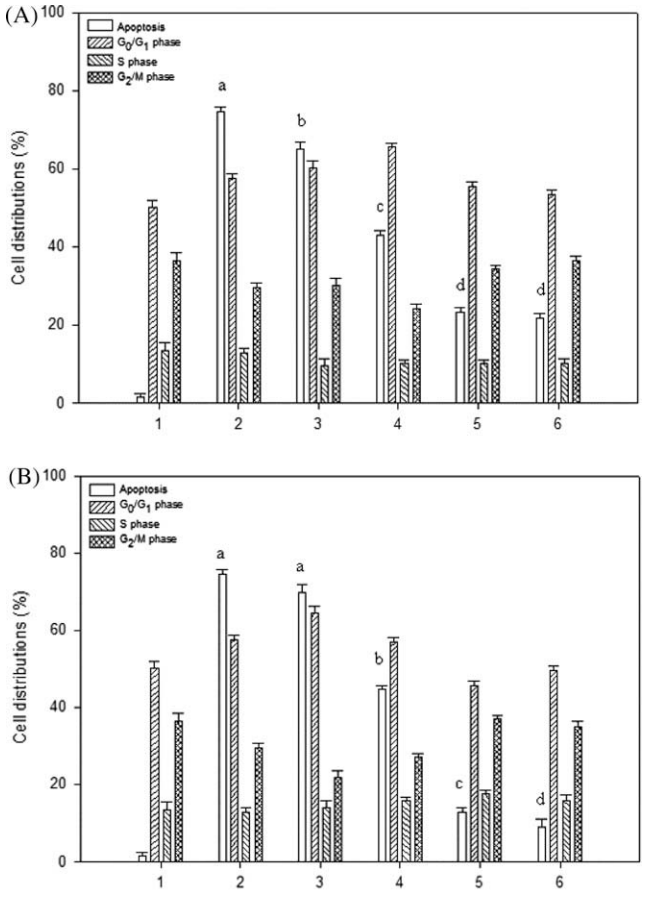

Fig. 3. Cell death and cell cycle of PC-12 cells after treating Protamex (A) and Maltogenase (B) extracts from *Artemisia annua* prior H_2O_2 treatment. The cells were treated with various concentrations of Protamex and Viscozyme extracts from *Artemisia annua* prior 1 mM H_2O_2 treatment for 24 h. Means ± SD of determinations were made in triplicate experiments. 1; control, 2; H_2O_2 1 mM, 3; 0.0625 mg/ml, 4; 0.125 mg/ml, 5; 0.25 mg/ml, 6; 0.5 mg/ml. (a–d) Values with different alphabets are significantly different at $p < 0.05$ as analysed by Duncan's multiple range test compared to H_2O_2 1 mM.

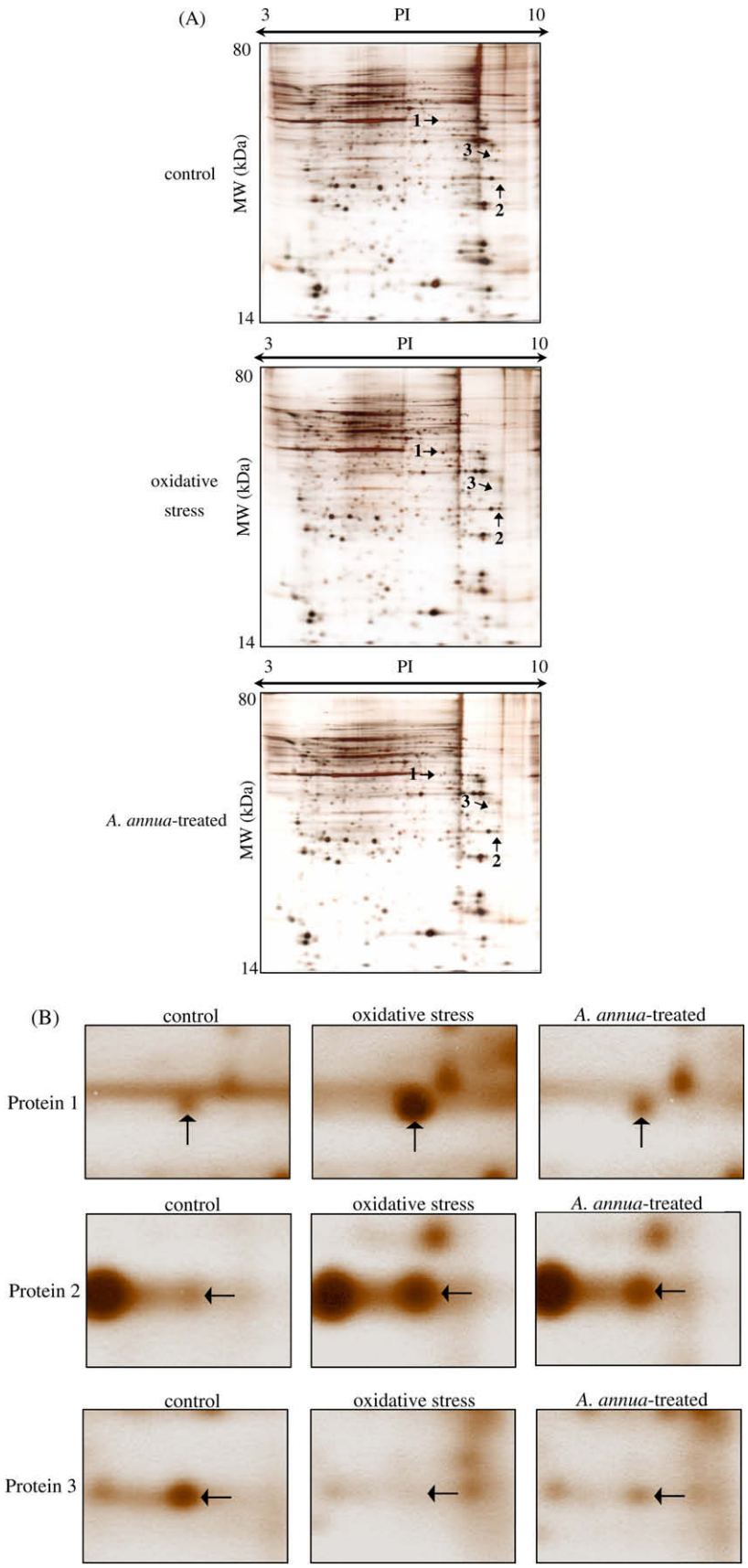

Fig. 4. Silver-stained 2-DE gels of rat neuronal cells proteins. A total of 80 μg of proteins were extracted and separated by 2-DE. A shows the control. The cells were treated without (oxidative stress) or with (*A. annua*-treated) 1 mg/ml enzymatic extracts from *Artemisia annua* prior 1 mM H_2O_2 for 24 h (A). It is showed enlarged-each protein (B). Arrows show proteins subsequently examined by MALDI-TOF/TOF. The numbers indicated in the gels correspond to the numbers in Table 2.

signals were observed with the dose increment of all the enzymatic extracts (Fig. 1C). All the proteases extracts from *A. annua* exhibited alkyl radical scavenging activities, and the scavenging activities of Pancreatic trypsin, Protamex, Neutrase, Alcalase and Flavourzyme were 84.40%, 81.63%, 80.32%, 80.24% and 78.93% at 0.03125 mg/ml, respectively (Fig. 1C). In addition, the extracts hydrolysed from A. annua by the seven types of carbohydrases such as AMG, Dextrozyme, Termamyl, Maltogenase, Celluclast, Viscozyme and Promozyme had alkyl radical scavenging activities of 81.97%, 80.31%, 79.85%, 78.93%, 78.49%, 78.38% and 77.13% at 0.03125 mg/ml, respectively (Fig. 1C). Furthermore, The Pancreatic trypsin and AMG extracts showed the highest alkyl radical scavenging activities among the various proteases and carbohydrases extracts, and the IC_{50} values were 5.98 and 7.23 μg/ml, respectively.

3.5. Protective effect of the enzymatic extracts from Artemisia annua against hydroxyl radical-induced DNA damage

The antioxidative effects of the enzymatic extracts from *A. annua* were evaluated by their protective effects against free radical-induced plasmid pBR 322 DNA damage in vitro. DNA is broken into three forms: supercoiled (SC), open circular (OC) and linear form (Linear) when exposed to hydroxyl radical derived from a Fenton reaction. A supercoiled circular DNA molecule comprises 4361 base pairs (Watson, 1988). This is a 'model' DNA system which has the advantage that damage may be easily assessed by means of a simple gel electrophoretic protocol (Bates & Maxwell, 2005). In the present study, the Protamex and Maltogenase extracts were selected to investigate the protective effects against hydroxyl radical-induced DNA damage because the two extracts had the highest hydroxyl radical scavenging activities among the various protease and carbohydrase extracts. The protective effects of the Protamex and Maltogenase extracts from *A. annua* against free radical-induced DNA damage were shown in Fig. 2. The SC form of the DNA was completely converted to the linear form under the treatment of the hydroxyl radicals generated from the Fenton reaction (Lane 2) when compared with the plasmid DNA control (Lane 1). According to the results, the Protamex and Maltogenase extracts from *A. annua* protected against hydroxyl radical-induced DNA damage at concentrations of 1.5 and 3.0 mg/ml, however, they had no effects at 0.75 mg/ml (Lanes 3–5).

3.6. Neuroprotective effect against H_2O_2-induced damage

Hydrogen peroxide has been reported to induce apoptosis in cells of the central nervous system (Chandra, Samali, & Orrenius, 2000). In this study, the Protamex and Maltogenase extracts were selected to investigate the neuroprotective effects against H_2O_2-induced damage because the extracts showed the highest protective activities at 1.0 mg/ml among the various protease and carbohydrase extracts in a preliminary experiment. Their protective effects on H_2O_2-induced neuronal cells damage were confirmed by flow cytometric analysis. The Protamex and Maltogenase extracts clearly reduced apoptosis in dose dependent manners, as shown in Fig. 3. The PC-12 cells were incubated with increasing concentrations of the Protamex extract from *A. annua* for 24 h. The percentages of apoptotic cells were 23.4%, 23.7%, 43.1% and 65% at 0.5, 0.25, 0.125 and 0.0125 mg/ml, respectively, whereas the percentage of apoptotic cells was 74.6% for the 1.0 mM H_2O_2 treated cells. When the PC-12 cells were incubated with 0.5, 0.25, 0.125 and 0.0125 mg/ml of Maltogenase extract from *A. annua* for 24 h, the percentages of apoptotic cells were 9.1%, 12.9%, 44.8% and 69.9%, respectively, whereas the percentage of apoptotic cells was 74.6% for the 1.0 mM H_2O_2 treated cells. These results indicate that the enzymatic extracts of *A. annua* exhibited protective effects against H_2O_2-induced neuronal cell damage.

3.7. Isolation and identification of proteins in neuronal cells

To identify the proteins responsive to oxidative stress in the neuronal cells, the cells were treated with 1.0 mg/ml of Maltogenase extract, which showed the highest hydroxyl radical scavenging activity, and were co-treated with 1.0 mM H_2O_2 after 1 h. Then after 24 h, the proteins were separated using the 2-DE technique. The separated proteins were visualised with silver staining and analysed with PDQuest software. Fig. 4A–C shows the typical 2-DE gels of total rat neuronal cell proteins for the control and with or without 1.0 mg/ml Maltogenase extract of *A. annua* exposure prior to the 1.0 mM H_2O_2 treatment. More than 80 proteins may be seen in each gel, with isoelectric pH values of pH 3–10 and MW of 14–18 kDa. The MALDI–TOF/TOF analyser identified 10 prominent proteins and we selected 3 proteins involved in cell survival. Table 2 identifies the proteins.

3.8. Neuronal cell proteins with changes in levels after Maltogenase extract of Artemisia annua treatment

The proteins markedly altered in expression by the 1.0 mg/ml Maltogenase extract treatment of *A. annua* prior to 1.0 mM H_2O_2 treatment were identified. The No. 1 protein, EF-Tu was altered by 0.337-fold in the Maltogenase extract-treated strips compared with the untreated strips (oxidative damage group). The main function of EF-Tu and its eukaryotic counterpart eEF1A are to deliver aminoacyl-tRNA to the A-site on the ribosome. In addition to this, multiple secondary functions have been reported for EF-Tu. EF-Tu is an essential host-donated subunit of the replicative complex of

Table 2
Summary of proteins prominently expressed in rat neuronal cells as identified by MALDI-TOF/TOF.

No.	DB	Score	Protein name	Accession No.	Mr. (Da)/ pI	Peptide sequence	Ratio of spot volume (*extracts/damage*)
1	NC	57	Translation elongation factor tu (EF-tu)	13358086	42875/ 5.83	YGFDGDNTPVIR	0.337
	SP	57		P50068	42875/ 5.83	YGFDGDNTPVIR	
2	NC	165	IgE binding protein	204728	15660/ 9.47	KGNDIAFHFNPR GNDIAFHFNPRV AVNDVHLLQYNHR	0.480
	SP	165		P08699	27053/ 8.62	KGNDIAFHFNPRGNDIAFHFNPRVAVNDVHLLQYNHR	
3	NC	99	Voltage dependent anion channel (VDAC-1)	13786200	30737/ 8.62	EHINLGCDVDFDIA GPSIRKLETAVNLA WTAGNSNTRYQVD PDACFSAK	377.107
	SP	99		Q60932	32331/ 8.55	EHINLGCDVDFDIA GPSIRKLETAVNLA WTAGNSNTRYQVD PDACFSAK	

the Q^{β} phage, and it may interact with the transcriptional apparatus as a positive regulator of RNA synthesis (Travers, Kamen, & Schleif, 1970). Recently, it has been shown that eEF1A is implicated in oxidative stress-induced apoptosis. In fact, in a cardiomyocites cell line, eEF1A levels undergo a rapid increase upon treatment with hydrogen peroxide, suggesting that the up-regulation of eEF1A plays an important role in the execution of the apoptotic program in response to an oxidative stress (Chen, Proestou, Bourbeau, & Wang, 2000; Duttaroy, Bourbeau, Wang, & Wang, 1998). The No. 2 protein, IgE was altered by 0.480-fold in Maltogenase extract-treated strips compared with the untreated strips (oxidative damage group). IgE is a protein that is released by our body in large amounts to battle the alleged invaders. In addition, the extracellular membrane-proximal domain (EMPD) of human membrane IgE is a key control element of apoptotic signalling delivered through the engagement of B cell receptors (BCR) within the context of a mature B cell. EMPDs play a key role in controlling the capacity of membrane-bound IgE to induce apoptosis in the context of a mature isotype-switched B cell (Poggianella, Bestagno, & Burrone, 2006). In this study, we assumed that IgE played essential roles in determining the apoptosis of cell signalling in the PC-12 cells. The No. 3 protein, VDAC-1 was altered by 377.107-fold in the Maltogenase extract-treated strips compared with the untreated strips (oxidative damage group). VDAC-1 is the predominant protein labelled in brain. VDAC-1 is shown to coimmunoprecipitate $GABA_A$ receptors, suggesting a functional relationship between steroid binding to VDAC-1 and modulation of $GABA_A$ receptor function (Darbandi-Tonkabon et al., 2004). Shimizu, Narita, and Tsujimoto (1999) hypothesised that apoptogenic proteins were released through the VDAC–Bax channels. The attenuated expression of VDAC in a human gastric cancer cell line (BGC823 cells) treated with DATS appears in agreement with the apoptotic process in these cells. However, VDAC-dependent apoptosis is regulated by a number of Bcl-2 proteins, some anti-apoptotic and some pre-apoptotic. Next steps will be investigating antioxidative protein derived Maltogenase extract in the mitochondrial pathway of apoptosis.

4. Conclusions

The scavenging effects on ROS of various enzymatic extracts prepared from *A. annua* were evaluated using an ESR spectrometer, DNA strand breaks and flow cytometer. The water soluble enzymatic extracts were effectively prepared by enzymatic hydrolysis with seven carbohydrases, including Promozyme, Celluclast, Maltogenase, Viscozyme, Termamyl, Dextrozyme and AMG, and five proteases including Protamex, Flavourzyme, Neutrase, Pancreatic trypsin and Alcalase. The enzymatic extracts of *A. annua* showed their highest scavenging activities against alkyl radicals. In addition, the Protamex and Maltogenase extracts showed a protective effect against hydroxyl radical-induced DNA damage, and the extracts reduced apoptosis induced by hydrogen peroxide in PC-12 cells. We also investigated the mechanism of this H_2O_2-induced apoptosis of neuronal cells using proteomic analysis. EF-Tu and IgE which are involved in many biological functions, such as cell growth and differentiation, basic metabolism and immune system were overexpressed in H_2O_2-treated group compared with control, but they were downregulated in enzymatic extract-treated group compared with H_2O_2-treated group. VDAC-1 which is involved membrane potential was downregulated in H_2O_2-treated group compared with control, but they were upregulated in enzymatic extract-treated group compared with H_2O_2-treated group. We surmise that enzymatic extracts reduce the damage against H_2O_2. Therefore, the enzymatic extracts from *A. annua* have the potential to inhibit the cell damage induced by hydrogen peroxide. And the molecular mechanism remains to be elucidated in future studies how EF-Tu, IgE and VDAC expressions and functions are mediated by enzymatic extracts from *A. annua*. Thus we concentrated on further confirmation of its effects on neuronal cells.

These result showed that EF-Tu, IgE and VDAC are the proteins regulated by treatment of enzymatic extract from *A. annua* in neuronal cells and the extract of *A. annua* were able to reduce biological damages against free radicals-induced cell damage.

Acknowledgement

This work was supported by the Regional Innovation Center of the Ministry of Commerce, Industry and Energy through the Bio-Food & Drug Research at Konkuk University, Republic of Korea.

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Food Chemistry 117 (2009) 241–247

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Ester synthesis by lactic acid bacteria isolated from goat's and ewe's milk and cheeses

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ARTICLE INFO

Article history:
Received 26 January 2009
Received in revised form 13 March 2009
Accepted 31 March 2009

Keywords:
Esters
Esterification
Alcoholysis
Lactic acid bacteria

ABSTRACT

The present work evaluates the ability of lactic acid bacteria isolated from goat's and ewe's milk and cheeses to synthesise short-chain fatty acid esters. In order to elucidate the mechanisms of ester synthesis involved, cell-free extracts were incubated in sodium phosphate buffer containing triglyceride plus ethanol (alcoholysis) and free fatty acid plus ethanol (esterification). After 24 h incubation at 37 °C esters were extracted and determined by gas chromatography. Strains evaluated were able to synthesise ethyl esters from 2 to 10 carbon atoms, mainly ethyl butanoate and ethyl hexanoate. A great variability amongst strains was observed. In general, higher ester-forming activities by esterification were detected. In enterococci strains the alcoholysis mechanism was also involved.

These strains could be useful as adjunct cultures for small ruminants' dairy products and would contribute to the development of fruity flavour notes. A deeper understanding of ester synthesis mechanisms involved would allow the control of flavour development.

1. Introduction

Goat's and ewe's milk cheeses produced in the northwest of Argentina are semi-hard varieties made from raw milk, without addition of starter cultures. These cheeses are greatly appreciated because of their particular organoleptic characteristics.

Lipolysis, the process of milk fat hydrolysis, plays a crucial role in the development of flavour in these cheeses (Freitas & Malcata, 1998; Macedo, Costa, & Malcata, 1996; Tavaria, Silva Ferreira, & Malcata, 2004). The major flavour compounds that are released during lipolysis are free fatty acids (FFA), which directly affect cheese flavour. FFA can also be transformed by microorganisms to other and often more potent flavour compounds, including methyl ketones, lactones, esters, secondary alcohols and aldehydes, which also directly affect flavour in different cheeses (Collins, McSweeney, & Wilkinson, 2003).

Esters are part of the aroma array of cheeses made from goats' and ewes' milk (e.g., Feta, Manchego, Serra da Estrela and Roncal), but the number and type of esters found vary between cheese varieties (Dahl, Tavaria, & Malcata, 2000; Horwood, Lloyd, & Stark, 1981; Le Quere, Pierre, Riaublanc, & Demaizieres, 1998; Martínez-Castro, Sanz, Amigo, Ramos, & Martín-Alvarez, 1991; Ortigosa, Torre, & Izco, 2001). Of the esters identified in these cheeses, the five ethyl esters of the straight-chain fatty acids of C2–C10 are most frequently found (Liu, Holland, & Crow, 2004). These esters, which are potent flavour compounds at less than 5 ppm, are important for development of the characteristic "fruity" type flavours such as ethyl butanoate and ethyl hexanoate (Moio & Addeo, 1998). Esters may also mask the impact of off-flavours (e.g., pungent, sharp) imparted by high levels of short-chain FFA. Excessive levels of ethyl esters of short-chain FFA (typically ethyl butanoate and ethyl hexanoate) cause a fruity flavour defect in some raw and pasteurised milks, and Cheddar cheese (Horwood, Stark, & Hull, 1987; Whitfield, Jensen, & Shaw, 2000).

Lactic acid bacteria (LAB) are used as starter or adjuncts cultures or are present as secondary microbial flora (non-starter LAB) in cheese fermentation (Crow, Curry, & Hayes, 2001). Indigenous LAB isolated from ewe's and goat's milk and artisanal cheeses manufactured in the provinces of northwest Argentina were identified as enterococci, lactococci, leuconostoc and lactobacilli. *Enterococcus faecium* and *Lactobacillus plantarum* were the most frequently isolated species from ewe's and goat's milk and cheeses (Medina, Katz, González, & Oliver, 2001; Oliszewski, González, & Pérez Chaia, 2006).

The contribution of cheese microflora to the formation of flavour compounds occurs *via* the esterase/lipase systems of lactic and propionic acid bacteria, non-starter LAB (NSLAB), surface microorganisms, yeasts and moulds (McSweeney & Sousa, 2000). Moreover, some volatile compounds arise from microbial amino acid catabolism (Tavaria & Malcata, 2003). We have previously demonstrated that LAB isolated from goat's and ewe's dairy prod-

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.03.105

ucts present very complex intracellular esterolytic systems able to release C2–C6 fatty acids and thus they actively contribute to flavour development in these products. (Abeijón, Medina, Katz, & González, 2006; Abeijón Mukdsi et al., 2009; Katz, Medina, González, & Oliver, 2002; Oliszewski, Medina, González, & Pérez Chaia, 2007).

Esterases are defined as the enzymes that hydrolyse carboxyl ester linkages in water-soluble substrates (e.g., slightly water-soluble short-chain glycerides and aliphatic esters) in aqueous solutions. These enzymes can also synthesise esters under certain conditions (Bornscheuer, 2002). The mechanisms of ester synthesis are: esterification (reaction of an acid and an alcohol), alcoholysis (reaction of an ester and an alcohol), acidolysis (reaction of an acid and an alcohol) and transesterification (reaction of two esters) (Holland et al., 2005; Malcata, Reyes, Garcia, Hill, & Amundson, 1992).

Presumably, esterases from starter and NSLAB are responsible for release of short-chain FFA from milk fat at elevated water activity (a_w) and synthesis of short-chain ethyl esters as a_w decreases with ripening (Holland et al., 2005). The equilibrium existing between these processes is dependent on the a_w, the enzymes present, pH, temperature and availability of substrates characteristic of each cheese variety (Ha & Lindsay, 1992; Moio & Addeo, 1998).

The acid or acyl CoA moieties of esters are formed from the action of the cheese microflora and their enzymes on lactose, lactate, lipids and proteins of cheese curd (Urbach, 1997). Ethanol in cheese is thought to result from the activity of obligatory heterofermentative lactobacilli and/or from yeasts (Chamba & Irlinger, 2004). We have previously detected ethanol production from citrate metabolism in goat's milk fermented by enterococci (Cabral, 2005).

Evidence is provided that esterases of LAB catalyse the synthesis of esters by esterification (Liu, Holland, & Crow, 1998) and alcoholysis (Liu, Holland, & Crow, 2003a; Liu, Baker, et al., 2004). It is not known whether esterases of LAB can catalyse acidolysis and transesterification.

Even though there are many reports concerning volatile compound formation in goat's and ewe's milk cheeses manufactured with starter LAB (Abeijón Mukdsi et al., 2009; Georgala et al., 2005; Randazzo, Pitino, De Luca, Scifò, & Caggia, 2008; Tavaria, Tavares, Silva-Ferreira, & Malcata, 2006), only a few studies on ester synthesis mechanisms by these bacteria have been carried out. Some reports about ester synthesis by lactobacilli, streptococci and lactococci have been made, but no information about enterococci is available.

The aim of the present work was to evaluate the ester synthesis ability of indigenous lactobacilli and enterococci strains from goat's and ewés dairy products, and to go deeper into the elucidation of the prevalent mechanisms of ester synthesis in aqueous medium.

2. Materials and methods

2.1. Microorganisms and growth conditions

Strains provided by Laboratorio de Ecofisiología Tecnológica of the Centro de Referencia para Lactobacilos (CERELA), were isolated from Argentinean goat's and ewe's milk and cheese (Medina et al., 2001; Oliszewski et al., 2006). *Lactobacillus fermentum* ETC1, *L. delbrueckii* subsp. *bulgaricus* ETC2, *L. rhamnosus* ETC14, *L. plantarum* ETC17, *L. casei* ETC19, *Enterococcus faecium* ETC124 and *E. faecium* ETC418 were isolated from goat's milk and cheeses. *Lactobacillus plantarum* Ov156, Ov161, Ov186, Ov236, *Enterococcus faecium* Ov157, Ov167, Ov178, Ov194, Ov242, Ov254, Ov426, Ov409 and *E. durans* Ov421 were isolated from ewe's milk and cheeses.

These strains were previously selected for their ability to produce flavour compounds from sugars, citrate and proteins, and for their esterase–lipase activities (Abeijón et al., 2006; Cabral, Abeijón Mukdsi, Medina, & González, 2007; Katz et al., 2002; Oliszewski et al., 2007). *Enterococcus* strains were previously tested for vancomycin/teicoplanin resistance and haemolysin and gelatinase production (Katz, 2005; Oliszewski, 2006).

All strains were frozen at −70 °C in MRS broth (Merck, Darmstadt, Germany) containing glycerol 20% (v/v), and grown in MRS broth for 16 h. Cultures were incubated at 37 °C.

2.2. Cell-free extract preparation

Cells cultured in 400 ml of MRS broth were harvested after 16 h by centrifugation at 10,000*g* for 10 min at 4 °C, washed twice with 100 mM sodium phosphate buffer, pH 7, and resuspended at 50% (w/v) of the same buffer. The cell suspensions were disrupted by three successive passes through a French pressure cell at 1000 psi (ThermoSpectronic, NY, USA). Cellular debris was removed by centrifugation (20,000*g* for 30 min at 4 °C) and the supernatant was used as cell-free extract (CFE).

2.3. Mechanisms of ester synthesis

2.3.1. Synthesis of ethyl esters by alcoholysis

These assays were performed according to the protocol of Liu et al. (2003a) with some modifications.

Ester synthesis by alcoholysis was analysed in an assay mixture containing 100 mM sodium phosphate buffer (pH 7), 100 mM ethanol, 33 mM triglyceride (tributyrin or tricaproin) and CFE (ca. 1–3 mg/ml). The assay mixtures were incubated statically at 37 °C for 24 h. A 1 ml sample was removed and added to 2 ml diethyl ether. Extraction was performed by shaking vigorously for about 2 min, followed by centrifugation (1300*g* for 5 min). The top solvent layer was then transferred to a gas chromatography (GC) vial. Controls lacking substrates were included to check for endogenous ester production. Controls lacking CFE were also included to test for the non-enzymatic formation of esters. Both controls were incubated and processed as described above.

2.3.2. Synthesis of ethyl esters by esterification

Ester synthesis by esterification was analysed in an assay mixture containing 100 mM sodium phosphate buffer (pH 7), 100 mM ethanol, 100 mM free fatty acid (butanoic acid or hexanoic acid) and CFE (ca. 1–3 mg/ml). The assay mixtures were incubated statically at 37 °C for 24 h. A 1 ml sample was removed and processed as described above. Controls mentioned above were also included.

2.4. Gas chromatography analysis

The following ethyl esters of short-chain fatty acids were determined with a gas chromatograph (Agilent 6890N, Agilent Technologies, CA, USA) equipped with a flame ionisation detector (FID): ethyl acetate (EtC2), ethyl butanoate (EtC4), ethyl hexanoate (EtC6), ethyl octanoate (EtC8), ethyl decanoate (EtC10). An HP-5 column (length 30 m, i.d. 0.32 mm, thickness 0.25 μm) (Hewlett-Packard, CA, USA) was used. The oven was temperature-programmed at 30 °C for 5 min, followed by increasing the temperature to 250 °C at 10 °C/min and held at 250 °C for 2 min. Sample injections of 1 μl were performed in split less mode. The injector and FID detector temperatures were 270 and 300 °C, respectively. Column flow rate was 1 ml/min using nitrogen as carrier gas. The FID output signal was recorded and processed using appropriate software (Agilent ChemStation Software, Agilent, CA, USA.)

Esters were quantified from the regression curve ($R^2 > 98\%$) of the corresponding standard (Sigma, MO, USA), using external standard calibration and the GC conditions described above. To check the recovery efficiency of each ester from the different assay mixtures, a known amount of standard solution was added to each mixture. Extraction and GC analysis were performed as described above. Correction factors were calculated and used to accordingly adjust ester concentration values.

A unit of ester-forming activity was defined as the amount of enzyme that formed 1 nmol of ester in 24 h. Specific ester-forming activity was defined as units per milligram of protein.

2.5. Protein determination

Protein concentrations were determined by the method of Bradford (1976), using a kit from Bio-Rad (CA, USA) and bovine serum albumin (Sigma, MO, USA) as standard.

2.6. Statistical analysis

All experiments were repeated twice. The results were submitted to analysis of variance (ANOVA). Tukey's test was performed for multiple mean comparisons ($P < 0.05$). Relations between strains and production level of each ester were studied by means of the Multiple Correspondence Analysis (MCA) method (Abdi & Valentin, 2007). For the MCA method, all quantitative data was transformed in categorical data. Results were expressed as production (ppm) and four categories (levels) were established according to minimal and maximal production level for each ester (Tables 1 and 2). Calculations and graphics were carried out with the Infostat Professional software 2004p1.

3. Results and discussion

3.1. Synthesis of ethyl esters

3.1.1. Synthesis of ethyl esters by alcoholysis

Ester-synthesising activity by alcoholysis (transferase activity) was determined on CFE in assay mixtures containing triglyceride (tributyrin or tricaproin) plus ethanol. Results are shown in Table 3.

Table 1
Categories established according to ester production by alcoholysis.

Level	Ester production (ppm)				
	EtC2	EtC4	EtC6	EtC8	EtC10
1	⩽1.30	⩽3.10	⩽1.80	⩽0.023	⩽0.40
2	⩽2.60	⩽6.20	⩽3.60	⩽0.046	⩽0.80
3	⩽3.90	⩽9.30	⩽5.40	⩽0.069	⩽1.20
4	⩽5.20	⩽12.40	⩽7.20	⩽0.092	⩽1.60

EtC2, ethyl acetate; EtC4, ethyl butanoate; EtC6, ethyl hexanoate; EtC8, ethyl octanoate; EtC10, ethyl decanoate.

Table 2
Categories established according to ester production by esterification.

Level	Ester production (ppm)			
	EtC4	EtC6	EtC8	EtC10
1	⩽17	⩽36	⩽2	⩽0.10
2	⩽34	⩽72	⩽4	⩽0.20
3	⩽51	⩽108	⩽6	⩽0.30
4	⩽68	⩽144	⩽8	⩽0.40

EtC2, ethyl acetate; EtC4, ethyl butanoate; EtC6, ethyl hexanoate; EtC8, ethyl octanoate; EtC10, ethyl decanoate.

Strains evaluated in this work showed a great variability in their ability to synthesise ethyl esters by alcoholysis. Ethyl esters of C4 and C6 were detected when CFE were incubated in presence of tributyrin and ethanol. Amongst lactobacilli strains the highest EtC4-synthesising activity was observed in *L. casei* ETC19 and *L. plantarum* ETC17 (49.18 ± 6.28 and 41.13 ± 4.33 U/mg, respectively). Enterococci strains showed higher EtC4-forming activity than lactobacilli, displaying *E. faecium* Ov194 and *E. faecium* ETC124 the highest activities (105.56 ± 11.23 and 104.88 ± 15.04 U/mg, respectively). Amongst all of the strains evaluated, only *L. plantarum* Ov161 showed significant EtC6-synthesising activity (48.92 ± 5.66 U/mg). *E. faecium* Ov178 was the only strain displaying EtC8-forming activity (0.40 ± 0.15 U/mg). None of the strains was able to synthesise ethyl esters of C2 and C10.

In the assay mixture containing tricaproin and ethanol, EtC2-forming activity was detected in *L. plantarum* ETC17, *L. plantarum* Ov186, *E. faecium* Ov167 and *E. faecium* Ov157. Other ethyl ester-forming activities were significantly lower in evaluated strains. Only two strains were able to produce EtC4 and four strains synthesised EtC8 (Table 3). *E. faecium* Ov254 was the only strain displaying EtC6-forming activity (0.63 ± 0.22 U/mg). *L. plantarum* Ov186 showed the highest EtC10-forming activity (7.41 ± 1.03 U/mg).

Liu et al. (2003a) reported that dairy LAB can synthesise substantial amounts of EtC4 from tributyrin and ethanol in aqueous systems *via* a transferase reaction (alcoholysis) in which butyryl groups from tributyrin are transferred directly to ethanol. They also found LAB to vary in their ability to produce esters *via* alcoholysis with *S. thermophilus* and *L. fermentum* displaying the highest transferase activities amongst a range of LAB studied. It was demonstrated that the enzymes that catalyse ester biosynthesis *via* the transferase reaction are indeed esterases that display acyltransferase activities (Liu, Baker, et al., 2004).

The highest ester-forming activities observed in enterococci strains would indicate that alcoholysis would be the main ester synthesis mechanism in this genus. This fact could be explained by the high esterolytic activities of these strains, which would allow the release of fatty acids from triglycerides and their concomitant esterification with ethanol (Abeijón et al., 2006; Katz et al., 2002; Oliszewski et al., 2007).

Oliszewski et al. (2007) have reported that enterococci strains isolated from goat's milk and cheeses showed the highest esterolytic activities on α-naphthyl (α-NA) derivatives of fatty acids of 4 and 6 carbon atoms. These results are in agreement with Katz et al. (2002), who observed that enterococci strains isolated from ewes' milk and cheese had higher esterolytic activity on α-NA butyrate and caproate than lactobacilli strains. Tsakalidou et al. (1994) concluded that enterococci strains show significantly higher esterolytic activity than strains of most other genera of LAB.

Other authors have reported significant EtC4-synthesising activity in *Streptococcus thermophilus* than other LAB. This correlates with both the high esterolytic activity detected in *S. thermophilus* (Crow, Holland, Pritchard, & Coolbear, 1994) and the perceived sweet/fruity flavour notes associated with this thermophilic starter (Law, 1998). Nevertheless, some authors have observed that there is no correlation between ester-hydrolysing and synthesising activities (Gandolfi, Gaspari, Franzetti, & Molinari, 2000).

A number of studies have demonstrated that water acts as a competitive inhibitor of lipase-catalysed esterification (Liu, Holland, et al., 2004), so ester synthesis by alcoholysis, in which fatty acid groups from acylglycerols are transferred to alcohols without direct involvement of water, would be the main mechanism involved in aqueous systems. The strains with high ester-forming activity by alcoholysis may be involved in the production

Table 3
Specific ester-synthesising activity[a] by alcoholysis detected in cell-free extracts of lactic acid bacteria isolated from goat's and ewe's milk and cheeses.

Strains	Tributyrin + ethanol					Tricaproin + ethanol				
	EtC2	EtC4	EtC6	EtC8	EtC10	EtC2	EtC4	EtC6	EtC8	EtC10
L. fermentum ETC1	n.d.	25.82 ± 4.32^{abc}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
L. delbrueckii subsp. *bulgaricus* ETC2	n.d.	11.21 ± 3.09^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
L. rhamnosus ETC14	n.d.	25.04 ± 5.19^{abc}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.42 ± 0.10^{b}	0.16 ± 0.08^{a}
L. plantarum ETC17	n.d.	41.13 ± 4.33^{bc}	n.d.	n.d.	n.d.	57.79 ± 9.25^{b}	n.d.	n.d.	n.d.	n.d.
L. casei ETC19	n.d.	49.18 ± 6.28^{c}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
L. plantarum Ov156	n.d.	16.65 ± 5.45^{ab}	1.94 ± 0.23^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	0.12 ± 0.05^{a}	0.10 ± 0.02^{a}
L. plantarum Ov161	n.d.	0.85 ± 0.12^{a}	48.92 ± 5.66^{b}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
L. plantarum Ov186	n.d.	4.82 ± 0.98^{a}	n.d.	n.d.	n.d.	28.88 ± 6.21^{ab}	n.d.	n.d.	n.d.	7.41 ± 1.03^{b}
L. plantarum Ov236	n.d.	25.49 ± 3.26^{abc}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
E. faecium Ov157	n.d.	40.43 ± 3.94^{bc}	n.d.	n.d.	n.d.	2.90 ± 1.01^{a}	n.d.	n.d.	n.d.	n.d.
E. faecium Ov167	n.d.	42.04 ± 5.05^{bc}	n.d.	n.d.	n.d.	35.69 ± 6.26^{ab}	n.d.	n.d.	n.d.	0.10 ± 0.04^{a}
E. faecium Ov178	n.d.	24.76 ± 2.92^{abc}	n.d.	0.40 ± 0.15	n.d.	n.d.	n.d.	n.d.	0.53 ± 0.05^{b}	n.d.
E. faecium Ov194	n.d.	105.56 ± 11.23^{d}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
E. durans Ov421	n.d.	14.08 ± 2.36^{ab}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.13 ± 0.02^{a}
E. faecium Ov242	n.d.	29.68 ± 3.65^{abc}	n.d.	n.d.	n.d.	n.d.	13.73 ± 1.07^{b}	n.d.	0.42 ± 0.08^{b}	n.d.
E. faecium Ov254	n.d.	53.39 ± 6.29^{c}	n.d.	n.d.	n.d.	n.d.	n.d.	0.63 ± 0.22	n.d.	n.d.
E. faecium Ov426	n.d.	95.87 ± 7.11^{d}	0.09 ± 0.05^{a}	n.d.	n.d.	n.d.	2.59 ± 0.88^{a}	n.d.	n.d.	n.d.
E. faecium Ov409	n.d.	98.02 ± 6.38^{d}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
E. faecium ETC124	n.d.	104.88 ± 15.04^{d}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
E. faecium ETC418	n.d.	24.62 ± 3.99^{abc}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

EtC2, ethyl acetate; EtC4, ethyl butanoate; EtC6, ethyl hexanoate; EtC8, ethyl octanoate; EtC10, ethyl decanoate.
n.d., Not detected.
[a] Results are expressed as U/mg of protein. Mean ± SD. Values in the same column with different superscript letters differ significantly ($P < 0.05$).

of these flavour-active compounds mainly in fermented milks, soft cheeses, and at the first stages of cheese ripening.

The multiple correspondence analysis (MCA) provides valuable information allowing the visualisation of the relations between strains and different production levels of each of the five ethyl esters analysed. The MCA of the results of ester synthesis by alcoholysis is shown in Fig. 1.

In the origin of the system (axes intersection delimiting the four quadrants) are situated all the strains having low levels of ester production (level 1). *L. plantarum* Ov186 differs significantly from the rest of the strains, being associated to the highest level of EtC10 (level 4) and intermediate level of EtC2 production (level 2) from tricaproin and ethanol. (Quadrant I).

Strains situated in the upper side of quadrant II are good producers of EtC4 from tributyrin and ethanol. *E. faecium* Ov409, Ov426, Ov194 and ETC124 showed the highest EtC4 production (level 4) when CFE were incubated in the presence of these substrates. Next to them are located some strains with level 2 production of this ester.

Strains situated in the quadrant III are good producers of ethyl esters from C2, 6 and 8. *L. plantarum* ETC17 and *E. faecium* Ov167 are associated with high production levels of EtC2 from tricaproin and ethanol (level 4 and 3, respectively). These strains are also associated with level 2 EtC4 production from tributyrin (Quadrant II). *L. plantarum* Ov161 is associated with level 4 EtC6 production. Strains associated with high EtC8 production in the medium containing tricaproin and ethanol are *E. faecium* Ov178, *E. faecium* Ov242 and *L. rhamnosus* ETC14 (level 4). *E. faecium* Ov178 is also related to level 4 EtC8 production from tributyrin and ethanol.

3.1.2. Synthesis of ethyl esters by esterification

Ester-synthesising activity by esterification was determined on CFE in assay mixtures containing free fatty acid (butanoic or hexanoic acid) plus ethanol. Results are shown in Table 4.

Large strain differences in the potential for ethyl ester formation amongst the LAB surveyed were observed. Liu et al. (1998) reported the formation of EtC4 from ethanol and butanoic acid by non-growing cells of several starter and non-starter LAB, which was both specific and strain dependent.

When CFE were incubated in an assay mixture containing butanoic acid and ethanol only EtC6-synthesising activity was observed. *L. casei* ETC19 was the only strain displaying EtC4-forming activity (88.54 ± 9.15 U/mg). In general lactobacilli strains showed higher ester-synthesising activities than enterococci when CFE were incubated with these substrates. Amongst lactobacilli strains *L. rhamnosus* ETC14 showed the highest EtC6-synthesising activity (679.20 ± 39.78 U/mg). *L. casei* ETC19 and *L. plantarum* Ov156 showed similar activities (210.12 ± 19.37 and 239.83 ± 31.20 U/mg, respectively). Only three strains of enterococci showed ester-synthesising ability: *E. faecium* ETC124, Ov178 and Ov194 (22.85 ± 2.88, 5.15 ± 1.44, 4.58 ± 0.89 U/mg of EtC6, respectively). None of the evaluated strains showed an ability to synthesise ethyl esters of C2, C8 and C10.

The fact that EtC6 was synthesised, whereas no EtC4 was formed in the presence of its immediate precursors (butanoic acid and ethanol) would indicate that other ester synthesis mechanism, besides esterification could be involved. Similar results were observed by Liu, Holland, and Crow (2003b), who reported that a commercial lipase produced EtC6 in addition to EtC4, when incubated in a cheese-based medium containing butanoic acid and ethanol. We reason that ester synthesis by acidolysis could be a tentative explanation. EtC6 can be formed by the reaction between the esters, formed by esterification between butanoic acid and ethanol, and the butanoic acid that remains in the medium. In addition, some authors suggested that cheese esters can be formed non-enzymatically (Liu, Holland, et al., 2004).

In the assay mixture containing hexanoic acid and ethanol, synthesising-activities of ethyl esters from C4 to C10 were observed, mainly EtC4 and EtC6. The highest EtC4-synthesising activities were observed in *E. faecium* ETC124 (569.46 ± 47.75 U/mg), *L. rhamnosus* ETC14 (470.43 ± 37.93 U/mg) and *L. plantarum* ETC17 (406.18 ± 42.33 U/mg).

Lactobacillus fermentum ETC1 displayed the highest EtC6-forming activity (991.38 ± 101.92 U/mg), whereas similar activities were observed in *L. rhamnosus* ETC14, *E. durans* Ov421 and *E. faecium* Ov242 (Table 4). Some strains showed EtC8 and EtC10-forming activities, being significantly lower than EtC4 and EtC6-synthesising activities (Table 4).

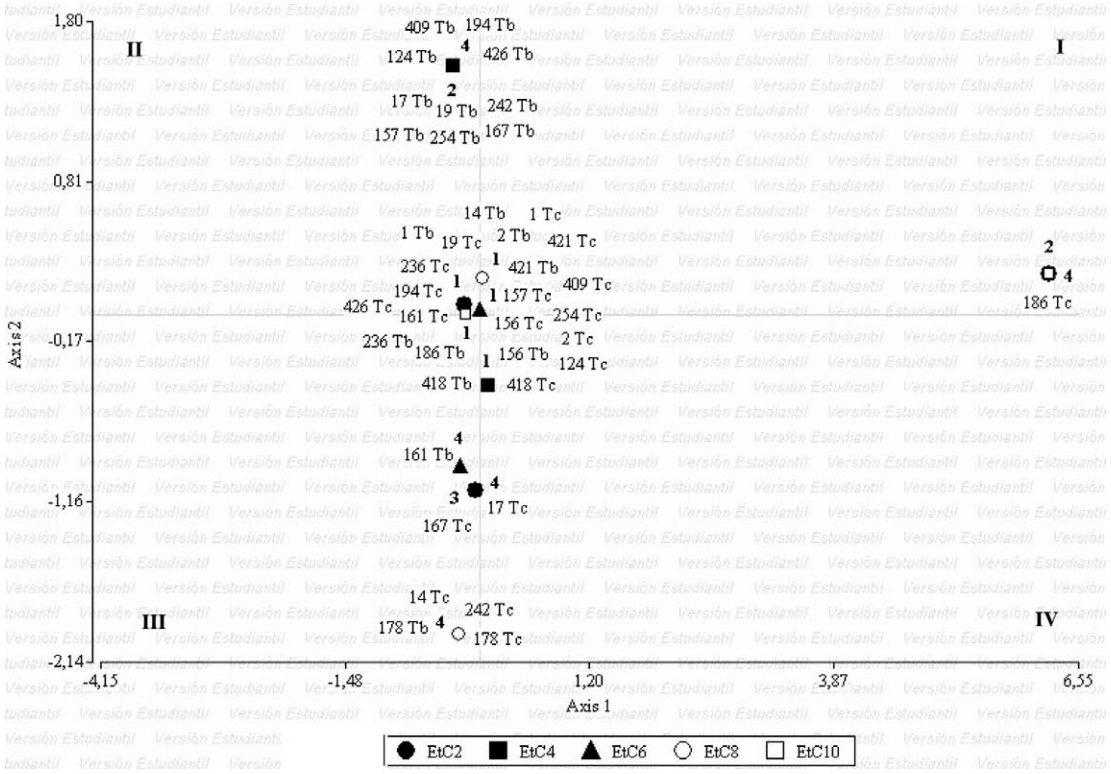

Fig. 1. Biplot obtained by Multiple Correspondence Analysis of ethyl esters of short-chain fatty acids produced by the mechanism of alcoholysis, expressed in ppm. Points are coded by strain (1, *L. fermentum* ETC1; 2, *L. delbrueckii* subsp. *bulgaricus* ETC2; 14, *L. rhamnosus* ETC14; 17, *L. plantarum* ETC17; 19, *L. casei* ETC19; 124, *E. faecium* ETC124; 418, *E. faecium* ETC418; 156, *L. plantarum* Ov156; 161, *L. plantarum* Ov161; 186, *L. plantarum* Ov186; 236, *L. plantarum* Ov236; 157, *E. faecium* Ov157; 167, Ov167; 178, Ov178; 194, Ov194; 242, Ov242; 254, Ov254; 426, Ov426; 409, Ov0409; 421, *E. durans* Ov421) and substrate (Tb, tributyrin; Tc, tricaproin). EtC2, ethyl acetate; EtC4, ethyl butanoate; EtC6, ethyl hexanoate; EtC8, ethyl octanoate; EtC10, ethyl decanoate. The position of some points was slightly modified to avoid overlapping of the labels.

Table 4
Specific ester-synthesising activity[a] by esterification detected in cell-free extracts of lactic acid bacteria isolated from goat's and ewe's milk and cheeses.

Strains	Butanoic acid + ethanol					Hexanoic acid + ethanol				
	EtC2	EtC4	EtC6	EtC8	EtC10	EtC2	EtC4	EtC6	EtC8	EtC10
L. fermentum ETC1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	267.17 ± 39.25^{efg}	991.38 ± 101.92^{f}	2.01 ± 0.08^{a}	1.78 ± 0.55^{b}
L. delbrueckii subsp. *bulgaricus* ETC2	n.d.	n.d.	3.36 ± 1.02^{a}	n.d.	n.d.	n.d.	52.81 ± 7.03^{abc}	198.59 ± 22.30^{abc}	1.12 ± 0.05^{a}	0.41 ± 0.10^{ab}
L. rhamnosus ETC14	n.d.	n.d.	679.20 ± 39.78^{c}	n.d.	n.d.	n.d.	470.43 ± 37.93^{hi}	605.63 ± 74.32^{e}	10.32 ± 2.05^{a}	n.d.
L. plantarum ETC17	n.d.	n.d.	45.01 ± 5.75^{a}	n.d.	n.d.	n.d.	406.18 ± 42.33^{gh}	n.d.	44.78 ± 6.21^{c}	n.d.
L. casei ETC19	n.d.	88.54 ± 9.15	210.12 ± 19.37^{b}	n.d.	n.d.	n.d.	n.d.	316.71 ± 25.09^{bcd}	0.30 ± 0.10^{a}	0.02 ± 0.01^{a}
L. plantarum Ov156	n.d.	n.d.	239.83 ± 31.20^{b}	n.d.	n.d.	n.d.	202.98 ± 31.09^{def}	553.86 ± 39.44^{de}	4.42 ± 0.88^{a}	0.98 ± 0.17^{ab}
L. plantarum Ov161	n.d.	n.d.	2.03 ± 0.90^{a}	n.d.	n.d.	n.d.	29.83 ± 1.92^{a}	112.54 ± 15.60^{ab}	0.31 ± 0.12^{a}	n.d.
L. plantarum Ov186	n.d.	n.d.	2.89 ± 1.23^{a}	n.d.	n.d.	n.d.	18.73 ± 2.50^{a}	1.64 ± 0.40^{a}	n.d.	n.d.
L. plantarum Ov236	n.d.	n.d.	11.43 ± 0.78^{a}	n.d.	n.d.	n.d.	n.d.	443.86 ± 33.07^{cde}	7.03 ± 1.22^{a}	0.77 ± 0.20^{ab}
E. faecium Ov157	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	32.85 ± 4.29^{ab}	n.d.	n.d.	n.d.
E. faecium Ov167	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	9.25 ± 1.52^{a}	n.d.	n.d.	n.d.
E. faecium Ov178	n.d.	n.d.	5.15 ± 1.44^{a}	n.d.	n.d.	n.d.	50.79 ± 8.58^{abc}	104.35 ± 12.84^{ab}	0.11 ± 0.05^{a}	n.d.
E. faecium Ov194	n.d.	n.d.	4.58 ± 0.89^{a}	n.d.	n.d.	n.d.	138.76 ± 21.15^{abcde}	433.68 ± 60.21^{cde}	$3.00 \pm 0,45^{a}$	n.d.
E. durans Ov421	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	106.12 ± 9.75^{abcd}	622.42 ± 57.94^{e}	4.76 ± 1.10^{a}	0.02 ± 0.01^{a}
E. faecium Ov242	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	689.89 ± 44.26^{e}	3.52 ± 0.22^{a}	n.d.
E. faecium Ov254	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	215.52 ± 28.34^{def}	n.d.	2.65 ± 0.45^{a}	n.d.
E. faecium Ov426	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	346.60 ± 29.12^{fgh}	n.d.	33.43 ± 2.89^{b}	n.d.
E. faecium Ov409	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	181.24 ± 23.65^{cde}	n.d.	3.63 ± 0.29^{a}	n.d.
E. faecium ETC124	n.d.	n.d.	22.85 ± 2.88^{a}	n.d.	n.d.	n.d.	569.46 ± 47.75^{i}	280.21 ± 30.05^{bc}	n.d.	n.d.
E. faecium ETC418	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	178.03 ± 19.45^{bcde}	n.d.	7.39 ± 1.03^{a}	1.54 ± 0.29^{b}

EtC2, ethyl acetate; EtC4, ethyl butanoate; EtC6, ethyl hexanoate; EtC8, ethyl octanoate; EtC10, ethyl decanoate.
n.d., Not detected.
[a] Results are expressed as U/mg of protein. Mean ± SD. Values in the same column with different superscript letters differ significantly ($P < 0.05$).

The MCA of the results of ester synthesis by esterification is shown in Fig. 2. Strains with low ester production levels are concentrated in the origin of the system. All of the strains are related to the lowest level of ester production in the assay mixture containing butanoic acid and ethanol, except for *L. rhamnosus* ETC14 which is related to level 3 production of EtC6. This strain is also related to level 3 production of this ester from hexanoic acid and ethanol (Quadrant IV). Very close situated in the same quadrant are

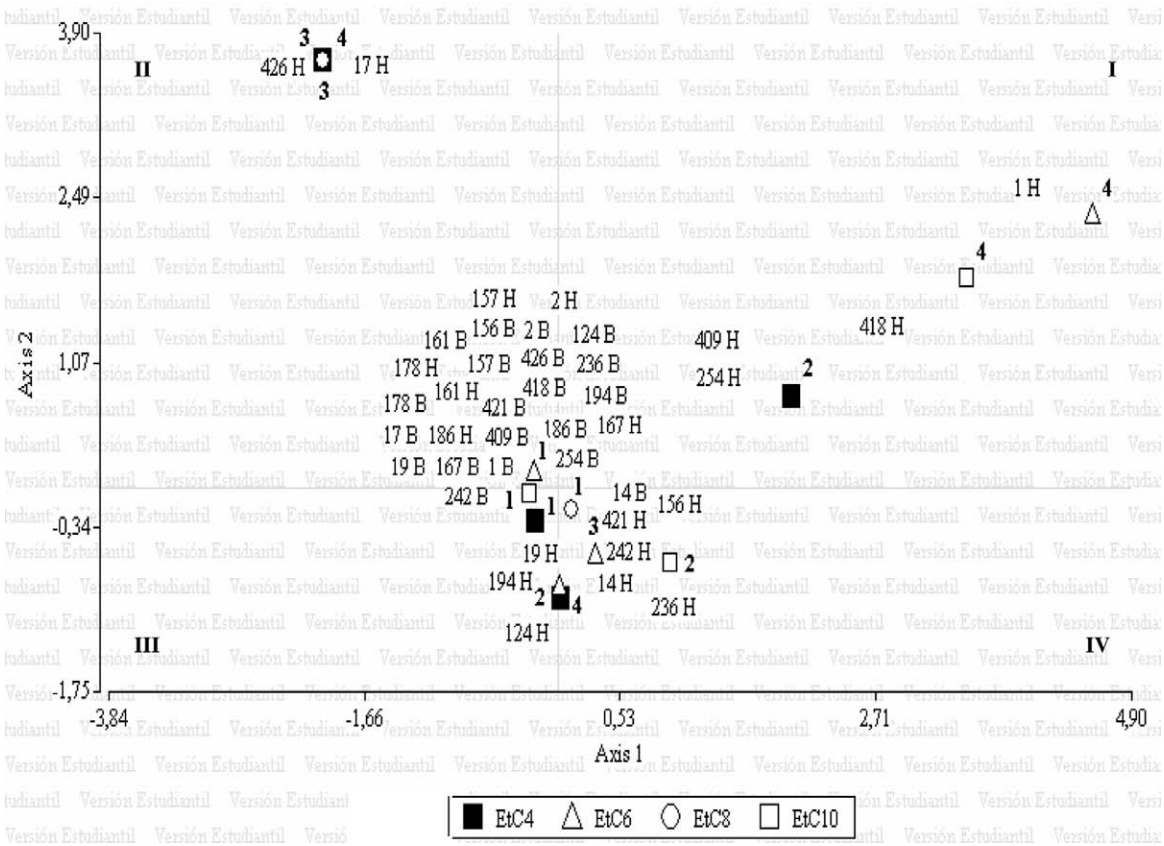

Fig. 2. Biplot obtained by Multiple Correspondence Analysis of short-chain fatty acids produced by the mechanism of esterification, expressed in ppm. Points are coded by strain (1, *L. fermentum* ETC1; 2, *L. delbrueckii* subsp. *bulgaricus* ETC2; 14, *L. rhamnosus* ETC14; 17, *L. plantarum* ETC17; 19, *L. casei* ETC19; 124, *E. faecium* ETC124; 418, *E. faecium* ETC418; 156, *L. plantarum* Ov156; 161, *L. plantarum* Ov161; 186, *L. plantarum* Ov186; 236, *L. plantarum* Ov236; 157, *E. faecium* Ov157; 167, Ov167; 178, Ov178; 194, Ov194; 242, Ov242; 254, Ov254; 426, Ov426; 409, Ov0409; 421; *E. durans* Ov421) and substrate (B, butanoic acid; H, hexanoic acid). EtC2, ethyl acetate; EtC4, ethyl butanoate; EtC6, ethyl hexanoate ; EtC8, ethyl octanoate; EtC10, ethyl decanoate. The position of some points was slightly modified to avoid overlapping of the labels.

E. durans Ov421, *E. faecium* Ov242 and *L. plantarum* Ov156, since they produce similar amounts of EtC6.

The strain *L. fermentum* ETC1 differs significantly from the rest, being associated to the highest levels of EtC6 and EtC10 production in the presence of hexanoic acid and ethanol (Quadrant I). *E. faecium* ETC418 is positioned not far but distinguishable from this first strain, in the same quadrant, since their EtC10 production is similar (both level 4).

Lactobacillus plantarum ETC17 and *E. faecium* Ov426 are very close situated in quadrant II, since they produce very similar amounts of EtC4 (level 3). They are also related to high levels of EtC8 production (level 4 and 3, respectively). *L. rhamnosus* ETC14 and *E. faecium* ETC124 are very closely located in the limit between quadrant III and IV, presenting the highest production of EtC4. This latter strain is also situated near to *E. faecium* Ov194 and *L. casei* ETC19, since they are all related to level 2 production of EtC6.

Enterococcus faecium ETC124 is associated to the highest EtC4 production by both mechanisms evaluated: alcoholysis (Fig. 1) and esterification (Fig. 2). These results could be explained by the presence of more than one esterase. Multiple esterases were found in several LAB (Abeijón et al., 2006; Katz et al., 2002; Oliszewski et al., 2007; Sarantinopoulos et al., 2001).

When comparing the ester-synthesising activity of the assayed strains by the two mechanisms evaluated (Tables 3 and 4), in general, higher ester-forming activities by esterification were observed. Enterococci strains were able to synthesise esters by alcoholysis in the presence of tributyrin, which would be related to their higher esterolytic activities. Other authors reported that in an aqueous environment, the yield of ester biosynthesis *via* alcoholysis was much higher than that of esterification (Liu, Baker, et al., 2004). In an environment where the water activity is low, such as ripened cheese, these strains may contribute to ester formation by both mechanisms (esterification and alcoholysis).

4. Conclusions

The results show that strains evaluated in this work were able to synthesise esters of short-chain fatty acids, mainly ethyl butanoate and ethyl hexanoate, which are desirable key odours in goat's and ewe's milk cheeses.

The highest ester-synthesising activities were observed when CFE were incubated in the presence of free fatty acids and ethanol, indicating that esterification is the prevalent mechanism in these strains. In enterococci strains the alcoholysis mechanism is also involved. These strains could be useful as adjunct cultures for small ruminants' dairy products and would contribute to the development of fruity flavour notes. Great variability in ester-forming activities between strains highlights the need for selecting appropriate cultures.

These results contribute to the knowledge of ester synthesis mechanisms by lactic acid bacteria and provide information about this topic in *Enterococcus* genus, which has not been reported at present. A deeper understanding of these mechanisms would allow to control ester production in fermented milk and cheese, assisting the manufacturers in their endeavour to obtain products with appropriate organoleptic characteristics.

Acknowledgements

Authors are grateful to Elena Bru for her collaboration with statistical analysis. This work was supported by Grants from Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT26/D429-2) and PIP: 00343.

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Food Chemistry 117 (2009) 248–253

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Occurrence of C16:1 isomers in milk fats from ewes fed with different dietary lipid supplements

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ARTICLE INFO

Article history:
Received 28 January 2009
Received in revised form 12 March 2009
Accepted 31 March 2009

Keywords:
Milk
Trans fatty acid
C16:1
β-Oxidation
Ewe
Dairy foods

ABSTRACT

The *trans* as well as the *cis* C16:1 isomer profiles were established in 43 ewe milk fats supplemented with different dietary lipid sources representative of the variety of unsaturated fatty acids found in nature such as olive, sunflower, linseed and fish oils. Fractionation by silver-ion solid phase extraction facilitated a rapid separation of the *trans*, *cis* and saturated FA before gas chromatography analysis took place. C16:1 isomers with a double bond in positions 7, 9 and 13 in the *cis* group and 8 and 9 in the *trans* fraction were the most abundant. Dietary lipid supplementation produced a noticeable increase in the total *trans* C16:1 content and elevated correlations were observed between *trans*-8 C16:1 and *trans*-10 C18:1 as well as *trans*-9 C16:1 and *trans*-11 C18:1. These results support the idea that altering the *trans* C18:1 profile affects *trans* C16:1 isomer composition consistent with the β-oxidation products from the *trans* C18:1 isomers.

1. Introduction

Dairy fat is one of the most complex dietary fats encountered in nature. This is particularly true with respect to our nutritional knowledge of the contents of *trans* fatty acids (TFAs) currently found in milk lipids, and is of great interest due to the possible role played by these TFAs in atherosclerosis and coronary heart disease though this is still subject to controversy. The TFA composition of ruminants reflects the biohydrogenation processes of the dietary fatty acids (FAs) taking place in the rumen. A multitude of research has reported the effects of feed, dietary regimens and lipid supplementation on *trans* isomer production, mainly with respect to 18 carbon atom unsaturated FAs (Chilliard et al., 2007; Harfoot & Hazlewood, 1997) but only a few articles have provided detailed information on other minor monounsaturated TFAs in milk fat. The first report that appeared about isomeric distribution of *trans* C16:1 isomers in bovine was published by Hay and Morrison (1970). Other studies in cow (Molkentin & Precht, 1997) and human (Precht & Molkentin, 2000) milk fat have been published more recently. In ewe milk fat the information is even scarcer. Destaillats, Wolff, Precht, and Molkentin (2000) observed that in different French cheeses made with cow, goat and ewe milks and purchased locally in supermarkets, the *trans* C16:1 isomers represented about 5% of the sum of *trans* C16:1 plus *trans* C18:1 isomers, whereas Goudjil et al. (2004) reported a mean content of *trans* C16:1 of 0.25% of total FA in ewe milk fat samples collected from five different breeds. Furthermore, hardly any data on individual *cis* C16:1 isomer FA content in ewe milk lipids have been published (Destaillats et al., 2000).

In the aforementioned studies the *trans* C16:1 content was determined by using a combination of silver ion thin-layer chromatography (Ag^+-TLC) and gas chromatography (GC), because an overlapping of *cis* C16:1 and branched C17:0 FA was frequently obtained if no pre-separation step was applied. However, as the Ag^+-TLC methodology has been deemed time-consuming and labour-intensive apart from the fact that it cannot be automated, GC combined with pre-fractionation by silver ion solid phase extraction (Ag^+-SPE) has very recently been shown to be a good alternative to Ag^+-TLC for isolating specific groups of FAs in milk fat. Kramer, Hernández, Cruz-Hernández, Kraft, and Dugan (2008) applied total fatty acid methyl esters (FAME) from different milks onto Ag^+-SPE cartridges and eluted with different solvents. The Ag^+-SPE method proved to be easier than Ag^+-TLC and yielded better FA resolution in different regions of the GC chromatogram.

Although the world production of ewe milk seems insignificant compared with the cow milk market, ewe cheeses are widely consumed in some geographical areas of the world such as the Mediterranean countries. Thus, it is useful to obtain more information about the composition of ewe milk fat, and especially about its minor TFAs and any factors which cause variations. Therefore, the aim of this work was to determine the *trans* C16:1 isomer composition of ewe milk fat and to study the variations in its composition relative to the source of lipids, using the new method of Ag^+-

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.03.107

SPE fractionation. Since there is little published information about other minor FA eluting in the same chromatographic region (*cis* C16:1 and branched C17:0), such information was also included in this study.

2. Materials and methods

2.1. Samples

This work was made up of 43 ewe milk fats originating from previous studies. All of them were frozen at −20 °C. Milk samples were selected to cover the effects of five types of dietary lipid supplements on the ewe milk fat samples (Table 1). Briefly, the first group dealt with the impact of a control diet supplemented with 6% dry matter (DM) of olive oil a source of oleic acid, the second with 6% DM of sunflower oil a source of linoleic acid, the third with 6 and 12% linseed DM a source of α-linolenic acid, the fourth with 2% of fish oil DM a source of long chain polyunsaturated fatty acids (PUFA) and finally the fifth a combination of sunflower and fish oil. Moreover, control samples from ewes fed without lipid supplementation were also studied (Table 1). These fats were used for the purpose of comparison with samples from supplemented diets.

2.2. Ag^+-SPE fractionation

Ewe milk fats were defrosted and derivatised to FAMEs. These derivatives were prepared by base-catalysed methanolysis of the glycerides (KOH in methanol) in accordance with the ISO-IDF procedure (2002). After derivatisation FAMEs were fractionated according to the number and geometry of the double bonds by Ag^+-SPE as described by Kramer et al. (2008). Ag^+-SPE cartridges were purchased from Supelco (Bellefonte, PA, USA). Before use, the cartridges were first conditioned with 4 ml of acetone to remove any moisture followed by hexane to equilibrate them. All the FAMEs were applied onto the column and eluted with 6 ml volumes of hexane containing increasing amounts of acetone: 99:1 (v/v) eluted saturated FAMEs, 96:4 (v/v) eluted mono-*trans* FAMEs plus the *cis–cis* and *trans–trans* FAME isomers of conjugated linoleic acid (CLA) and 90:10 (v/v) eluted mono-*cis* plus *cis/trans* of CLA. All fractions were taken to dryness in a stream of nitrogen and then reconstituted in an appropriate volume of hexane for analysis by GC.

2.3. Gas chromatography

The total FA profile was recorded by analysis of FAMEs on a gas–liquid chromatograph (Agilent 6890N Network System) with auto injector, fitted with a flame ionisation detector onto a CP-Sil 88 fused silica capillary column (100 m × 0.25 mm, Varian, Middelburg, The Netherlands). Helium was the carrier gas at an inlet pressure of 193.5 kPa and a split ratio of 1:100. The injector and detector temperature was 250 °C. Linear flow velocity of carrier gas in GC was 1.0 ml/min. Total time of the chromatographic programme was 180 min: the initial oven temperature being 45 °C. After 4 min, the oven temperature was raised at a rate of 13 °C min^{-1} to 150 °C. After 98 min, the oven temperature was raised 10 °C min^{-1} to 210 °C and then held for 64 min. Saturated, mono *cis* and mono *trans* FA fractions were analysed as FAMEs on the same equipment and conditions but the split ratio was 1:2.

2.4. Identification, calibration and quantitation of fatty acids

The FA profiles of milk fat samples were compared with a FAME reference standard mixture (GLC-461, Nuchek, Elysian, MN, USA). *Iso* and *anteiso* C17:0 methyl ester standards were available from Larodan Fine Chemicals (Malmö, Sweden). Identification of the 18:1, 16:1 *cis* and *trans* FA was based on principles of fractionation by argentation and GC, selected isomers from Nuchek, and by comparison with previously published reports.

Calibration of the individual FAs was carried out using the reference material CRM-164 obtained from the Community Bureau of Reference (Commission of the European Communities, Brussels, Belgium). The quantitation of positional isomers of *trans*-16:1 and *trans*-C18:1 present in the *trans* monoene fraction was achieved with the *trans*-9 C16:1 and *trans*-16 C18:1, respectively, which had been quantitated by stearic acid in the chromatography of the unfractionated FAMEs. Furthermore, the *cis* C16:1 and *cis* C18:1 isomers were calibrated by C17:1 and *cis*-12 C18:1, respectively, which were also determined in the total FAME GC analysis.

2.5. Statistical analysis

Statistics were performed by SPSS 17.0 for Windows XP. A bivariate analysis with Pearson correlations ($p < 0.05$ level, 2-tailed) was conducted with the data to determine the relationship between different FAs.

3. Results and discussion

Table 2 contains the *trans* C16:1 total content for the different groups of samples. Milk fat from ewes fed unsupplemented diets exhibited a mean content of total *trans* C16:1 of 0.30% with an interval of variation between 0.12 and 0.41%. These results coincide with data previously reported by Destaillats et al. (2000) for different cheeses made with ewe milk purchased in French supermarkets as well as with those of Goudjil et al. (2004) for bulk ewe milk from five herds. These values (Table 2) are greater than those observed in goat and cow milk fat where the total *trans* C16:1 content was never above 0.25% (Destaillats et al., 2000; Molkentin & Precht, 1997). Even so, it should be stressed that *trans* C16:1 isomers represent less than 10% of the sum of all *trans*-monoenoics apart from being very minor isomers in ewe milk fat too.

However, regardless of the lipid source, dietary supplementation provoked an increase in the total *trans* C16:1 content (Table 2). These increases were more striking in those milks coming from animals fed sunflower oil as well as from those fed combined sun-

Table 1
Ewe milk fat samples analysed.

Group	No of samples	Type of dietary lipid supplementation (% in dry matter)	Forage/concentrate proportion
1	8	Olive oil (6%)	20/80
2	7	Sunflower oil (6%)	20/80
3a	4	Linseed (6%)	60/40
3b	4	Linseed (12%)	60/40
4	4	Fish oil (1%)	20/80
5	4	Fish oil (1%) + sunflower oil (2%)	20/80
6a	10	None	20/80
6b	2	None	60/40

Table 2
Mean, maximum and minimum contents of the total and *trans* C16:1 isomers (g/100 g total fatty acid methyl esters) of milk fat from ewe fed with different dietary lipid sources as determined using silver ion solid phase extraction combined with gas chromatography. *n*: number of samples analysed; ND: not detected.

Isomer		Source of lipid supplementation					
		None *n* = 12	Olive *n* = 8	Sunflower *n* = 7	Linseed *n* = 8	Sunflower + fish *n* = 4	Fish *n* = 4
trans-4	Mean	0.01	0.01	ND	ND	0.01	0.01
	Maximum	0.01	0.02	ND	ND	0.01	0.01
	Minimum	ND	0.01	ND	ND	0.01	ND
trans-5	Mean	0.01	0.02	0.01	ND	0.02	0.02
	Maximum	0.02	0.03	0.02	ND	0.02	0.03
	Minimum	0.01	0.01	0.01	ND	0.01	0.01
trans-6 + *trans*-7	Mean	0.03	0.07	0.04	ND	0.06	0.07
	Maximum	0.05	0.10	0.06	ND	0.07	0.09
	Minimum	ND	0.06	0.02	ND	0.05	0.06
trans-8	Mean	0.03	0.08	0.10	0.05	0.13	0.08
	Maximum	0.04	0.11	0.15	0.05	0.25	0.15
	Minimum	0.01	0.03	0.06	0.04	0.03	0.02
trans-9	Mean	0.11	0.08	0.46	0.23	0.48	0.24
	Maximum	0.16	0.15	0.63	0.30	0.66	0.41
	Minimum	0.01	0.05	0.10	0.16	0.29	0.13
trans-10	Mean	0.01	0.02	0.01	0.01	0.02	0.02
	Maximum	0.05	0.02	0.02	0.02	0.02	0.02
	Minimum	ND	0.01	0.01	0.01	0.02	0.02
trans-11 + *trans*-12	Mean	0.04	0.04	0.04	0.07	0.06	0.05
	Maximum	0.07	0.06	0.07	0.08	0.08	0.07
	Minimum	0.01	0.04	0.01	0.06	0.05	0.03
trans-13	Mean	0.03	0.04	0.03	0.03	0.02	0.01
	Maximum	0.07	0.05	0.08	0.04	0.03	0.02
	Minimum	0.01	0.02	0.01	0.02	0.01	0.01
trans-14	Mean	0.02	0.03	0.02	0.01	0.02	0.03
	Maximum	0.03	0.04	0.05	0.02	0.03	0.05
	Minimum	0.01	0.02	0.01	0.01	0.02	0.01
Total *trans* C16:1	Mean	0.30	0.39	0.71	0.40	0.81	0.52
	Maximum	0.41	0.43	0.92	0.47	0.95	0.74
	Minimum	0.12	0.33	0.26	0.32	0.72	0.40

flower and fish oil supplements. In these samples, total *trans* C16:1 values were nearer to 1%, reaching in some cases percentages similar to those for *cis* C16:1 (Table 3). The presence of elevated amounts of *trans* C16:1 in ruminant milks has barely been documented (Cruz-Hernández et al., 2007), but, it is well known (Bauman et al., 2006; Harfoot & Hazlewood, 1997) that dietary lipid supplementation with sources of PUFA can generate high percentages of total *trans* C18:1 FA in milk fat. Its origin is the result of incomplete biohydrogenation of the dietary C18 PUFA in the rumen. Some of these *trans* C18:1 FAs produced in the rumen are transferred to milk fat. Nevertheless the occurrence of *trans* C16:1 isomers in milk fat would not be easy to explain, since the ruminant diet did not contain relevant amounts of *cis* hexadecenoate FAs susceptible to isomerisation and/or hydrogenation in the rumen. This fact, together with the high correlation ($R^2 = 0.81$; $P < 0.01$) observed between total *trans* 16:1 and total *trans* C18:1 content could be evidence of a possible conversion pathway from C18 to C16 FA groups.

Fig. 1 shows the typical gas partial chromatograms of the C16:1 area from ewe milk fat after Ag^+-SPE fractionation. Applying of this technique in combination with GC resulted in the separation of almost all *cis* and *trans* C16:1 isomers of ewe lipids. The *cis* C16:1 isomer group is much simpler than the corresponding *trans* fraction. As expected (Table 3), *cis*-9 C16:1 (palmitoleic acid) is by far the major isomer followed by *cis*-7 and *cis*-13. The rest of the isomers were detected in very low concentrations corresponding with the values reported for ewe milk cheese fat by Destaillats et al. (2000). Palmitoleic acid in milk fat is mostly formed by the action of delta-9 desaturase in the mammary gland using the same mechanism employed to convert C18:0 to *cis*-9 C18:1. This enzyme is located in the endoplasmic reticulum of the mammary gland cells and its primary substrates are palmitic and stearic acid. Although mammalian desaturase is not always entirely specific for the 9 position, it seems likely that the remaining *cis* isomers are principally the product of rumen microbial desaturases (Hay & Morrison, 1970).

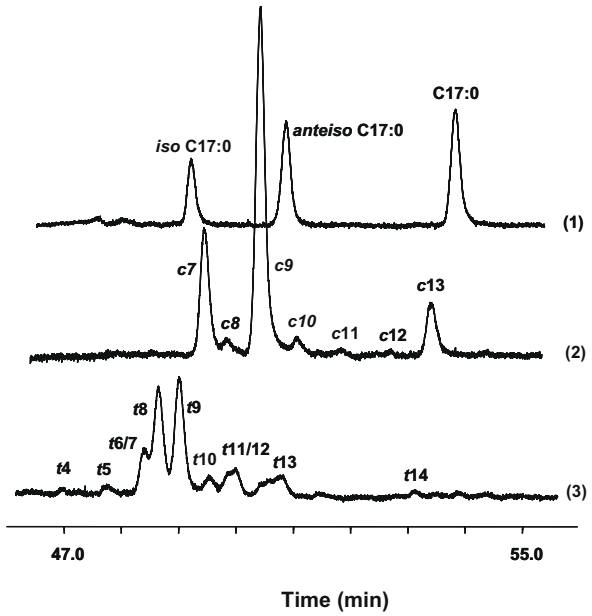

Fig. 1. Partial gas chromatograms of silver-ion solid phase extraction fractions showing saturated (1), *cis* C16:1 (2) as well as *trans* C16:1 (3) fatty acids methyl esters of ewe milk fat. *c*: *cis*; *t*: *trans*.

Milk fat from ewes fed lipid supplement diets showed lower percentages of saturated FA whereas contents of omega-3 FA were enhanced when linseed or fish oil was included in the ration (Table

Table 3
Mean, maximum and minimum contents of the total saturated fatty acids (SCFA), total omega-3 fatty acids, and *cis* C16:1 isomers as well as branched C17:0 fatty acids (g/100 g total fatty acid methyl esters) of milk fat from ewe fed with different dietary lipid sources, as determined using silver ion solid phase extraction combined with gas chromatography. *n*: number of samples analysed; ND: not detected.

Isomer		Source of lipid supplementation					
		None *n* = 12	Olive *n* = 8	Sunflower *n* = 7	Linseed *n* = 8	Sunflower + fish *n* = 4	Fish *n* = 4
cis-7	Mean	0.19	0.21	0.13	0.14	0.18	0.17
	Maximum	0.29	0.31	0.17	0.18	0.22	0.18
	Minimum	0.10	0.09	0.07	0.11	0.16	0.15
cis-8	Mean	0.01	0.01	0.01	ND	0.01	0.01
	Maximum	0.02	0.01	0.01	ND	0.01	0.01
	Minimum	ND	ND	ND	ND	ND	ND
cis-9	Mean	0.71	0.56	0.30	0.42	0.71	0.74
	Maximum	1.07	0.72	0.38	0.54	0.80	0.79
	Minimum	0.26	0.27	0.14	0.31	0.62	0.65
cis-10	Mean	0.02	0.01	0.02	0.03	0.01	0.01
	Maximum	0.03	0.02	0.02	0.04	0.01	0.01
	Minimum	0.01	0.01	0.01	0.02	0.01	0.01
cis-11	Mean	0.01	0.02	0.01	0.02	0.02	0.02
	Maximum	0.03	0.03	0.02	0.03	0.03	0.02
	Minimum	0.01	0.01	0.01	ND	0.01	0.01
cis-12	Mean	0.01	0.01	0.01	0.01	0.02	0.01
	Maximum	0.03	0.03	0.02	0.03	0.02	0.02
	Minimum	ND	0.01	0.01	ND	0.01	0.01
cis-13	Mean	0.10	0.04	0.02	0.05	0.06	0.09
	Maximum	0.18	0.07	0.05	0.07	0.09	0.10
	Minimum	0.03	0.02	0.01	0.03	0.05	0.08
Total *cis* C16:1	Mean	1.18	0.91	0.54	0.80	1.08	1.11
	Maximum	1.80	1.21	0.70	0.98	1.27	1.17
	Minimum	0.48	0.46	0.26	0.62	0.94	1.05
C17:0 iso	Mean	0.22	0.14	0.14	0.21	0.20	0.17
	Maximum	0.35	0.17	0.18	0.26	0.22	0.18
	Minimum	0.10	0.10	0.06	0.19	0.17	0.16
C17:0 anteiso	Mean	0.34	0.24	0.18	0.22	0.29	0.28
	Maximum	0.42	0.27	0.23	0.27	0.31	0.29
	Minimum	0.17	0.19	0.08	0.18	0.25	0.27
Total SCFA	Mean	73.18	60.24	54.21	59.96	60.12	69.77
	Maximum	75.91	62.92	56.68	64.44	62.32	71.01
	Minimum	70.29	57.98	52.21	53.98	58.87	68.47
Total omega-3	Mean	0.37	0.17	0.43	1.73	0.88	1.17
	Maximum	0.54	0.19	0.46	2.31	1.03	1.39
	Minimum	0.17	0.14	0.39	1.32	0.72	0.98

3). On average, milk fats from animals fed unsupplemented diets contained about 0.2 and 0.3% of *iso* and *anteiso* C17:0, respectively (Table 3). These proportions do not substantially differ from those reported for cow milk (Kramer et al., 2008). Percentages of *iso* and *anteiso* C17:0 were lower in milk fat samples from ewe fed lipid supplements than control diets (Table 3). It is well known that secretion of odd and branched-chain FAs in milk, often resulting from microbial FA metabolism in the rumen, tends to diminish or significantly decrease with oil supplemented diets (Vlaeminck, Fievez, Cabrita, Fonseca, & Dewhurst, 2006). Ruminant diets high in lipids can contribute towards changes in the digestive microflora as well as rumen metabolism thereby modifying the milk branched FA profile.

In milk fat from ewes fed without lipid supplementation *trans*-9 (palmitelaidic acid) is the most common isomer (0.11% of total FAMEs) followed by *trans*-11 *plus* *trans*-12, whilst the rest of the *trans* C16:1 isomers appear in lower proportions (Table 2). *trans*-9 C16:1 in milk from ewes on an unsupplemented diet represented almost 40% of total *trans* C16:1, a higher level to that previously reported in cow and ewe dairy fat (Destaillats et al., 2000; Kramer et al., 2008) but in accordance with the data of the first study by Hay and Morrison (1970) in bovine milk.

There was a noticeable rise in the percentages of some *trans* C16:1 isomers (Table 2) as a result of lipid supplementation. The most remarkable increases were observed for *trans*-9, *trans*-8 and, to a lesser degree, for both *trans*-6/*trans*-7 and *trans*-11/*trans*-12 pairs. Unfortunately, due to a lack of information in literature in this field, reliable comparisons cannot be made. However, very recently, Kramer et al. (2008) observed enhanced proportions of some *trans* C16:1 isomers in milk fat from cows fed a mixture of 4.5% sunflower oil and 0.5% fish oil. The main increases corresponded to *trans*-8 and *trans*-9 C16:1 isomers as has been the case in the present study (Table 2).

A potential source of *trans* C16:1 isomers could be the result of β-oxidation, a single step 2-carbon degradation of the *trans* C18:1 FA. Peroxisomal β-oxidation is a well-recognised process in FA metabolism in cells in which FA chains are shortened (Osmundsen, Bremer, & Pedersen, 1991). *trans* Hexadecenoates would appear to originate from the shortening of *trans* octadecenoate chains as was previously suggested in different tissues (Wood, 1979). The predominance of the *trans*-9 hexadecenoate may be related to the abundance of the substrate, *trans*-11 C18:1 (Fig. 2). The same pattern could be true for *trans*-8 C16:1 and its potential precursor, *trans*-10 C18:1.

The similarity between *trans* C16:1 and *trans* C18:1 profiles suggests that the same biosynthetic pathways could be responsible for the formation of the *trans* C16:1 acids. Theoretically, it would be impossible to decide whether they originate from *trans* C18:1 isomers through β-oxidation, by biohydrogenation of dietary C16:3 or C16:2, or as a result of both mechanisms operating together. However, as was mentioned above, it is unlikely that the *trans* 16:1 isomers were formed by biohydrogenation from C16 PUFA precursors, analogous to linoleic or α-linolenic acid forming *trans* C18:1 in ruminants, since C16 PUFA are not rich in ruminant diets or lipid supplements. In this context the evaluation of correlations between such isomeric contents would be of importance.

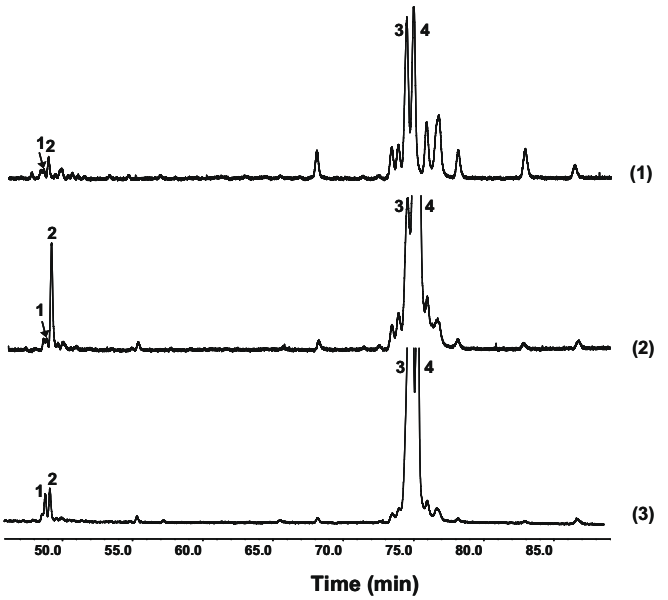

Fig. 2. Partial gas chromatograms including C16:1 and C18:1 regions of *trans* fatty acid methyl esters from control milk fat (1), *trans*-11 C18:1 (2) and *trans*-10 C18:1 (3) enriched milk fats. Control milk fat was derived from ewes fed unsupplemented diet whereas samples (2) and (3) came from ewes fed a 2% sunflower plus 1% fish oil supplement. Peak numbering: 1: *trans*-8 C16:1; 2: *trans*-9 C16:1; 3: *trans*-10 C18:1; 4: *trans*-11 C18:1.

Linear regression analysis was carried out establishing relationships between pairs of the quantitatively most relevant hexadecenoic and octadecenoic FAs present in the 43 ewe milk fat samples. The statistically highest positive correlations were found for *trans*-8 C16:1 and *trans*-10 C18:1 (R^2 = 0.97; $P < 0.01$) as well as for *trans*-9 C16:1 and *trans*-11 C18:1 (R^2 = 0.97; $P < 0.01$) (Fig. 3). From these correlations, it could be possible to deduce the existence of a conversion pathway from *trans* C18:1 to *trans* C16:1 based on a two carbon shortening mechanism of the octadecenoic TFA molecules. Such a mechanism could explain, from a quantitative point of view, how those *trans*-11 C18:1 enriched samples, the most abundant intermediate generated during linoleic and α-linolenic acid biohydrogenation (sunflower oil and linseed characteristic FAs), derived the most relevant growth of *trans*-9 C16:1. This argument would also explain why milk fats with the highest *trans*-10 C18:1 content, as a result of changes in biohydrogenation pathways associated with elevated dietary proportions of concentrate, elevated lipid supplementation or the presence of fish oil, also had the highest percentages of *trans*-8 C16:1.

Among the lipid supplements assayed in the ovine diets, only olive oil did not increase *trans*-9 C16:1 content (Table 2). The explanation for this behaviour could also be found in the biohydrogenation pathways of oleic acid. Results from experiments in ewe milk fat (Gómez-Cortés et al., 2008), which coincided with previous evidence obtained in in vitro studies (Mosley, Powell, Riley, & Jenkins, 2002), supported the idea that biohydrogenation pathways of *cis*-9 C18:1 in the rumen involved the formation of relevant amounts of a variety of *trans* monoene C18:1, except for *trans*-11. Thus, if the formation of *trans*-11 C18:1 is not promoted in the rumen, the contents of *trans*-9 C16:1 generated by β-oxidation in the tissues and subsequently excreted in the milk fat would not be elevated. The levels of *trans*-8 C16:1 in milk from ewes fed with olive oil could be justified by taking into account the production of large amounts of *trans*-10 C18:1 in the rumen from oleic acid as a precursor.

In line with that argument, the biohydrogenation pathways of α-linolenic acid should not be forgotten when considering the levels of *trans*-8 C16:1 in linseed samples, hardly different from those measured in control samples (Table 2). Among the *trans* monoene C18:1 intermediates of C18:3 in the rumen, only the *trans*-11 isomer is generated in remarkable amounts. In contrast, *trans*-10 C18:1 and other *trans* C18:1 are not frequently associated with α-linolenic acid biohydrogenation pathways (Chilliard et al., 2007; Harfoot & Hazlewood, 1997). Therefore, in linseed samples, important increases in *trans*-9 C16:1 and no changes in *trans*-8 C16:1 content were to be expected.

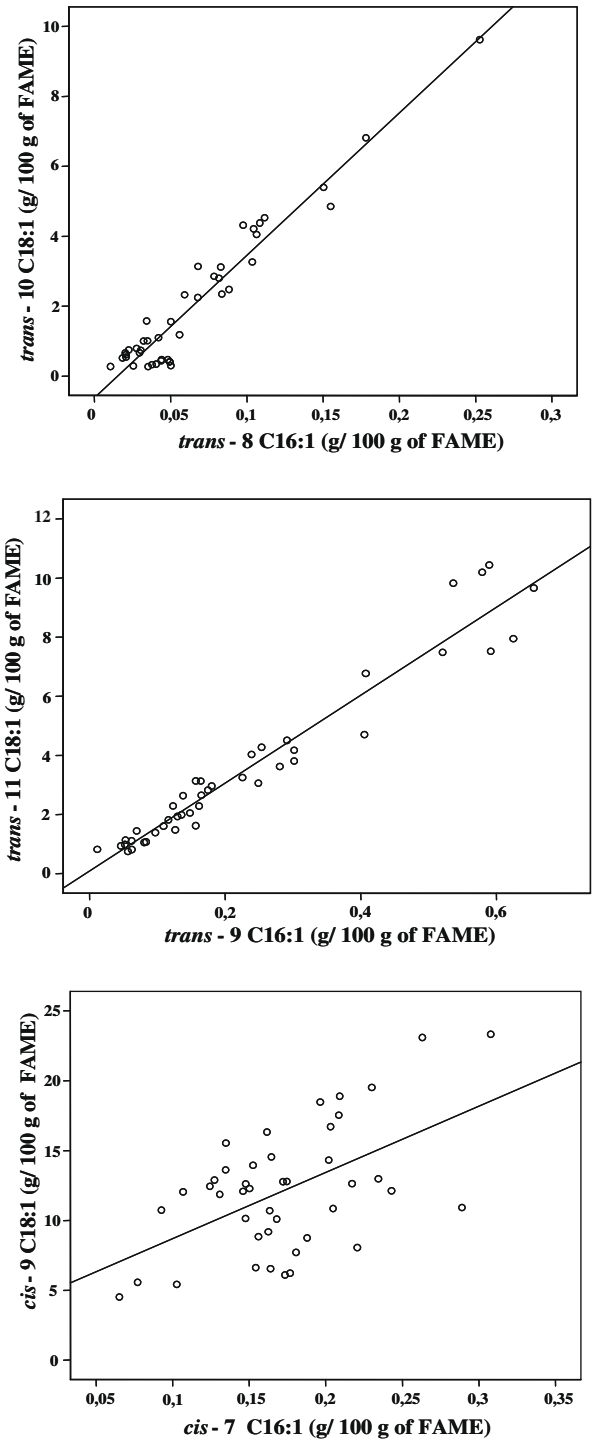

Fig. 3. Linear regression analysis establishing relationships between the pairs *trans*-8 C16:1 and *trans*-10 C18:1, *trans*-9 C16:1 and *trans*-11 C18:1, and *cis*-7 C16:1 and *cis*-9 C18:1 in 43 ewe milk fats obtained from ewes fed with different diets. FAME: fatty acid methyl esters.

Some chain shortening of the *cis* octadecenoate isomers could also occur, though to a much lesser degree than with the *trans* octadecenoates. The correlation between *cis*-7 C16:1 and oleic acid (*cis*-9 C18:1), its potential precursor, was clearly weaker ($R^2 = 0.55$, $P < 0.01$) than that reported between *trans* FAs (Fig. 3). This conclusion is also based upon the assumption that the *cis*-9 hexadecenoate that predominated in the *cis* fraction of the triglycerides was not derived to any significant extent from the chain shortening of the *cis*-11 octadecenoate isomer.

4. Conclusions

This work confirms that levels of hexadecenoic acids in ewe milk fat are similar to those found in other ruminant species. The total *trans* C16:1 content in milk obtained from ewes fed with lipid unsupplemented diets was low, but the inclusion of different sources of unsaturated FAs in ovine diets promoted increases in these FAs in milk fat, mainly *trans*-8 C16:1 and *trans*-9 C16:1 isomers. The strong correlations observed between both the *trans*-8 C16:1/*trans*-10 C18:1 pair and the *trans*-9 C16:1/*trans*-11 C18:1 pair would reveal the existence of a biosynthetic pathway linking both types of monounsaturated FAs.

Acknowledgements

This work was supported by the *Ministerio de Ciencia e Innovación* (Projects AGL2008-04805-CO2-01 and Consolider CSD2007-063) and the *Comunidad Autónoma de Madrid* (Project S-505/AGR-0153). The authors thank to Dr. G. Hervás from the *Instituto de Ganadería de Montaña* (León, Spain), Dr. A. Bach from *Institut de Recerca i Tecnologia Agroalimentàries* (Barcelona, Spain) and A. García and J.C. Rodríguez (Lodyn S.L., Ciudad Real, Spain) for supplying ewe milk samples. The authors also wish to thank Laura Barrios for the statistical analysis.

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Food Chemistry 117 (2009) 254–260

Contents lists available at ScienceDirect

Food Chemistry

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From apple to applesauce: Processing effects on dietary fibres and cell wall polysaccharides

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ARTICLE INFO

Article history:
Received 14 October 2008
Accepted 31 March 2009

Keywords:
Malus domestica Borkh
Post-harvest maturity
Puree
Pectins
Cooking
Refining

ABSTRACT

Modifications in dietary fibre content of apples during industrial processing into applesauce were investigated. Samplings with different post-harvest storage times were performed at five different processing steps (apple sorting, cooking, refining, sugaring and pasteurisation) and the samples examined for their insoluble, soluble and total fibre contents, following the AOAC method. Total fibres were also estimated through preparation of alcohol-insoluble solids and polysaccharide compositions of the various fibre residues were determined. Total fibre content decreased from apple to applesauce from 2.4 to 1.7 g for 100 g of fresh weight and the soluble fraction increased. Fibre loss and soluble/insoluble redistribution occurred during processing. Refining triggered a loss of insoluble polysaccharides whilst cooking and, surprisingly, sugaring led to pectin solubilisation and further degradation due to prolonged heating. These pectic changes, moreover, seemed to be dependent on the post-maturity stage of the apples.

1. Introduction

Dietary fibres are defined today by the Association of Official Analytical Chemists (AOAC) as "the polysaccharides and remnants of plant materials that are resistant to hydrolysis (digestion) by human digestive enzymes" (Cho, Devries, & Prosky, 1997). This definition includes non-starch polysaccharides, resistant starch, lignin, and minor components, such as waxes, cutin and suberin, and has been adopted by many European countries. They may be divided into two fractions: one is soluble in water at 100 °C and one insoluble (Grigelmo-Miguel & Martin-Belloso, 1999). The methodologies based on such a definition (such as the enzymatic–gravimetric methods) are now the standards used for labelling of numerous food products. Many studies have highlighted that the consumption of foods rich in dietary fibre may improve health: recommended intake reaches 30–45 g per day and person yet current consumption of fibre in the West is only around 20 g/day person (Figuerola, Hurtado, Estevez, Chiffelle, & Asenjo, 2005; Grigelmo-Miguel & Martin-Belloso, 1999; Thebaudin, Lefebvre, Harrington, & Bourgeois, 1997).

Apple has been extensively studied as it is a widely consumed fruit and also an interesting source of insoluble (cellulose, some xyloglucans) and soluble dietary fibres (mainly pectic polysaccharides), both being mostly related to the cell-wall (Aprikian et al., 2001; Gheyas, Blankenship, Young, & McFeeters, 1997; Massiot & Renard, 1997). Industrial food products based on apple, such as applesauce, are thus nowadays often presented as a healthy and convenient alternative to candy or chocolate bars, often eaten between meals, especially in the case of children. However, few data are available on the persistence of the original apple dietary fibre content in such products. More precisely, the effects of the whole processing involved for production of apple-based foods are not known. Indeed, processing effects on dietary fibres are often studied through unit operations such as heating, drying or even enzymatic treatment (Chang & Morris, 1990; Nyman, Palsson, & Asp, 1987; Renard & Thibault, 1991). Heating effects (implicated in canning or blanching operations) have been the most studied, especially on carrot, and have been shown to trigger degradative effects on its pectic polysaccharides (Albersheim, Neukom, & Deuel, 1960; Lo, Grun, Taylor, Kramer, & Fernando, 2002; Massiot, Guiller, Baron, & Drilleau, 1992; Ng & Waldron, 1997; Sajjaanantakul, van Buren, & Downing, 1989; Sila, Smout, Elliot, Loey, & Hendrickx, 2006; Sila, Smout, Vu, Loey, & Hendrickx, 2005). Fruit processing also often involves peeling operations that may, as well,

Abbreviations: AOAC, Association of Official Analytical Chemists; TDF, total dietary fibre; IDF, insoluble dietary fibre; SDF, soluble dietary fibre; AIS, alcohol-insoluble solids; DM, dry matter.

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doi:10.1016/j.foodchem.2009.03.109

be detrimental for fibre content. Indeed, the peel (epidermis) and the core zones (carpels) contain a higher proportion of cell-wall than does the flesh (Massiot & Renard, 1997). Moreover, the concentration and type of fibre-related polysaccharide differ from one tissue to the other, carpels being rich in glucans and xyloglucans and flesh containing a high proportion of pectic components (Massiot, Baron, & Drilleau, 1994). Finally, both skin and seeds appear to be rich in non-polysaccharide insoluble material (respectively, waxes and lignin). Therefore, both processing operations themselves, as well as their arrangement during the complete processing, may play a role in fibre content and in the type of fibre remaining in the final product.

Beyond the impact of processing itself, the original maturity stage of the fruit may affect the final fibre and cell wall polysaccharide content of the product. Indeed, variations in raw material quality and composition affect industrial processing of fruits. Nowadays, apples are processed all year long, mostly from the raw material remaining after sorting of table quality, thus including the use of fruits stored under controlled atmosphere. Impact of storage on apple composition is now well documented. Through maturation and post-harvest storage, losses in arabinose and galactose and solubilisation of galacturonic acid polymers of high molecular weight may be observed (Fischer & Amado, 1994; Knee, 1973; Massiot, Baron, & Drilleau, 1996; Wechsler, Strasser, & Amado, 1996). Cellulose and xyloglucan contents remain mostly unaffected (Bartley, 1976; Knee, 1973; Percy, Melton, & Jameson, 1997). However, interaction of this well-studied fruit evolution with processing impact is yet to be determined. The aim of this study was thus to investigate the response of apple dietary fibres and cell-walls to each unit operation of processing into applesauce, also taking storage into account. Two batches, corresponding to two different storage times (beginning and end of controlled atmosphere storage), were thus considered. To get a better insight of the modifications triggered by processing and storage time, cell-wall and total dietary fibres, as well as both soluble and insoluble fibre contents, were determined at each industrial step. Residues were analysed for neutral sugars and galacturonic acid.

2. Materials and methods

2.1. Industrial processing and sample preparation

Applesauce processing was carried out by a French manufacturer, using a hot-break process. The process was composed of several steps, summarised in Fig. 1: apples were first sorted and fruits presenting major visual defects were discarded. The fruits were roughly cut, then cooked (85 °C, 15 min), before refining (sieve opening 1.2 mm). A 15% proportion of glucose–fructose syrup (85 °Brix) was then added to the puree and the product was kept in a flow-regulating tank (85 °C, around 30 min) before pasteurising (90 °C, 2–3 min). In order to study the evolution of fibre content during the five main processing steps (sorting, cooking, refining, sugaring and pasteurisation), samplings were taken after each step. Samples were composed of 30 apples cut into large pieces or about 1 l of product.

To take into account the variations in apple composition, the sampling procedure was carried out twice: at the beginning (December 20th, 2005) and at the end (February 17th, 2006) of the controlled atmosphere storage period of apples. At each date, the sampling procedure was repeated twice, in order to include processing variability itself: two series of samplings were performed, one in the morning and one in the afternoon. In total, 20 different samples were thus obtained (5 processing steps × 2 dates × 2 sampling hours). Processing on December 20th will be further referred as "batch A" and processing on February 17th as "batch B". For both processing days, apple batches had the same composition: 90% of Golden apples and 10% of bicolour apples.

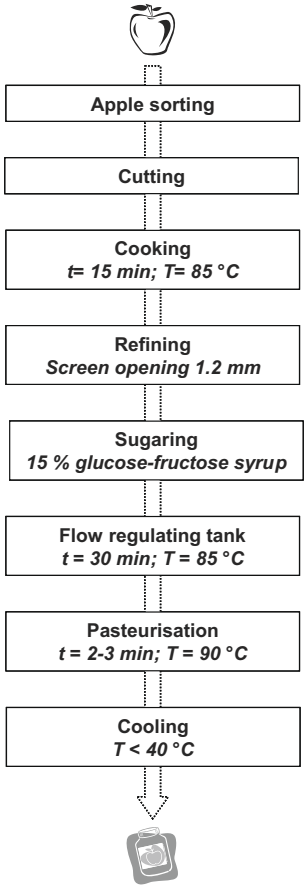

Fig. 1. Applesauce processing.

Samples were stored at −20 °C before freeze-drying of aliquots and milling in liquid nitrogen. The apple powders obtained were prepared in duplicate for each different sample, giving a total of 40 powdered samples, and stored in a desiccator under vacuum prior to analysis. Residual water content after freeze-drying was measured on each powdered sample by drying overnight at 105 °C. The final dry matter (DM) of the samples was then obtained after correction of the freeze-drying yields for this residual water content. Freeze-drying of the sweetened samples proved to be efficient, with less that 5% residual water in the powders.

2.2. Dietary fibre preparation

Insoluble (IDF), soluble (SDF) and total dietary fibre contents (TDF) were determined according to the enzymatic–gravimetric (phosphate buffer) AOAC methods (labelled 991.42, 993.19 and 985.29, respectively) with slight modifications (Cho et al., 1997). Sample duplicates (1 g of each duplicate powdered sample) were gelatinized with a thermo-stable α-amylase (Termamyl 120L, Novo Nordisk, Denmark) and successively treated with a protease (from *Bacillius licheniformis*, P3910, Sigma–Aldrich, France) and an amyloglucosidase (from *Aspergillius niger*, A9913, Sigma–Aldrich, France) to remove protein and starch, respectively. SDF and IDF were separated by filtration on a 75 ml Sep-pack prep column (Interchim, France) equipped with a sinter of porosity 20 μm. The residue obtained after filtration, containing IDF, was dried by solvent exchange (ethanol, acetone) and overnight at 105 °C and weighed. The IDF weight was corrected for protein (automated Dumas and Kjeldhal methods, protein content estimated as N × 6.25) and ash

contents (500 °C, 5 h). The filtrate, containing SDF, was precipitated with four volumes of 96% ethanol preheated to 60 °C. The SDF fraction was then recovered after filtration on a 75 ml Sep-pack prep column, dried by solvent exchange and overnight at 105 °C and weighed. A duplicate blank was run, together with each series of eight duplicated samples and SDF weight was corrected for blank weight. TDF was estimated as the sum of SDF and IDF. For each batch and processing step, a total of four estimations of dietary fibre content were obtained (duplicated sample × 2 sampling hours).

2.3. Alcohol-insoluble solids preparation

Alcohol-insoluble solid (AIS) content was determined on each duplicate powdered sample according to Renard (2005b). Apple powder was extracted with 70% ethanol at room temperature and filtered on the 75 ml Sep-pack prep columns (20 μm sinter) until the filtrate was sugar-free, as attested by the method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956). The residue was then dried by solvent exchange and overnight at 40 °C. A total of four estimations of AIS content were obtained for each batch and processing step.

2.4. Chemical analysis

2.4.1. Acid hydrolysis of fibre samples

The IDF and AIS residues were submitted to acid pre-hydrolysis (13 M H_2SO_4, 1 h, 25 °C), followed by acid hydrolysis (1 M H_2SO_4, 3 h, 100 °C). The SDF samples were first solubilised in distilled water (2 mg/ml) before acid hydrolysis (1 M H_2SO_4, 3 h, 100 °C).

2.4.2. Neutral sugar analysis

The individual neutral sugars were analysed by gas chromatography (TRB-225 column, 30 m × 0.32 mm × 0.25 μm, AIT, France) after hydrolysis and derivatisation to alditol acetates, following the method described by Thomas, Crépeau, Rumpunen, and Thibault (2000). Myo-inositol was used as internal standard. Runs were performed at 200 °C, using nitrogen as carrier gas. Analysis was duplicated for each AIS residue and performed once on the IDF and SDF residues.

2.4.3. Galacturonic acid analysis

Galacturonic acid content was determined using the metahydroxydiphenyl assay (Thibault, 1979) on the IDF and AIS residues hydrolysed, as described in Section 2.4.2. The solubilised SDF residues were first submitted to saponification (0.05 M NaOH, 30 min, 25 °C), neutralised (0.05 M HCl), then analysed. Galacturonic acid was used as internal standard. Analysis was duplicated for each AIS residue and performed once on the IDF and SDF residues.

2.5. Data correction for process-added sugar

As the water content of the samples was likely to vary during processing, and because the studied applesauce was sweetened, processing effects were studied on a dry weight basis and after correction for the process-added sugar for sweetened samples (i.e. those corresponding to the sugaring and pasteurisation steps).

Apple fraction (Af, in %) of the sweetened samples was calculated, knowing the amount of sugar added (15% of glucose–fructose syrup for 85% apple puree) and its dry matter (85 g/100 g) and the dry matter of the studied sample (DM, $\text{sweetened}_{\text{sample}}$, in g/100 g), according to the formula:

$$\text{Af}(\%) = [\text{DM,sweetened}_{\text{sample}} - (15 \times 85/100)]/\text{DM,sweetened}_{\text{sample}} \times 100$$

Fibre content (on a dry weight basis), measured on these sweetened samples was then corrected according to its true apple fraction by multiplying fibre content by 100/Af.

Moreover, the dry matter relative to the apple fraction (DM, apple, in g/100 g) was also calculated for the sweetened samples, by considering that the dry matter of the sweetened samples came from the addition of the dry weight provided by 15% syrup and 85% apple puree:

$$\text{DM apple} = \text{Af} \times \text{DM sweetened}_{\text{sample}}/85$$

Statistical analyses were applied to these corrected values.

2.6. Statistical analysis

Analysis of variance (ANOVA) and the tests of mean comparison, according to Fisher least significant difference (LSD) were applied to instrumental data with the Statgraphics Plus v. 5.1 programme (Sigma Plus, France). Level of significance (α) was 0.05. Standard deviations were calculated for each series of measurement, using the sum of individual variances given by the sum of individual degrees of freedom.

3. Results

3.1. Fibre content of the fresh products

Fibre contents obtained with both AOAC and AIS methods for fresh apples and for applesauce (the finished product) are presented Table 1. On this fresh weight basis, fibre contents of unpeeled apples were similar, whatever the method, reaching 2.3 (AIS content) to 2.4 (TDF content) in g/100 g, which was consistent with values found in literature (2.0–2.7 g/100 g) (Gheyas et al., 1997; Marlett & Vollendorf, 1994; Renard, 2005b; Renard & Thibault, 1991). Around 28% of the dietary fibres were soluble: the calculated SDF/IDF ratio obtained was close to that found by Renard and Thibault (1991) on cored Golden apples and by Gheyas et al. (1997) on peeled apples (about 0.35). In the final sweetened applesauce (15% added sugar), fibre content was lower than in the fresh apples, reaching 1.7–1.8 g/100 g. Few studies report the fibre content of applesauce, yet values obtained here were slightly higher than those reported by Marlett and Vollendorf (1994) (1.4 g/100 g) but the authors did not mention whether their product was sweetened or not. The AOAC data base (Cho et al., 1997), however, mentions a TDF content of 1.2 g/100 g for unsweetened applesauce, which would give a lower fibre content than those measured here. Though the total fibre content decreased between apple and applesauce, the SDF content remained similar, indicating an increase in the soluble fibre fraction (39% in the case of applesauce).

Processing thus modified both the dietary fibre content and the ratio between soluble and insoluble fibre.

Table 1
Average fibre content (g/100 g of fresh weight) of fresh apples and applesauce, calculated on batches A and B. Standard deviations ($n = 8$) are presented in brackets.

Fibre content (g/100 g fresh weight)	Fresh apple	Applesauce
AIS	2.43 (0.09)	1.76 (0.05)
TDF	2.33 (0.09)	1.68 (0.13)
IDF	1.69 (0.08)	1.02 (0.07)
SDF	0.64 (0.08)	0.66 (0.07)
SDF/IDF ratio	0.38 (0.06)	0.65 (0.04)

Table 2
Fishers' *F*-values and *p*-values associated with two-way ANOVAs (processing step and apple-batch effects, with interaction) performed on dietary fibre content (expressed in dry weight and corrected for sugar content) and on the dry matter relative to apple content of the samples (apple DM).

Variable	Processing step effect		Apple-batch effect		Processing step × apple-batch effect	
	F	*p*	*F*	*p*	*F*	*p*
Apple DM (g/100 g)	5.4	0.002	21.9	0.0001	1.2	0.3
AIS (g/100 g)	75.8	<0.0001	3.9	0.06	2.3	0.09
TDF (g/100 g)	23.0	<0.0001	3.0	0.09	3.0	0.03
(%) IDF	34.2	<0.0001	0.0	0.98	3.6	0.02

3.2. Processing and apple-batch effects on dry matter and fibre content

Processing and batch effects were studied on a dry weight basis and after correction for the process-added sugar for sweetened samples (corresponding to the sugaring and pasteurisation steps). Two-way ANOVAs (processing step and batch effects, with interactions) were performed on the product dry matter relative to its apple fraction and on the corrected fibre contents (Table 2).

The effect of processing step was highly significant ($p < 0.001$), whatever the type of fibre content measured, whereas apple batch effect was not significant. However, some significant interactions appeared between processing step and apple batch for TDF and for the soluble/insoluble fraction. The dry matter of the product, relative to its apple fraction, was also significantly affected by both processing step and apple batch, without significant interactions. Overall, given the presence of some significant interactions, the overall modifications in fibre content and dry matter during processing are presented for each batch separately in Table 3.

The dry matter, relative to the apple fraction, showed slight but significant changes during processing (Table 3): refining (i.e. seed and skin removal by extruding of the apple slurry through a sieve) mostly led to a decrease in the dry matter (visible yet not significant in the case of batch B) which increased again at the sugaring step. The overall dry matter was lower for batch B than for batch A. AIS content modifications during processing were the same for both batches: AIS increased after cooking (+20%) then decreased at refining (−16%) and after sugaring (−18%). The TDF content for batch A followed a similar pattern but these modifications were much less marked for batch B, for which cooking and sugaring steps had no significant impact. TDF modifications were followed by variations in the soluble/insoluble ratio. For batch A, TDF increase at cooking was due to both soluble and insoluble fibres. At refining, the redistribution between soluble and insoluble fractions indicated a predominant gain in soluble fibres, whilst the slight total fibre loss at sugaring did not change the soluble/insoluble ratio. In the case of batch B, redistributions between soluble and insoluble fractions occurred at cooking and sugaring, without any significant TDF loss. At refining; however, the decrease in TDF took place without modification of the soluble/insoluble ratio.

Overall, the general impact of processing was an increase in total fibres (AIS and TDF) at cooking (however not significant for TDF content of batch B) and a decrease at refining and sugaring (again not significant for TDF content of batch B). The modifications in the soluble/insoluble fibre fraction during processing were, however, different from one batch to another. Most noticeably, the less pronounced modifications observed here for TDF content of batch B were likely to come from some marked opposite variations in the SDF and from IDF contents of this batch.

3.3. Processing and apple-batch effects on fibre composition

The compositions of AIS, IDF and SDF in apple samples during processing were calculated on original dry substances (i.e. fibre yield in mg/g × fibre composition in mg/g of fibre). Total polysaccharides content was estimated as the sum of neutral sugars and galacturonic acid. In the case of SDF, analysis of the blanks run with the samples, during dietary fibre extraction, showed that they contained significant amounts of mannose (130 mg/g) and of glucose (20 mg/g). Both sugars probably mostly arose from the various enzymatic treatments applied during the extraction procedures. These sugars were thus not considered for the study of polysaccharide content. For the other sugars, concentrations were calculated from crude SFD yield (i.e. without blank correction) to individual sugar content in the crude SDF.

Two-way ANOVAs (processing step and batch effects, with interactions) were performed on polysaccharide content and sugar composition. Except for total polysaccharide and xylose contents, the apple batch effect was always highly significant ($p < 0.0001$) and more important than the processing effect (lower *p*-values) (data not shown). However, as some significant interactions occurred, data are presented for each batch separately in Tables 4 and 5.

Table 3
Modifications during processing of dry matter relative to apple content (apple DM, in g/100 g of fresh weight), AIS and TDF contents (in g/100 g of dry weight corrected for process-added sugar) and IDF fractions (in % of TDF). IDF values are followed in brackets by contents in g/100 g of dry weight corrected for process-added sugar. Different letters in the same series indicate significant difference at the 0.05 level according to Fisher's LSD test. SD, standard deviation of the mean (degrees of freedom: 35).

Batch	Processing step	Apple DM (g/100 g fresh weight)	Fibre content (g/100 g dry weight corrected for process-added sugar)		
			AIS	TDF	(%) IDF (g/100 g)
Batch A	Sorting	16.4^{b}	14.9^{b}	13.9^{b}	72 (9.9)^{a}
	Cooking	16.0^{b}	19.1^{c}	17.4^{c}	70 (12.2)^{a}
	Refining	14.7^{a}	15.5^{b}	14.0^{b}	60 (8.3)^{b}
	Sugaring	16.5^{b}	12.6^{a}	12.6^{ab}	57 (7.2)^{b}
	Pasteurisation	16.5^{b}	12.5^{a}	11.6^{a}	60 (6.9)^{b}
Batch B	Sorting	15.0	16.1^{b}	16.0^{b}	73 (11.7)^{a}
	Cooking	13.9	18.2^{c}	16.7^{b}	67 (11.2)^{b}
	Refining	14.3	15.9^{b}	13.2^{a}	66 (8.7)^{bc}
	Sugaring	15.5	13.1^{a}	13.1^{a}	52 (6.8)^{d}
	Pasteurisation	15.3	13.6^{a}	13.5^{a}	61 (8.2)^{c}
	SD	1.0	0.9	1.2	3.8 (0.9)

Table 4
Composition of AIS during processing, calculated on original apple dry substance (expressed in g of dry weight corrected for process-added sugar). Different letters in the same series indicate significant difference at the 0.05 level according to Fisher's LSD test. SD, standard deviation of the mean (degrees of freedom: 35).

		Sorting	Cooking	Refining	Sugaring	Pasteurisation	SD
Batch A	Polysaccharides[A](mg/g)	105[b]	138[d]	118[c]	102[ab]	96.4[a]	8.5
	Relative composition (mol%)[A]						
	Rha	1.9[abc]	2.0[c]	2.0[bc]	1.8[a]	1.8[ab]	0.1
	Fuc	1.2[a]	1.3[b]	1.4[c]	1.4[bc]	1.3[bc]	0.1
	Ara	17.0[c]	16.2[c]	13.7[b]	11.7[a]	12.6[ab]	1.3
	Xyl	7.7	7.7	7.6	7.6	7.7	0.6
	Man	2.4	2.5	2.4	2.6	2.4	0.3
	Gal	10.2[c]	9.2[a]	9.8[bc]	9.3[ab]	9.5[ab]	0.6
	Glc	34.0[a]	35.7[ab]	37.8[bc]	39.2[d]	37.9[cd]	2.0
	GalA	25.6	25.3	26.3	26.3	26.8	2.6
Batch B	Polysaccharides[A] (mg/g)	107[b]	125[c]	117[c]	97.2[a]	98.5[a]	8.1
	Relative composition (mol%)[A]						
	Rha	1.8	1.8	1.8	1.7	1.7	0.1
	Fuc	1.5	1.4	1.5	1.4	1.4	0.1
	Ara	13.0[c]	13.0[c]	11.8[b]	11.3[b]	10.2[a]	0.8
	Xyl	7.9[bc]	8.0[c]	8.0[c]	7.5[a]	7.7[ab]	0.3
	Man	2.3	2.3	2.4	2.2	2.2	0.1
	Gal	8.0	8.4	8.5	8.1	8.3	0.4
	Glc	34.4[a]	34.5[a]	36.1[b]	38.8[c]	40.3[d]	0.8
	GalA	31.1[d]	30.5[cd]	29.8[bc]	28.9[ab]	28.1[a]	1.2

[A] Expressed in the original dry substance, and with correction from process-added sugar.

Table 5
Composition of IDF and SDF during processing, calculated on original apple dry substance (expressed in g of dry weight corrected for process-added sugar). Different letters in the same series indicate significant difference at the 0.05 level according to Fisher's LSD test. SD, standard deviation of the mean (degrees of freedom: 15).

		Sorting		Cooking		Refining		Sugaring		Pasteurisation		SD	
		SDF	IDF	SDF	IDF	SDF	IDF	SDF	IDF	SDF	IDF	SDF	IDF
Batch A	Polysaccharides[A] (mg/g)	35.8[a]	75.4[c]	40.9[ab]	89.3[d]	44.0[b]	70.8[bc]	59.0[c]	62.8[ab]	56.1[c]	58.0[a]	5.1	5.9
	Relative composition (mol%)[A]												
	Rha	3.0[a]	1.9	3.0[ab]	1.9	3.2[c]	1.9	3.1[abc]	1.9	3.1[bc]	1.9	0.1	0.1
	Fuc	1.9[a]	1.2[a]	2.0[a]	1.2[a]	2.2[b]	1.3[b]	2.2[b]	1.3[b]	2.3[b]	1.3[b]	0.1	0.0
	Ara	25.7[c]	16.5[d]	24.0[b]	15.1[c]	21.0[a]	12.8[b]	19.9[a]	12.1[a]	20.7[a]	12.4[ab]	0.8	0.5
	Xyl	11.6[a]	7.5	12.3[b]	7.7	12.3[b]	7.5	12.4[b]	7.5	13.2[c]	7.9	0.4	0.3
	Man	–	2.4[a]	–	2.4[ab]	–	2.5[c]	–	2.5[bc]	–	2.5[bc]	–	0.1
	Gal	15.6[b]	10.0[b]	13.9[a]	8.7[a]	15.5[b]	9.5[b]	15.6[b]	9.4[ab]	15.9[b]	9.5[b]	0.7	0.5
	Glc	–	33.5[a]	–	34.8[b]	–	36.3[c]	–	37.1[d]	–	37.8e	–	0.4
	GalA	42.3[a]	27.1	44.9[b]	28.2	45.9[b]	28.0	46.8[b]	28.3	44.8[b]	26.8	1.5	1.0
Batch B	Polysaccharides[A] (mg/g)	36.4[a]	80.0[c]	51.0[b]	80.1[c]	50.9[b]	70.1[b]	59.2[c]	56.8[a]	58.7[c]	59.6[a]	3.2	6.1
	Relative composition (mol%)[A]												
	Rha	2.8	1.8	2.8	1.8	2.8	1.7	2.9	1.7	2.9	1.7	0.2	0.1
	Fuc	2.3	1.5	2.3	1.4	2.4	1.5	2.4	1.4	2.4	1.4	0.2	0.1
	Ara	20.1[b]	12.7[d]	19.7[b]	12.4[cd]	18.7[ab]	11.5[bc]	19.0[ab]	11.1[ab]	17.6[a]	10.1[a]	1.0	0.7
	Xyl	12.4	7.8	12.7	8.0	12.9	7.9	12.6	7.4	13.2	7.6	0.6	0.4
	Man	–	2.4	–	2.3	–	2.3	–	2.3	–	2.3	–	0.1
	Gal	12.5[a]	7.9	12.8[ab]	8.1	13.6[bc]	8.4	13.7[bc]	8.1	14.3[c]	8.3	0.7	0.4
	Glc	–	34.6[a]	–	34.6[a]	–	36.0[b]	–	39.0[c]	–	40.0[c]	–	0.9
	GalA	49.9	31.4[b]	49.7	31.4[b]	49.5	30.5[b]	49.3	29.0[a]	49.5	28.6[a]	1.2	0.9

In SDF, Glc and Man are not taken into account as they mostly arose from enzymes used during fibre analysis.
[A] Expressed in the original dry substance, and with correction from process-added sugar.

AIS composition of unpeeled apples was consistent with data found in the literature, with large amounts of glucose and galacturonic acid, followed by high proportions of arabinose and galactose (Massiot & Renard, 1997; Massiot et al., 1994; Renard, 2005b) (Table 4). The major component in the insoluble fraction was glucose but high amounts of galacturonic acid were also detectable (Table 5). The soluble fraction was composed of polymers, mainly containing galacturonic acid, arabinose and galactose, probably pectic polymers. This fraction contained less polysaccharides than did the IDF fraction, since blank weight accounted for almost 50% of the crude SDF recovered after filtration. Fruits from batch A mainly contained more arabinose and galactose and less galacturonic acid than did apples from batch B (Table 4). The same trends were also visible with the SDF and IDF fractions (Table 5), with an overall soluble polysaccharide content higher for batch B. These differences are consistent with apple cell-wall modifications reported by several authors during maturation and storage (Bartley, 1976; Jonhston, Hewett, & Hertog, 2002; Knee, 1973; Wechsler et al., 1996) and thus reflect the more advanced post-harvest maturity stage of batch B (processed two months after batch A and hence stored for two months more). The higher proportion of galacturonic acid in batch B is possibly the direct result of arabinose and galactose loss: with the loss of these pectic side-chain-related sugars (arabinans and arabino(galactans)), cell-walls are relatively more rich in homogalacturonans.

Total polysaccharide content of AIS mostly increased at cooking, then decreased at sugaring (Table 4). A decrease was also visible at refining for both batches but not significant in the case of batch B.

The most relevant changes in polysaccharide composition were related to arabinose and glucose. Arabinose decreased at refining and sugaring whilst glucose increased. The polysaccharide increase upon cooking did thus not lead to marked modifications in polysaccharide composition, except a slight decrease in the galactose fraction for batch A.

As was the case for the changes in the soluble/insoluble fraction, polysaccharide content modifications during processing differed from one batch to another (Table 4). The proportion of soluble polysaccharides increased at cooking but mostly at refining for batch A, whilst it only increased at cooking for batch B. An increase at sugaring was, however, observed for both batches. Insoluble polysaccharides increased at cooking for batch A then kept decreasing during the other processing steps. For batch B, insoluble polysaccharide content started to decrease after refining and during the next processing steps but in a more pronounced way than for batch A. Sum of soluble and insoluble polysaccharide contents gave a close estimation of AIS polysaccharide content (Table 4), except at sugaring (and pasteurisation), where it gave a higher estimation, leading to a different behaviour: an increase at this step whereas AIS polysaccharide content decreased. The polysaccharides involved in soluble and insoluble modifications mainly contained arabinose, galactose, glucose and galacturonic acid (Table 5). Arabinose decreased from the insoluble fraction throughout processing, whilst glucose increased. In the case of batch B, galacturonic acid content also slightly decreased, whilst a loss of galactose was observed at cooking for batch A. In the soluble fraction, arabinose decreased at cooking and refining (mostly for batch A). Slight modification of the galactose content occurred as well: an overall decrease during processing for batch B, and a short drop at cooking for batch A. Finally, galacturonic acid content increased at cooking for batch A.

4. Discussion

During processing of applesauce, two main mechanisms may affect the dietary fibre content and composition:

- heat treatment, leading to pectic depolymerisation;
- mechanical separation of more resistant fractions, namely seeds, skin and carpels.

Two main heat treatments occurred during processing: at cooking and at sugaring, during the storage of applesauce in the flow-regulating tank. The fibre and total polysaccharide contents surprisingly increased at cooking (Tables 3 and 4). As there was no excess water added, this could not be related to leaching of non-fibre components, such as was observed by Nyman et al. (1987) for carrots cooked in water. Two possible explanations are (i) still loss of some internal soluble matter (by dripping of sugar solution) or (ii) a sampling bias, i.e. a change in the skin/flesh/seed ratio between the fresh apples (layering of apples on the belt carrier, with smallest fruit and densest pieces, such as seeds, on the lowest part) and the slurry of cooked apples. SDF and soluble polysaccharides increased at cooking (Table 3): the temperature applied (85 °C) may have contributed to solubilisation by depolymerisation of cell wall polysaccharides previously linked to the insoluble fraction, most probably pectins (Lo et al., 2002; Massiot et al., 1992; Ng & Waldron, 1997; Sila et al., 2005, 2006). The pH of apple fruit (3.5–4.0), moreover, supports the hypothesis of pectic acid hydrolysis (Fraeye et al., 2007; Sajjaanantakul, van Buren, & Downing, 1989).

Despite a slight product concentration during sugaring, the AIS content decreased and was linked to the loss of polymers containing rhamnose and arabinose and consequent gain in glucose (Table 4), consistent with heat-related pectic solubilisation and fragmentation. The prolonged heating could then have led to pectic fragments too small to be retained and thus estimated during the AIS extraction procedures. This was not visible at cooking, either because this step was too short or because it was masked by the change in the peel/flesh/seed ratio. A cumulative temperature effect could also be visible when the sugaring step is reached. Decrease of insoluble and increase of soluble polysaccharides (Table 5) also indicates pectic solubilisation. However, pectic fragmentation due to processing can be dampened by some artefactual pectin β-elimination. Indeed, the pH and temperature conditions applied for the AOAC method is likely to trigger β-elimination (Kravtchenko, Arnould, Voragen, & Pilnik, 1992), leading to pectin fragmentation and possible overestimations of the initial SDF content. In the present case, the decrease in insoluble polysaccharides is largely compensated by an increase of the soluble polysaccharide content (Table 5) whereas total AIS polysaccharide content showed a decrease at this step (Table 4).

Mechanical separation occurred at refining, which unsurprisingly led to a slight decrease in the dry matter following the loss of low-water content tissues. It also triggered a decrease in the total fibre content (AIS and TDF) and, to some extent, in the insoluble fibre fraction (Table 3) and insoluble polysaccharide (Table 5) contents. Indeed, epidermis and carpels both contain more AIS material than do parenchyma, and probably more insoluble fibres (Massiot & Renard, 1997; Massiot et al., 1994). Interestingly, although epidermis and carpels contain high amounts of non-polysaccharidic insoluble components (suberin, cutin, waxes and lignin), their loss did not lead to a relative increase in the polysaccharide content of the fibres. This might be due to the high loss of polysaccharides induced by the discarding of carpels. AIS, IDF and SDF all not only contained proportionally less arabinose but also more glucose (AIS and IDF) or more galactose (SDF) after refining. The pectic profile could thus have been modified following the tissue change, leading to soluble polymers containing more galactose and less arabinose.

Apart from these mechanical and heat treatments, two other phenomena may influence the dietary fibre evolution:

- maturity stage of the fruits;
- co-precipitation of proteins and polyphenols from the apple fruit.

Fruits from batches A and B differed by their post-maturity storage time, with apples from batch B likely to have undergone some pectic solubilisation and pectic side-chain losses during storage. This influenced the impact of heat-processing steps. Indeed, at cooking, the soluble fraction was enriched in polymers containing galacturonic acid (possibly homogalacturonans) for batch A apples whilst the different pectic-related sugar proportions (arabinose, galactose and galacturonic acid) remained constant for batch B (Table 5). As apples from batch B have already experienced pectic solubilisation, the cooking-induced solubilisation did not significantly modify the proportions of the various pectic constituents. In the case of batch A, homogalacturonans seem to have been preferably solubilised. Ng and Waldron (1997) have already observed (on cooked carrots) that pectic fractions rich in galacturonic acid are the first solubilised, branched polymers being possibly more connected with the cell-wall matrix. At sugaring, as well, the soluble/insoluble fibre ratio was modified for batch B yet not for batch A. The insoluble fraction was enriched in glucose, with a slight loss of the pectic-related sugars, galactose and arabinose, and of galacturonic acid in the case of batch B. These differences may again be linked to sample susceptibility to pectin degradation, consequent to the post-harvest maturity stage. For batch A, the loss in insoluble fibres (pectic solubilisation) was equivalent to that of

soluble fibres (pectic intense fragmentation). For batch B, however, the gain in soluble fibre was more intense and compensated the loss of insoluble fibres, indicating that pectins were solubilised but less fragmented, maybe because the pectic profile was already modified by post-harvest storage. Yet again, the bias induced by the AOAC method could also have enhanced the differences between the two batches.

One may finally mention the formation of processing-induced insoluble complexes, possibly increasing IDF content. This includes formation of not only Maillard products during cooking but also interaction of polyphenols, especially procyanidins, with cell wall polysaccharides once the apples are cut and crushed (i.e. between sorting and cooking steps) and during cooking (Le Bourvellec, Guyot, & Renard, 2004; Le Bourvellec & Renard, 2005; Renard, Baron, Guyot, & Drilleau, 2001). Such a cooking effect on AIS content has also been reported on pear by Renard (2005a), who also observed higher AIS yields during early cooking stages (up to 1 h of cooking). In the present case, both flesh/peel/seed ratio and insoluble aggregates formation may have contributed together to an increase in insoluble polysaccharide and IDF contents at cooking, more or less compensating (depending on the batch) the insoluble polysaccharide loss by pectic solubilisation.

In conclusion, apple processing into applesauce only induced a limited loss in dietary fibres (30%, on a fresh weight basis), yet triggered redistribution between the soluble and insoluble fractions, the soluble fraction increasing from 28% to 39% of the total fibre content. The most important changes occurred at refining and sugaring (or more precisely temporary storage of apple purée in a flow-regulating tank). These steps, respectively, caused a loss of insoluble fibres through mechanical treatment and some pectic solubilisation through heat-treatment. Susceptibility to heat-processing differed according to the post-harvest maturity stage of the fruits, the intensity of pectin solubilisation and the nature of the polysaccharides involved (homogalacturonans or branched arabinans and arabinogalactans) being different. Overall, this soluble/insoluble redistribution might affect the effects of dietary fibre *in vivo*.

Acknowledgements

This research was supported by a doctoral grant provided by "Angers Loire Métropole". The authors are very grateful to Marie-Jeanne Crépeau (INRA, Nantes) for her assistance in determination of galacturonic acid content and to Marie-Christine Aumond (SONAS, Angers) for her help with the gas chromatography. Special thanks are also due to M. Le Moigne and L. Perrin for their advice on statistical treatment of the data.

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Food Chemistry 117 (2009) 261–264

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Degradation of organophosphorus pesticides in wheat during cookie processing

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ARTICLE INFO

Article history:
Received 30 July 2008
Received in revised form 12 March 2009
Accepted 31 March 2009

Keywords:
Malathion
Chlorpyrifos-methyl
Wheat
Cookies

ABSTRACT

For investigating carryover of some organophosphorus pesticide residues in the cereal food chain from grain to consumer, a study was set up on wheat bran, flour and cookies, with and without bran. Special emphasis was given to malathion and chlorpyrifos-methyl residues in cookies for better protection of consumers. Pesticide-free wheat was placed in a small-scale model of a commercial storage vessel and treated with these pesticides. The residue levels of insecticides were determined in wheat, as well as in bran, flour and cookies produced from stored wheat at various time intervals during storage. A multiresidue analysis was performed using GC–NPD and GC–MS. Malathion and chlorpyrifos-methyl residue levels were higher than the maximum residue limits (MRLs) in wheat after 240 days of storage. MRLs established by the EC for malathion and chlorpyrifos-methyl in wheat are 8 and 3 mg kg^{-1}, respectively. The residue levels of insecticides in flour samples also exceeded the MRL (2 mg kg^{-1} for both insecticides). Eight months of storage were not effective for reducing the residues in wheat to the levels below MRLs. Although, considerable amounts of the insecticides remained in the bran and flour, the cookie processing significantly reduced the concentrations in general. Chlorpyrifos-methyl was more persistent than was malathion and comparatively less degradation occurred during milling and cookie processing due to its physicochemical properties.

1. Introduction

There are increasing concerns over the use of organophosphorus insecticides linked to health, which are widely used for protecting stored commodities from pests (Collins, 2006). The grain storage pesticides were identified as among the key pesticide residues in cereals, based on their frequency of occurrence in surveys. The main residues detected were storage insecticides, such as chlorpyrifos-methyl and malathion (Balinova, Mladenova, & Obretenchev, 2006). They are applied post-harvest to wheat, as grain protectants, because of their relatively low rates of degradation in the grain under practical storage conditions. Chlorpyrifos-methyl is listed as a product unlikely to present acute toxicity and malathion is listed as a slightly hazardous compound in the WHO's recommended classification of pesticides by hazard (WHO, 1997). Malathion is a neurotoxin and toxic to aquatic organisms and certain species of birds and mammals at concentrations that may be attained in the open environment (ATSDR, 2000).

Despite the considerable amount of research that has been carried out on the application of malathion to wheat, there are limited investigations on the persistence of chlorpyrifos-methyl as residues in stored grains (Uygun, Senoz, & Koksel, 2008). Unit processes, such as washing, milling and baking, are important factors leading to reduction of residues left on grain after harvest or post-harvest treatment. These can often substantially reduce the residue levels in crops (Uygun, Koksel, & Atlı, 2005). However, in some cases, more toxic transformation products or metabolites can be formed during processing. There is still not sufficient information on the role of the baking process in degrading pesticide residues in flour and bran.

Cookies are one of the best known snack products. Consumption in Turkey currently runs at around 0.5 million tones per year, which is roughly equivalent to 40 packets (200 g) per person per year (Anonymous, 2003). They are a substantive part of children's diet. For better protection of the health of consumers, particularly children, a special emphasis has been given in this study to residues of pesticides in cookies.

The purpose of the study was to investigate the fate and magnitude of malathion and chlorpyrifos-methyl residues in wheat under local conditions of storage and to examine the reduction of the residues in flour, bran and cookies prepared according to the AACCI standard method (AACCI, 2000). The cookies, with bran (10%) and without bran, were prepared at each storage period.

2. Material and methods

2.1. Materials

Pesticide and metabolite standards, malathion, malaoxon, isomalathion and chlorpyrifos-methyl, were purchased from

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doi:10.1016/j.foodchem.2009.03.111

Promochem Ltd. (Germany). Commercial malathion (Gold malathion 20EC) was purchased from Safa Tarim (Turkey) and chlorpyrifos-methyl (Reldan 2E) was obtained from Dow Agro Science (UK). The wheat sample (cv. Bayraktar) with known origin was supplied by Field Crops Improvement Center, Ankara, Turkey. The sample was cleaned on a Carter Dockage Tester before the treatment.

2.2. Sample treatment with the insecticides

A small-scale model (65 × 45 × 45 cm) of a commercial storage vessel was built for laboratory experiments. The inner surfaces of wooden stores were covered with thin metal sheet. Wheat was treated with malathion and chlorpyrifos-methyl according to good application practice which is regulated by the Registered Agrochemicals for Pest Management Warehouse Pest Recommendations of the Turkish Ministry of Agriculture. Malathion (190 g/l) and chlorpyrifos-methyl (227 g/l) emulsions were prepared at the doses of 650 ml/100 m^2 and 425 ml/100 m^2, respectively, and then sprayed onto the surfaces of the stores. After standing for half an hour to evaporate the excess of solvent, the wheat (10 kg) samples were placed into storage vessels by mixing to provide a homogeneous distribution of pesticide on grain. Time-zero samples were taken for the analysis after 2 h. The rest of the samples were stored at ambient temperature for three months. The grain was analysed at various time intervals during storage.

2.3. Cookie processing

The wheat samples (1 kg) were milled after each storage period in a Brabender Quadrumat Junior laboratory mill (Duisburg, Germany) to obtain straight-grade flour and bran consisting of endosperm and the outer layers of grain, respectively. Duplicate samples from whole ground grain, flour and bran were used for analysis. The cookies were prepared according to the wire-cut cookie formulation of AACCI (Method No: 10–54) (AACCI, 2000). In bran cookies, bran was substituted for a portion of wheat flour at the level of 10%.

2.4. Extraction

Wheat (50 g), bran (12.5 g) and flour (50 g) samples were homogenised with ethyl acetate (100 ml) and anhydrous sodium sulphate (5 g) in a high-speed blender for 2 min. The homogenates were filtered and the extracts were concentrated to 2 ml in vacuo at 40 °C, using a rotary evaporator. The extracts were applied onto GC and GC–MS systems without a clean-up step. The cookie samples (25 g) were extracted with ethyl acetate (100 ml), standing overnight. Then the samples, dried by anhydrous sodium sulphate, were cleaned according to the EN 1528/3 method (EN, 1996) and the cleaned extracts were concentrated to 2 ml in vacuo at 40 °C, using a rotary evaporator.

2.5. GC–NPD and GC–MS

Gas chromatography was performed using an Agilent 6890 gas chromatograph equipped with a nitrogen phosphorus detector and capillary column (DB-5 30 m × 0.32 mm ID, 0.25 μm film thickness), using helium carrier gas at a flow rate of 1 ml min^{-1}. The oven temperature programme was: initial temperature isothermal at 50 °C, then from 50 to 150 °C at 25 °C min^{-1}, from 150 to 170 °C at 10 °C min^{-1}, from 170 to 200 °C at 2 °C min^{-1}, from 200 to 250 °C at 10 °C min^{-1}, then held for 2 min at 250 °C. Injector and detector temperatures were 250 and 300 °C, respectively. Gas chromatography–mass spectrometry was performed using an Agilent 5973 mass detector under the same conditions as for gas chromatography. GC–MS was used for confirmation of the pesticide identification. Electron ionisation (EI mode) was used to obtain the mass spectra of the samples.

Identification and confirmation of the pesticides were based on their GC retention times and comparison of their sample mass spectra with the characteristic ions in the reference standards mass spectra. Single-point calibration was used to estimate pesticide concentrations (SANCO, 2003). In GC–NPD analyses, limits of detection were calculated by using a signal-to-noise ratio of 3 and determined as 0.02 and 0.01 mg kg^{-1} for malathion and chlorpyrifos-methyl, respectively.

2.6. Recovery

The recovery studies were performed by using blank samples of untreated wheat grain and pesticide-free cookies. The weights of blanks were the same as the weights of analytical samples of treated grains. Fortifications were carried out by the addition of calculated volumes of working standard solutions, in order to obtain three concentration levels of the insecticides studied: at MRLs, half of the MRLs and twice the MRLs. The fortified samples were analysed according to the method described above. Data derived from these experiments are presented in Table 1.

2.7. Statistical analysis

Data were statistically evaluated by the one-way analysis of variance (ANOVA) procedure. When significant differences were found, the LSD (least significant difference) test was used to determine the differences among means.

3. Results and discussion

3.1. Degradation of malathion in wheat during storage, milling and cookie processing

The effects of storage, milling and processing on breakdown of malathion were examined at various times during eight months of storage at ambient temperature. The results of the pesticide degradation during storage and processing are presented in Table 2. The residues in wheat were dissipated relatively quickly during the first two weeks of storage, then slightly increased the first month; thereafter, they decreased at a slower rate until the end of storage. The increase might be related to migration of a proportion of the lipophilic insecticide through the bran and germ, which have high levels of triglycerides. Although, 63% of the malathion residue in wheat was degraded after eight months, the amount left was still high (Fig. 1). Similarly, malathion residue levels in stored bran increased gradually with storage time and this was explained by the penetration of malathion into the seeds during storage (Lalah & Wandiga, 1996). In another study, the pesticide residues in faba beans and soybeans increased during storage. The residues showed a slow but definite increase with storage period, and a higher percentage was observed in stored soybeans, due to their oily nature, than in faba beans (Zayed, Farghaly, Mahdy, & Soliman, 2007).

The residue concentrations were greatly reduced during the cookie processing. The conditions used in the cookie process, such as high temperature (205 °C) and degree of moisture loss are important for the quantitative effects on residue levels. Malathion in the cookies and bran cookies, produced from the wheat stored for eight months, decreased by about 45% and 60%, respectively (Fig. 1). Rates of degradation and volatilisation of the residues were increased by the heat involved in the process.

Although, the amount of degradation was high, the levels of malathion residue were still excessive in the cookies, particularly

Table 1
Recoveries and standard deviations of the pesticides in wheat samples at different fortification levels ($n = 6$).

Insecticides	Fortification (mg kg^{-1})	Recoveries (%)				LOD (mg kg^{-1})
		Wheat	SD±	Cookies	SD±	
Malathion	16 (2 × MRL)	79	1.63	80	1.67	
	8 (MRL)	84	2.11	79	1.46	
	4 (0.5 × MRL)	86	3.53	80	1.29	0.02
Average		**83**	**3.87**	**80**	**1.27**	
Chlorpyrifos-methyl	6 (2 × MRL)	79	3.01	76	1.52	
	3 (MRL)	78	2.00	76	1.59	
	1.5 (0.5 × MRL)	80	3.69	76	0.74	0.01
Average		**79**	**3.12**	**76**	**1.37**	

Table 2
Residue levels of malathion in stored wheat and its products at various times during storage (mg kg^{-1}).

Time (days)	Wheat	Bran	Flour	Cookies	Bran cookies
0	61.25a	102.50a	7.20a	1.95a	3.07a
14	32.85c	77.35b	5.73bc	1.35b	2.80a
30	39.60b	50.95c	6.46ab	1.75a	3.08a
90	29.05d	50.05c	5.19c	1.38b	1.64b
150	27.10d	29.60d	5.20c	1.14bc	1.30b
240	22.80e	25.85d	4.78c	1.10c	1.23b

Data are the means of four replicates and expressed on a dry basis.
Values followed by the same letter in the same column are not significantly different ($p < 0.05$).

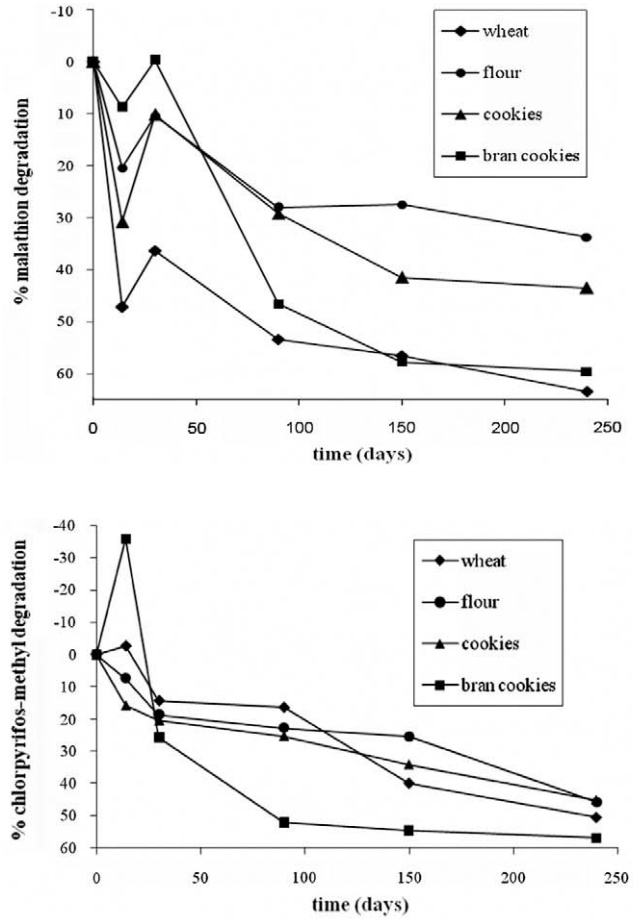

Fig. 1. Percent residue degradation of malathion and chlorpyrifos-methyl in wheat, cookies and bran cookies.

in the bran cookies. Since the residues were concentrated in the bran, the residue levels were found to be approximately 1.5–2 times higher in the bran cookies than in cookies without bran. In the cookies, the residue concentrations significantly increased during the first month of storage, in accordance with the rise in the wheat during the same period. These results were quite comparable to the results obtained from previous work carried out by Uygun et al. (2005).

The metabolites of malathion were also studied. Fortunately, no isomalathion or malaoxon was detected in any of the samples. This is probably due to the pesticide application method, in which the pesticide was applied on the surface of the store, not directly onto the grain.

3.2. Degradation of chlorpyrifos-methyl in wheat during storage, milling and cookie processing

The results showed that chlorpyrifos-methyl levels of wheat, as well as of its milling and baking products, decreased towards the end of storage (Table 3). After eight months of storage, the residues of chlorpyrifos-methyl in wheat were degraded by about 50%. The persistence of chlorpyrifos-methyl residues in wheat was found to be higher than that of malathion under the same conditions. Similar to the results of this study, it was reported that chlorpyrifos-methyl was more persistent than was malathion in stored grains (Holland, Hamilton, Ohlin, & Skidmore, 1994; Matthews, 1990). This might be related to its higher octanol–water partition coefficient and lower vapour pressure. Both malathion and chlorpyrifos-methyl are lipophilic compounds. However, log Kow values reported in the literature are 2.74 and 4.23 for malathion and chlorpyrifos-methyl, respectively (Tomlin, 1994). Therefore, higher carryover percentages from wheat to cookies of chlorpyrifos-methyl might be due to its higher log Kow value.

3.3. Carryover of the pesticides from wheat to cookies

At the beginning of storage, the carryover percentages of malathion residues were found to be about 3% and 5% from wheat to

Table 3
Residue levels of chlorpyrifos-methyl in stored wheat and its products at various times during storage (mg kg^{-1}).

Time (days)	Wheat	Bran	Flour	Cookies	Bran Cookies
0	18.8ab	32.9ab	3.69a	1.46a	2.41b
14	19.3a	35.4a	3.42ab	1.22b	3.29a
30	16.0bc	31.5bc	3.00ab	1.16bc	1.80bc
90	15.8c	29.0c	2.85bc	1.08bc	1.16 cd
150	11.3d	13.3d	2.75bc	0.95 cd	1.10 cd
240	9.35d	10.0d	2.01c	0.80d	1.04d

Data are the means of four replicates and expressed on a dry basis.
Values followed by the same letter in the same column are not significantly different ($p < 0.05$).

Table 4
Carryover percentages of malathion and chlorpyrifos-methyl from wheat to flour and cookies.

Time (days)	% Carryover Wheat → flour		% Carryover Flour → cookies		% Carryover Wheat → cookies		% Carryover Wheat → bran cookies	
	M	CM	M	CM	M	CM	M	CM
0	12	20	27	40	3	8	5	13
14	18	18	24	36	4	6	9	17
30	16	19	27	39	4	7	8	11
90	18	19	27	38	5	7	6	7
150	19	24	22	35	4	9	5	10
240	21	22	23	40	5	9	5	11

M: malathion; CM: chlorpyrifos-methyl.

cookies and bran cookies, respectively (Table 4). As the level of malathion decreased during storage, the carryover of the residues from wheat to cookies slightly increased. This has been interpreted as being due to the residues tending to move into the deeper layers of the grain (Holland et al., 1994).

Although, the initial concentration of chlorpyrifos-methyl in wheat was significantly lower than that of malathion, carryover percentages of chlorpyrifos-methyl from wheat to the cookie samples were higher than those of malathion (Table 4). This could be explained by lipophilic properties of the pesticide that was retained by the fat in the cookies. The retention mechanism of lipophilic pesticides in cookies could be compared with that in oil seeds. It has been reported that the dominant feature in the processing of oil seeds is the retention of lipophilic pesticides in the oil or fat fraction. Furthermore, higher residue concentrations are produced by post-harvest treatment against storage pests that can lead to elevated residue levels in the oils (Holland et al., 1994).

4. Conclusion

In this study, the approved doses of pesticides for stored grain were applied in a small-scale storage vessel. Although, the approved doses of insecticides for stored grain were used, the residue levels exceeded the MRLs during the whole storage period. MRLs for malathion and chlorpyrifos-methyl in wheat, established by the EC, are 8 and 3 mg kg^{-1}, respectively (European Commission, 2005). In 2008, the Codex Alimentarius Commission proposed new MRLs for malathion (0.5 mg kg^{-1} and 0.2 mg kg^{-1} in wheat and white flour, respectively) and 10 mg kg^{-1} for chlorpyrifos-methyl in wheat (FAO/WHO, 2008). The proposed reduction in the MRL for malathion initiated new discussions and signified the requirement for comprehensive studies on this matter.

The storage period was generally not effective enough to reduce the residues in wheat to levels at or below the MRLs of the insecticides. Malathion and chlorpyrifos-methyl residue levels were higher than the MRLs established by the EC in wheat after 240 days of storage.

For white flour, the MRLs of malathion and chlorpyrifos-methyl are 0.2 and 2 mgkg^{-1}, respectively (FAO/WHO, 2008). The residue levels in all flour samples exceeded the limits excessively. This might be due to application of pesticides on wheat at pilot scale. The chlorpyrifos-methyl residues in the flour sample produced from the wheat stored for 240 days were very close to the MRL.

In general, the cookie processing significantly reduced the concentrations of the insecticides. But, there are no official limits for pesticide residues in cookies for comparison. If the MRL for malathion in white flour proposed by Codex (0.2 mgkg^{-1}) is considered, the residue levels of the pesticides in all cookie samples were above the limit. On the other hand, if the MRL for chlorpyrifos-methyl in white flour, established by Codex (2 mgkg^{-1}), is considered, the residue levels of the pesticides in all cookie samples were below the limit, except in the bran cookies produced from the wheat stored for 0 and 14 days.

Acknowledgements

The authors thank The Field Crops Research Center of Turkey for supplying the pesticide-free wheat.

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Food Chemistry 117 (2009) 265–271

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Volatile compounds produced by the probiotic strain *Lactobacillus plantarum* NCIMB 8826 in cereal-based substrates

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ARTICLE INFO

Article history:
Received 3 July 2008
Received in revised form 11 March 2009
Accepted 31 March 2009

Keywords:
Lactic acid bacteria
Lactobacillus plantarum
Probiotic
Cereal-based media
Volatile compounds
Flavour

ABSTRACT

The production of volatile compounds by the probiotic strain, *Lactobacillus plantarum* NCIMB 8826, in cereal-based media (oat, wheat, barley and malt) was investigated. Sixty compounds, including fatty acids and their esters, amides, alcohols, aldehydes, aromatic hydrocarbons, furans, ketones, peroxides and pyrans, were identified. *L. plantarum* significantly changed the aroma profile of the four cereal broths, and each substrate showed a specific volatiles profile. Oat and barley media were the substrates more influenced by the fermentation process. The most abundant volatiles detected in oat, wheat, barley and malt were oleic acid, linoleic acid, acetic acid, and 5-hydroxymethylfurfural, respectively. Analysis of these products confirmed the heterofermentative pathway of *L. plantarum*. Maillard compounds were not detected during sterilisation and fermentation. This study is the first to report the volatile composition of probiotic drinks produce with non-supplemented cereal-based media and the results obtained could contribute to the development of new non-dairy probiotic formulations.

1. Introduction

Consumers, worldwide, are becoming increasingly aware of the relationship between food or food constituents and health. Functional products, *i.e.* foods or beverages offering specific health benefits beyond basic nutrition, are increasingly valued.

Within functional foods, probiotics, with prebiotics, represent one of the fastest growing sectors. Probiotic foods are fermented formulates containing sufficient numbers of selected live microorganisms to beneficially modify the intestinal microbiota of the host (Fuller, 1989; Havenaar & Huis in't Veld, 1992a,b).

Commercial probiotic foods are primarily dairy-based, although in recent years other substrates have been explored to develop new probiotic formulations (Prado, Parada, Pandey, & Soccol, 2008). Among the tested raw materials, cereals are becoming one of the promising substrates for future developments.

Cereals are grown over 73% of the total world harvested area and account for 60% of world food production (FAO, 2006). Cereal grains and their fractions contain many functional compounds, such as essential fatty acids, phytosterols, phenolic compounds or resistant starch, and the consumption of whole grains has been associated with lower incidences of certain cancers and cardiovascular diseases (Baublis, Lu, Clydesdale, & Decker, 2000; Hudson, Dinh, Kokubun, Simmonds, & Gescher, 2000; López-Varela, González-Gross, & Marcos, 2002; Peterson, 2001; Truswell, 2002; Welch, 1995). In addition, cereals and cereal fractions have been proved to support growth of probiotic lactic acid strains (Charalampopoulos, Wang, Pandiella, & Webb, 2002a; Kedia, Vázquez, & Pandiella, 2008a, 2008b).

For centuries, fermentation with lactic acid microorganisms has been employed to preserve or upgrade the digestibility and stability of foods. Fermentation can also change the flavour and texture of the substrates and has been used to improve the organoleptic quality of foods (Gélinas & McKinnon, 2000).

These facts suggest that the use of cereals as substrates for probiotic cultures could produce a fermented food with health-promoting properties that could fulfil consumer expectations. However, the acceptance of a new probiotic formulate is not only determined by its health-promoting qualities. Although consumers are increasingly demanding products with functional properties, the consumption patterns of new products are strongly determined by their sensorial characteristics (Breslin, 2001). Flavour is one of the most important characteristics in the sensorial profile of a specific food and plays a decisive role in consumer acceptability. The analysis of volatile flavour components tends to be one of the main parameters to take into account in the development of a novel fermented food (McFeeters, 2004).

The use of oat, wheat, barley and malt substrates to support the growth of *Lactobacillus plantarum* to probiotic levels has been

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doi:10.1016/j.foodchem.2009.03.112

previously reported (Charalampopoulos, Pandiella, & Webb, 2002b; Kedia et al., 2008b). The media used in this work are suitable for the formulation of probiotic beverages. Alternatively, flavour characterisations of some probiotic dairy products and studies of the volatile compounds of several cereals and cereal-based products have been previously reported (Grosch & Schieberle, 1997; Maga, 1978; Modzelewska-Kapituła, Kłębukowska, & Kornacki, 2008; Pelletier, Faurie, François, & Teissier, 2007; Saarela, Mogensen, Fondén, Mättöa, & Mattila-Sandholm, 2000; Zhou, Robards, Glennie-Holmes, & Helliwell, 1999; Østlie, Helland, & Narvhus, 2003). However, attempts to characterise cereal-based probiotic drinks have not yet been reported.

In this work, the direct culture of a probiotic lactic acid strain, *L. plantarum* NCIMB 8826, grown in oat, wheat, barley, and malt broths without additional growth enhancers, has been studied. The main objective was to identify and compare the volatile compounds of natural occurrence in the different media, as well as to study the production of flavour compounds by the probiotic bacteria in the cereal-based substrates.

2. Materials and methods

2.1. Cereal substrates

Three different cereals, wheat (*Triticum aestivum*), oat (*Avena sativa*), and barley (*Hordeum vulgare*), together with spray-dried malt extract (Muntons PLC, UK), were used in this study. Cereals were harvested at ripening stage and stored at 8 °C. No spoilage due to contamination from insects or fungi was detected during storage.

2.2. Microorganism and inoculum

A selected *L. plantarum* bacterial strain (NCIMB 8826; National Collection of Industrial and Marine Bacteria, UK), isolated from human saliva, was used as inoculum in this study. The strain was preserved in 40% glycerol at −30 °C. A frozen ampoule containing the lactic bacteria was thawed and transferred to a 25 ml screw-capped glass bottles with 20 ml of MRS broth (Oxoid, UK). The bottles were incubated at 37 °C to the exponential phase of growth (24 h). The bacterial suspensions were then used to inoculate the fermenting media at 1% (v/v). In all cases, the initial microbial concentration was approximately 7.5 $\log_{10}$ CFU/ml.

2.3. Fermentation

The cereal mashes used as fermentation substrates were prepared from whole-grain flour (or dried malt extract) and water at a concentration of 5% (w/w). Grains were ground in a hammer mill (Laboratory Mill 120, Perten Instruments, Sweden) equipped with a 0.8 mm sieve to produce homogeneous samples. After milling, the flour was stored in sealed bags at −30 °C to prevent contamination and to halt the action of cereal enzymes. The cereal flour without water was autoclaved (121 °C, 20 min) and afterwards mixed with sterilised distilled water to form the fermentation broth. The slurries were then inoculated and incubated for 24 h at 37 °C to produce the cereal-based probiotic drinks. All the fermentations were carried out in 500 ml screw-capped glass bottles with 400 ml of medium. Samples were taken at the end of the fermentation and kept at −30 °C for further analysis.

2.4. Volatile compounds extraction

A 35 ml sample with 20% w/v of sodium chloride (Fluka, Switzerland) was centrifuged at 4500*g* and 2 °C for 5 min. Twenty-five millilitre of the supernatant were placed in a 80 ml screw-cap flask, mixed in a proportion a 1:1 with ethyl acetate (Sigma–Aldrich, Germany) and stirred for 40 min. Then the mixture was centrifuged again (2000*g*, 2 °C, 5 min). The organic phase was transferred

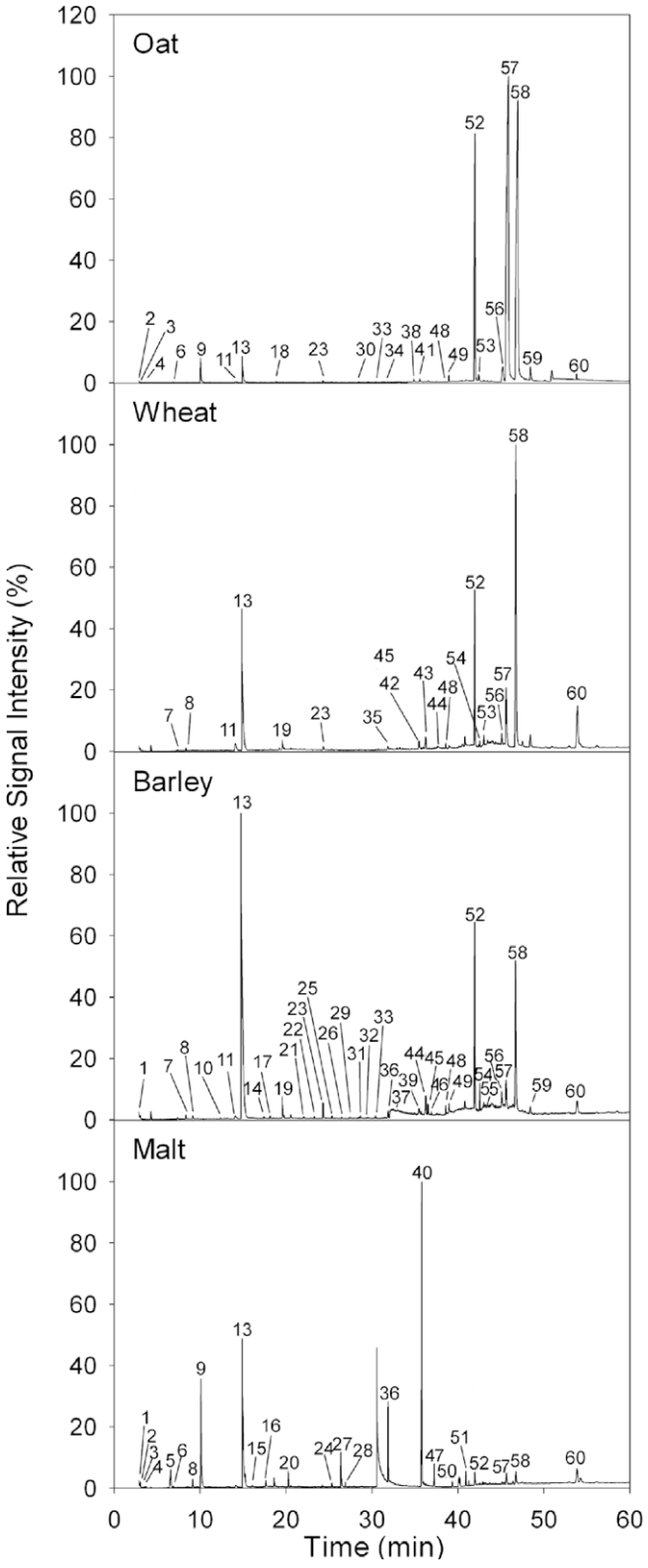


Fig. 1. Gas chromatograms of the volatile compounds produced by *L. plantarum* NCIMB 8826 in the cereal-based media: (a) oat; (b) wheat; (c) barley; (d) malt. Peak identities are shown in Table 1.

to a 100 ml round-bottom flask and concentrated to 1.5 ml using a vacuum rotary evaporator (Buchi, Switzerland) at 40 °C.

2.5. Gas chromatography–mass spectrometry (GC–MS)

GC–MS analysis was carried out with an Agilent 6890 N Series gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 5973 Series quadrupole mass-selective spectrometer, operated in electron impact mode (70 eV). A 30 m × 0.25 mm (ID) DB-Wax capillary column (Agilent Technologies), with 0.1 μm film thickness, was used.

The sample (1 μl) was injected in split mode (1:50) and the injector temperature was 230 °C. Helium was used as carrier gas at a constant flow of 1.1 ml/min. The oven temperature was programmed as follows: at 40 °C for 7 min, increased at 5 °C/min to 220 °C, and then held constant for 17 min.

Table 1
Relative abundances* of volatile compounds detected in cereal-based media before fermentation (*B.F.*) and after fermentation (*A.F.*) with *L. plantarum* NCIMB 8826.

Peak number	Compound	Oat		Wheat		Barley		Malt	
		B.F.	*A.F.*	*B.F.*	*A.F.*	*B.F.*	*A.F.*	*B.F.*	*A.F.*
1	Isobutanol	–	–	–	–	–	0.06	–	0.28
2	Ethylbenzene	–	0.007	–	–	–	–	–	0.07
3	1,4-Xylene	–	0.007	–	–	–	–	–	0.08
4	1,2-Xylene	–	0.01	–	–	–	–	–	0.14
5	Isoamyl alcohol	–	–	–	–	–	–	–	3.23
6	2-Ethoxyethanol	–	0.02	–	–	–	–	–	0.28
7	1-Oxiranylethanone	0.04	0.03	0.8	0.3	3.4	0.4	–	–
8	Acetoin	–	–	–	0.04	–	0.3	–	0.94
9	2-Ethoxyethyl acetate	–	1.4	–	–	–	–	7.3	15.72
10	2-Cyanoethyl hexyl ether	–	–	–	–	–	0.1	–	–
11	n-Tetradecane	0.01	0.08	–	1.8	2.7	0.3	–	0.64
12	Furfural	–	–	–	–	–	–	0.8	2.33
13	Acetic acid	2.9	1.5	17.5	17.3	39.3	47.3	14.4	20.83
14	Propionic acid	–	–	–	–	–	0.2	–	–
15	1-(2-Furyl)ethanone	–	–	–	–	–	–	–	0.14
16	2,3-Butanediol	–	–	–	–	–	–	0.5	1.03
17	Isobutyric acid	–	–	–	–	–	0.2	–	–
18	2,2-Dichloroethanol	0.1	0.05	–	–	–	–	–	–
19	Butyric acid	0.4	0.1	2.9	0.9	1.0	1.7	1.0	0.28
20	2-Furanmethanol	–	–	–	–	–	–	0.8	1.43
21	Valeric acid	–	–	–	–	–	0.2	–	–
22	Methyl laurate	–	–	–	–	–	0.2	–	–
23	Caproic acid	0.2	0.09	–	0.4	0.2	1.6	–	–
24	2-Phenylethanol	–	–	–	–	–	–	–	0.34
25	Butylated hydroxytoluene	–	0.02	0.03	0.05	–	0.2	–	–
26	Heptanoic acid	–	–	0.1	0.3	–	0.2	–	–
27	Maltol	–	–	–	–	–	–	4.7	4.08
28	2-(Methoxycarbonyl)furan	–	–	–	–	–	–	0.1	0.52
29	Isoamyl salicylate	–	–	–	–	–	0.2	–	–
30	Octanoic acid	–	0.02	–	–	–	–	–	–
31	Amyl salicylate	–	–	–	–	–	0.2	–	–
32	1,4-Dimethylpiperazine	–	–	–	–	–	0.1	–	–
33	Nonanoic acid	0.01	0.02	–	–	–	0.2	–	–
34	Methyl palmitate	–	0.03	–	–	–	–	–	–
35	Ethyl palmitate	–	–	–	0.5	–	–	–	–
36	Pyranone	–	–	–	–	–	0.6	12.5	8.07
37	2-Butanol	–	–	–	–	–	1.1	–	–
38	Methyl octadec-9-enoate	0.01	0.4	–	1.2	–	–	–	–
39	2-Dodecanol	–	–	–	–	–	0.8	–	–
40	5-Hydroxymethylfurfural	–	–	–	–	–	–	38.3	27.35
41	Methyl linoleate	0.1	0.2	–	–	–	–	–	–
42	1-Octadecene	–	–	–	0.3	–	–	–	–
43	9,17-Octadecadienal	–	–	–	0.5	–	–	–	–
44	Bis(2-methylpropyl)- 1,2-benzenedicarboxylate	0.3	0.05	–	1.4	–	1.9	–	–
45	Vanillin	0.04	0.1	–	–	–	0.8	–	–
46	2-Methoxyethylbenzene	–	–	–	–	–	0.3	–	–
47	2-Thiopheneethanol	–	–	–	–	–	–	–	2.03
48	Dibutyl phthalate	–	0.06	–	0.5	–	0.6	–	–
49	Myristic acid	0.2	0.3	–	–	–	0.7	–	–
50	3-(m-amino benzoyl)-2-methyl propanoate	–	–	–	–	–	–	–	0.73
51	Benzocaine	–	–	–	–	–	–	–	0.57
52	Palmitic acid	14.2	0.4	18.6	11.7	21.4	14.2	5.4	1.39
53	Palmitoleic acid	–	0.6	–	0.2	–	–	–	–
54	1,4-Dihydroxy-2-methoxybenzene	–	–	–	0.4	–	1.7	–	–
55	Tetradecanamide	–	–	–	1.3	1.6	1.0	–	–
56	Octadecenoic acid	1.7	1.5	1.5	1.0	2.6	2.0	–	–
57	Oleic acid	41.9	50.6	13.2	7.8	7.1	3.4	4.9	1.78
58	Linoleic acid	36.4	40.9	43.5	42.3	13.9	13.8	5.1	2.07
59	Linolenic acid	0.7	1.1	–	–	–	1.1	–	–
60	Oleamide	0.7	0.4	1.8	9.9	6.7	2.5	4.1	3.65

–, Not detectable.
* Relative abundance of the volatile compounds is expressed as $(compound_{peak\ area}/total\ compounds_{peak\ area}) \times 100$. Results are the averages of triplicates.

Table 2
Bibliographic information of the flavour properties of some volatile compounds detected.

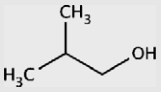
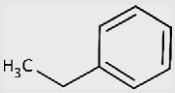
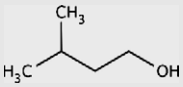
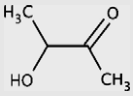
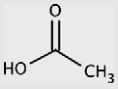
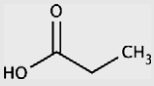
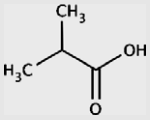
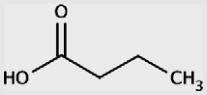
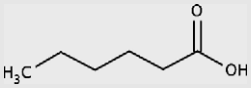
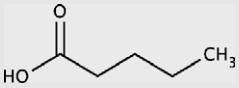
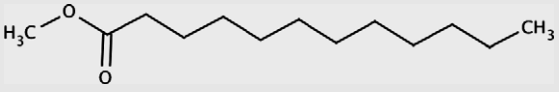
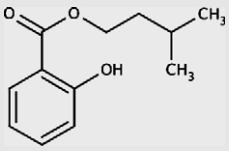
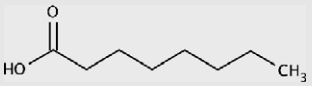
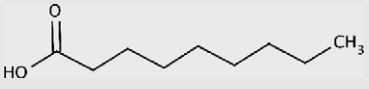
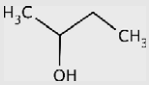
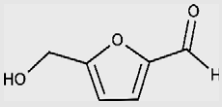
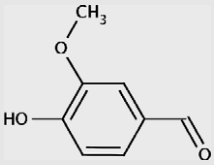
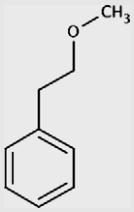
Compound	Structure	Flavour attribute	References
Isobutanol		Acidic, fruity, floral	c
Ethylbenzene		Fruity	a
Isoamyl alcohol		Pungent	c
Acetoin		Floral, wet	m, f
n-Tetradecane		Astringent, harsh	b
Acetic acid		Acidic, vinegary	u
Propionic acid		Acidic, aromatic	e
Isobutyric acid		Sweaty	k
Butyric acid		Sour, rancid, cheesy	v, p, i
Caproic acid		Sweaty, fatty	c, p
Valeric acid		Sweaty	k
Methyl laurate		Fruity	t, g
Isoamyl salicylate		Fruity	s
Octanoic acid		Fatty-acid, dry	l
Nonanoic acid		Sweet, butter-like	o

Table 2 (*continued*)

Compound	Structure	Flavour attribute	References
2-Butanol		Leaves, grasses	d
5-Hydroxymethylfurfural		Bitter, astringent	q, r
Vanilla		Vanilla	j
2-Methoxyethylbenzene		Fruity	h

[a] Aparicio, Morales, and Alonso (1997); [b] Borse et al. (2006); [c] Clary, Gamache, Cliff, Fellman, and Edwards (2006); [d] Cortes, Gil, and Fernández (2005); [e] Dieguez, Diaz, De La Pena, and Gómez (2002); [f] Escudero, Campo, Farina, Cacho, and Ferreira (2007); [g] Ferrari et al. (2004); [h] Kawasaki (2005); [i] Kishimoto, Wanikawa, Kono, and Shibata (2006); [j] Lee and Noble (2003); [k] Lee and Noble (2006); [l] Pérez-Prieto, López-Roca, and Gómez-Plaza (2003); [m] Romano and Suzzi (1993); [n] Rychlik, Sax, and Schieberle (2006); [o] Shiratsuchi, Yoshimura, Shimoda, Noda, and Osajima (1995); [p] U et al. (2007); [q] Vernin and Parkanyi (1982); [r] Vernin and Vernin (1982); [s] Vlasova et al. (1995); [t] Wang, Xu, Zhao, and Li (2004); [u] Xu, Fan, and Qian (2007); [v] Zea, Moyano, Moreno, Cortes, and Medina (2001).

The data acquisition was performed in scan mode. The following MS parameters were applied: m/z range 29–350; scans/s 4.44; EI source temperature 230 °C; quadrupole temperature 150 °C; EM voltage + 294 V relative to the value established by the tune programme (2788). Compounds were identified by the MSD ChemStation library of mass spectra (Data Analysis Application 2003, Agilent Technologies).

All analyses were performed in triplicate. Results were reported as relative abundance expressed as $(compound_{peak\ area}/total\ compounds_{peak\ area}) \times 100$.

3. Results

Gas chromatograms of the volatile compounds in the fermented wheat, oat, barley, and malt broths after 24 h of incubation are shown in Fig. 1. Details about the peak identities and relative abundances of the volatile compounds expressed as $(compound_{peak\ area}/total\ compounds_{peak\ area}) \times 100$ are shown in Table 1. Among the identified organic compounds there are acids, amides, alcohols, aldehydes, aromatic hydrocarbons, esters, furans, ketones, peroxides, and pyrans.

High concentrations of several volatile compounds were found in all cereal media before the fermentation (Table 1), some of them detected in significant amounts in all substrates and others in specific media:

(1) Compounds found in all the substrates: oleic acid (4.9–41.9%) and linoleic acid (5.1–43.5%) showed significant peaks in all chromatograms, particularly in wheat and oat, where the sum of the two compounds represented more than 57% and 78%, respectively. Other organic acids found in all substrates were palmitic acid (5.4–21.4%) and acetic acid (2.9–39.3%).

(2) Exclusive compounds found in malt media: 5-hydroxymethylfurfural (38.3%), pyranone (12.5%), 2-ethoxyethyl acetate (7.3%) and maltol (4.7%). These four compounds account for 63% of the total volatile compounds in the malt broth before inoculation.

Other chemicals were found in smaller amounts. Butyric acid (0.4–3%) and oleamide (0.7–6.7%) were observed in all substrates. Furfural (0.8%) and 2-furanmethanol (0.8%) were present only in malt, and 1-oxiranylethanone (0.04–3.4%) and octadecenoic acid (1.5–2.6%) were found in all the media but malt.

Small amounts of 2,2-dichloroethanol, methyl 9-octadecenoate, methyl linoleate, oleic acid, bis-(2-methylpropyl)-1,2-benzenedicarboxylate, vanillin, myristic acid, nonanoic acid and linolenic acid were detected in oat, heptanoic acid, and butylated hydroxytoluene in wheat, tetradecanamide in barley, 2,3-butanediol and 2-(methoxycarbonyl)furan in malt, and caproic acid and n-tetradecane in oat and barley broths.

All the substances detected in significant amounts in the raw media were still present in the fermented product, also at considerable relative concentrations (Table 1). However, the relative concentration of these compounds dropped in most media. Only certain compounds showed a clear increase after fermentation: acetic acid in barley (39.4 → 47.3%) and malt (14.4 → 20.8%); oleic acid (41.9 → 50.6%) and linoleic acid (36.4 → 40.9) in oat; and 2-ethoxyethyl acetate in malt (7.3 → 15.7%).

A number of new compounds appeared in the fermented product. None of these were detected in all media and, in most cases, they had relatively low concentrations. Moderate amounts of isoamyl alcohol (3.23%) and 2-thiophenethanol (2.03%) were also detected in the fermented malt medium; 2-butanol (1.1%) in barley; and 1,4-dihydroxy-2-methoxybenzene in wheat and barley (0.4% and 1.7%, respectively).

Table 3
Values of pH in the cereal-based substrates before fermentation (*B.F.*) and after (*A.F.*) with *L. plantarum* NCIMB 8826.

Media	pH	
	B.F.	*A.F.*
Oat	5.96	4.34
Wheat	5.26	4.85
Barley	5.33	4.27
Malt	4.25	3.55

Lower levels of octanoic acid and methyl palmitate were exclusively detected in oat, ethyl palmitate, 1-octadecene, and 9,17-octadecadienal in wheat, 2-cyanoethyl hexyl ether, propionic acid, isobutyric acid, valeric acid, methyl laurate, isoamyl salicylate, amyl salicylate, 1,4-dimethylpiperazine, 2-dodecanol, 2-methoxyethylbenzene in barley, and 1-(2-furyl)ethanone, 2-phenylethanol, 3-(m-amino bezoyl)-2-methyl propanoate and benzocaine in malt. Small amounts of isobutanol, ethylbenzene, 1,2-xylene, 1,4-xylene, 2-ethoxyethanol, acetoin, dibutyl phthalate and palmitoleic acid were detected in several media.

The available information in the literature regarding the flavour properties of some of the detected volatile compounds is summarised in Table 2. Table 3 shows the changes of the pH in the four cereal-based broths after 24 h of fermentation with *L. plantarum*. In all cases, the pH dropped slightly, and the biggest change was observed in oats (ΔpH = −1.62). The wheat fermented drink had a small pH decrease of 0.41 units. pH drops of 1.06 and 0.7 were observed in the barley and malt broths, respectively.

4. Discussion

The aim of this study was to test the suitability of several cereal matrices to produce probiotic formulations with acceptable organoleptic properties. Previous work by this group suggests that the cereal tested can produce lactic acid bacterial populations with higher cell concentrations than the minimum requirement for a probiotic drink (10^6 CFU/ml, Sanders & Huis in't Veld, 1999). The production of major metabolites, e.g. lactic acid and acetic acid, has also been previously studied and modelled (Charalampopoulos, Pandiella, & Webb, 2003; Kedia et al., 2008a, 2008b), but knowledge regarding other volatiles that could affect flavour remains scarce.

Volatiles produced by *L. pantarum* in oat, wheat, barley and malt were extracted in ethyl acetate and later measured by GC. The fermented products show macro- and microscopical changes with respect to the raw ingredients. As expected, volatile compounds of natural occurrence in the raw cereal grains and substances generated during processing were found (see Table 1).

Cereal milling, storage conditions, enzymatic hydrolysis and the Maillard reaction during autoclaving could be responsible for the production of flavour active compounds. The Maillard reaction is actually a complex set of chemical reactions involving aminoacids and reducing sugars under heating conditions. The final products of the Maillard reaction are cyclic and polycyclic molecules that can react again and break down to form even more new compounds. These molecules are dependent on the aminoacid involved in the reaction and, depending on the substrate, hundreds of different flavour compounds can be formed.

Since all the cereal flours rich in sugars and proteins suffered a thermal treatment (121 °C, 20 min), a wide variety of Maillard compounds was expected. However, only in the malt media were compounds from thermal degradation were found. Pyranone, maltol, furfural, 2-furanmethanol, 2-(methoxycarbonyl)furan and 5-hydroxymethylfurfural, all previously reported as Maillard compounds (Kato, Nakayama, Sugimoto, & Hayase, 1982; Mori & Ito, 2004), were detected in the fermented malt media but not in other cereals. It is likely these compounds were already present in the malt before autoclaving. These aromas could have been generated during the kilning of the malt, a high temperature drying stage that takes place after germination in the malting process. Maillard compounds play a key role in the quality of foods as they are strongly related to the colour, aroma, and taste of the end-product (Adams, De Kimpe, & Van Boekel, 2008). Nevertheless, these thermal-induced compounds can enhance or negatively affect the flavour, depending on their reactive nature (Schutter et al., 2008). The impact of the Maillard compounds detected in this study on the sensory characteristics of the drinks produced could not be determined. Further characterisations by sensorial panels should be carried out in order to understand the relationship between the presence of Maillard compounds and the flavour of the probiotic drinks.

The Maillard reaction in aqueous systems is very slow, which could explain the absence of Maillard compounds generated during fermentation (Eichner & Karel, 1972). In addition, all media were acidic (see Table 3) and in these environments the amino groups are protonated and have a lower nucleophilicity, making difficult any reaction with the carbonyl group of sugars.

Another group of flavour compounds could be generated during processing due to the enzymatic activity of the lactic bacteria. According to metabolic pathways during fermentation, lactic acid bacteria can be classified in two different groups: homofermentative and heterofermentative. In homofermentative lactic acid bacteria, glucose is metabolised by the Embden–Meyerhof pathway and lactic acid is the sole final product. Heterofermentative lactic acid bacteria metabolise the glucose mainly through the pentose phosphate pathway, producing lactic acid, ethanol, and CO_2. However, the heterofermentative metabolism is affected by the presence of maltose and electron acceptors, such oxygen or fructose, and the metabolic pathways can be diverted towards acetic acid production (Sedewitz, Schleifer, & Gotz, 1984; Stolz, Vogel, & Hammes, 1995). Our results suggest that the four broths have undergone a heterofermentative process. This pathway yields less lactic acid than does the homofermentative one.

The high concentrations of acetic acid could be explained by the aerobic metabolism of *L. plantarum*. Sedewitz et al. (1984) have showed that *L. plantarum* during the stationary phase, under aerobic conditions and low glucose concentration, can consume the previously secreted lactic acid and an equivalent amount of acetic acid is released. Similar behaviour of *L. plantarum* in residual media was also reported by Vázquez, Docasal, Prieto, González, and Murado (2008). The high relative concentrations of acetic acid found in the four cereal-based broths suggest that part of the lactic acid produced could have been metabolised to acetic acid.

Inoculation with the probiotic lactic acid bacteria caused a significant change in the aroma profile of the four cereal broths. In particular, oat, and barley broths were more affected by the fermentation process. The oat medium showed a significant increment in the contents of flavour active volatiles. In barley, considerable amounts of new volatiles were generated after the fermentation. In general, the volatile production depends more on the substrate than on the microorganism. None of the new volatiles detected after fermentation is common for all media. The results obtained add to the knowledge of the volatile composition of cereals and cereal-based products, and provide useful information for further development of suitable non-dairy probiotic drinks.

Acknowledgements

Ivan Salmeron thanks CONACYT (Mexico) for the financial Support. Pablo Fuciños thanks *Xunta de Galicia* for the mobility grant

and the financial support provided through the "Ánxeles Alvariño Program".

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Food Chemistry 117 (2009) 272–275

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Seasonal variations in total fatty acid composition of muscles and eye sockets of tucunaré (*Cichla* sp.) from the Brazilian Amazon area

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ARTICLE INFO

Article history:
Received 31 July 2008
Received in revised form 30 March 2009
Accepted 31 March 2009

Keywords:
Freshwater fish
ω3 and ω6 fatty acids
Amazon fish

ABSTRACT

Seasonal variations in total fatty acid compositions of tucunaré, *Cichla* sp. in the Janauacá Lake of the Amazon basin were determined by GC. Saturated fatty acids (SFAs) were found to be at higher concentrations than monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) in two seasonal periods. Major fatty acids were palmitic (16:0, 48.5–51.6% of total SFA), oleic (18:1ω9, 43.9–50.2% of total MUFA), docosahexaenoic (DHA, 22:6ω3, 13.5–27.9% of total PUFA) and arachidonic acid (AA, 20:4ω6, 16.0–19.6% of total PUFA). Polyunsaturated fatty acids were the most abundant in muscle during the flood period, and more especially fatty acids 22:6ω3 (10%) and 20:4ω6 (7%). There were no significant variations amongst total lipid SFA, MUFA, and PUFA from eye sockets. The ω6 fatty acid percentages were always higher than the ω3 fatty acid percentages in tucunaré, with ω6/ω3 ratios varying between 1.02 and 1.6 in the flood and drought periods.

1. Introduction

The nutritional importance of fish consumption is closely associated with the ω3 fatty acid content of each species (Ackman, 1989; Rasoarahona, Barnathan, Bianchini, & Gaydou, 2005; Rice, 1996; Simopoulos, 1991). The quantity and composition of fatty acids from lipids are not only associated with the species, but also depend on diet, temperature, seasonality, age and gender (Ackman, 1989).

Interest in long-chain polyunsaturated fatty acids, particularly of the ω3 series (eicosapentaenoic-EPA and docosahexaenoic-DHA) stems from the fact that increased intake levels reduce blood total triacylglyceride concentrations and biochemical risk factors associated with cardiovascular diseases, arthritis, psoriasis and cancer, and also because they act directly on the processes of human growth and development (Eilander, Hundscheid, Osendarp, Transler, & Zock, 2007; Siguel, 1996; Simopoulos, 1991; Weaver & Holob, 1988). However, studies have revealed high concentrations of arachidonic acid (AA), of the ω6 series, and DHA in the milk of nursing mothers in the first weeks of breastfeeding, indicating that these nutrients are required by the newborn, and that their presence decreases at a rate directly proportional to the presence of these fatty acids in the diets of nursing mothers (Jensen, 1999; Martin et al., 2006).

Nutritionists believe that the ω6:ω3 ratio in daily nutrition should be 5:1 and that the addition of PUFA ω3 could improve nutritional condition, thus helping to prevent diseases (FAO, 1995; Muggli, 1997; Rice, 1996).

Fish and fish oil are considered the best ω3 PUFA sources, and are capable of providing balanced amounts of EPA and DHA. A comparative study, with volunteers, between vegetable-oil and fish-oil diets, showed that the ingestion of 18:3 ω3-rich flaxseed oil did not produce a sufficient amount of long-chain ω3 fatty acids, which is part of human daily requirements (Layne et al., 1996).

In Brazil, the greatest diversity of freshwater fish is found in the Amazon Basin, with over 1500 ichthyic species already described (Junk, Soares, & Saint-Paul, 1997). As a result, the *per capita* fish intake in the region has been the highest of Brazil. In the city of Manaus alone, fish consumption varied between 55 and 38.5 kg person^{-1} year^{-1} (Giugliano, Shirimpton, Arkcoll, Giugliano, & Petrere, 1978) while, in the interior of the State of Amazonas, it ranged from 510 to 600 g person^{-1} day^{-1} (Batista, Inhamuns, Freitas, & Freire-Brasil, 1998). Tucunaré (*Cichla* sp.) is an ichthyophagous fish from the Amazon Basin and amongst the most sought-after species by the local population, both for consumption and sport fishing. The meat achieves high commercial value, with an estimated production in 2003 of 1.172 tons in the main fishing discharge ports of the Amazon region (Souza et al., 2007).

The river water volume oscillations throughout the year determine harvest and off-season periods in the Amazon region. Consequently, this seasonal variation directly influences the diet and biology of animal species (Junk et al., 1997; Rasoarahona et al.,

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0308-8146/$ - see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2009.03.113

2005). Contreras-Guzman (1994) referred to climatic variations as being responsible for the great variability in the diet and habitat of ichthyic species throughout the year, which, in turn, cause variations in the lipid composition of fish, directly affecting fat taste and stability to oxidation, which are technological parameters of great importance in the fish industry.

Knowledge of the lipid composition, in different seasonal periods of the various fish species sold, is essential, since diets can be formulated more precisely through fatty acid quantification in tissues; it also allows processing procedures to be adequately dimensioned, considering the nutritional value and the high susceptibility to oxidation of polyunsaturated fatty acids. The residues generated by industrial processing, such as fish heads, especially the fat accumulated in the eye sockets, can be used for the production of ω3 concentrates.

2. Materials and methods

2.1. Sampling

The tucunaré (*Cichla* sp.) lots used in this study (by weekly sampling) were collected during different seasons in the Amazon Basin at the Janauacá lake (3°23′S, 60°16′W), near the city of Manaus/AM, one in the drought (ebbing) period and the other in the rainy (flood) period in the months of peak, in other words June in the flood period and December in the drought period, in a total of 25 monthly collected samples, divided into five lots.

The dorsal muscles and orbital fatty tissue from the eye socket of each sampling lot were removed, and freeze-dried, and had their total lipids extracted (Bligh & Dyer, 1959), and were then stored at −18 °C under a N_2 atmosphere for later analysis. As the samples were dehydrated, the respective percentage of water was added, close to the solvents, during the lipids extraction.

2.2. Gas chromatography analyses

Derivatization of fatty acids from total lipids was performed according to the procedure described by Joseph and Ackman (1992). The chromatographic analysis was carried out in a VARIAN, Mod 3300 gas chromatograph, equipped with a flame ionisation detector, split injector, and a DB-WAX 20 M fused silica capillary column (30 m × 0.247 mm × 0.25 μm) (WCOT, SGE, Australia), with the following operating parameters: detector temperature 280 °C; injector temperature 250 °C; column temperature 170 °C for 16 min and the apparatus was programmed at 2 °C per minute until 210 °C, remaining stable for 25 min; hydrogen was used as carrier gas at 1 ml/min, linear velocity of 38 cm/s, and an oxygen filter attached to the gas line; nitrogen was used as make up gas at 30 ml/min, with synthetic air 300 ml/min; split injection technique 1:100; 1 μl of FAME was injected. All steps, from transesterification to the injections, were performed under a nitrogen atmosphere. Retention time and the area of peaks were recorded with a Varian Mod 4290 integrator. Quantification was expressed as area percentage.

2.3. Fatty acids identification

In order to identify the fatty acids, comparisons were made between peak retention times (tr) in the sample chromatograms and retention time values of methyl ester standards (Sigma, St. Louis, MO). Equivalent chain length (ECL) values were used and calculated from the corrected retention times (tr′) for the fatty acid methyl esters (Maia, 1992; Stránsky, Jursík, & Vitek, 1997; Thompson, 1996). Identification was confirmed by fragmenting the fatty acids via electron impact at 70 eV in a Shimadzu QP 5000 gas chromatograph–mass spectrometer.

2.4. Statistical analysis

The area percentage values were submitted to analysis of variance (ANOVA) by the general linear models procedure (PROC GLM – Duncan's test at the 5% significance level), with one factor (season of the year or body part) and two levels (drought/flood or muscle/eyes). The SAS statistical package (Cary, NC) was used in this analysis.

3. Results and discussion

The captured tucunaré samples varied between 28 and 29 cm, with weights between 288 and 348 g in the flood period and 30–32 cm and 443–520 g in the drought period. The average moisture contents varied between 76% ± 1 and 78% ± 1, with average total lipid contents between 0.8% ± 0.2 and 2.1% ± 0.2 in both seasons, with a typical lean-species lipid composition (Ackman, 1989) (Table 1).

The lipid contents in fish can vary, depending on the species, age, seasonality, geographical origin, sexual maturity, body region, type of muscle and diet (Contreras-Guzman, 1994; Rasoarahona et al., 2005). In the flood period, lipid contents were equal to or lower than 1%, probably due to the high energy intake of piscivorous species in the search for food dispersed in inundated lowlands of rivers and lakes, and due to the reproduction period (Goulding, 1979). In the drought period, as the flooded area becomes smaller, food is abundant and capture is facilitated, resulting in accumulation of adipose tissue. The variation in lipid contents was inversely proportional to the variation in moisture content for both periods, which may create processing and storage problems, in addition to negatively affecting organoleptic properties (Contreras-Guzman, 1994).

Table 2 presents the fatty acids from total lipids of tucunaré in different seasonal periods. Eighty-two fatty acids (of which eight were not identified) and two dimethyl acetals were found. When tucunaré dorsal muscle and eye sockets were compared, it was observed that saturated (SFAs) and monounsaturated fatty acids (MUFAs) were predominant in the eye sockets, varying between 47.1% and 47.4%, and between 29.8% and 31.3%, respectively. Polyunsaturated fatty acids (PUFAs) were predominant in the dorsal muscle in both seasons, varying between 24.2% and 35.8%. The predominant fatty acids in both periods were: palmitic-16:0 (48.5–51.6% of total SFA), oleic-18:1ω9 (43.9–50.2% of total MUFA), docosahexaenoic-22:6ω3 (13.5–27.9% of total PUFA) and arachidonic acid-20:4ω6 (16.0–19.6% of total PUFA).

In both seasonal periods, saturated (SFAs) and monounsaturated fatty acids (MUFAs) were predominant in eye socket fat. In the flood period, major fatty acids with significantly different contents were 15:0, 17:0, 18:0, 16:1ω7, 18:1ω7, and 18:1ω9 while, in the drought, only 15:0 was significantly different.

However, polyunsaturated fatty acids were significantly higher in muscle tissue lipids in both seasons; major fatty acids of the ω3 series with significant differences in the flood period were 22:5ω3,

Table 1
Biometric index, moisture, and lipid determined for tucunaré (*Cichla* sp.) fillets in different seasonal periods.

Flood period (January–June)			Drought period (July–December)		
BI[a]	Moisture[b] (%)	Lipid[b] (%)	BI	Moisture (%)[b]	Lipid (%)[b]
2.34 ± 0.8	78 ± 1	0.8 ± 0.1	2.99 ± 0.9	76 ± 1	2.1 ± 0.2

[a] Biometric index (g weight by cm size of 25 samples for seasonal period.
[b] Means and standard deviations of duplicate analyses (five lots).

Table 2
Seasonal variations in total fatty acid composition (%) of tucunaré (*Cichla* sp.) from the Amazon region.

Fatty acid*	Flood period		Drought period	
	Muscle	Eyes	Muscle	Eyes
10:0	0.1 ± 0.01	tr	tr	tr
11:0	tr	tr	0.11 ± 0.03	tr
12:0	0.18 ± 0.01^{a}	0.24 ± 0.01^{a}	0.3 ± 0.1^{a}	0.28 ± 0.02
i 13:0	tr	tr	tr	nd
13:0	0.27 ± 0.03	0.36 ± 0.02	0.26 ± 0.03	0.34 ± 0.02
i 14:0	tr	0.10 ± 0.01	tr	0.12 ± 0.01
14:0	1.8 ± 0.1^{aa}	2.2 ± 0.2^{bb}	2.6 ± 0.2^{aa}	2.9 ± 0.1^{bb}
i 15:0	0.45 ± 0.04^{a}	0.72 ± 0.03^{a}	0.66 ± 0.08^{a}	0.83 ± 0.05
15:0	1.5 ± 0.2^{a}	2.0 ± 0.2^{a}	1.7 ± 0.1^{b}	1.98 ± 0.06^{b}
ai 15:0	0.14 ± 0.01	0.16 ± 0.01	0.15 ± 0.02	0.16 ± 0.01
i 16:0	0.30 ± 0.02^{a}	0.43 ± 0.03^{a}	0.35 ± 0.03^{a}	0.46 ± 0.03^{b}
16:0DMA	0.96 ± 0.01^{a}	0.25 ± 0.01^{a}	0.28 ± 0.05^{a}	0.31 ± 0.01
16:0	20.0 ± 2.0^{a}	23.0 ± 1.0	23.0 ± 1.0^{a}	24.1 ± 0.6
i 17:0	0.8 ± 0.1^{a}	1.04 ± 0.06^{a}	0.95 ± 0.08^{a}	0.98 ± 0.05
ai 17:0	0.29 ± 0.02^{a}	0.43 ± 0.04^{a}	0.4 ± 0.1^{a}	0.49 ± 0.01
17:0	1.64 ± 0.04^{a}	2.1 ± 0.1^{a}	1.74 ± 0.06	2.0 ± 0.05
i 18:0	0.16 ± 0.01	0.2 ± 0.01^{a}	0.13 ± 0.01	0.14 ± 0.01^{a}
18:0	9.9 ± 0.3^{a}	11.9 ± 0.5^{b}	10.1 ± 0.3^{a} =	10.6 ± 0.3^{b}
i 19:0	0.33 ± 0.03^{a}	0.52 ± 0.04^{a}	0.24 ± 0.06^{a}	0.28 ± 0.01
19:0	0.43 ± 0.04^{aa}	0.47 ± 0.05^{bb}	0.32 ± 0.01^{aa}	0.25 ± 0.02^{bb}
20:0	0.51 ± 0.04^{a}	0.70 ± 0.06^{a}	0.56 ± 0.03	0.62 ± 0.01
21:0	tr	tr	tr	tr
22:0	0.31 ± 0.03^{a}	0.48 ± 0.03^{a}	0.35 ± 0.02^{b}	0.41 ± 0.04^{a}
24:0	0.26 ± 0.02^{a}	0.4 ± 0.4^{b}	0.20 ± 0.01^{a} =	0.16 ± 0.01^{b}
∑SFA	38.8	47.4	44.5	47.1
14:1ω9	tr	nd	tr	tr
14:1ω7	0.13 ± 0.01	0.16 ± 0.02	0.14 ± 0.03	0.14 ± 0.01
14:1ω5	tr	nd	tr	tr
15:1ω9	tr	tr	nd	tr
15:1ω7	tr	tr	tr	nd
16:1ω9	1.0 ± 0.1	1.17 ± 0.07	1.33 ± 0.07	1.1 ± 0.0
16:1ω7	5.0 ± 1.0^{a}	7.6 ± 1.0^{a}	6.29 ± 0.03^{a}	7.0 ± 0.1
16:1ω5	0.24 ± 0.01^{a}	0.33 ± 0.01^{a}	0.28 ± 0.03^{a}	0.35 ± 0.02
17:1ω9	0.71 ± 0.07^{a}	0.89 ± 0.06^{a}	0.81 ± 0.07^{a}	0.87 ± 0.04
17:1ω6	0.19 ± 0.01	nd	tr	0.11 ± 0.01
18:1DMA	tr	tr	tr	tr
18:1ω11	0.15 ± 0.01^{a}	0.29 ± 0.03^{a}	0.19 ± 0.02	0.21 ± 0.01 ^{a}
18:1ω9	10.0 ± 1.0^{a}	13.4 ± 1.0^{b}	15.0 ± 0.4^{a} =	15.7 ± 0.2^{b}
18:1ω7	4.2 ± 0.3^{a}	5.2 ± 0.3^{a}	4.2 ± 0.3	4.8 ± 0.2
18:1ω6	0.14 ± 0.01	0.20 ± 0.01	0.10 ± 0.01	0.12 ± 0.01
18:1ω5	0.12 ± 0.01^{a}	0.19 ± 0.01^{a}	0.14 ± 0.01	tr^{a}
18:1ω3	0.22 ± 0.01	0.23 ± 0.02	0.22 ± 0.06	0.18 ± 0.01
19:1ω7	0.14 ± 0.01	0.16 ± 0.01^{a}	0.15 ± 0.01	tr^{a}
20:1ω11	0.20 ± 0.03^{a}	0.41 ± 0.04^{a}	0.5 ± 0.01^{a}	0.38 ± 0.02
20:1ω9	0.29 ± 0.01^{a}	0.48 ± 0.03^{a}	0.43 ± 0.02^{a}	0.32 ± 0.01^{a}
20:1ω7	0.12 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.13 ± 0.01
22:1ω11	tr	tr	tr	tr
22:1ω9	tr	nd	tr	tr
24:1ω9	tr	tr	tr	tr
∑MUFA	22.8	29.8	30	31.3
16:2ω7	0.31 ± 0.03^{a}	tr^{a}	0.10 ± 0.01^{a}	tr
16:2ω4	0.16 ± 0.01	0.13 ± 0.01	tr	0.17 ± 0.01
16:3ω6	0.16 ± 0.01^{a}	0.32 ± 0.02^{a}	0.17 ± 0.03	0.15 ± 0.01^{a}
16:4ω3	1.9 ± 0.2^{a}	0.38 ± 0.01^{a}	0.4 ± 0.1^{a}	0.1 ± 0.0^{a}
16:4ω6	tr^{a}	0.26 ± 0.02^{a}	0.24 ± 0.06^{a}	0.17 ± 0.01^{a}
17:2ω5	0.14 ± 0.01	tr	nd	nd
17:2ω4	0.12 ± 0.01	tr	tr	tr
18:2ω6	3.5 ± 0.4^{a}	4.2 ± 0.4	5.0 ± 0.3^{a}	4.75 ± 0.02
18:2ω4	0.11 ± 0.01	0.16 ± 0.02	0.11 ± 0.01	tr
18:3ω6	0.15 ± 0.01^{aa}	0.18 ± 0.02^{bb}	0.33 ± 0.02^{aa}	0.32 ± 0.00^{bb}
18:3ω4	0.11 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	0.14 ± 0.01
18:3ω3	1.3 ± 0.1^{aa}	1.6 ± 0.1^{bb}	2.6 ± 0.2^{aa}	2.4 ± 0.1^{bb}
18:4ω3	0.15 ± 0.04	0.12 ± 0.02	0.13 ± 0.01	tr
19:2ω7	nd	0.15 ± 0.01	tr	nd
20:2ω6	0.47 ± 0.02	0.37 ± 0.03^{a}	0.47 ± 0.04	0.44 ± 0.02 ^{a}
20:3ω6	0.81 ± 0.04^{a}	0.64 ± 0.05^{a}	0.67 ± 0.03^{a}	0.54 ± 0.01
20:4ω6	7.0 ± 1.0^{a}	3.4 ± 0.4^{a}	4.5 ± 0.4^{a}	3.2 ± 0.2^{b}
20:3ω3	0.31 ± 0.02^{aa}	0.33 ± 0.02^{bb}	0.51 ± 0.02^{aa}	0.47 ± 0.02^{bb}
20:4ω3	0.33 ± 0.02^{a}	0.26 ± 0.02^{a}	0.32 ± 0.03	0.29 ± 0.03
20:5ω3	0.9 ± 0.06^{a}	0.51 ± 0.05^{a}	0.60 ± 0.02^{a}	0.57 ± 0.03
21:3ω3	nd	tr	tr	tr
21:4ω3	tr	tr	tr	tr

Table 2 (*continued*)

Fatty acid*	Flood period		Drought period	
	Muscle	Eyes	Muscle	Eyes
21:5ω3	0.11 ± 0.01	tr	tr	tr
22:2ω6	0.13 ± 0.01	tr	tr	tr
22:4ω6	2.3 ± 0.1^{a}	1.38 ± 0.02^{a}	1.5 ± 0.1^{a}	1.4 ± 0.1
22:5ω6	3.2 ± 0.2^{a}	1.12 ± 0.03^{a}	1.4 ± 0.1^{a}	1.0 ± 0.2
22:4ω3	nd	tr	tr	tr
22:5ω3	2.2 ± 0.2^{a}	1.37 ± 0.03^{a}	1.24 ± 0.07^{a}	1.1 ± 0.1
22:6ω3	10.0 ± 1.0^{a}	3.6 ± 0.3^{a}	3.8 ± 0.2^{a}	2.68 ± 0.06^{b}
∑PUFA	35.8	20.3	24.2	20
∑AGω3	17.3^{a}	8.1^{a}	9.5^{a}	7.7
∑AGω6	17.7^{a}	11.9^{a}	14.3^{a}	12.0
∑ω6 / ∑ω3	1.02	1.5	1.5	1.6
EPA + DHA	10.9	4.11	4.4	3.25
Unidentified	0.3	0.7	1.0	0.6

* Analyses of 25 samples coming from five lots in each seasonal period. Abbreviations: i, iso; ai, anteiso; tr, trace (mean value below 0.1%); DMA, dimethyl acetal. Conditions: column DB-WAX 20 M (30 m × 0.247 mm × 0.25 mm); 170 °C/16 min. programmed to increase at 2 °C/min. up to 210 °C/25 min. Significant difference at a 5% level is designated by 'a' and 'b': different letters in the same period indicate a significant difference between muscle and eyes. The same letters in different periods indicate a significant difference in the referenced part of the body. Double letters (aa or bb) indicate a significant difference only between the periods. =, No significant difference in the referenced period, even with the presence of letters 'a' or 'b'. The absence of letters indicates no significant difference amongst the observations.

20:5ω3 and 16:4ω3, 22:5ω6 and 22:4ω6, while the 22:6ω3 and 20:4ω6 acids were significant in both periods and, in proportional terms, even exceeded monounsaturated fatty acids in the flood period. Polyunsaturated fatty acids varied between the seasonal periods studied, with significant difference for muscle tissue fatty acids in the flood period, with a high relative percentage when compared with the drought period.

When tucunaré polyunsaturated fatty acids were analysed, a high proportion of docosahexaenoic acid (22:6ω3) (10 ± 1%) was found in muscle during the flood period, with a low proportion of eicosapentaenoic acid (20:5ω3) (0.9 ± 0.1%), but with a high arachidonic acid content (22:4ω6) (7 ± 1%) (Fig. 1). In general, the ω6-series fatty acids showed higher proportions than did the ω3 in both seasonal periods, especially linoleic (18:2ω6) and arachidonic acid, which contributed to balance the ω6/ω3 ratio in the muscle and eyes, despite the high DHA content (22:6ω3) detected in muscle during the flood period.

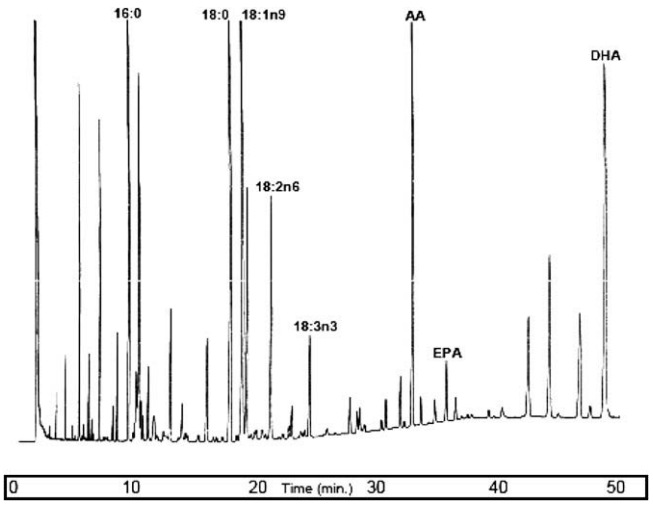

Fig. 1. Methyl esters chromatogram of tucunaré (*Cichla* sp.) fatty acids in DBWAX 20 M column (170 °C for 16 min, programmed to increase at 2 °C/min up to 210 °C with a final holding time of 25 min).

Similar results were reported by Maia et al. (1998), who analysed fatty acids in Amazonian fish without seasonal distinction and found high proportions of the 22:6ω3 (7.2%) and 20:4ω6 acids (6.5%) in tucunaré (*Cichla* sp.) muscle, with very close values for both, keeping the ω6/ω3 ratio near 1. They also observed that leaner fish, such as tucunaré, had higher polyunsaturated fatty acid contents than had fatter fish.

In seasonal studies on fatty acids of mapará muscle (*Hypophthalmus* sp.) in the Amazon region, no significant differences were found for polyunsaturated fatty acids throughout the year; however, ω6/ω3 ratios ranged from 0.6 to 0.8 (Inhamuns & Franco, 2001). In a similar study, five popular marine and freshwater fish species, sardine (*Sardinella* sp.), croaker (*Micropogonias furnieri*), tilapia (*Oreochromis* sp.), curimbatá (*Prochilodus* sp.), and shrimp (*Xiphopenaeus kroyeri*), were analysed for their fatty acids contents in two seasonal periods. The greatest ω6/ω3 ratio variations occurred in the freshwater species tilapia (*Oreochromis* sp.) and curimbatá, with values varying from 0.4 to 1.3 and from 0.8 to 1.02, respectively (Luzia, Sampaio, Castellucci, & Torres, 2003).

Rasoarahona et al. (2005) analysed the fatty acids of three tilapia species, *Oreochromis niloticus*, *O. macrochir*, and *Tilapia rendalli*, and found high AA and DHA contents, with ω6/ω3 ratios varying from 1.2 to 2.2, 0.6 to 2.0, and 0.8 to 1.7, respectively, in three seasonal periods. Özogul, Özogul, and Alagoz (2007) studied the muscle tissue composition of marine and freshwater species, and also observed that ω6 PUFA contents were higher in freshwater species.

The higher proportion of polyunsaturated fatty acids in the flood period detected in tucunaré muscle was probably due to a high energy intake during that period, as revealed by lower total lipid contents, since saturated and monounsaturated fatty acids constitute a source of energy readily available in fish, while polyunsaturated fatty acids are preserved, as they are structural constituents important for the metabolic functions of organs and tissues (Contreras-Guzman, 1994).

Tucunaré can be considered a rich source of essential fatty acids of the ω3 and ω6 series, with greater potential in muscle tissue and during the flood period in the Amazon Basin. The ω6/ω3 ratio, which varied between 1:1 and 1:6 in all periods and fractions analysed, proved to be balanced between these fatty acid groups. This result suggests that including this species in the diet may contribute to maintain the ω6/ω3 ratio recommended by FAO (1995), that is, between 5:1 and 10:1, considering the inclusion of vegetable oils frequently associated with fish consumption.

The industrial residues generated during tucunaré processing, such as heads and offal can be sent for oil extraction and concentration, to be used as nutraceuticals.

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Food Chemistry 117 (2009) 276–281

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Antioxidant activity of polyphenol and anthocyanin extracts from fruits of *Kadsura coccinea* (Lem.) A.C. Smith

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ARTICLE INFO

Article history:
Received 17 February 2009
Received in revised form 9 March 2009
Accepted 1 April 2009

Keywords:
Kadsura coccinea
Fruits
Polyphenols
Anthocyanins
Antioxidant activity

ABSTRACT

Polyphenols and anthocyanins were extracted from *Kadsura coccinea* fruit. The contents of total phenolics and anthocyanins, influences of pH and temperature on anthocyanins, and antioxidant activities of extracts were further analyzed. The results showed that the total phenolic content of peel extracts was superior to that of pulp extracts. The total anthocyanin content of peel extracts was 180 ± 2.91 mg/100 g of fresh peel tissues. *K. coccinea* anthocyanins were stable below pH 4, whilst high temperature and extended heating time induced their degradation. Metal-chelating capacity (MCC) assay indicated that no ortho-dihydroxy aromatic moiety existed in the B-ring of the anthocyanin molecules, but there might be a catechol group in the B-ring of the polyphenol molecules. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity and reducing power of extracts decreased in the following order: peel polyphenol extracts > anthocyanin extracts > pulp polyphenol extracts. It would be worthwhile to introduce this rare fruit into more countries.

1. Introduction

Oxidation is an essential biological process for energy production in many living organisms. However, excessive reactive oxygen species, produced *in vivo* during some oxidative reactions, are not only strongly associated with lipid peroxidation but also involved in the development of some diseases (Moskovitz, Yim, & Choke, 2002). Recently, increasing numbers of reports confirm that many fruits and vegetables may afford protection against some chronic diseases caused by oxidative stress. This is mainly attributed to their antioxidant constituents, including vitamin C, vitamin E, carotenoids, flavonoids and anthocyanins (Heim, Tagliaferro, & Bobilya, 2002; Seifried, 2007). Polyphenols and anthocyanins are important components in fruit tissues. These compounds are thought to be instrumental in combating oxidative stress. They can prevent some oxidation-related diseases such as atherosclerosis, cardiovascular and neurodegenerative diseases and cancer (Kaliora, Dedoussis, & Schmidt, 2006; Pietta, Minoggio, & Bramati, 2003; Ramassamy, 2006; Wang & Stoner, 2008).

Kadsura coccinea (Lem.) A.C. Smith, a plant indigenous to South China, belongs to the family Schisandraceae. *K. coccinea* fruit is known as 'black tiger' in China. It is a novel and rare fruit with high nutritional value. The mature fruit of *K. coccinea*, with 3.5–10.5 cm cross diameter and 3.0–9.5 cm vertical diameter, is aggregated by about 30–65 small berries. Each small berry has a fruit aroma similar to apple, and peel and pulp colours similar to litchi. By atomic absorption spectrophotometry, a high content of essential trace elements, Mn, Ca, Fe, Mg, Cu and Zn, is observed in this fruit, indicating that it possesses benefits for human health (Huang, Long, Wen, & Li, 2006). In recent years, *K. coccinea* fruit has been introduced and cultivated in some provinces of China, such as Guangxi, Guangdong, Guizhou, Yunnan, Hunan and Sichuan. At present, this fruit is mainly consumed in the form of fresh fruit, juice and fruit wine. Due to its good taste and health-beneficial effect, this fruit is relatively expensive in markets. The high commercial value attracts more and more people to study and develop it. Previous researches revealed that some lignans and triterpenoids in stem or seed extracts from *K. coccinea* were effective as antitumor, anti-HIV, anti-lipid peroxidative, cytotoxic and anti-hepatitis agents (Gao et al., 2008; Liu & Li, 1995). However, there are still no reports on antioxidant activity of polyphenols and anthocyanins from *K. coccinea* fruit. *K. coccinea* fruit is rich in polyphenols and anthocyanins in its edible portion, which has a potential health benefit. The objective of the present study is to determine polyphenol and anthocyanin contents in this fruit and fur-

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doi:10.1016/j.foodchem.2009.04.001

ther analyze their antioxidant activity. The research results will be useful for elucidating the nutritional and health values of *K. coccinea* fruit and popularising this novel fruit in countries outside China.

2. Materials and methods

2.1. Plant materials

The mature aggregate fruits of *K. coccinea* with dark purplish-red fleshy and edible peels were harvested from the northwest area of Guangxi province of China in October, 2008. The individual small berries, without defects and diseases, were selected and deseeded. Peel and pulp tissues were collected and weighed, respectively. All samples were stored at −20 °C prior to further extraction and analysis.

2.2. Chemicals and reagents

Folin–Ciocalteau reagent, gallic acid, ferrozine, 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$), 2,4,6-tripyridyl-s-triazine (TPTZ) and ascorbic acid standard were purchased from Sigma–Aldrich (St. Louis, MO, USA). Other chemicals and reagents were of analytical grade.

2.3. Extraction and partial purification of polyphenols and anthocyanins

Polyphenols and anthocyanins were extracted from crushed fresh peel and pulp tissues (20 g) of *K. coccinea* fruit, according to the modified method of Zhang, Quantick, and Grigor (2000) and Zheng, Wang, Wang, and Zheng (2003). The extraction was performed twice at 20 °C in a shaking incubator (ZHWY-200B, Zhicheng Analytical Co., Shanghai, China). Extracting time was 30 min, and extracting solvent was 100 ml of methanol/acetone/water (3.5:3.5:3, v/v/v) containing 1% formic acid. All extracts were combined and filtered through two layers of cheesecloth. The collected filtrate was centrifuged for 15 min at 7000 *g*. The supernatant was collected and evaporated under vacuum at 35–40 °C to remove methanol and acetone. Lipophilic pigments were then eliminated from the aqueous phase by two successive extractions in a separatory funnel with a twofold volume of petroleum ether. The aqueous phase was collected and further extracted three times by ethyl acetate (ethyl acetate: aqueous phase = 1:1, v/v) in the separatory funnel. Three ethyl acetate phases were collected, evaporated and dried under vacuum at 35 °C. The residue was re-dissolved in 2 ml of ethanol and used as a partially purified polyphenol sample. The aqueous phase was also dried under vacuum at 55 °C and used as an anthocyanin sample. All samples were stored at −20 °C prior to further assays.

2.4. Determination of total phenolic content

Total phenolic content was determined using the Folin–Ciocalteau method described by Singleton and Rossi (1965) with some modifications. One millilitre of 10-fold diluted extracts by methanol/acetone/water (3.5:3.5:3, v/v/v), containing 1% formic acid, was thoroughly mixed with 1 ml of Folin–Ciocalteau reagent and stood for 3 min at the room temperature. Then, 3 ml of sodium carbonate (75 g/l) were added to the mixture, which was allowed to stand for 2 h at the room temperature. Sample aliquots were filtered through a 0.45 μm filter prior to the determination of total phenolics using a UV–visible Spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan), monitoring at 760 nm. Total phenolic content was standardised against gallic acid and expressed as microgrammes of gallic acid equivalents (GAE) per millilitre.

2.5. Determination of total anthocyanin content

Total anthocyanin content of *K. coccinea* peel extracts was measured using the pH differential method described by Wrolstad and Giusti (2001) and Elisia, Hu, Popovich, and Kitts (2007) with minor modifications. The crude anthocyanin extracts were dissolved in potassium chloride buffer (KCl, 0.025 M, pH 1.0) and sodium acetate ($CH_3CO_2Na \cdot 3H_2O$, 0.4 M, pH 4.5) with a pre-determined dilution factor. The absorbances of measured samples were read at 520 and 700 nm against a blank cell containing deionized water (dd H_2O). The absorbance (A) of the diluted sample was then calculated as follows: $A = (A_{\lambda vis\text{-}max} - A_{700nm})$ pH 1.0 $- (A_{\lambda vis\text{-}max} - A_{700nm})$ pH 4.5. The monomeric anthocyanin pigment concentration in the original sample was calculated according to the following formula:

$$\text{Anthocyanin content (mg/l)} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times 1}$$

in which cyanidin-3-glucoside molecular weight (MW = 449.2), the dilution factor or dilution multiple (DF = 50) and the molar absorptivity constant (ε = 29,600) were used.

2.6. Effects of pH values and heat on anthocyanin properties

Anthocyanin solutions at pH 1, 4, 7, 10 and 12 were prepared using 0.1 M HCl and 0.1 M NaOH at 25 °C. All samples were spectrophotometrically scanned from 400 to 700 nm. By comparing variations of spectrum shapes, absorption peaks, colours and maximum absorbances within 3 min, the effects of different pH values on *K. coccinea* anthocyanins were determined.

Thermal stability of anthocyanins was analyzed according to the modified method of Dyrby, Westergaard, and Stapelfeldt (2001). Five millilitres of aqueous anthocyanin solution were respectively sealed into four tubes that were well capped to avoid evaporation. These tubes were placed in thermostatic water baths operating at 20, 40, 60 and 80 °C. One anthocyanin sample at each temperature was removed after 1, 2 and 3 h, and then rapidly cooled in ice-water. The thermal degradation of anthocyanin was spectrophotometrically measured at 520 nm. The residual amounts of anthocyanin (%) = $(A_{1,\ 2,\ or\ 3h}/A_{0h}) \times 100$, where A_{0h} was the absorbance of anthocyanin sample without undergoing heating treatment, and $A_{1,\ 2,\ or\ 3h}$ was the absorbance of anthocyanin sample after heating for 1, 2 and 3 h at different temperatures.

2.7. Metal-chelating capacity (MCC)

The metal-chelating capacity was determined by the modified method of Dinis, Madeira, and Almeida (1994). Aliquots (1 ml) of 0 (control), 25, 50, 100, 250, 500 and 1000 μg/ml of polyphenol and anthocyanin extracts dissolved in ethanol were added to 2.8 ml distilled water and then mixed with 50 μl of 2 mM $FeCl_2 \cdot 4H_2O$ and 150 μl of 5 mM ferrozine. The mixture was centrifuged at 120 *g*. After 10 min, the Fe(II) was monitored by measuring the formation of ferrous ion–ferrozine complex at 562 nm. The metal-chelating capacity was calculated as follows: chelating capacity (%) = (1 − absorbance of sample/absorbance of control) × 100.

2.8. DPPH radical-scavenging activity

The DPPH radical-scavenging activity was estimated by the modified method of Bondet, Brand-Williams, and Berset (1997). Aliquots (0.5 ml) of 0 (control), 25, 50, 100, 250, 500 and 1000 μg/ml of polyphenol and anthocyanin extracts dissolved in ethanol were added to 2.5 ml of 0.2 mM $DPPH^{\cdot}$ solution in ethanol. The absorbance at 517 nm of samples was measured after 30 min of incubation at room temperature in the dark. $DPPH^{\cdot}$-scavenging activity (%) = $[1 - (A - B)/A_0] \times 100$, where A = absorbance of sam-

ple, B = absorbance of 0.5 ml of extracts + 2.5 ml of ethanol, and A_0 = absorbance of control. The 50% inhibition concentration (IC_{50}), i.e. the concentration of extracts that was required to scavenge 50% of radicals, was calculated.

2.9. Ferric reducing/antioxidant power (FRAP)

This procedure was conducted according to the modified method of Benzie and Strain (1996). The working FRAP reagent was produced by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution and 20 mM $FeCl_3 \cdot 6H_2O$ in a 10:1:1 ratio prior to use and heated to 37 °C in a water bath. A total of 3.0 ml of FRAP reagent was added to a cuvette and a blank reading was taken at 593 nm, using the spectrophotometer. In total, 100 μl of polyphenol and anthocyanin extracts and 300 μl of distilled water were added to the cuvette. After addition of the sample to the FRAP reagent, a second reading at 593 nm was performed after 40 min of incubation at 37 °C in a water bath. The changes in absorbance after 40 min from the initial blank reading were compared with a standard curve. Standards of known Fe(II) concentrations were run using several concentrations ranging from 100 to 1000 μM. A standard curve was then prepared by plotting the FRAP values of each standard versus its concentration. The final result was expressed as the concentration of antioxidant having a ferric reducing ability in 1 g of sample (μM/g).

2.10. Data analysis

All experiments were performed in triplicate ($n = 3$), and an ANOVA test (using SPSS 13.0 statistical software, SPSS Inc., Chicago, USA) was used to compare the mean values of each treatment. Significant differences between the means of parameters were determined by using the LSD test ($P < 0.05$). The results represented means ± standard error (SE) of three replicated determinations.

3. Results and discussion

3.1. Contents of total phenolics and anthocyanins

In the present study, the total phenolic content of extracts (by methanol/acetone/water/formic acid) from *K. coccinea* peel tissues (179 ± 1.95 μg/ml GAE) was superior to that from its pulp tissues (81.1 ± 1.60 μg/ml GAE). In addition, it was observed that no anthocyanin existed in *K. coccinea* pulp tissues. The total anthocyanin content of fleshy peel extracts, determined by pH differential method, was 180 ± 2.91 mg/100 g of fresh peel tissues. The result of the spectral scan showed that the anthocyanin extracts dissolved in acidified distilled water (pH 2) exhibited four absorption peaks at 280, 310, 340 and 520 nm in the wavelength range from 250 to 650 nm (Fig. 1A). This result was consistent with the reported spectral character of anthocyanins (Fig. 1B), which showed distinctive Band I peaks in the 450–560 nm region (due to the B-ring hydroxyl cinnamoyl system) and Band II peaks in the 240–280 nm region (due to the A ring benzoyl system) (Rice-Evans, Miller, & Paganga, 1996). Peaks at 310 and 340 nm further suggested that the acyl group existed in *K. coccinea* anthocyanin molecules (Yoshitama, 1978).

3.2. Effects of pH values on Kadsura coccinea anthocyanins

The variation of pH values influences anthocyanin properties, such as colour, stability, spectral shape and absorption peak. Naturally, anthocyanins contribute to the range of red to purple colours found in many fruits, vegetables, flowers and other plant tissues. The same anthocyanin may have different colours in different

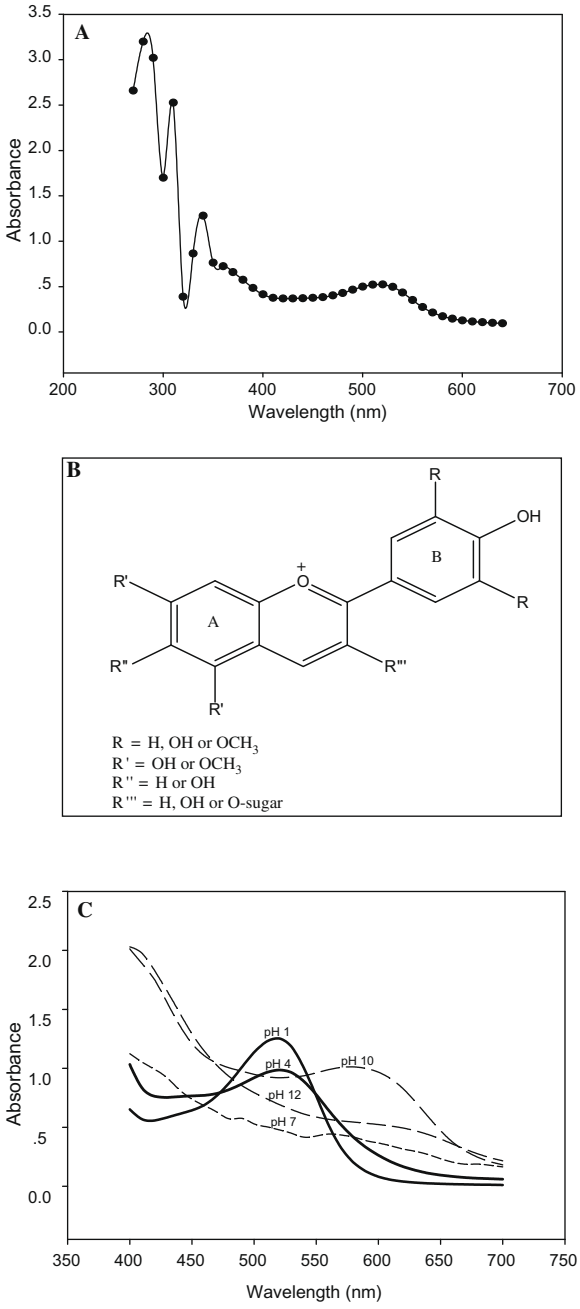

Fig. 1. Spectral character of *Kadsura coccinea* anthocyanins. (A) Absorption spectrum of *Kadsura coccinea* anthocyanin extracts dissolved in acidified distilled water (pH 2), (B) a general structure of anthocyanins and (C) absorption spectra of *Kadsura coccinea* anthocyanin extracts at different pH values.

plants, which depends virtually on the pH values of organelles (Dao, Takeoka, Edwards, Berrios, & De, 1998; Janna, Khairul, & Maziah, 2007). It is observed from Fig. 1C that the anthocyanin extracts from *K. coccinea* peels had maximum absorption around 520 nm at pH 1 and 4. Anthocyanin solutions, at both pH values, exhibited bright-red colour, and their absorbances at 520 nm varied slightly within 3 min. However, when pH values of anthocyanin solutions were above four, there were no obvious absorption peaks in the 450–560 nm regions. The colours of anthocyanin solutions gradually changed from moderate pink to brown from pH 7–12. Their absorbances at 520 nm continuously increased within 3 min. Moreover, the experimental results also showed that the colours of anthocyanin solutions presented reversible change between acidic and alkaline pH values, which was due to the high reactivity

of the flavylium cation toward nucleophilic reagents (including OH^- and H^+), resulting in the variation of chemical structures under different pH conditions (this was a reversible pH-dependent reaction) (Cooke, Steward, Gescher, & Marczylo, 2005; Março, Levi, Scarminio, Poppi, & Trevisan, 2005). By the above analysis, the anthocyanins from *K. coccinea* peels, as the antioxidants, were stable and maintained their original bright-red colour below pH 4.

3.3. Effects of heat on Kadsura coccinea anthocyanins

Heat treatment is one of the most important factors that influence anthocyanin stability (Wang & Xu, 2007). Anthocyanins are not stable at high temperature. Their thermal degradation has been found in many fruits, e.g. grape (Morais, Ramos, Forgács, Cserháti, & Oliviera, 2002), strawberry (García-Viguera & Zafrilla, 1999), sour cherry (Cemeroğlu, Velioğlu, & Işik, 1994), pomegranate (Martí, Pérez-Vicente, & García-Viguera, 2001) and blackberry (Wang & Xu, 2007). From Fig. 2, temperature and time of heat treatment affect the stability of *K. coccinea* anthocyanins. It was observed that the stability of anthocyanin in solutions gradually declined with increase of the heating temperature and extension of heating time. Placing the anthocyanin solution for 3 h in water baths at 20 °C, it was stable and retained red colour. However, heated at 40, 60 and 80 °C, the anthocyanins could break down, and their residual amounts rapidly declined within 1 h. After 3 h, the residual amounts were 92.3 ± 0.2% for 40 °C, 88.1 ± 0.7% for 60 °C and 88.0 ± 0.4% for 80 °C, which were significantly ($P < 0.05$) lower than those for 20 °C (97.8 ± 0.5%). The results suggested that *K. coccinea* anthocyanins tended to degrade at high temperatures when they were used as antioxidants.

3.4. Antioxidant properties of polyphenol and anthocyanin extracts

3.4.1. MCC assay

The MCC method depends on the ability of samples to chelate transition metals, which possess the ability to catalyse hydrogen peroxide decomposition and Fenton-type reactions (Du, Li, Ma, & Liang, 2009). In the present study, *K. coccinea* anthocyanin extracts had no metal-chelating capability, indicating that no ortho-dihydroxy aromatic moiety (the key structure for chelating transition metals) existed in the B-ring of the anthocyanin molecules. However, polyphenol extracts from peel and pulp tissues of *K. coccinea* fruit exhibited some metal-chelating capability (Fig. 3), suggesting that there was a catechol group in B-ring of these polyphenol molecules (Brouillard & Dangles, 1993). By the regression analysis, no

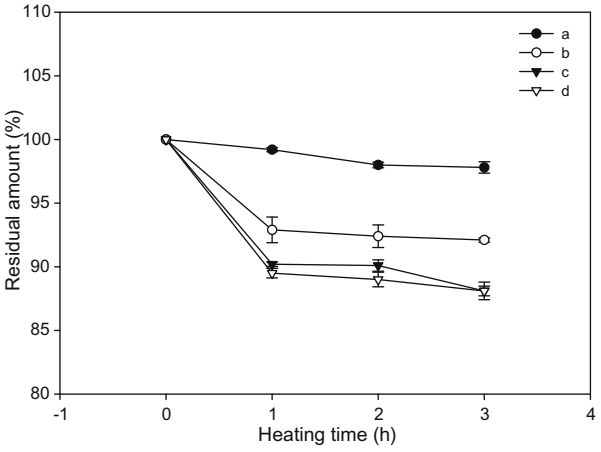

Fig. 2. Residual amounts of *Kadsura coccinea* anthocyanins at different temperatures. a, 20 °C; b, 40 °C; c, 60 °C; d, 80 °C.

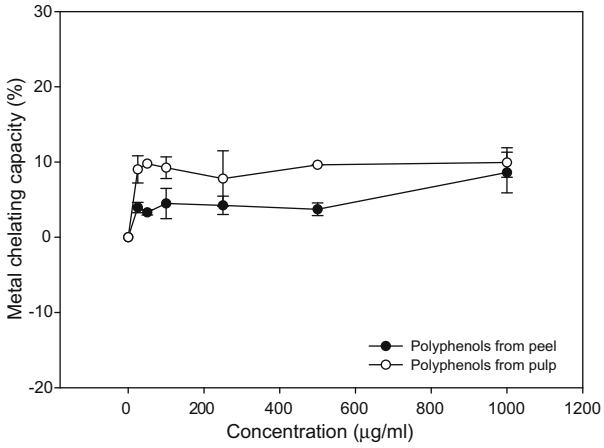

Fig. 3. Metal-chelating capacity of polyphenol extracts from peel and pulp tissues of *Kadsura coccinea* fruit.

significant correlation was found between the concentrations of polyphenol components and MCC.

3.4.2. DPPH radical-scavenging activity

DPPH radical-scavenging activity has been extensively used for screening antioxidants, such as polyphenols and anthocyanins, from fruits. $DPPH^·$ is scavenged by polyphenols and anthocyanins through the donation of hydrogen, forming the reduced DPPH–$H^•$. The colour changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm (Bondet et al., 1997; Chang et al., 2007). Fig. 4 shows the DPPH radical-scavenging activity of polyphenol and anthocyanin extracts in *K. coccinea* fruit. A well-known antioxidant compound, ascorbic acid, was used as positive control. From Fig. 4, when sample concentration was above 25 μg/ml, the DPPH radical-scavenging activity of ascorbic acid was higher than that of polyphenol and anthocyanin extracts from *K. coccinea* fruit. Peel polyphenol extracts exhibited stronger DPPH radical-scavenging activity than did anthocyanin extracts and pulp polyphenol extracts in the concentration ranges from 0 to 1000 μg/ml. The scavenging activity of pulp polyphenol extracts was lowest amongst four antioxidants. Furthermore, in the present study, DPPH radical-scavenging activity was linearly correlated (positive) with the concentrations of peel polyphenol extracts and anthocyanin extracts in the range 0 to 1000 μg/ml and ascorbic acid in the range 0–100 μg/ml, respectively. The corresponding correlation coefficients were 0.9838 for peel polyphenol extracts ($Y = 0.0602X + 4.3267$), 0.9722 for anthocyanin extracts ($Y = 0.0255X + 1.1749$) and 0.9858 for ascorbic acid ($Y = 0.9507X + 3.93$). By the calculation of 50% inhibition concentration, it was further confirmed that the DPPH radical-scavenging activity of samples decreased in the following order: ascorbic acid (IC_{50} 48.5 ± 0.745 μg/ml) > peel polyphenol extracts (IC_{50} 759 ± 19.0 μg/ml) > anthocyanin extracts (IC_{50} 1915 ± 43.1 μg/ml) > pulp polyphenol extracts (IC_{50} 22,302 ± 2614 μg/ml).

3.4.3. FRAP assay

FRAP assay depends upon the reduction of ferric tripyridyltriazine (Fe(III)–TPTZ) complex to ferrous tripyridyltriazine (Fe(II)–TPTZ) with an intensive blue colour by a reductant at low pH (Benzie & Strain, 1996). Reductants polyphenols and anthocyanins have strong electron-donating capacity, which can induce the formation of a blue coloured Fe(II)–TPTZ from the colourless oxidised Fe(III) form (Benzie & Strain, 1996). From Fig. 5, in the concentration range 0–1000 μg/ml, the FRAP value of samples decreased in the following order: ascorbic acid > peel polyphenol extracts >

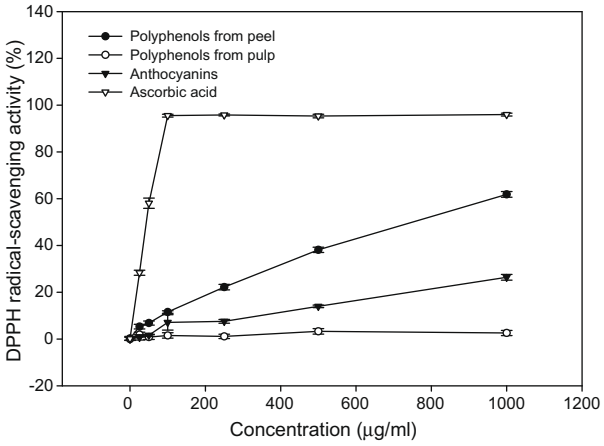

Fig. 4. DPPH radical-scavenging activity of polyphenol and anthocyanin extracts from *Kadsura coccinea* fruit.

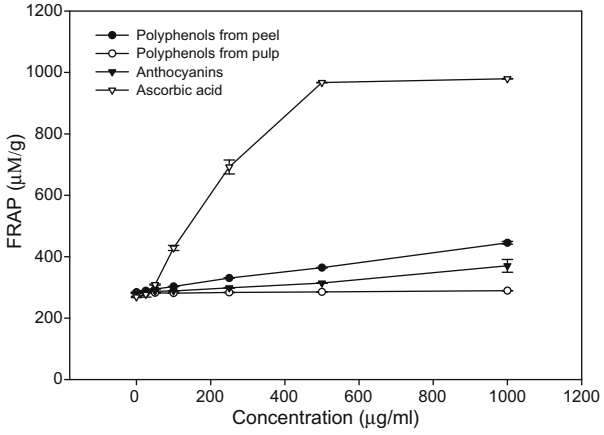

Fig. 5. Reducing power (FRAP values) of polyphenol and anthocyanin extracts from *Kadsura coccinea* fruit.

anthocyanin extracts > pulp polyphenol extracts, indicating that the ability of samples to reduce Fe(III) to Fe(II) (i.e. reducing power of samples) also decreased in the above order. In addition, by linear regression analysis, the FRAP value was positively correlated with the concentrations of peel polyphenol extracts, anthocyanin extracts and pulp polyphenol extracts in the range 0–1000 μg/ml and ascorbic acid in the range 0–500 μg/ml, respectively. The corresponding correlation coefficients were 0.9986 for peel polyphenol extracts ($Y = 0.1592X + 286.34$), 0.9789 for anthocyanin extracts ($Y = 0.0851X + 280.19$), 0.9718 for pulp polyphenol extracts ($Y = 0.0084X + 281$) and 0.984 for ascorbic acid ($Y = 1.4647X + 264.67$).

4. Conclusion

In the present study, polyphenols and anthocyanins in *K. coccinea* fruit, a novel and rare fruit with high nutritional value in South China, were extracted from fresh peel and pulp tissues. The total phenolic content of peel extracts was superior to that of pulp extracts. The total anthocyanin content of fleshy peel extracts was determined to be 180 ± 2.91 mg/100 g of fresh peel tissues. The influences of pH values and temperatures on properties of *K. coccinea* anthocyanins showed that they were stable under pH 4, whilst higher heating temperature and extended heating time induced their degradation. By measuring metal-chelating capacity, it could be estimated that no ortho-dihydroxy aromatic moiety existed in the B-ring of *K. coccinea* anthocyanin molecules, but there might be a catechol group in the B-ring of polyphenol molecules from peel and pulp tissues of this fruit. Moreover, the DPPH radical-scavenging activity and reducing power of *K. coccinea* extracts decreased in the following order: peel polyphenol extracts > anthocyanin extracts > pulp polyphenol extracts. The above results indicated that *K. coccinea* fruit possessed health benefits due to its polyphenol and anthocyanin components with antioxidant activities. It is worth developing this novel fruit to introduce it to other countries and areas.

Acknowledgements

The financial support provided by Scientific Research Foundation for Doctoral Personnel from Guangxi Academy of Agricultural Sciences was appreciated. The authors are grateful to Mr. Qingguo Wu for field management on *Kadsura coccinea* (Lem.) A.C. Smith.

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Food Chemistry 117 (2009) 282–289

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Diversity in properties of seed and flour of kidney bean germplasm

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ARTICLE INFO

Article history:
Received 25 September 2008
Received in revised form 31 March 2009
Accepted 1 April 2009

Keywords:
Kidney bean
Flour
Physicochemical
Pasting
Texture

ABSTRACT

The genetic diversity in seeds (physicochemical, hydration, textural and cooking properties) and flours (pasting and gel texture) among kidney bean lines was studied. A wide range of variation was observed for yield and yield-related traits. Seed weight, volume, density, hydration capacity, hydration index, swelling capacity, cooking time and amylose content ranged from 10.2 to 51.7 g/100 seeds, 14 to 46 ml/100 seeds, 0.51 to 2.15 g/ml, 0.03 to 0.62 g/seed, 0.16 to 0.97, 1.24 to 1.93 ml/seed, 50 to 120 min, and 0.09% to 5.02%, respectively. Hardness, cohesiveness, gumminess, springiness and chewiness of hydrated seeds ranged from 0.81 to 2.03 g, 0.18 to 0.48, 0.20 to 0.97 g, 0.31 to 0.51 and 0.08 to 0.43 g, respectively. Pasting temperature, peak viscosity, breakdown, final viscosity and setback ranged from 79 to 95 °C, 402 to 3235 cP, 9 to 393 cP, 862 to 5311 cP, and 363 to 2488 cP, respectively. Hardness, cohesiveness, gumminess, springiness, chewiness and adhesiveness of flour gels ranged from 3.9 to 5.3 g, 0.52 to 0.76, 1.47 to 23.52 g, 0.91 to 0.99, 3.21 to 23.91 and 13.2 to 178.5 g s, respectively.

1. Introduction

Legumes are the edible fruits or seeds of pod-bearing plants belonging to the order *Leguminosae* and are widely grown throughout the world. Legumes have been considered as the most significant food source for people of low incomes (Bressani & Elias, 1979). Legumes have a high food value and store well, play an important role in the diet of most of the people of the world, being second only to cereals as a source of human and animal food (Singh, Kaur, Sandhu, & Sodhi, 2004). Legumes not only add variety to the human diet, but also serve as an economical source of supplementary proteins for the large human populations in developing countries, such as India, where the majority of the population is vegetarian (Kaur & Singh, 2007b). In general, legumes are sources of complex carbohydrate, protein and dietary fibre, having significant amounts of vitamins and minerals, and high energetic value (Tharanathan & Mahadevamma, 2003). Legumes are generally soaked before cooking to ensure uniform expansion of the seed coat and cotyledon and to ensure their tenderness (Hoff & Nelson, 1965). Kidney bean (*Phaseolus vulgaris* L.) is the most widely produced and consumed food legume in Africa, India, Latin America and Mexico (FAO, 2002). This bean usually contains 20–30% protein on a dry basis, and the protein has a good amino acid composition but is low in sulphur-containing amino acids (notably methionine) and tryptophan (Gueguen & Cerletti, 1994; Sathe, 2002). Dry beans have recovered prestige in the diets of developed countries. This is due, in part, to health problems related to meat consumption, as well as the discovery of the benefits of legumes in the diet and the protection they afford against colon disease (Champ, 2001; Hangen & Bennink, 2003; Lee, Prosky, & DeVries, 1992; Mathres, 2002). Kidney beans have numerous health benefits, e.g., they reduce heart and renal disease risks, lower glycaemic index for persons with diabetes, increase satiation, and prevent cancer. Furthermore, kidney beans are regarded as an important source of protein and minerals for livestock feed production, as well as potential raw materials for processing into human food (Shimelis & Rakshit, 2007). A wide variation in chemical, thermal, pasting and textural properties of seeds, flour and starches of black gram and chickpea lines has been reported previously (Singh et al., 2004; Kaur & Singh 2005).

The objectives of the present investigation were to evaluate (i) physicochemical, hydration and textural properties of seeds from different kidney bean germplasm lines, (ii) composition, pasting and textural properties of flours and (iii) relationship between different properties of seeds and flours.

2. Materials and methods

2.1. Materials

The seed material for the present experiment comprised 50 diverse germplasm accessions (36 exotic and 14 indigenous) of kidney bean, namely EC18639, EC28758, EC45816, EC57004, EC116177, EC398501, EC398523, EC398548, EC405199, EC405179, EC405203, EC405213, EC405230, EC493713, EC498445, EC506078, EC530944,

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doi:10.1016/j.foodchem.2009.04.002

EC530952, EC530955, EC530966, EC530968, EC530969, EC530974, EC530977, EC530978, EC530991, EC530993, EC530996, EC540796, IC11660, IC199223, IC202271, IC262749, IC278582, IC313249, IC313257, IC341755, NC2162, NC58584, PI163833, PI197967, PI204719, PI240554, PI301808, PI312296, PI339501, PLB10-1, Laxmi, Vaspa and Triloki, which were grown at the Regional Station of the National Bureau of Plant Genetic Resources (NBPGR) located in Phagli area of Shimla at latitude of 31°.06′N and longitude of 77°.10′E in the year 2006–2007. The lines for quality analysis of seed and flour were selected on the basis of genetic variability observed for various characteristics but primarily for seed size and colour. The agroclimatic conditions of the area where crop was grown: rainfall (1350 mm), topography (submountainous) and temperature (14 °C), soils are shallow to moderately shallow, having loam to clay loam texture with varying proportions of gravel.

2.2. Seed weight

Seed weight was determined in triplicate. Kidney bean grains were randomly selected and 50 kernels of kidney bean grains were counted. The counted grains were then weighed and expressed in grams.

2.3. Seed volume

Seed volume was determined in triplicate (Williams et al., 1983). Fifty seeds were transferred to a 50-ml measuring cylinder and 25 ml distiled water were added. The difference in the volume was noted down and divided by 50 to calculate the volume per seed.

2.4. Bulk density

Kidney bean grains were gently added to a 100-ml graduated cylinder, previously tared. The bottom of the cylinder was gently tapped on a laboratory bench, several times, until there was no further diminution of the sample level after filling to the 100 ml mark. Bulk density was calculated as weight of sample per unit volume of sample (g/ml). All the measurements were in triplicate.

2.5. Hydration capacity

Fifty seeds were transferred to a 125-ml Erlenmeyer flask and water was added to 100 ml. The flask was lightly stoppered and left overnight at room temperature. Next day the grains were drained, superfluous water was removed with absorbent paper and the swollen seeds were reweighed. Hydration capacity per seed was recorded as:

$$(\text{weight after soaking} - \text{weight before soaking})/50.$$

2.6. Hydration index

Hydration index was calculated as:

$$\text{hydration capacity per seed}/\text{weight of one seed}(g)$$

2.7. Swelling capacity

Swelling capacity was calculated by reweighing the soaked seeds. Seeds were transferred to a 100-ml measuring cylinder and 50 ml water were added. Swelling capacity per seed was recorded as:

$$\frac{\text{volume after soaking} - \text{volume before soaking}}{50}$$

2.8. Swelling index

Swelling index was calculated as swelling capacity per seed/volume of one seed (ml).

2.9. Textural properties of soaked seeds

Texture profile analysis (TPA) of soaked kidney bean lines was performed on a single soaked grain from each variety, using a TA/XT texture analyser (Stable Microsystems, Crawley, UK). The grain was subjected to 75% compression with a probe (P/75) at a speed of 1 mm/s. The textural parameters of hardness (maximum height of the force peak on the first compression cycle), springiness (ratio of the time elapsing between the end of first bite and the start of second bite), cohesiveness (ratio of the positive force areas under the first and second compressions), gumminess (product of hardness and cohesiveness) and chewiness (product of gumminess and springiness) were determined. Twenty repeated measurements were performed on each sample.

2.10. Cooking time

For the determination of cooking time, about 250 ml distiled water were brought to boiling point in a 500-ml beaker fitted with condenser to avoid evaporation losses during boiling and then 25 g seed was added. Boiling was continued, and boiled seeds at intervals of 2 min were drawn and tested for their softness by pressing between the forefinger and thumb. The time taken to achieve the desirable softness was recorded as the cooking time of the sample.

2.11. Preparation of kidney bean flour

About 30 g of seeds from different legume lines were ground to pass through sieve No. 72 (BIS) to obtain flour, which was packed in airtight containers.

2.12. Proximate composition

Flour samples were evaluated for their moisture, ash, fat and protein (% $N \times 6.25$) contents by employing standard methods of analysis (AOAC, 1990). Studies were conducted in triplicate.

2.13. Amylose (%)

Amylose content of the isolated starch was determined by using the method of William, Kuzina, and Hlynka (1970). A starch sample (20 mg) was taken and 10 ml of 0.5 N KOH were added to it. The suspension was thoroughly mixed. The dispersed sample was transferred to a 100 ml volumetric flask and diluted to the mark with distiled water. An aliquot of test starch solution (10 ml) was pipetted into a 50-ml volumetric flask and 5 ml of 0.1 N HCl were added, followed by 0.5 ml of iodine reagent. The volume was diluted to 50 ml and the absorbance was measured at 625 nm. The measurement of the amylose was determined from a standard curve developed using amylose and amylopectin blends.

2.14. Pasting properties of kidney bean flours

Pasting properties of legume flour gels were evaluated using Rapid Visco Analyzer (RVA4, Newport Scientific Pvt. Ltd., Warriewood, Australia) from different legume lines. Viscosity profiles of flours were recorded using flour suspensions (29 g total weight). The temperature–time conditions included a heating step from 50 to 95 °C at 6 °C/min (after an equilibration time of 1 min at 50 °C), a holding phase at 95 °C for 5 min, a cooling step from 95 to 50 °C at 6 °C/min and a holding phase at 50 °C for 2 min. A typical RVA curve for

a kidney bean flour is shown in Fig. 1. Parameters recorded were pasting temperature, peak, trough (minimum viscosity at 95 °C), final (viscosity at 50 °C), breakdown (peak-trough viscosity), and setback viscosity (final-trough viscosity). All the measurements were in triplicate. Pasting temperature is the temperature at the onset of rise in viscosity. It provides an indication of the minimum temperature required to cook the flour. Final viscosity indicates the ability of the material to form a viscous paste. Setback viscosity measures the retrogradation tendency of flours upon cooling of cooked flour pastes. Breakdown viscosity measures the ease with which the swollen granules can disintegrate.

2.15. Textural properties of flour pastes

The flour pastes prepared in the RVA were poured into aluminium canisters and stored at 4 °C to cause gelation. Textural properties of RVA gels were determined using a TA/TX2 texture analyser (Stable Microsystems). Each canister was placed upright on a metal platform and the gel was compressed at a speed of 0.5 mm/s, using a 5-kg load cell to a distance of 10 mm with a cylindrical plunger (5 mm diameter). Hardness is defined as maximum height of the force peak on the first compression cycle (first bite). The compression was repeated twice to generate a forcetime curve, from which hardness (height of first peak) and springiness (ratio of the time elapsed between the end of first bite and the start of second bite) was determined. Cohesiveness was calculated as the ratio between the area under the second peak and the area under the first peak (Bourne, 1978). Gumminess was determined by multiplying hardness and cohesiveness. Chewiness was derived from gumminess and springiness and was obtained by multiplying these two. Ten repeated measurements were performed for each sample and their average was taken.

2.16. Statistical analysis

Statistical analysis of the results was done using Minitab Release 14 Statistical Software (Minitab Inc., State College, PA). Pearson correlation coefficients (r), for the relationships between various flour properties and grain parameters, were determined.

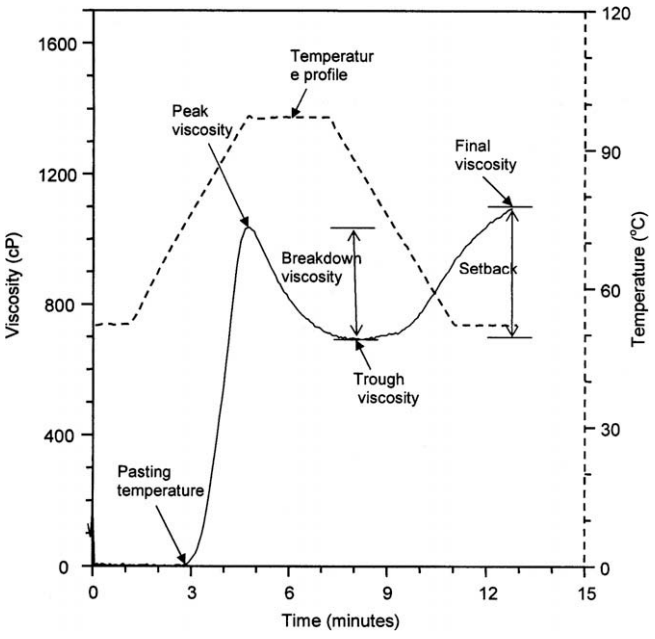

Fig. 1. Typical Rapid Visco Analyzer (RVA) Pasting Curve.

3. Results and discussions

3.1. Plant characteristics

The period for completion of 50% flowering ranged from 41 to 82 days. IC202271 completed 50% flowering in the shortest period (41 days) whereas Vaspa required the longest period (82 days). Leaflet length varied from 68 to 177 mm and it was smallest for EC498445 and longest for Vaspa and Triloki. Width of leaflet varied from 52 to 138.5 mm, EC506078 showed the smallest width (52 mm) and IC313249 had the greatest width (138.5 mm). Pod length also varied significantly among lines; IC313257 had the smallest pod length (76 mm) while Triloki had the longest (168 mm). Pod width ranged from 8 mm (EC530974) to 16 mm (EC530966, IC11660). Number of pods per plant varied from 9 to 34. EC506078 had the least pods whereas IC313257 had the highest. Days to 50% maturity ranged from 94 days for EC493713 to 140 days for EC405213. Seed width and length varied from 3.4 mm (EC116177) to 7.8 mm (EC493713), and 5.2 mm (EC498445) to 19.6 mm (PLB10–1), respectively. EC405203 gave highest yield (40 g/plant), while EC498445 gave lowest seed yield (4 g/plant). The number of seeds/pod varied between 4 (EC57004, EC398523, EC405199) and 7 (IC11660).

3.2. Physicochemical and cooking properties of grains

Various physical parameters evaluated for different lines of kidney beans were seed weight, seed volume, seed density, hydration capacity, hydration index, swelling capacity, swelling index and cooking time. PLB10-1 had the highest 100 seed weight (51.68 g/100 seeds) and seed volume (46 ml /100 seeds), whereas EC530969 showed the lowest seed weight (10.16/100 seeds). Deshpande, Sathe, and Salunkhe (1984) reported 100 seed weight of dry bean cultivars in the range between 15.0 and 50.3 g. Kaur and Singh (2007a) reported the 100 seed volume to be 34 ml for kidney beans. Lowest seed volume, i.e., 14 ml/100 seeds was observed for EC18639, EC28758, EC493713 and PI163833 whereas the highest seed volume (46 ml/100 seeds) was observed for PLB10-1. Density ranged between 0.51 to 2.15 g/ml, the lowest density was observed for EC530969 and the highest for PI163833. Deshpande et al. (1984) reported density of dry bean cultivars in the range of 1.18 to 1.36 g/ml. The cotyledon with higher density has been reported to have slower water uptake resulting in longer cooking time (Seena & Sridhar, 2005).

Hydration capacity ranged between 0.03 and 0.62 g/seed, EC530991 showed the lowest (0.03 g/seed) and PLB10-1 showed the highest hydration capacity (0.62 g/seed). Seena and Sridhar (2005) reported hydration capacity/seed of 0.08 g and hydration index of 0.1 for some under explored legumes. The higher water absorption of PLB10-1 may be attributed to the greater permeability of its seed coat and softer cotyledons (Singh et al., 2004). Hydration index ranged between 0.16 and 1.97; PI301808 showed the least and PI197967 showed the highest. These differences may be attributed to difference in size, seed coat thickness and water absorption characteristics of seeds (SefaDedah & Stanley, 1979). Swelling capacity ranged from 1.24 to 1.93 ml/seed; EC530968, EC530978, and EC530991 showed the lower swelling capacity (1.24 ml/seed) whereas PLB10-1 showed the higher swelling capacity. PI197967 possessed the lowest swelling index (4.65) whereas PI163833 the highest (11.34). Water-absorbing capacity of seeds depends on cell wall structure, composition of seed and compactness of the cells in the seed (Muller, 1967). Cooking time for different kidney bean cultivars varied significantly, ranging from 50 to 120 min. The lowest cooking time was observed for EC28758 and the highest for Vaspa. The difference in cooking times among legumes could be related to the rate at which cell

separation occurs, due to loosening of the intercellular matrix of the middle lamella upon cooking (Rockland & Jones, 1974). The varietal differences in cooking quality have been reported to exist even in the same legume (Narasimha & Desikachar, 1978). The longer cooking time requirement for PI204719 could be attributed to its larger seed weight, as seed size governs the distance to which water must penetrate in order to reach the innermost portion of seeds (SefaDedah & Stanley, 1979). Amylose content ranged between 0.09% and 5.02%; EC530974 showed the lowest while EC530968 showed the highest. The frequency distribution of physicochemical properties showed that 100 seed weight of most of the lines falls in the range between 20 and 30 g, seed volume from 20 to 24 ml/100 seeds, seed density from 1 to 1.4 g/ml, hydration capacity from 0.12 to 0.24 g/seed and swelling capacity from 1.28 to 1.60 (Fig. 2a).

3.3. Texture profile analysis (TPA) of soaked grains

The rate and degree of softening differed significantly among seeds from different kidney bean lines. Hardness of soaked kidney bean seeds ranged from 0.81 to 2.03 g. EC530993 showed the lowest hardness and PI339501 the highest. Singh, Sekhon, Bajwa, and Gopal (1992) reported that constituents like fibre, lignin, cellulose and hemicellulose are the factors responsible for hardness of grains, and lines with lower contents of these constituents had greater hardness and vice versa. Kaur and Singh (2007a) reported a hardness of 1.7 g for kidney beans. SefaDedah and Stanley (1979) reported that seed coats of lines possessing good hydration properties facilitate rapid softening of the seed during soaking.

Cohesiveness ranged between 0.18 and 0.48, EC28758 and EC398523 showed the lowest value and PI339501 showed the highest. Gumminess ranged from 0.20 g (PI197967) to 0.97 g (PI339501). Springiness was found to be the lowest for Laxmi (0.31) and the highest for EC45816 (0.51). Chewiness varied from 0.08 to 0.43 g, EC398523 showed the lowest chewiness whereas PI339501 showed the highest. Kaur and Singh (2007a) reported cohesiveness, gumminess, springiness and chewiness values of 0.30, 0.49 g, 2.55 and 1.25 g, respectively for kidney beans. The frequency distribution of textural properties of hydrated kidney bean seeds showed most lines have a hardness in the range of 1.2 to 1.5 g, cohesiveness 0.18 to 0.40, gumminess 0.25 to 0.55 g, springiness 0.40 to 0.43 and chewiness in the range of 0.1 to 0.20 g (Fig. 2b).

3.4. Proximate composition of flours

The proximate composition of flours varied significantly among different kidney bean lines. Ash content varied from 3% to 6%. EC405213, EC540796, EC530974, IC313249, PI163833, PI204719 and Triloki showed lower ash content (3%) than EC405230, IC199223 and IC313257 (6%). Protein content ranged between 16% and 26%, with the lowest for EC530974 and the highest for EC116177. Fat content ranged from 0.60% (IC11660) to 2.38% (EC540796). Seena and Sridhar (2005) reported an ash content, protein content and fat content of 3.3%, 32% and 1.9 %, respectively, for legume flour. The frequency distribution of composition of kidney bean flour showed a maximum number of lines have ash content in the range between 3.5% and 5.5%, fat 1% and 1.8%, protein 18% and 23% (Fig. 3).

3.5. Pasting properties of flour

The pasting properties of flours from different kidney bean lines differ significantly, with a range between 402 and 3235 cP, 31 and 414 cP, 362 and 2488 cP 862 and 5310 cP, and 79 and 95 °C, respectively, for pasting viscosity, breakdown, setback, final viscosity and pasting temperature. The behaviour of the viscosity during cooking (from 50 to 90 °C) reflects the capacity of the starch to absorb water and swell (Sandhu, Singh, & Malhi, 2007). Among various lines studied, EC540796 showed the highest peak viscosity (3235 cP), trough viscosity (2823 cP), final viscosity (5310 cP) and setback (2488 cP). EC398501 showed the lowest breakdown, whereas PI301808 showed the highest breakdown. The lowest breakdown of EC398501 flour indicated its high thermal stability. EC530996 showed the lowest final viscosity of 862 cP. EC530993 showed the lowest setback (362 cP), indicating

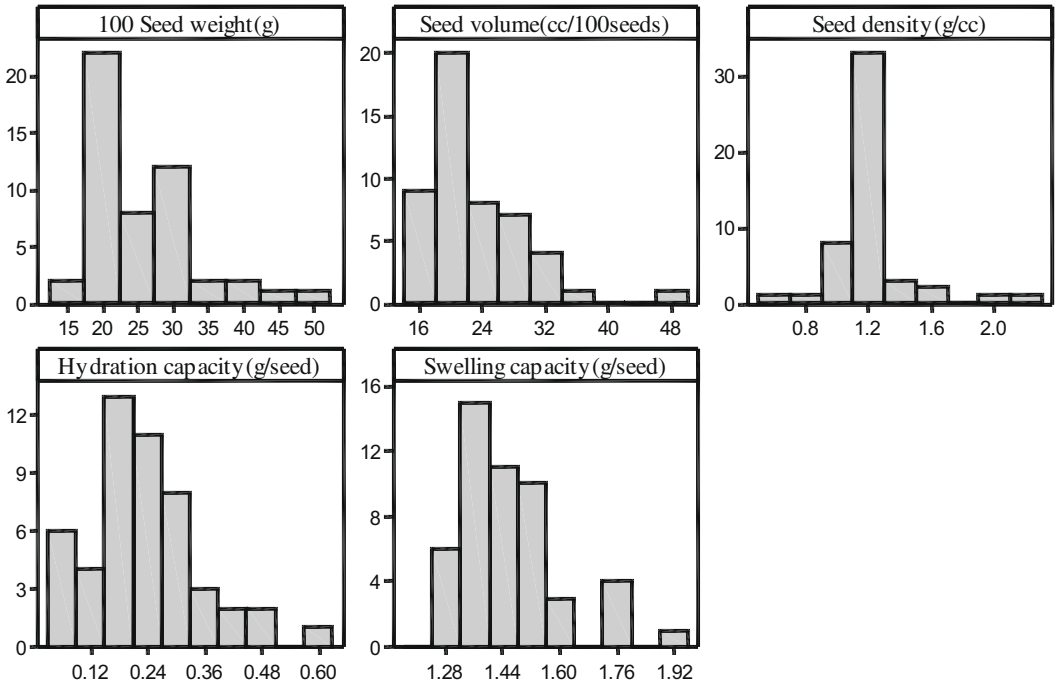

Fig. 2a. Frequency distributions for physicochemical properties of seeds of kidney bean germplasm accessions.

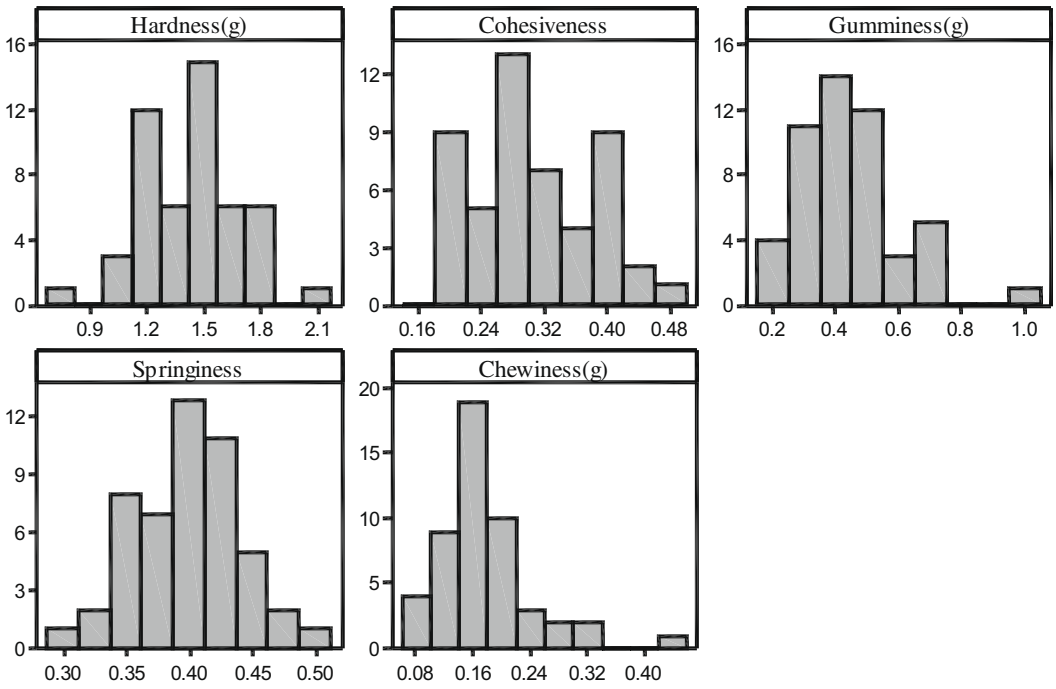

Fig. 2b. Frequency distributions for textural properties of hydrated seeds of kidney bean germplasm accessions.

its lower tendency to retrogradation (Sandhu et al., 2007). EC530966 and EC530978 showed the lowest pasting temperatures and EC530955 and NC2162 showed the highest. Pasting temperature provides an indication of the minimum temperature required to cook, as well as the temperature at which the viscosity begins to increase during the heating process. The majority of the lines showed pasting temperatures around 82 °C. A gradual increase in viscosity with increase in temperature may be attributed to the removal of water from the exuded amylose by the granules on swelling (Ghiasi, Varriano-Marston, & Hoseney, 1982). During cooling (from 95 to 50 °C), the viscosity increased, owing to the alignment of the chains of amylose (Flores-Farias et al., 2000). The frequency distribution histogram showed that the majority of lines had peak viscosity and final viscosity from 1600 to 2000 cP and 2500 to 3500 cP, respectively (Fig. 3). Setback showed a wide diversity, ranging from 363 to 2488 cP, however, the majority of lines had setback between 1200 and 1400 cP (Fig. 4).

3.6. Gel texture properties of flours

The textural parameters of gels from different kidney bean lines varied significantly. Gel hardness ranged from 2.7 to 39 g, NC2162 showed the lowest and EC530996 showed the highest. The gel firmness is mainly caused by retrogradation of starch, which is associated with the synaeresis of water and crystallisation of amylose, leading to harder gels (Miles, Morris, Orford, & Ring, 1985). Cohesiveness varied from 0.50 to 0.77, EC506078 had the highest and IC313257 had the lowest. NC2162 showed the lowest gel gumminess, with a value of 1.47, whereas EC530996 showed the highest gel gumminess (23.52). Springiness ranged between 0.86, for EC498445, and 0.99 for IC313249. Chewiness varied between 1.48 and 23.9 g. NC2162 showed the lowest chewiness, whereas EC530996 showed the highest chewiness. Adhesiveness of flour gel ranged from 13.3 to 165 g s. EC530944 showed the lowest adhesiveness, whereas NC2162 showed the highest. The results clearly showed that the lines with higher amylose content had the higher adhesiveness and vice versa. The frequency distribution of textural properties of kidney bean flour showed most lines have hardness in the range of 6 to 12 g, cohesiveness 0.6 to 0.76, gumminess 4 to 8 g, springiness 0.94 to 0.98, chewiness 2 to 7 g and adhesiveness in the range of 30 to 80 g s (Fig. 5).

3.7. Pearson correlations between different properties of grains and flours

Pearson correlation coefficients between various physicochemical, pasting and textural properties of different kidney bean lines are shown in Table 1. Seed volume was positively correlated with hydration capacity ($r = 0.563$, $p \leqslant 0.005$), swelling capacity ($r = 0.688$, $p \leqslant 0.005$), and cooking time ($r = 0.275$, $p \leqslant 0.05$), consistent with earlier reported results (Singh et al., 2004) for black gram, and negatively correlated with swelling index ($r = 0.830$, $p \leqslant 0.005$). This is in accordance with the findings reported by Khairwal, Berwal, and Sharma (1997). Seed weight was positively correlated with hydration ($r = 0.617$, $p \leqslant 0.005$) and swelling capacity ($r = 0.832$, $p \leqslant 0.005$), as reported earlier by Khattak, Khattak, Mahmood, Bibi, and Ihsanullah (2006), as well as with seed density ($r = 0.360$, $p \leqslant 0.05$), and negatively correlated with swelling index ($r = 0.444$, $p \leqslant 0.005$). Swelling capacity was positively correlated to hydration capacity ($r = 0.893$, $p \leqslant 0.005$), hydration index ($r = 0.462$, $p \leqslant 0.005$), as observed earlier by Khairwal et al. (1997) for pearl millet, and negatively correlated with amylose ($r = 0.293$, $p \leqslant 0.05$), gel hardness ($r = 0.434$, $p \leqslant 0.005$), gel gumminess ($r = 0.422$, $p \leqslant 0.005$) and gel adhesiveness ($r = 0.328$, $p \leqslant 0.05$). The water absorption of seeds depends on cell wall, seed composition and tightness of the cells in the seeds (Muller, 1967). Cooking time had a positive correlation with seed gumminess ($r = 0.546$, $p \leqslant 0.005$), also observed by Singh et al. (2004), seed cohesiveness($r = 0.575$, $p \leqslant 0.005$), seed), seed chewiness ($r = 0.550$, $p \leqslant 0.005$), gel hardness ($r = 0.288$, $p \leqslant 0.005$), gel gumminess ($r = 0.295$, $p \leqslant 0.05$), gel chewiness ($r = 0.300$, $p \leqslant 0.05$), and was negatively correlated with swelling index ($r = 0.292$, $p \leqslant 0.05$) and hydration index ($r = 0.303$, $p \leqslant 0.05$) as also reported early by Kaur, Singh, and Sodhi (2005). Swelling index was positively correlated with seed density($r = 0.594$, $p \leqslant 0.005$).

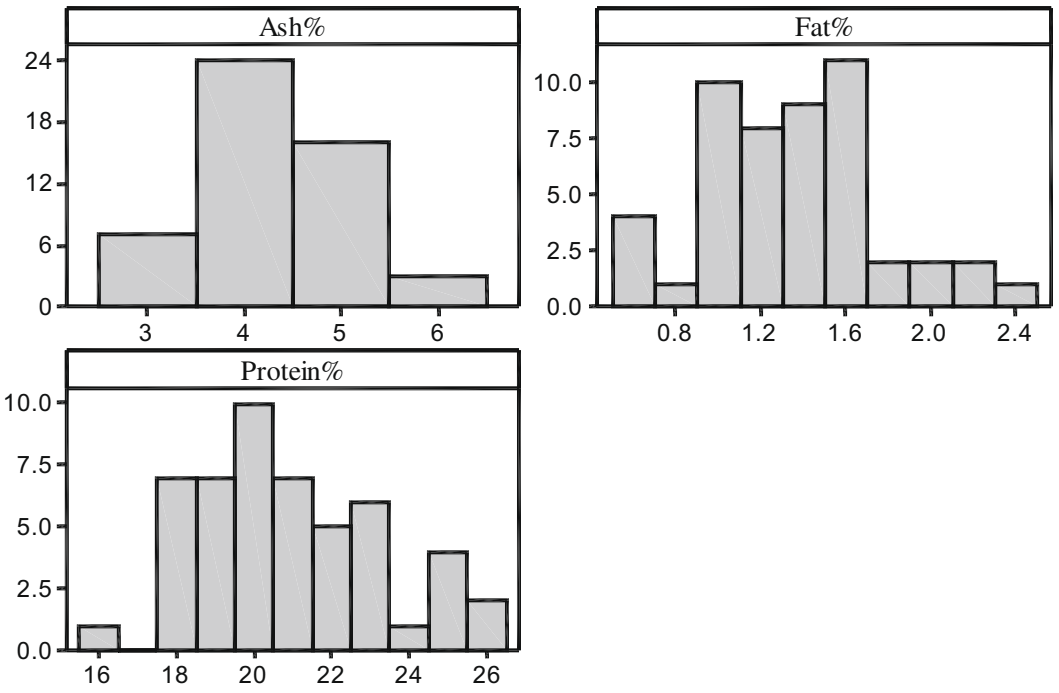

Fig. 3. Frequency distributions for chemical constituents of flours from kidney bean germplasm accessions.

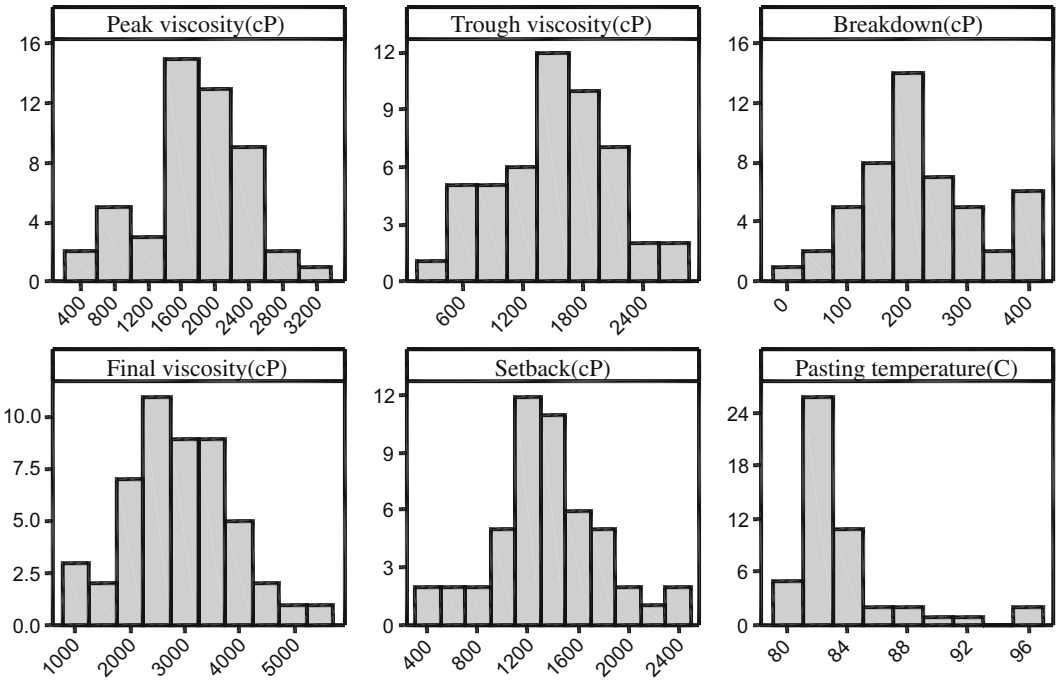

Fig. 4. Frequency distribution for pasting properties of flour from kidney bean germplasm accessions.

Hydration index was positively correlated with hydration capacity($r = 0.750$, $p \leqslant 0.005$) and negatively correlated with gel chewiness ($r = 0.397$, $p \leqslant 0.005$). Hardness of soaked seed was positively correlated with seed gumminess ($r = 0.692$, $p \leqslant 0.005$) as observed also by Kaur et al. (2005) and chewiness ($r = 0.688$, $p \leqslant 0.005$). Seed cohesiveness was positively correlated with seed gumminess ($r = 0.862$, $p \leqslant 0.005$), chewiness ($r = 0.805$, $p \leqslant 0.005$), gel hardness ($r = 0.351$, $p \leqslant 0.05$), gel gumminess ($r = 0.332$, $p \leqslant 0.05$), gel chewiness ($r = 0.343$, $p \leqslant 0.05$). Seed gumminess was positively correlated with seed chewiness ($r = 0.963$, $p \leqslant 0.005$), as observed also by Kaur et al. (2005), gel hardness ($r = 0.291$, $p \leqslant 0.05$), gel adhesiveness ($r = 0.284$, $p \leqslant 0.05$), gel gumminess ($r = 0.278$, $p \leqslant 0.05$) and gel chewiness ($r = 0.2763$, $p \leqslant 0.05$). Seed springiness was positively correlated with seed chewiness ($r = 0.308$, $p \leqslant 0.05$). Above correlations indicated a significant interdependence of these parameters.

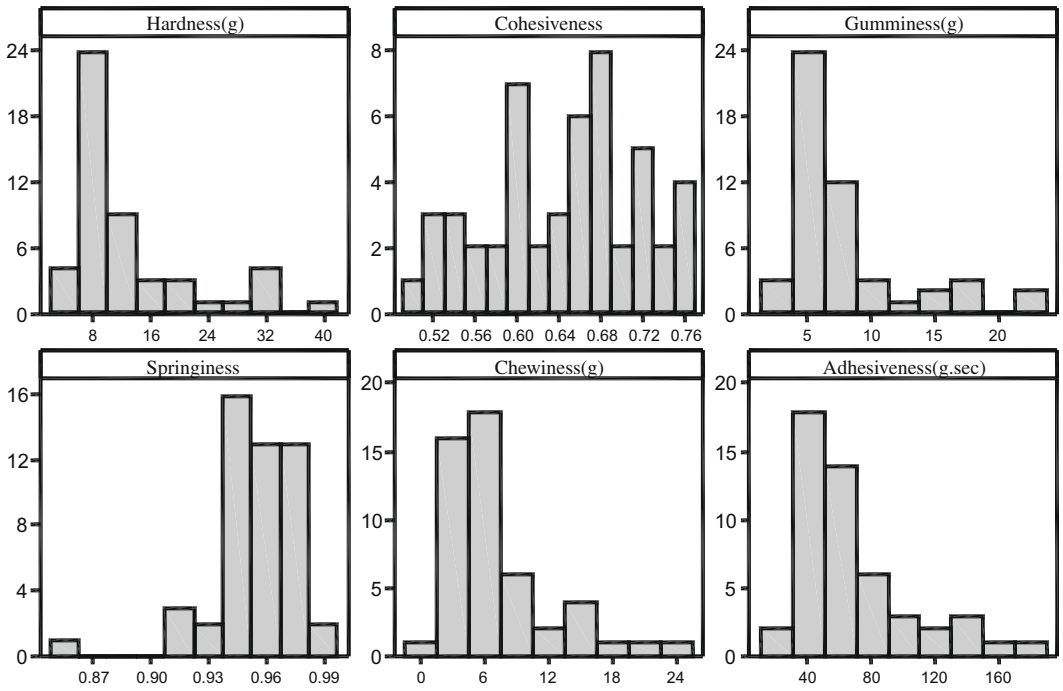

Fig. 5. Frequency distributions of gel textural properties of flours from kidney bean germplasm accessions.

Table 1
Pearson correlation coefficients between various physicochemical, pasting and textural properties of different kidney bean lines.

Parameters	*r*-Values
Swelling Index vs. seed density	0.594***
Cooking time vs. seed volume	0.275**
Cooking time vs. swelling index	0.292**
Cooking time vs. hydration index	0.303**
Amylose vs. swelling capacity	0.293**
Seed gumminess vs. cooking time	0.546***
Seed chewiness vs. cooking time	0.550***
Setback viscosity vs. fat	0.284**
Gel hardness vs. hydration index	0.407***
Gel hardness vs. swelling capacity	0.434***
Gel hardness vs. cooking time	0.288***
Gel adhesiveness vs. hydration capacity	0.275**
Gel adhesiveness vs. hydration index	0.362**
Gel adhesiveness vs. swelling capacity	0.328**
Gel adhesiveness vs. amylose	0.411***
Gel adhesiveness vs. seed gumminess	0.284**
Gel cohesiveness vs. seed volume	0.288***
Gel cohesiveness vs. setback	0.403***
Gel gumminess vs. hydration index	0.393***
Gel gumminess vs. swelling capacity	0.422***
Gel gumminess vs. cooking time	0.295**
Gel gumminess vs. amylose	0.303**
Gel chewiness vs. hydration index	0.397***
Gel chewiness vs. swelling capacity	0.425***
Gel chewiness vs. cooking time	0.300**
Gel adhesiveness vs. peak viscosity	0.454***
Gel adhesiveness vs. trough viscosity	0.479***
Gel adhesiveness vs. final viscosity	0.402**
Gel adhesiveness vs. pasting temperature	0.330**

** $p \leqslant 0.05$.
*** $p \leqslant 0.005$.

Trough viscosity of flour gel was positively correlated to peak viscosity ($r = 0.987$, $p \leqslant 0.005$), breakdown ($r = 0.479$, $p \leqslant 0.005$), amylose ($r = 0.302$, $p \leqslant 0.05$), final viscosity ($r = 0.946$, $p \leqslant 0.005$), setback ($r = 0.769$, $p \leqslant 0.005$), and negatively correlated with pasting temperature ($r = 0.532$, $p \leqslant 0.005$). Setback viscosity was positively correlated with fat ($r = 0.284$, $p \leqslant 0.05$). Similar relationships between setback and fat were reported earlier for rice starch by Liang, King, and Shih (2002). Setback viscosity was also positively correlated with peak viscosity ($r = 0.798$, $p \leqslant 0.005$), breakdown viscosity ($r = 0.596$, $p \leqslant 0.005$), final viscosity ($r = 0.924$, $p \leqslant 0.005$), gel cohesiveness ($r = 0.403$, $p \leqslant 0.005$) and negatively correlated with pasting temperature ($r = 0.494$, $p \leqslant 0.005$). Peak viscosity was positively correlated with amylose ($r = 0.286$, $p \leqslant 0.05$), breakdown ($r = 0.601$, $p \leqslant 0.005$), final viscosity ($r = 0.958$, $p \leqslant 0.005$), similar results were reported for black gram by Singh et al. (2004). Peak viscosity was also positively correlated with gel adhesiveness ($r = 0.454$, $p \leqslant 0.005$) and negatively correlated with pasting temperature ($r = 0.515$, $p \leqslant 0.005$). Final viscosity was positively correlated with breakdown viscosity ($r = 0.569$, $p \leqslant 0.05$), gel adhesiveness ($r = 0.102$, $p \leqslant 0.05$) and negatively correlated with pasting temperature ($r = 0.531$, $p \leqslant 0.005$).

Hardness of gel was positively correlated with seed chewiness ($r = 0.273$, $p \leqslant 0.05$), gel gumminess ($r = 0.981$, $p \leqslant 0.005$), gel chewiness ($r = 0.983$, $p \leqslant 0.005$), gel adhesiveness ($r = 0.764$, $p \leqslant 0.005$) and negatively correlated with hydration index ($r = 0.407$, $p \leqslant 0.005$). Gel gumminess was positively correlated with amylose ($r = 0.303$, $p \leqslant 0.05$), gel chewiness ($r = 0.997$, $p \leqslant 0.005$), gel adhesiveness ($r = 0.763$, $p \leqslant 0.005$), and negatively correlated with hydration index ($r = 0.393$, $p \leqslant 0.005$). Adhesiveness of flour gels was positively correlated with amylose ($r = 0.411$, $p \leqslant 0.005$), and gel chewiness ($r = 0.732$, $p \leqslant 0.005$), and negatively correlated with hydration capacity ($r = 0.275$, $p \leqslant 0.05$), hydration index ($r = 0.362$, $p \leqslant 0.05$), pasting temperature ($r = 0.330$, $p \leqslant 0.05$), and gel springiness ($r = 0.358$, $p \leqslant 0.05$).

4. Conclusion

This study demonstrated the diversity in physicochemical, pasting and textural properties of seeds and flours of different kidney bean lines. The study is useful in selecting lines with specific desired characteristics. PLB10-1 showed the highest hydration and swelling capacity, and hence, was most suitable for processing.

Acknowledgements

The financial assistance from Department of Science and Technology (DST), Ministry of Science and Technology, Govt. of India, to N.S. is acknowledged.

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Food Chemistry 117 (2009) 290–295

Contents lists available at ScienceDirect

Food Chemistry

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Inhibition of oxidation of omega-3 polyunsaturated fatty acids and fish oil by quercetin glycosides

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ARTICLE INFO

Article history:
Received 26 January 2009
Received in revised form 29 March 2009
Accepted 1 April 2009

Keywords:
Omega-3
PUFA
Fish oil
Lipid oxidation
TBARS
Quercetins
Flavonols
BHT
α-Tocopherol

ABSTRACT

The antioxidant properties of naturally occurring flavonols, quercetin glycosides, were examined and compared with common food antioxidants butylated hydroxytoluene (BHT) and α-tocopherol. Antioxidants were incorporated into selected polyunsaturated fatty acids (PUFA) or fish oil in aqueous emulsions and bulk oil systems. The effectiveness of quercetin was similar to or greater than quercetin glycosides in inhibiting lipid oxidation in the oil-in-water emulsion systems when oxidation was induced by heat, light, peroxyl radical or ferrous ion. In bulk fish oil, C-3 glycosylation enhanced the antioxidant activity of quercetin. The effectiveness of quercetin and its glycosides was greater than that of α-tocopherol in the emulsions. Quercetin and quercetin-3-*O*-glucoside exhibited a better antioxidant activity than BHT in bulk fish oil; however, the reverse was observed in the emulsions of omega-3 PUFA and fish oil systems in agreement with the polar paradox theory. Quercetin and its glycosides were more effective than α-tocopherol in emulsion systems.

1. Introduction

Dietary lipids, fatty acid profiles and their balance in food and within the body have received considerable attention in recent years. This is due to a better recognition that low levels of omega-3 fatty acids may be implicated in several chronic diseases (Shahidi & Miraliakbari, 2004, 2005; Simopoulos, 2002). As a result, omega-3 fatty acid-containing functional foods and nutraceuticals have been introduced into the market. However, highly unsaturated fatty acids (HUFA) in such oils are vulnerable to oxidation, thus producing various aldehydes and ketones that render unacceptable colours, odours and flavours in polyunsaturated fatty acid (PUFA) containing foods and nutraceutical products (Nawar, 1996). Moreover, products of lipid oxidation, such as propanal, acrolein and malonaldehyde, among others, possess adverse health effects due to their cytotoxic and genotoxic effects (Esterbauer, Schaur, & Zollner, 1990; Fang, Vaca, Valsta, & Mutanen, 1996). The high rate of oxidation of PUFA can be controlled by the addition of synthetic or natural antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ) and α-tocopherol. Recently, consumer health consciousness has led to a demand for 'natural' alternatives to synthetically produced food antioxidants.

Flavonols are a sub-group of flavonoids, found ubiquitously in fruits, vegetables and many medicinal and aromatic plants (Rupasinghe, 2008). Quercetin, a common flavonol, has been shown as an effective antioxidant in several *in vitro* systems such as the oxygen radical absorbance capacity (ORAC) (Ou, Hampsch-Woodill, & Prior, 2001), the ferric reducing antioxidant power (FRAP) (Pulido, Bravo, & Saura-Calixto, 2000) and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assays (Kemertelidze, Tsitsishvili, Alaniya, & Sagareishvili, 2000). When compared to other flavonoids, quercetin has been shown to prolong the lag time before the initiation of low density lipoprotein (LDL) oxidation effectively (Safari & Sheikh, 2003). As well, quercetin has also been shown to inhibit lipid oxidation in cereal grains (Viscidi, Dougherty, Briggs, & Camire, 2004) and marine oils rich in PUFA (Wanasundara & Shahidi, 1998; Montero, Giménez, Pérez-Mateos, & Gómez-Guillén, 2005; Nieto et al., 1993). Heat-induced cholesterol oxidation can also be reduced by incorporation of 0.002% (w/w) quercetin (Chien, Hsu, & Chen, 2006).

In plants, quercetin occurs in the glycosylated forms such as glucoside, galactoside, rhamnoside, arabinoside and rutinoside (Herrera & Luque de Castro, 2004). In fruits such as apples, glyco-

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doi:10.1016/j.foodchem.2009.04.007

sylation is commonly occur at the C-3 position (Rupasinghe, 2008) but the influence of C-3 glycosylation and the type of sugar moiety on its ability to inhibit omega-3 PUFA oxidation is not clear. Therefore, the objective of this research was to examine the inhibition of oxidation of omega-3 PUFA in emulsion and fish oil by selected naturally occurring quercetin glycosides and to compare their ability with common food antioxidants such as BHT and α-tocopherol, under different oxidation-induced conditions.

2. Materials and methods

2.1. Materials and chemicals

The authentic flavonol compounds were obtained as follows: quercetin-3-*O*-rhamnoside (quercitrin) and quercetin-3-*O*-galactoside (hyperin) were from Indofine Chemical Company (Hillsborough, NJ, USA); quercetin-3-*O*-glucoside (isoquercitrin) and quercetin aglycone were from Sigma–Aldrich (St. Louis, MO, USA); and quercetin-3-*O*-rutinoside (rutin) was from ChromaDex (Santa Ana, CA, USA). Methyl linolenate (MLN) and docosahexaenoic acid (DHA) were obtained from NuChek (Elysian, MN, USA). The bulk fish oil (Canadian Food Inspection Agency [CFIA] registration number 3529) was a gift from Ocean Nutrition Canada, Dartmouth, NS, Canada. The fish oil was devoide of any antioxidants. The composition of the oil was 17.6%, monounsaturates; and 77.6% polyunsaturates [61% eicosapentaenoic acid (EPA), 4.3% DHA] by weight of total fatty acids. Approximately 51% of EPA and DHA were in the triacylglycerol form. BHT, trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA) and α-tocopherol were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2,2′-Azobis(2-amidinopropane)dihydrocholride (AAPH) was acquired from Wako Chemicals (Richmond, VA, USA). Other chemicals were obtained from Fisher Scientific (Ottawa, ON, Canada).

2.2. The aqueous emulsion (oil-in-water) model system

The MLN, DHA and fish oil model systems were adapted from those of Okuda, McClements, and Decker (2005) and Boadi, Iyere, and Adunyah (2003) as follows: MLN or DHA (1.5 mg per mL) was suspended in a buffer solution (0.05 M TRIS–HCl, 0.15 M KCl, 1% Tween 20, pH 7.0) by homogenisation for 20 s using a Polytron homogeniser (Kinematica GmbH, Kriens, Switzerland) and placed in 13 × 100 mm borosilicate glass tubes. For fish oil emulsion (10 mg per mL), sonication (20 kHz, model 750D, VWR International, Mississauga, ON, Canada) for 30 s was performed to create the emulsion. For all experiments the test tubes were incubated using a shaker rotating horizontally at approximately 150 rpm to maintain the emulsion. For the experiments using $FeSO_4$ as a source of Fe^{2+}, the buffer solution was adjusted to pH 5. The stock emulsion was divided into individual experimental units (test tubes) before antioxidants were added. The concentration dependence of quercetin, quercetin-3-*O*-glucoside and BHT was examined at three levels (10, 50 and 100 μM) for the MLN and DHA systems. The concentration dependence for the fish oil system was examined at four levels (0.1, 1, 10 and 100 μM). The tubes were capped with test tube closures which allowed the exchange of air, vortexed and then placed on a rotational shaker (150 rpm) either at room temperature, or at 50 °C. All tests were performed in triplicate and the experiments were conducted twice, independently.

2.3. Inductions of oxidation

The oxidation systems were optimised for determination of antioxidant properties of quercetin glycosides, α-tocopherol and BHT under various oxidation–induction conditions. MLN or DHA emulsions (1 mL final volume) were incorporated with antioxidants and then subjected to oxidation under different conditions: heat, light, iron (100 μL of 10 mM $FeSO_4$ for the MLN and 100 μL of 1 mM $FeSO_4$ for the DHA), or peroxyl radical (generated using 100 μL of 100 mM AAPH). Heat-induced oxidation for emulsions was carried out by incubation of test tubes at 50 °C for 10 h using an incubator-shaker oven (model Apollo HP50, CLP Tools, San Diego, CA, USA). Light-induced induction was performed using a 13 W light source (300–640 nm with peaks at approximately 440, 490, 540, 590 and 610 nm; Model Repti Glo 2.0uvB; HAGEN, China) at a distance of 12 cm. The incubation periods for peroxyl radical- and iron-induced oxidation were 20 and 3 h, respectively. These optimum induction periods were based on preliminary experiments that were conducted to study the time-oxidation relationships of the MLN model system (data not presented).

2.4. The bulk fish oil model system

The bulk fish oil model system was prepared by oxidising 90 μL of the fish oil in 13 × 100 mm borosilicate glass tubes with caps. Antioxidants were incorporated by placing 100 μL of the desired concentration of the specific compound in methanol in each test tube, drying the solvent completely under nitrogen, and then mixing with 10 μL of ethanol and 90 μL of fish oil. The final concentrations of quercetin glycosides used were 0.1, 0.5, 1 and 5 mM. Fish oil samples were incubated using the same shaker rotating horizontally (150 rpm) at 70 °C for 3 h.

2.5. Effect of the type of sugar moiety of quercetin glycosides on the inhibition of PUFA oxidation

The antioxidant properties of four naturally occurring quercetin glycosides (quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-rutinoside), quercetin, BHT and α-tocopherol were determined using 1 mL of emulsion systems of MLN and DHA (mentioned above) with peroxyl radical-induced oxidation (100 μL of 100 mM AAPH). The MLN and DHA emulsions were incorporated with 100 μL of 0.1 and 0.5 mM, respectively, of the antioxidant compounds. The final concentrations of antioxidants, 10 μM for MLN and 50 μM for DHA, were chosen based on the concentration effect as shown in Fig. 1.

2.6. Thiobarbituric acid reactive substances (TBARS) assay

After completion of the duration of the oxidation-induction treatment, TBARS were quantified by a modified method of Boadi et al. (2003) and Okuda et al. (2005), as follows: One-hundred microlitres of 2% BHT in ethanol were added to all test tubes to stop the oxidation process immediately. The TBA reagent (1 mL of 15% (w/v) TCA and 0.375% (w/v) TBA in 0.25 M HCl) was then added and vortexed. The reaction mixture was placed in an 80 °C water bath for 15 min. At the end of this time, the samples were brought to room temperature and centrifuged at 2000*g* for 10 min (Model 300 Precision, Durafuge, Winchester, VA, USA). The absorbance of the supernatant was then measured at 532 nm using 96-well microplates in the FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA). Percent inhibition of oxidation was calculated based on percentage of the total oxidation experienced by the system without the protection of antioxidants using the following equation:

$$\%\ \text{Inhibition of oxidation} = [1 - (\text{sample absorbance}/\text{control absorbance})] \times 100$$

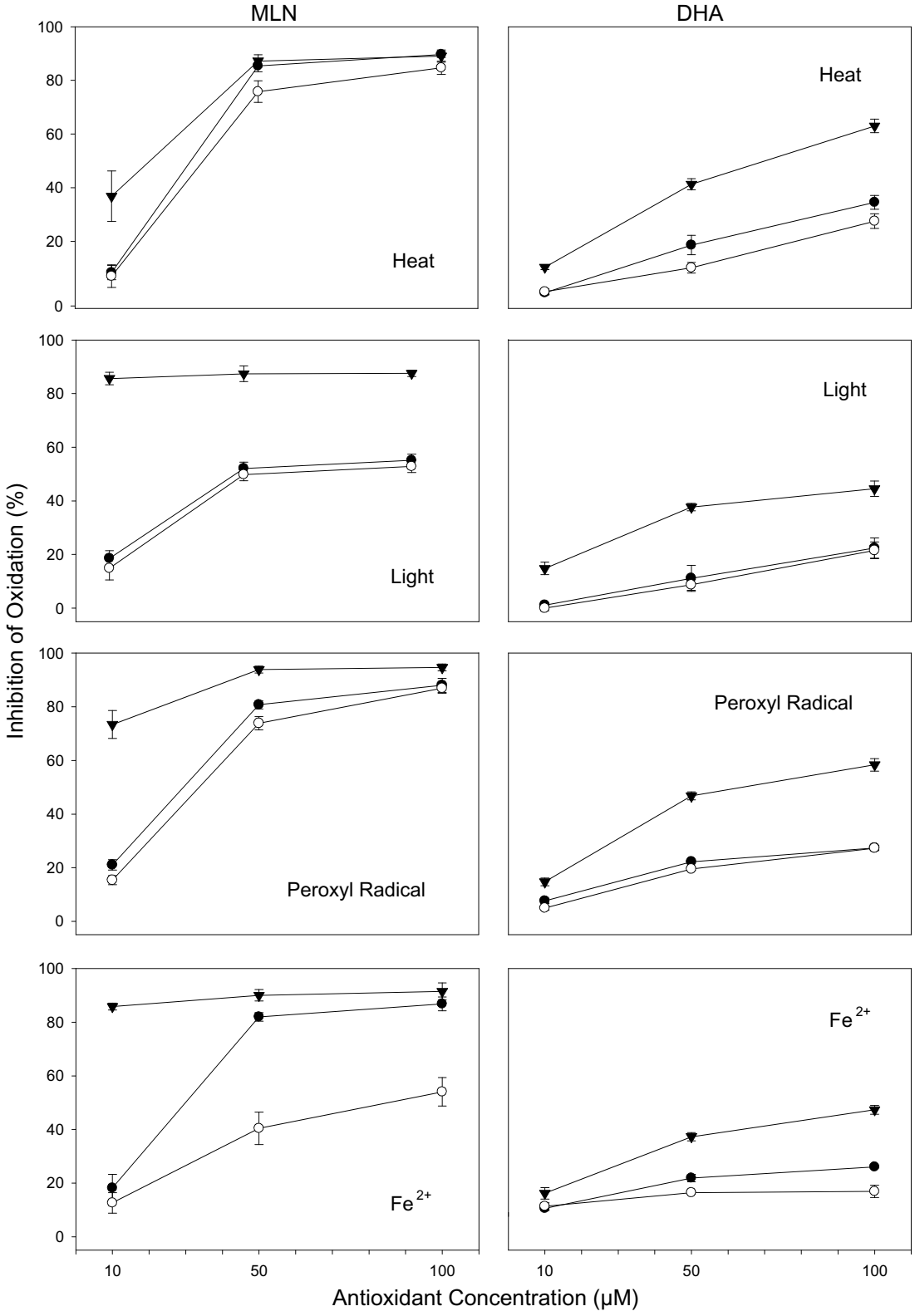

Fig. 1. Effect of different concentrations of BHT (▼), quercetin (●) and quercetin-3-*O*-glucoside (○) on the oxidation of the MLN and DHA emulsions. Oxidation was measured by the TBARS assay. Error bars represent standard error (SE). Data represent means of two independently conducted experiments in triplicate.

2.7. Experimental design and statistical analysis

The experimental design was a completely randomised one with treatments being the type and concentration of antioxidants employed. All experiments were conducted in triplicate, independently twice. A one-way analysis of variance blocked by experimental run and least significant difference (LSD) multiple means analysis was used to determine significant differences among the glycoside treatments, $\alpha = 0.05$, and assignment of letter groupings with SAS V8 (Cary, NC, USA). Graphical representations were made

using SigmaPlot 10.0 (San Jose, CA, USA). Error bars in the graphs represent standard error.

3. Results

3.1. Inhibition of PUFA oxidation by quercetin and quercetin-3-O-glucoside under various inductions

To examine the effect of C-3 glycosylation of quercetin on inhibition of PUFA oxidation, 10, 50 and 100 μM quercetin, quercetin-3-*O*-glucoside and BHT were incorporated into the aqueous emulsions of MLN and DHA and subjected to the oxidation by heat, light, peroxyl radical and Fe^{2+}, as inducers. In general, the higher the concentration of antioxidants, greater was the inhibition of oxidation of MLN and DHA (Fig. 1). In general, BHT was more effective in its inhibition of lipid oxidation than quercetin and its glucoside. However, quercetin and its glucoside at 100 μM were as effective as BHT in inhibiting heat-induced oxidation of MLN. Regardless of the type of antioxidant and induction method, inhibition of oxidation was lower in emulsions of DHA than that of MLN (Fig. 1). Meanwhile, the antioxidant activity of quercetin was similar or better than that of quercetin-3-*O*-glucoside in the omega-3 PUFA emulsions. The influence of C-3 glycosylation was more distinct when the oxidation was induced by ferrous ion.

3.2. Effect of type of sugar moiety at C-3 on the inhibition of PUFA oxidation

The effect of the type of sugar moiety at C-3 of quercetin was further studied by comparing the antioxidant activity of four quercetin glycosides (quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-rutinoside) with their aglycone, BHT and α-tocopherol using the peroxyl radical-induced oxidation of emulsions of MLN and DHA. For the emulsions containing DHA, 50 μM antioxidants were used instead of 10 μM due to the low inhibition of oxidation at the latter level (Fig. 1). Depending on the fatty acid used for the emulsion and the type of sugar moiety, C-3 glycosylation of quercetin had either no influence or a lower effect for inhibiting the peroxyl radical-induced oxidation (Fig. 2). When the sugar moiety was a disaccharide (rutin), its antioxidant activity in DHA emulsion was lower than the corresponding monosaccharide. BHT was significantly more effective in suppressing oxidation than flavonols and α-tocopherol (Fig. 2). α-Tocopherol exhibited a significantly lower inhibition of oxidation of MLN and DHA when compared to other antioxidants tested (Fig. 2).

3.3. Inhibition of heat-induced oxidation of fish oil emulsion and bulk fish oil by quercetin and quercetin-3-O-glucoside

Incorporation of antioxidants in fish oil emulsions exhibited a concentration-dependent antioxidant activity similar to that of MLN and DHA emulsions, but BHT was more effective than flavonols in inhibiting lipid oxidation (Fig. 3A). Quercetin and quercetin-3-*O*-glucoside at three tested concentrations rendered similar effects (Fig. 3A). In bulk fish oil, quercetin-3-*O*-glucoside at 100 and 500 μM was more effective than quercetin and BHT ($p < 0.001$, $\alpha = 0.05$), but at the 1000 μM concentration both quercetin and quercetin-3-*O*-glucoside were equally effective (Fig. 3B). The effectiveness of BHT in bulk fish oil was lower than that of quercetin and its glucoside.

4. Discussion

The C-3-glycosylated-quercetins examined in this study are commonly found in fruits and their processing by-products (Rupasinghe, 2008; Rupasinghe & Kean, 2008). An emulsion model system of MLN, DHA and fish oil was used to resemble food products which contain lipids dispersed in an aqueous phase (oil-in-water) such as milk, salad dressings, beverages, soups and sauces (McClements, 2004). Though a concentration-dependent inhibition of oxidation of the omega-3 PUFA and fish oil containing emulsions by quercetin, quercetin-3-*O*-glucoside and BHT was observed, the antioxidants tested were more effective in MLN emulsion compared to DHA and fish oil emulsions. The observed differences could be, in part, due to the fact that DHA has twice as many double bonds when compared with MLN. The oxidation of PUFA and their esters in aqueous emulsions was found to de-

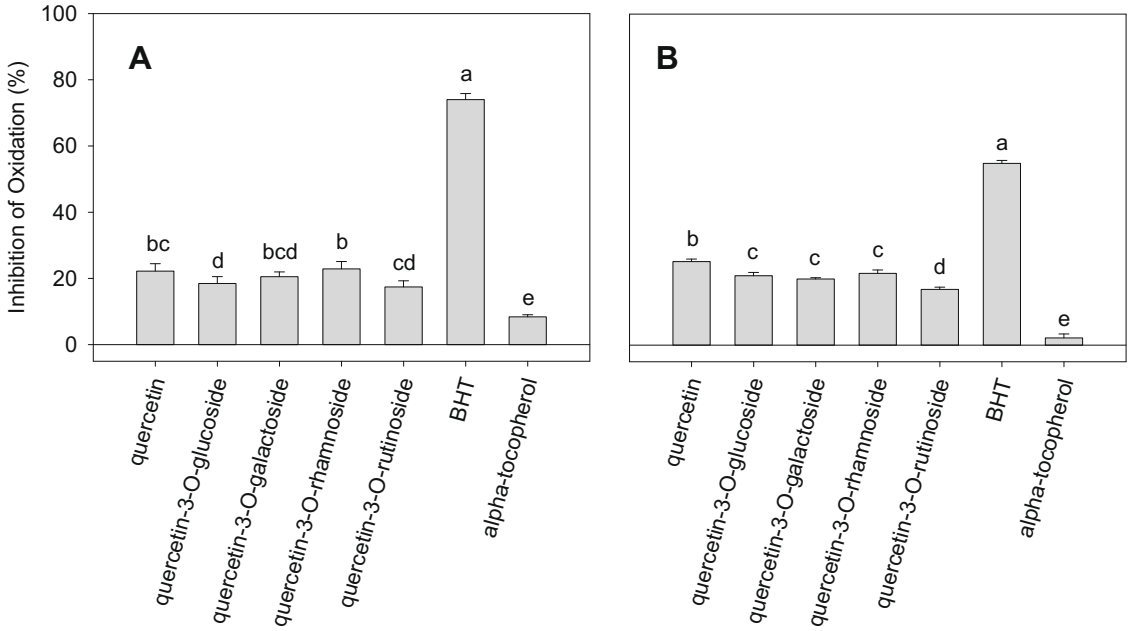

Fig. 2. Comparison of the antioxidant activity of flavonols with BHT and α-tocopherol (10 μM for MLN and 50 μM for DHA) for peroxyl radical-induced oxidation of MLN (A) and DHA (B) emulsions. Oxidation was measured by the TBARS assay. Significantly different values are represented by different letters.

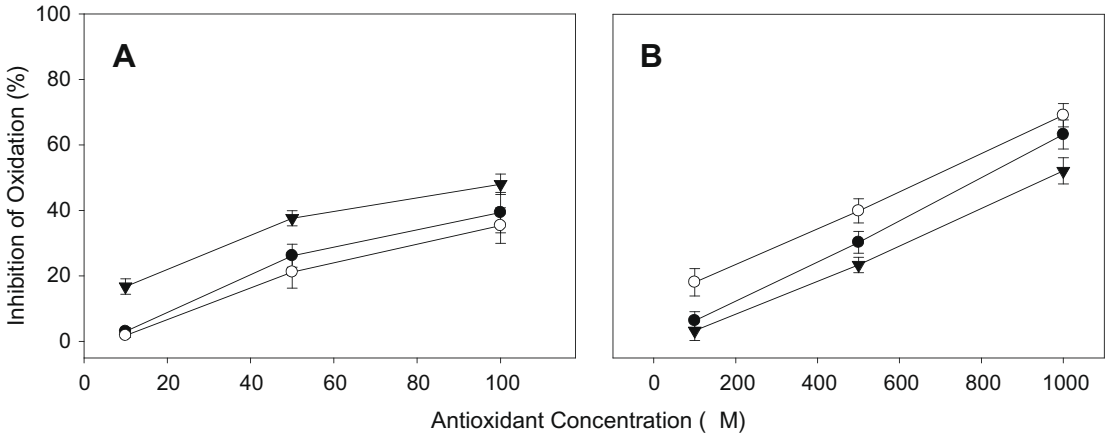

Fig. 3. Effect of different concentrations of BHT (▼), quercetin (●) and quercetin-3-*O*-glucoside (○) on the heat-induced oxidation of the fish oil emulsion (A) and the bulk fish oil. Oxidation was measured by the TBARS assay. Error bars represent standard error (SE).

crease with an increase in the number of bis-allylic positions in the fatty acids involved (Miyashita, Nara, & Ota, 1993). A higher level of UVB-radiation-induced oxidation in arachidonic acid (four double bonds) micelles than MLN micelles was also reported by Carini et al. (1998).

In the emulsion systems, quercetin-3-*O*-glucoside showed a similar or a lesser effect on the inhibition of oxidation depending on the concentration and oxidation-induction method when compared to quercetin itself. In addition, the type of sugar attached at C-3 of quercetin influenced the effectiveness of inhibition of PUFA oxidation in the emulsion systems. For example, when the oxidation of PUFA was induced by light, inhibition of oxidation by quercetin-3-*O*-glucoside and its aglycone at all tested concentrations was similar. However, a lesser effect of quercetin-3-*O*-glucoside compared to its aglycone was observed when the oxidation was induced by Fe^{2+}. It may be speculated that C-3 glycosylation could hinder the ability of quercetin to chelate ferrous ion. In general, similar to the present results of heat- and Fe^{2+}-induced oxidation, quercetin-3-*O*-rutinoside has been reported to be less effective in inhibiting lipid oxidation than its aglycone in other lipid oxidation systems i.e. Fe^{2+}- and peroxyl radical-induced oxidation in a liposomal system (Arora, Nair, & Strasburg, 1998) and Cu^{2+}-induced oxidation of an LDL emulsion system (Brown, Khodr, Hider, & Rice-Evans, 1998), which also agrees with 'the polar paradox' theory (Frankel, 1996; Porter, 1980). The polar paradox theory states that lipophilic antioxidants are more effective at inhibiting oxidation in oil-in-water emulsions, whereas hydrophilic antioxidants are more effective in bulk oil systems. This difference is thought to be due to the interfacial phenomenon, such that the hydrophilic antioxidants are positioned between the oil-air interface and the lipophilic antioxidants are dissolved in the oil droplets and thus at the water-oil interface (Frankel, 1996; Porter, 1980). Glycosylation with the disaccharide moiety, glucorhamnoside, decreased the partition coefficient (1.2–0.37) in an octanol/water mixture, or in other words, increased the hydrophilicity of the molecule (Brown et al., 1998). According to the polar paradox theory, the aglycone should have been more effective than its glycosides in both MLN and DHA emulsion systems employed. However, fatty acid emulsion models produce micellar systems as opposed to droplet systems (Decker, Warner, & Richards, 2005). Quercetin glycosides have also been shown to be less effective than their aglycone in inhibiting oxidation of PUFA in a non-emulsion system and a peroxyl radical-induced oxidation of phospholipid bilayer system (Ioku, Tsushida, Takei, Nakatani, & Terao, 1995).

Compared to α-tocopherol, the effectiveness of flavonols was greater in the MLN and DHA emulsions. However, α-tocopherol is known to be less polar than flavonols (Frankel, 1996) which according to the polar paradox theory is expected to be a more effective antioxidant in the emulsion system. This discrepancy could be due to the potential micellar form of PUFA emulsions (Decker et al., 2005). Similar to our finding, Becker, Ntouma, and Skibsted (2007) found that quercetin was as effective, if not more so, than α-tocopherol in peroxyl radical-induced oxidation of oil-in-water emulsion, phospholipid liposome and bulk oil. Carini et al. (1998) also showed that a flavonoid mixture containing catechin, catechin oligomers and gallic acid catechin ester from grape seed was as effective as α-tocopherol in a UVB-induced oxidation of PUFA micellar system.

In contrast to the activity of the tested antioxidants in emulsions, the flavonols used were more effective than BHT in bulk fish oil, which is also in agreement with the polar paradox theory in that the more polar antioxidants are more effective in the bulk oil. Similar to the present results, quercetin was shown to be a more effective antioxidant than BHT in heat-induced oxidation of fish oil (Wanasundara & Shahidi, 1998). Naturally occurring flavonols or glycosylation of quercetin seem to increase the antioxidant activity due to the enhanced polarity of the aglycone. The antioxidant nature of phenolic compounds has been shown to provide a balance between the ability of the compound to enter the lipid droplet for preventing oxidation and the amount of phenolic-metal interactions within the aqueous phase (Mei, McClements, & Decker, 1999). Also, the presence of 3′,4′-dihydroxy (catechol structure) in B ring, the C2–C3 double bond and the 4-keto group seems to be the most important structural features in scavenging free radicals or chain breaking antioxidant activity (Amic, Davidovic-Amic, Beslo, & Trinajstic, 2003) since the C-3 glycosylation did not diminish the ability of quercetin to inhibit PUFA oxidation. However, further experiments required to understand the effect of the positioning of sugar moiety of flavonols on the ability to inhibit PUFA oxidation in emulsion and bulk oil systems.

5. Conclusions

Although flavonols were found to be less effective in oil-in-water emulsions than, BHT, they were as effective as α-tocopherol. Interestingly, flavonols exhibited a better antioxidant activity in bulk fish oil than BHT. Considering the consumer preference for 'natural' products and their existence in a wide array of fruits and vegetables, naturally sourced flavonols such as quercetin glycosides could be used as effective antioxidants for stabilising omega-3 PUFA-containing foods and nutraceuticals while providing potential additional health benefits.

Acknowledgements

This research was funded by the Natural Science and Engineering Research Council (NSERC) of Canada and the Advancing Canadian Agriculture and Agri-Food (ACAAF) program of Agriculture and Agri-Food Canada (AAFC).

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Food Chemistry 117 (2009) 296–301

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Isolation and identification of one kind of yellow pigments from model reaction systems related to garlic greening

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ARTICLE INFO

Article history:
Received 8 January 2009
Received in revised form 24 February 2009
Accepted 1 April 2009

Keywords:
2-(1*H*-Pyrrolyl) acetic acid
Garlic greening
Pyruvic acid
Yellow pigment
Structure

ABSTRACT

It was established that green pigment(s) responsible for garlic greening is composed of yellow and blue species, and pyruvic acid (a product from 1-PeCSO or 2-PeCSO under the action of alliinase) reacted with pigment precursor (PP) model compounds, 2-(1*H*-pyrrolyl) carboxylic acids to produce yellow pigments. However, the structure of the yellow pigments is unknown. In present paper, we identified three yellow pigments (Y1, Y2 and Y3) from three reaction systems containing pyruvic acid and 2-(1*H*-pyrrolyl) acetic acid (P-Gly) or 1-(2′-methyl-1′-carboxy-propyl) pyrrole (P-Val) or 1-(2′-methyl-1′-carboxy-butyl) pyrrole (P-Ile), respectively, by LC-ESI MS/MS and IT-TOF mass spectrometry. The three pigments have a UV/visible maximum absorbance between 400 and 434 nm and might be formed by dimerisation of the three corresponding PP under participation of pyruvic acid, molecular formula of which are $C_{16}H_{16}N_2O_4$ (Y1), $C_{22}H_{28}N_2O_4$ (Y2) and $C_{24}H_{32}N_2O_4$ (Y3), respectively.

1. Introduction

Garlic is processed as powders, purees and juices, granules and oleoresin. During processing, garlic greening occurs, representing a major concern because of loss of the economic value of these products (Adams & Brown, 2007; Kim, Cho, & Kim, 1999). In contrast, the formation of green colour is desirable and required in the traditional home-made Chinese garlic product, "Laba" garlic (Bai, Chen, Liao, Zhao, & Hu, 2005; Bai, Li, Hu, Wang, & Zhao, 2006). Based on previous studies, it was established that the mechanism of garlic greening is similar to that of onion redding, and that the discolouration is a multistep process consisting of enzymatic and nonenzymatic stages (Imai, Akita, Tomotake, & Sawada, 2006b; Joslyn & Peterson, 1960; Lee & Parkin, 1998; Shannon, Yamaguchi, & Howard, 1967a, 1967b). The first step corresponds to the conversion of S-(1-propenyl)-L-cysteine sulfoxide (1-PeCSO) into di(1-propenyl) thiosulfinate (Imai, Akita, Tomotake, & Sawada, 2006a) or 1-propenyl containing thiosulfinate (Kubec, Hrbáčová, Musah, & Velíšek, 2004; Kubec & Velíšek, 2007) under the action of alliinase. Indeed, Lukes found that 1-PeCSO is a key compound involved in the discolouration with a positive correlation with the degree of garlic greening (Lukes, 1986). Consistent with above observation, Kubec et al. suggested that discolouration occurs upon tissue disruption of any *Allium* species that contain a high enough content of 1-PeCSO (Kubec et al., 2004). Step 2 corresponds to the formation of the pigment precursors (PP) from a reaction of the di(1-propenyl) thiosulfinate and protein amino acids. Further support for this conclusion came from previous results showing that all of protein amino acids except for cysteine and proline were able to form coloured products when mixed with 1-propenyl-containing thiosulfinates (Kubec & Velíšek, 2007), and that thiosulfinate consumption in the pickling solution of "Laba" garlic is proportional to the formation of the pigments (Bai et al., 2005). Imai et al. isolated and characterised 2-(3,4-dimethyl-1*H*-pyrrolyl)-3-methylbutanoic acid (PP-Val) and 2-(3,4-dimethyl-1*H*-pyrrolyl) propanoic acid (PP-Ala) from the reaction of di(1-propenyl) thiosulfinate with L-valine or L-alanine. These two compounds were proposed to be pigment precursors due to findings that pigments were produced from the reaction of PP-Val and di(2-propenyl) thiosulfinate under heating (Imai et al., 2006a). Recent studies showed that 2-(1*H*-pyrrolyl) carboxylic acids (model compounds of PP-Val) can turn garlic puree (which was prepared from freshly harvested garlic bulbs) green (Wang, Nanding, Han, Chen, & Zhao, 2008), confirming that PP-Val is a pigment precursor for garlic greening. The catalytic conversion of 2-PeCSO by alliinase into di(2-propenyl) thiosulfinate represents the third step. Finally, formed thiosulfinate(s) reacts with PP to produce green pigment(s), corresponding to the fourth step.

However, it was found that the green pigment(s) consists of yellow and blue species which exhibited two maximal UV/visible absorbances at ~440 and ~590 nm, respectively (Bai et al., 2005; Kubec & Velíšek, 2007; Wang et al., 2008). Thus, above step 4 actually corresponds to the formation of the blue pigment(s). In parallel, a reaction between the pigment precursor (PP) and pyruvic acid resulted in the formation of the yellow species, representing a pathway of the formation of the yellow species. The combination of

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.04.021

both yellow and blue pigments finally creates the green colouration (Wang et al., 2008). However, so far, there is no information on the structure of the yellow species produced from pyruvic acid and PP. In the present study, three yellow pigments were isolated from three model systems by using column chromatography, and their corresponding structures were identified with mass spectrometry.

2. Materials and methods

2.1. Chemicals

All solvents/chemicals above were of analytical grade or purer. L-Glycine, L-valine, L-isoleucine, hydrochloric acid, acetic acid, sodium acetate anhydrous, ethyl acetate, sodium sulphate anhydrous and potassium hydroxide were obtained from Beijing Chemistry Co. (Beijing, China). 2,5-Dimethoxytetrahydrofuran was purchased from Fluka (Beijing, China). Pyruvic acid was obtained from Sinopharm Chemical Reagent Co. (Beijing, China). Formic acid ($\geqslant$98%) was supplied by Sigma–Aldrich (Chemie GmbH, Germany), and methanol and acetone were purchased from Honeywell Burdick and Jackson (SK Chemicals, Korea), and they were chromatography grade. The solid phase extraction (SPE) cartridges cleanert ODS C_{18} (12 mL, 2000 mg) were purchased from Agela Technologies (Beijing, China). Syringe filter units 0.45 and 0.22 μm were supplied by Hercules (Beijing, China).

2.2. Sample preparation

P-Gly, P-Val and P-Ile were synthesised as previously described (Wang et al., 2008). ^{1}H NMR spectra were performed with a dpx-300 MHZ NMR spectrometer (Brucker Co., Germany). Deuterium dimethyl sulfoxide (DMSO-d_6) was used as a solvent with tetramethylsilane (TMS) as an internal standard. Mass spectra were obtained by using LC–MS/MS (Alliance2695/Quattro Micro API, Waters Co., USA), and detection was performed in the positive mode. Their ^{1}H NMR spectra and MS data were identical to those in literature (Wang et al., 2008), confirming their purity and structures.

To prepare the yellow pigment(s), both P-Gly (20 mM) and pyruvic acid (20 mM) in water were mixed thoroughly with a volumetric ratio of 1:1, and then stood at room temperature (23–25 °C) for 48 h. Resultant solution was filtered through a 0.45 μm syringe filter for clean-up. In contrast, the reaction of P-Val or P-Ile with pyruvic acid was carried out by dissolving them in methanol instead of water followed by standing at 45 °C for 72 h. Other procedures are the same as those used as above for P-Gly and pyruvic acid.

2.3. Fractionation by ODS C_{18} SPE cartridge

ODS C_{18} SPE cartridge was used for further clean-up. SPE cartridge was conditioned consecutively with 8 mL of methanol and 8 mL of water. One millilitre of resultant solution mentioned above was loaded on the SPE cartridge and effluent was collected. Subsequently, a wash step was followed to elute yellow pigment retained on the SPE column. Water, acetone and methanol consecutively were subjected to the column for eluting. Only methanol eluent was collected and concentrated to a small volume with a rotary evaporator. Finally, resulting solution was filtered through a 0.22 μm syringe filter for LC–MS analysis.

2.4. LC–MS analysis

The LC–MS analysis of these yellow pigments was performed by an Alliance 2695 Separations Module containing an autosampler (Waters, Milford, MA, USA) coupled to a Micromass Quattro Micro triple-quadrupole mass spectrometer (Micromass, Manchester, UK) with MassLynx software. Twenty microlitre of the reaction solution containing P-Gly was injected onto an analytical scale reversed ASB-C_{18} column (250 × 4.6 mm, 5 μm, Venusil, Agela, USA) maintained at 29 °C. The elution mode was isocratic with a mixture of 40% methanol and 60% water containing 0.2% formic acid as mobile phase at a flow rate of 0.4 mL/min for LC–MS analysis.

The reaction solution containing P-Val or P-Ile (20 μL) was loaded to an reversed MP-C18 column (250 × 4.6 mm, 5 μm, Venusil, Agela, USA), which was maintained at 29 °C. The elution mode was also isocratic using a mixture of 45% methanol and 55% water containing 0.2% formic acid as mobile phase at a flow rate of 0.4 mL/min for LC–MS analysis. PDA detection ranging between 200 and 800 nm was performed. Yellow pigments were detected using electrospray ionisation in the positive ion mode. Used MS parameters were as follows: capillary voltage, 2.5 kV; cone voltage, 10 V; scan ranger, m/z 100–1000; source temperature, 110 °C; desolvation temperature, 400 °C; desolvation gas flow, 700 L/h nitrogen; cone gas flow, 50 L/h. High-resolution electrospray ionisation mass spectra were acquired on an FT-MS Bruker APEX IV (7.0 T) (Bruker Co., Germany) equipped with an ESI source in positive ion mode.

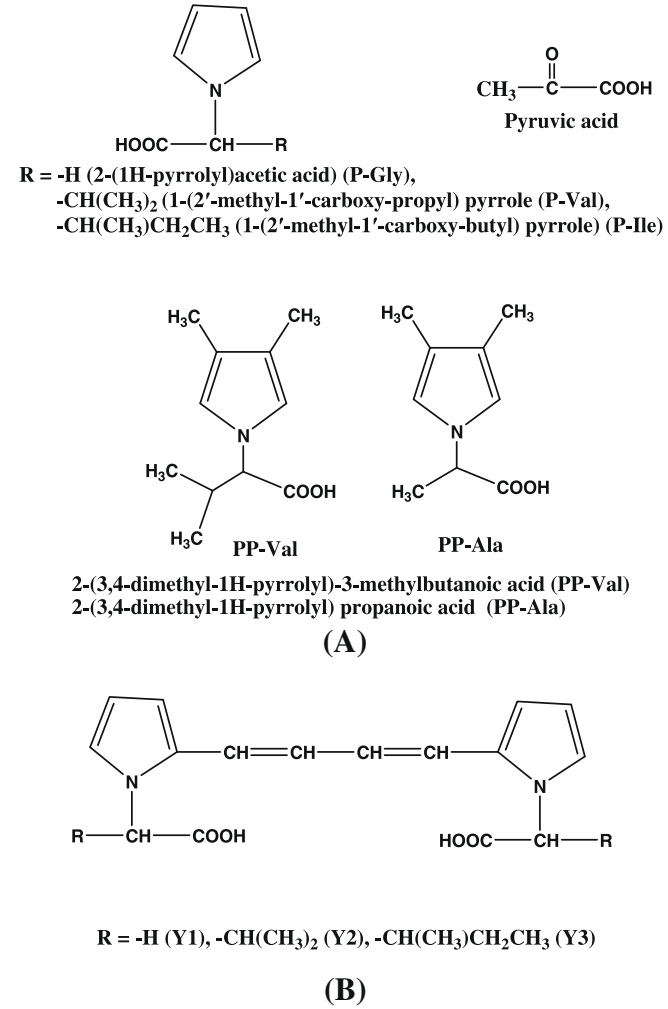

Fig. 1. (A) Structures of 2-(1*H*-pyrrolyl) acetic acid (P-Gly), 1-(2′-methyl-1′-carboxy-propyl) pyrrole (P-Val), 1-(2′-methyl-1′-carboxy-butyl) pyrrole (P-Ile) 2-(3,4-dimethyl-1*H*-pyrrolyl)-3-methylbutanoic acid (PP-Val) and 2-(3,4-dimethyl-1*H*-pyrrolyl) propanoic acid (PP-Ala) and pyruvic acid. (B) The proposed structure of the yellow pigments.

3. Results and discussion

Three model compounds P-Gly, P-Val and P-Ile (Fig. 1) were prepared mainly based on the method recently described (Wang et al., 2008). All of them have very similar structures to those of PP-Val and PP-Ala (pigment precursors, Fig. 1) (Imai et al., 2006a). Therefore, all three compounds were chosen to react with pyruvic acid, respectively, to produce the yellow species. Resultant reaction mixture of P-Gly or P-Val or P-Ile and pyruvic acid resolved only available with polar solvents, so water, acetone and methanol were used to consecutively elute the ODS C_{18} SPE cartridge which had retained the analyte above through absorption. Three kinds of solvent separated the sample into three different fractions. Since a visible yellow fraction can only be obtained through methanol eluting, this fraction was further purified by high-performance liquid chromatography (HPLC).

To achieve efficient HPLC resolution of the yellow fraction, the analytical condition first was optimised. The best separation condition was obtained on an analytical ASB-C_{18} column for Y1, and MP-C_{18} column for Y2 and Y3. A mixture of 40% methanol and 60% water containing 0.2% formic acid as mobile phase at a flow rate of 0.4 mL/min was used for Y1 while a mixture of 45% methanol and 55% water containing 0.2% formic acid was used as mobile phase for both Y2 and Y3. HPLC chromatograph of the yellow fraction containing Y1 with 400 nm as a detection wavelength was shown in Fig. 2a while HPLC spectra of the yellow fraction containing Y2 and Y3 at 440 nm as a detection wavelength were given in Fig. 2b and c. It was observed that all three yellow fractions are complex mixtures which consist of several components, a result reflecting the complexity of garlic greening. This observation is in accordance with that reported by Kubec et al. (2004), Kubec and Velíšek (2007). Since peak at 9.13 min (Fig. 2a), peak at 12.67 min (Fig. 2b) and peak at 24.63 min (Fig. 2c) were completely separated from other peaks in HPLC, they further were analysed by LC–MS/MS in conjunction with a diode array detector. UV/visible spectra of these three peaks performed by a diode array detector were displayed in Fig. 3, which were characterised by only one absorbance at 400.9 nm (Y1), 432.9 nm (Y2) and 433.9 nm (Y3) in visible region, respectively. Their absorptions were in good agreement with that of yellow pigment (440 nm) obtained from either a pickling solution of "Laba" garlic or an extraction solution of garlic puree (Bai et al., 2005; Kubec et al., 2004, Kubec & Velíšek, 2007). According to the polarity of mobile phase, it could observe that a methanol/acidic water ratio used for Y1 was less than that for Y2 and Y3; and Y1 had shorter retention time than Y2 or Y3 did, indicating that Y1 has the strongest polarity among the three compounds while Y3 exhibits the weakest. This finding is in good agreement with the polarity order of reactants, namely, P-Gly > P-Val > P-Ile.

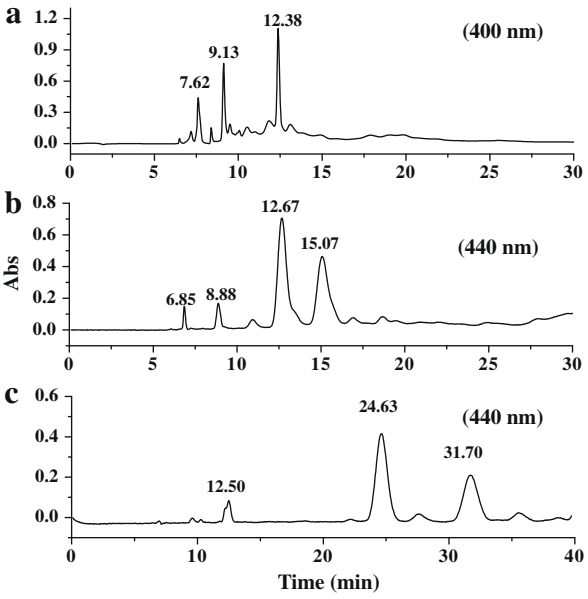

Fig. 2. HPLC chromatogram of the yellow fraction upon SOD C_{18} SPE cartridge clean-up. (a) The fraction from reaction containing pyruvic acid and P-Gly, detected at 400 nm. (b) The fraction from reaction containing pyruvic acid and P-Val, detected at 440 nm. (c) The fraction from reaction containing pyruvic acid and P-Val, detected at 440 nm.

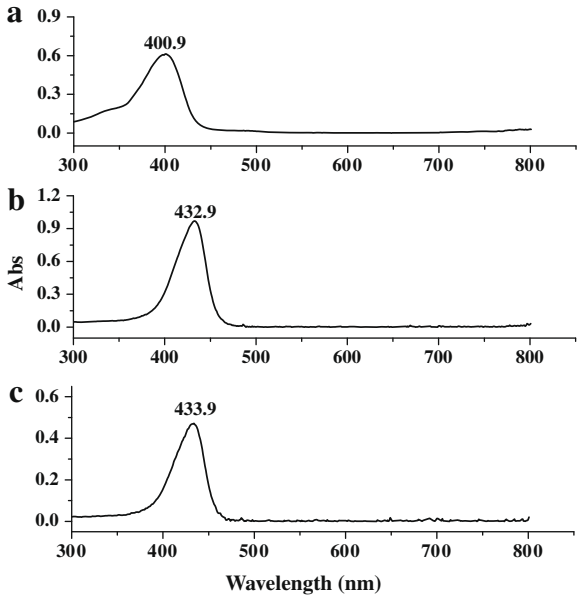

Fig. 3. UV/vis spectra of the yellow pigments. (a) The pigment (Y1) isolated from the reaction system containing pyruvic acid and P-Gly. (b) The pigment (Y2) isolated from the reaction system containing pyruvic acid and P-Val. (c) The pigment (Y3) isolated from the reaction system containing pyruvic acid and P-Ile.

Furthermore, both MS^n and high-resolution mass spectrometry were used to detect relative purity and molecular mass of the compounds. Firstly, Y1 was detected in the positive ion mode with experimental conditions as follows: capillary voltage, 2.5 kV; cone voltage, 10 V; scan ranger, m/z 100–1000, and result was given in Fig. 4a. The method used above was performed in a wider mass range scan with a lower voltage. Under present conditions, all components could be detected as their protonated adducts (Pasch, Pizzi, & Rode, 2001). There is a single prominent protonated molecular ion peak at m/z 301 $[M+H]^+$ in the MS spectrum of Y1. The accurate mass of this ion was determined to be 301.11917 by high-resolution MS (Table 1), which gives a possible molecular formula as $C_{16}H_{16}N_2O_4$. The difference in mass between the measured mass and a value calculated from their assigned formula has the least experimental error which was within 0.89 mDa unit, indicating that this formula could be the most reliable. Consistent with this formula, the number of carbon atoms was calculated to be ~16 by the observed intensity of the M+1 peak over contribution per carbon atom (1.08%) (Harris, 2003). In addition, the molecular weight of Y1 (~300) is even, suggesting that this yellow species contains an even number of N atoms in this molecule. All these data are consistent with the above proposed formula. The same MS conditions were used to analyse Y2, MS spectrum of which exhibited a single prominent protonated molecular ion peak at m/z 385 $[M+H]^+$, confirming its purity (Fig. 4b). Since Y2 was detected in the positive ion mode, its molecular weight was 384.

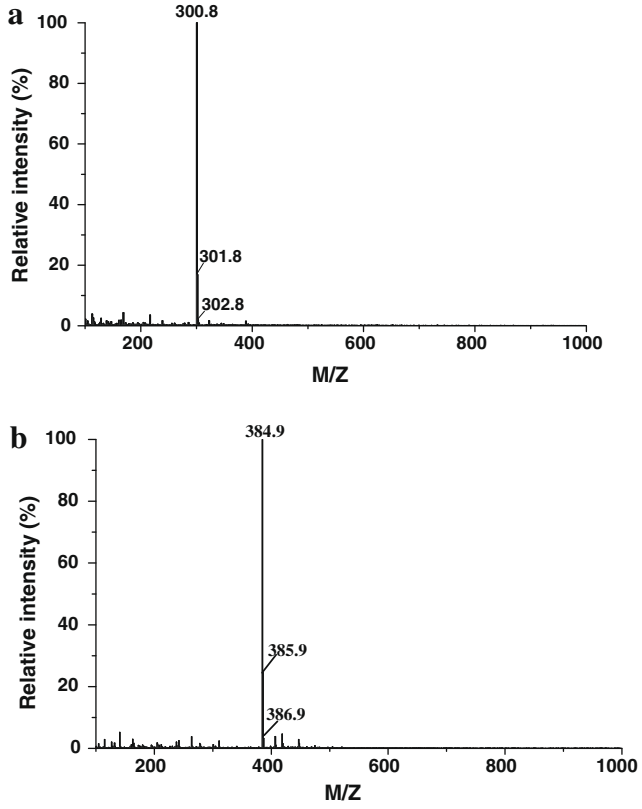

Fig. 4. MS spectra of the yellow pigments at cone voltage 10. (a) MS spectrum of Y1. (b) MS spectrum of Y2.

Molecular formula of Y2 was determined through the high-resolution MS analysis to be $C_{22}H_{28}N_2O_4$ (Table 1). Likewise, the high-resolution MS analysis for Y3 gave its molecular formula as $C_{24}H_{32}N_2O_4$ (Table 1). Based on their formula, we calculated the number of rings + double bonds (R + DB) in the molecule according to rings + double bonds formula (Eq. (1)) (Harris, 2003). The R + DB of all Y1, Y2 and Y3 were 10.

$$R + DB = c - h/2 + n/2 + 1 \quad (1)$$

From the structure of P-Gly and P-Val, it was observed that the difference in molecular weight between these two model compounds is around 42, corresponding to the mass difference between $-CH(CH_3)_2$ and –H groups, C_3H_6. Interestingly, the difference in M.W. between Y1 and Y2 is ~84, exactly the same as the mass of two C_3H_6, a resulting indicating that these two yellow species Y1 and Y2 was formed likely through dimerisation of P-Gly and P-Val, respectively. However, either Y1 (300) or Y2 (384) is not the simple adduct of two P-Gly (125) or P-Val (167) molecules according to their M.W. In addition, Y1 and Y2 have yellow colour but not contain metals (data not shown). These results suggested that both Y1 and Y2 are dimmers of their corresponding compounds, P-Gly and P-Val bridged by two conjugated double bonds as shown in Fig. 1B. Agreeing with this conclusion, the same features were found between Y2 and Y3, and between Y1 and Y3. The three new yellow species Y1, Y2 and Y3 were named as {2-[4-(1-carboxymethyl-1*H*-pyrrol-2-yl)-1-buta-1,3-dienyl]-pyrrol-1-yl}-acetic acid, 2-(2-{4-[1-(1-carboxy-2-methyl-propyl)-1*H*-pyrrol-2-yl]-buta-1,3-dienyl}-pyrrol-1-yl)-3-methyl-butyric acid and 2-(2-{4-[1-(1-carboxy-2-methyl-propyl)-1*H*-pyrrol-2-yl]-buta-1,3-dienyl}-pyrrol-1-yl)-3-methyl-pentanoic acid, respectively.

To prove above conclusion, compound Y3 was chosen to analyse its structure with MS/MS in positive mode. Increasing cone voltage could accelerate the collision of positive ions with N_2 molecules to break into a few fragments (Harris, 2003). Small cone voltage favours molecular ions whereas large voltage creates more fragments that aid in identification of analyte. Therefore, the degree of fragmentation can be controlled by adjusting the cone voltage. MS spectra with changing cone voltages in a wide range were shown in Fig. 5A. At low cone voltage (10 V), only a single peak at *m*/*z* 413 appeared, suggesting that peak 24.63 min contains one compound, Y3 (Fig. 2c). The difference in M.W. is ~1 unit between Y3 and recently reported compound purified from garlic green puree, M.W. of which is about ~411 and might contain sulphur (Lee, Cho, Kim, & Lee, 2007), a result indicating that Y3 is distinct from the reported species.

If the proposed structure of Y3 were correct, we would expect that the C–N bond between pyrrole ring moiety and α-carbon atom in Y3 be broken first due to its most instability. Indeed, with increasing the cone voltage to 70 V, this mother ion 413 $[M+H]^+$ produced its first daughter ion at *m*/*z* 298 through losing one group having mass as 413 − 298 = 115, indicating that lost fragment was the side chain of Y3, $-CH(COOH)CH(CH_3)CH_2CH_3$. The second positive ion was produced at *m*/*z* 232, a result indicating that the second group eliminated from this mother ion is pyrrole ring moiety with mass as 298 − 232 = 66. These results are in agreement with above proposed structure of Y3 (Fig. 1B). When the cone voltage increased to 80 V, three new peaks at *m*/*z* 283, 183 and 117 appeared except for the two above peaks. The peak at *m*/*z* 283 corresponds to loss of $-CH_3$ from ion with *m*/*z* 298. Cation at *m*/*z* 183 was produced through losing another side chain from the ion at *m*/*z* 298. Cation at *m*/*z* 117 was produced by several pathways depicted in Fig. 5B. Thus, all MS/MS spectra are in accordance with the proposed structures. Although Y1, Y2 and Y3 were yellow pigments, it is not known whether they were predominant yellow pigments relating to garlic greening. Previous studies showed that unidentified yellow pigments were also generated from degradation of blue species (Bai et al., 2005). However, the elucidation of the molecular structures of Y1, Y2 and Y3 would obtain insights into the mechanism of garlic greening. According to the structure of three yellow species, a possible formation pathway was proposed, which likely contains two main steps as depicted in Fig. 6. Step 1 corresponds to an nucleophilic addition reaction occurring between carbon atom in carbonyl of pyruvic acid and C-2 in pyrrole moiety of 2-(1*H*-pyrrolyl) carboxylic acid, resulting in the formation of intermediate I, which loses its –COOH group to produce intermediate II. This proposal is consistent with the fact that the reactivity of C-2 is greater than that of C-3 of 2-(1*H*-pyrrolyl) carboxylic acid due to its higher acidic property, and carbon atom of carbonyl group of pyruvic acid takes some positive charge. Step 2 corresponds to the formation of final yellow species through a dimerisation reaction in which 2 mol of intermediate II were polymerised followed by other reactions into 1 mol of corresponding yellow pigments.

Table 1
Formula of the yellow pigments observed from high-resolution electrospray ionisation mass spectra.

Pigment	Formula	Measured (*m*/*z*)	Calculated (*m*/*z*)	Error (mDa)	Error (ppm)
Y1	$C_{16}H_{16}N_2O_4$	301.11917	301.11828	0.89	2.94
Y2	$C_{22}H_{28}N_2O_4$	385.21289	385.21218	1.82	0.7
Y3	$C_{24}H_{32}N_2O_4$	413.24354	413.24348	0.15	0.06

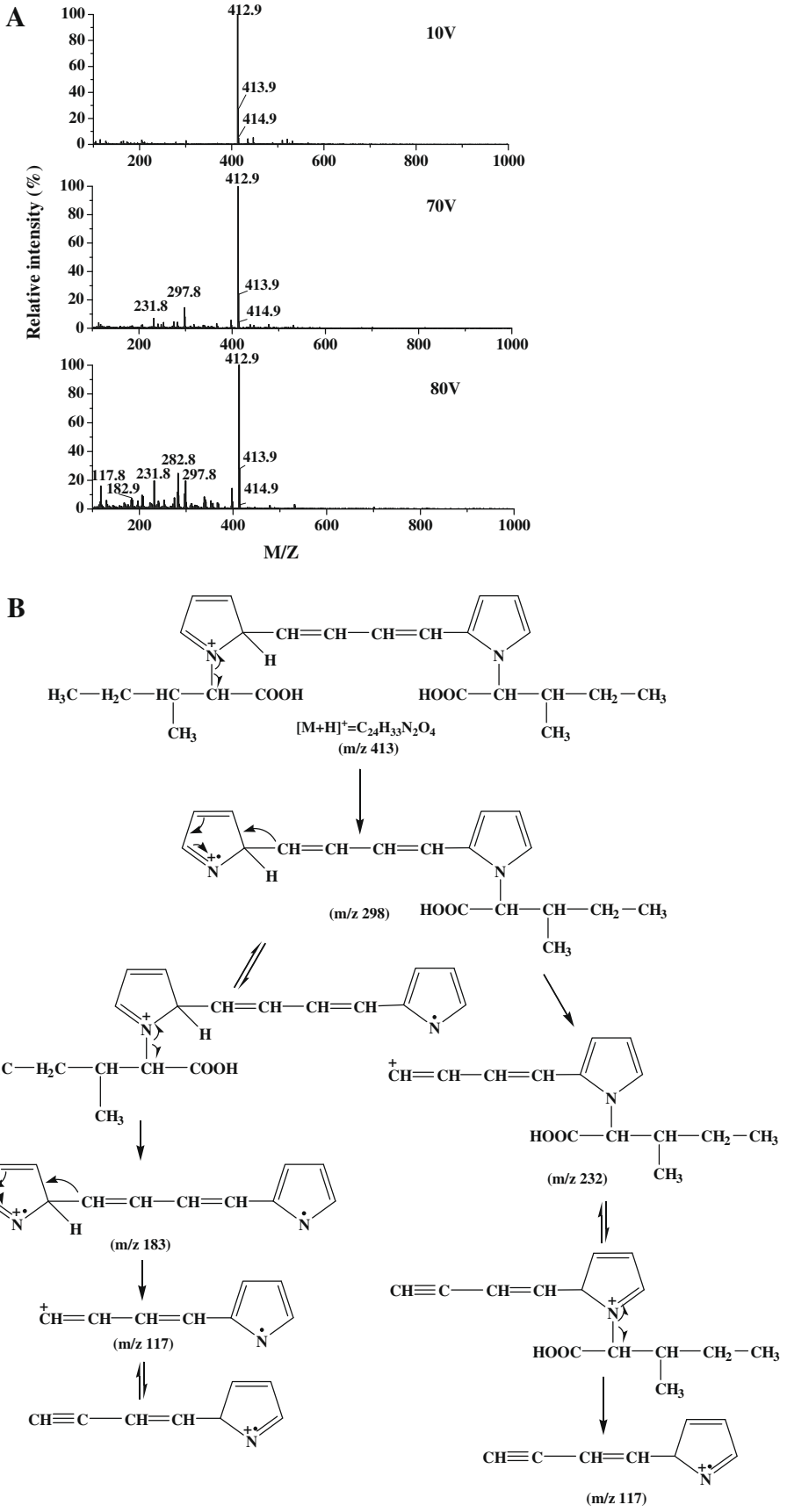

Fig. 5. (A) MS/MS spectra of the Y3 at different cone voltages 10, 70 and 80 V. (B) The proposed guideline for mass spectral data (m/z) of Y3.

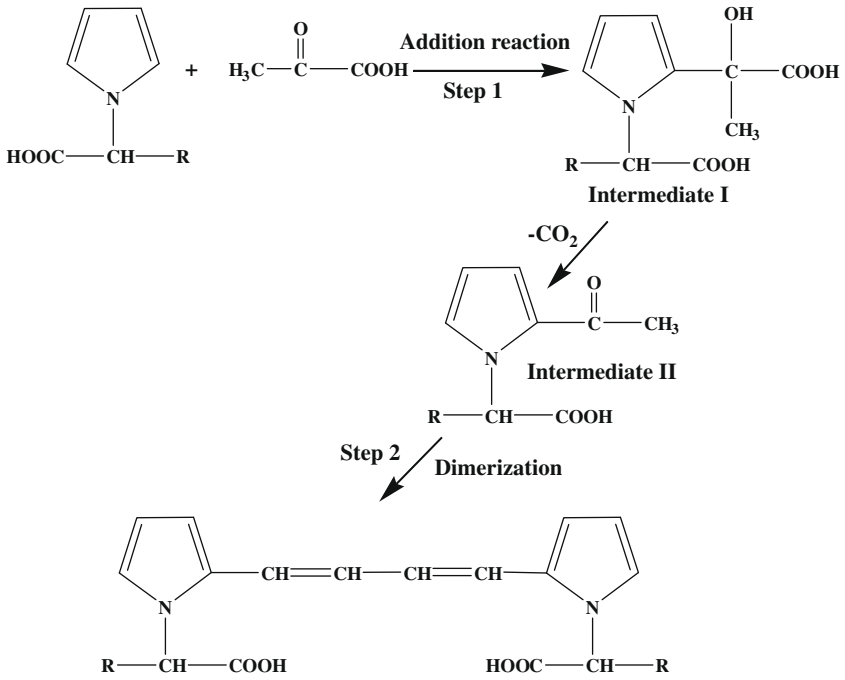

Fig. 6. Proposed two-step pathway of the formation of yellow pigments related to garlic greening.

Although the above two-step pathway was proposed, other pathways can not be excluded. More detailed mechanism about the formation of the yellow species is under investigation.

4. Conclusion

In this study, three yellow pigments from model systems were isolated and identified by HPLC, LC–MS/MS and high-resolution MS. The molecular formula of Y1, Y2 and Y3 was $C_{16}H_{16}N_2O_4$, $C_{22}H_{28}N_2O_4$ and $C_{24}H_{32}N_2O_4$, respectively. All of them contain two pyrrole ring moieties bridged by two conjugated double bonds. The elucidation of the three yellow species would benefit to understand the mechanism of garlic greening.

Acknowledgements

This work was supported by China High-Tech (863) Project (2007AA10Z333), the National Natural Science Foundation of China (30570181) and the Ministry of Education of the People's Republic of China, Specialised Research Fund for the Doctoral Program of Higher Education (20070019004).

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Food Chemistry 117 (2009) 302–305

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Food Chemistry

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Metal contents in "oloroso" sherry wines and their classification according to provenance

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ARTICLE INFO

Article history:
Received 14 October 2008
Received in revised form 1 April 2009
Accepted 2 April 2009

Keywords:
Oloroso
Sherry wine
Metals
Linear discriminant analysis

ABSTRACT

"Oloroso" sherry wines, belonging to the denominations of origin Jerez-Xérès-Sherry (JX), Montilla-Moriles (MM) and Condado de Huelva (CH) (Andalusia, Spain), were analysed for their contents of Ca, Mg, Fe, Cu, Mn and Zn by flame atomic absorption spectrophotometry, and Na, K, Al and Sr by flame atomic emission spectrophotometry. Among the three denominations of origin, differences in the mean contents were found for Na, Mg, Fe, Al and Sr. Cluster analysis showed that these differences were significant. Linear discrimination analysis, using Na, Mg, Fe, Al and Sr, gave 100% recognition ability and 93% prediction ability.

1. Introduction

Wine is a widely consumed beverage with thousands of years of tradition. It is a complex matrix, which, besides water and alcohol, contains a great variety of inorganic and organic components. The composition of wine is due to many factors, related to the specific production area, such as grape variety, soil and climate, culture, yeast, wine-making practices, transport and storage. All of them have important influences on the quality of wine, and they are very important in the characterisation and differentiation of wines, with applications to the detection of frauds (Lara, Cerutti, Salonia, Olsina, & Martínez, 2005; Urbano, Luque de Castro, Pérez, García-Olmo, & Gómez-Nieto, 2006).

The quantity of inorganic ions in wine is of great interest, because of their influence on wine technology, as well as their toxic effects (Galani-Nikolakaki, Kallithrakas-Kontos, & Katsanos, 2002). However, one of the main interests is use of the mineral content to characterise wines by their geographical origin, taking into account the relationship between the metallic content in samples and soil composition (Frías, Conde, Rodríguez-Bencomo, García-Montelongo, & Pérez-Trujillo, 2003). Metal elements have been widely used for differentiating Spanish wines according to their geographical origin (Álvarez, Moreno, Jos, Cameán, & González, 2007a; Barbaste, Medina, Sarabia, Ortiz, & Pérez-Trujillo, 2002; Frías et al., 2003; Moreno et al., 2007; Pérez-Magariño, Ortega-Heras, González-San José, & Boger, 2004; Pérez-Trujillo, Barbaste, & Medina, 2003; Rebolo et al., 2000).

Oloroso is a type of sherry wine produced according to the dynamic ageing system formerly called the "Soleras and Criaderas" method, which is used to blend the different vintages. But, in contrast to fino sherry wine, an initial fortification to 17% by volume prevents the development of the "yeast flor film" (biological ageing), and thus the wine slowly ages in constant contact with oxygen as it descends through the traditional criaderas and solera system (oxidative ageing) (www.sherry.org). This kind of wine can be made only in certain traditional wine regions (Jerez-Xérès-Sherry, Montilla-Moriles and Condado de Huelva DOs) located in the south of Spain.

The production region of the wines protected by the Jerez-Xérès-Sherry DO includes the cities of Jerez de la Frontera, El Puerto de Santa María and Sanlúcar de Barrameda. The grapes used for their wines belong mainly to the Palomino fino variety, but also Pedro Ximenez and Muscat are used. Typical vineyard soil is "albariza", rich in limestone and clay.

Condado de Huelva DO comprises a vast region located in the southeast of the province of Huelva. At present, this DO encompasses 14 municipal districts, being the most important area of the vineyards Bollullos Par del Condado. The Zalema grape variety is the most cultivated, followed by Palomino fino and Listan. The vineyard soil is rather diverse: sandy with limestone components in the south; just gritty towards the north, and "albarizas" in the most septentrional zone. The climate is very similar to Jerez, with slight differences in pluviosity.

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.04.006

Montilla-Moriles DO is located some 50 km south of Córdoba. Montilla is the main production centre of this DO. With respect to their neighbouring DOs, most of the Montilla wines are produced with the Pedro Ximenez variety, which has a much higher sugar content than have Palomino Fino and Zalema varieties, and therefore gives wines with naturally higher alcohol contents. Typical vineyard soils are "albero" and "albariza". These types of soil correspond to the upper limit of the Pliocene period, which is characterised by the presence of white marls (argile-containing limestone), typical of the Guadalquivir basin. The climate of the Montilla-Moriles zone is mainly Mediterranean but with some continental features due to altitude and location.

Accordingly, soil differences and differences in grape varieties could be reflected in the metal contents of the three DO wines. Consequently, the aim of this paper is the determination of the metal content profile of oloroso wines (considering the elements Ca, Mg, Na, K, Fe, Cu, Mn, Zn, Al and Sr) for discriminating between the three DOs considered. These same mineral elements have been determined for the differentiation of "fino" wines from Jerez-Xérès-Sherry and Condado de Huelva (Álvarez et al., 2007a) and for the study of the mineral profile of Montilla-Moriles "fino" wines by Álvarez, Moreno, Jos, Cameán, and González (2007b).

2. Materials and methods

2.1. Apparatus

A Perkin Elmer AAnalyst 100 (Norwalk, CT, USA) atomic absorption spectrometer was used for metal determination, involving atomic absorption spectrometry (AAS) and atomic emission spectrometry (AES). Table 1 lists the instrumental conditions for the determination of each element.

2.2. Reagents

Panreac (Barcelona, Spain) AA standard solutions of about 1000 mg l^{-1} were used as stock solution for calibration. Other reagents were of analytical grade. Milli-Q treated water was used throughout.

2.3. Samples

Forty-one samples of oloroso wines belonging to the three DOs were used. The distribution of samples was as follows: Jerez-Xérès-Sherry (15 samples), Montilla-Moriles (12 samples) and Condado de Huelva (14 samples). All samples were provided by the Regulatory Council of each DO, to ensure the geographic origin of the wines, or purchased in markets. The alcoholic contents ranged from 18% to 19% v/v of ethanol for all samples. Each sample was identified with a code referring its origin class (JX, MM, CH), together with the sample number.

Table 1
Instrumental conditions for the determination of each element.

Element	Wavelength (nm)	Type of flame
Ca	422.7	Air/acetylene
Mg	285.2	Air/acetylene
Na	589.0	Air/acetylene
K	760.5	Air/acetylene
Fe	248.3	Air/acetylene
Cu	324.8	Air/acetylene
Mn	279.5	Air/acetylene
Zn	213.9	Air/acetylene
Al	309.3	Acetylene/nitrous oxide
Sr	460.7	Acetylene/nitrous oxide

The containers used for storing and treating the samples were cleaned to avoid contamination of the samples with traces of any metal. Containers were treated with chromic acid mixture, followed by two washes with milli-Q water.

Once opened, wine samples were digested according to the following procedure: 25 ml of wines with 15 ml of hydrogen peroxide were heated at 80 °C until a volume of about 20 ml was achieved. Then 1 ml of nitric acid was added, and the heating continued to reach a final volume of about 2 ml. This volume was mixed with milli-Q treated water up to the starting volume. Three replicated digestions were done for each sample.

Hence, from each wine sample (bottle), three equivalent aliquots were digested and analysed. The triplicates gave insight about differences in sample treatment and response, but not about sample variability, because all of the aliquots were drawn from the same bottle and the wine samples were asumed to be homogeneous. Accordingly, we used the average value from the triplicates as a central measurement of the metal content of the sample.

Metals Ca, Mg, Fe, Cu, Mn and Zn were determined by AAS, and Na and K were measured by AES, using an air/acetylene flame. Al and Sr were determined by AES, using an acetylene/nitrous oxide flame.

2.4. Statistical analysis

Univariate and multivariate analyses were carried out by using the STATISTICA 7 package of Stafsoft (2005). A data matrix was built, consisting of 10 columns (the analysed elements) and 41 rows (the samples of the three categories, DOs). Each sample was labelled with a code corresponding to its class membership, e.g., 1, 2 and 3 for CH, JX and MM DO's, respectively.

3. Results and discussion

3.1. Mineral content

The metal contents of the three different DO wines were determined. The results, always expressed in milligrammes per litre, were the averages of triplicate measurements. The corresponding descriptive basic statistic for each DO is shown in Table 2. The mean contents for the majority of these metals in the three DOs wines are consistent with the values described in the literature by other authors (Cabanis, 2000; Mareca Cortés, 1983; Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2002; Zoecklein, Fugelsang, Gump, & Nury, 1995). In the case of sodium, our results, especially in Jerez samples, yielded greater values than those described in the majority of literature consulted. However, our values were similar to those obtained using wines made from vines exposed to considerable influence from the sea, as in Jerez samples (Frías et al., 2003).

3.2. Univariate analysis

A Kruskall–Wallis non-parametrics test was performed in order to establish the discriminant capacity of each variable, using the DO as a category. The Tukey HSD *post-hoc* test revealed which categories were particularly different from each other. The results are presented in Table 3, showing that strontium sodium, iron, aluminium and magnesium, were the most discriminant variables. Strontium is the only metal that completely differentiates the samples of each category.

Therefore, to select the most important variables for discriminating categories (JX, MM and CH), a rough feature selection, based on the Cooman's weight was performed. Thus, strontium can be

Table 2
Metal concentrations in Jerez wine samples (n = 15), Montilla-Moriles samples (n = 12) and Condado de Huelva samples (n = 14 samples).

Element	"Jerez samples"		"Montilla-Moriles samples"		"Condado de Huelva samples"	
	Mean ± SD ($mg\ l^{-1}$)	Range of quantified values ($mg\ l^{-1}$)	Mean ± SD ($mg\ l^{-1}$)	Range of quantified values ($mg\ l^{-1}$)	Mean ± SD ($mg\ l^{-1}$)	Range of quantified values ($mg\ l^{-1}$)
Ca	83.9 ± 25.7	42.7–159	87.0 ± 14.4	63.8–111	90.7 ± 24.4	57.0–144
Mg	113 ± 31.5	77.5–209	127 ± 30.1	79.8–157	96.7 ± 24.8	71.0–146
Na	90.6 ± 20.3	61.5–133	48.9 ± 16.6	21.9–84.2	54.7 ± 20.5	34.0–118
K	1011 ± 247	567–1417	1144 ± 116	946–1330	1285 ± 444	883–2471
Fe	2.76 ± 1.08	1.40–4.85	3.75 ± 1.43	2.39–6.44	5.13 ± 1.56	2.27–8.43
Cu	1.06 ± 0.69	0.21–3.03	0.79 ± 0.33	0.44–1.58	0.82 ± 0.46	0.12–1.84
Mn	1.09 ± 0.22	0.77–1.58	1.24 ± 0.35	0.67–1.65	1.01 ± 0.41	0.69–2.12
Zn	3.58 ± 5.55	0.10–18.0	1.32 ± 1.31	0.54–4.43	0.86 ± 0.43	0.34–1.61
Al	2.32 ± 1.03	0.90–5.07	2.27 ± 0.82	1.12–3.32	3.29 ± 0.93	1.95–5.04
Sr	4.30 ± 0.61	2.95–5.28	1.20 ± 0.34	0.81–1.93	2.67 ± 1.19	2.03–4.18

Table 3
Kruskall–Wallis and Tukey HSD study according to denomination of origin (DO).

Element	p-level	Significant differences
Ca	0.5937	–
Mg	0.0403	CH-MM
Na	0.0000	CH-JX, MM-JX
K	0.1141	–
Fe	0.0004	CH-JX, CH-MM
Cu	0.4976	–
Mn	0.1972	–
Zn	0.0585	–
Al	0.0103	CH-JX, CH-MM
Sr	0.0000	CH-MM, CH-JX, JX-MM

considered the most important feature for discriminating between JX and CH, JX and MM and CH and MM categories.

3.3. Multivariate analysis

Metal profiles of wines have been used as chemical descriptors for DO classification. For multivariate analysis, only those variables which showed some differences between the categories were used (in our case, Sr, Na, Fe, Al and Mg). Although our research begins from *a priori* knowledge of the class membership of wines, a cluster analysis (CA) has been applied. CA is an unsupervised technique that uses the information obtained from the measured variables to reveal the natural clusters existing between the studied samples. The dendrogram (Fig. 1) sets out the results obtained after using Ward's hierarchical method and City's block (Manhattan) distance as criteria of similarity. Two main clusters can be observed: the first one contains two subclusters, the first mainly constituted of samples of the MM category and the second of the JX samples. In the second cluster, two subclusters can also be observed, the first for JX category samples, and the second for CH samples. Accordingly, it seems that the variables used have sufficient explanatory power to detect the DO.

Linear discriminant analysis (LDA) is a widespread parametric method for classification purposes that assumes an *a priori* knowledge of the number of classes and sample class membership. The classification was performed according to the denominations of origin: Jerez (JX), Montilla-Moriles (MM) and Condado de Huelva (CH). The variables selected were strontium, sodium, iron, aluminium and magnesium. Two statistically significant discriminant functions were obtained, in which strontium, in the first function, and aluminium and magnesium, in the second, are the most important variables. The plot of both functions is represented in Fig. 2, in which a good differentiation between JX and MM categories can be visualised according to function 1, and CH category can be differen-

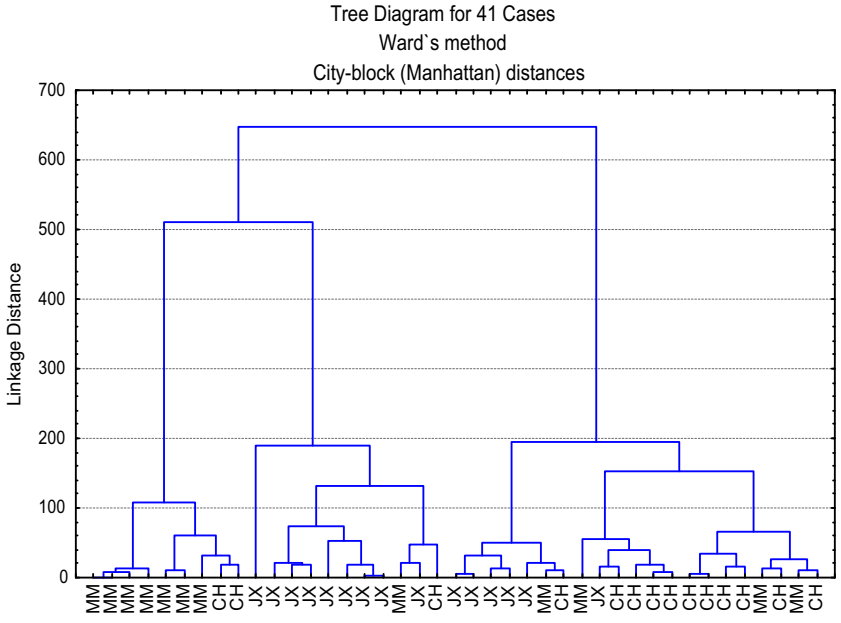

Fig. 1. Dendrogram of cluster analysis of oloroso wine samples of Condado de Huelva, Jerez and Montilla-Moriles, corresponding to classes CH, JX and MM, respectively.

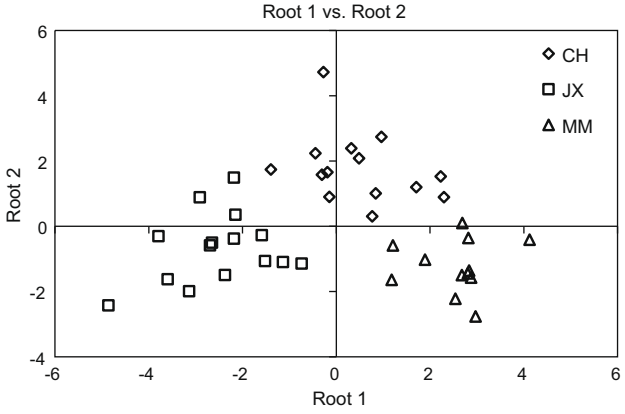

Fig. 2. Discriminant scatter plot of wine samples of Condado de Huelva, Jerez and Montilla-Moriles, corresponding to classes CH, JX and MM, respectively.

Table 4
Classification of the samples in the denominations of origin (JX, MM and CH) and recognition ability using sodium, magnesium, iron, aluminium and strontium, and LDA technique.

	Recognition ability (%)	JX	MM	CH
Jerez-Xérès-Sherry (JX)	100.0	15	0	0
Montilla-Moriles (MM)	100.0	0	12	0
Condado de Huelva (CH)	100.0	0	0	14
Total	100.0			

Table 5
Classification of the samples in the denominations of origin (JX, MM and CH) and prediction ability using sodium, magnesium, iron, aluminium and strontium, and Leave-one-out cross validation technique.

	Prediction ability (%)	JX	MM	CH
Jerez-Xérès-Sherry (JX)	93.3	14	1	0
Montilla-Moriles(MM)	100	0	12	0
Condado de Huelva (CH)	85.7	12	1	1
Total	93.0			

tiated according to function 2. Strontium contributes to differentiate between wines from JX and MM, and aluminium and magnesium to differentiate wines of CH from JX and MM. The recognition ability was 100% for the three categories (Table 4). Validation of these results was performed using leave-one-out cross-validation. During this test, a sample is removed from the data set. The classification model is rebuilt and the removed sample is classified in this new model. All the samples were sequentially removed and reclassified. The classifications obtained are presented in Table 5. The prediction ability for Montilla-Moriles is 100% and the total prediction ability is 93%.

4. Conclusions

Ten elements were used to characterise oloroso wines from Jerez-Xérès-Sherry, Montilla-Moriles and Condado de Huelva Andalusian denominations of origin. The results obtained show that the metallic contents were similar to those described in the literature, with the exception of sodium, whose content in Jerez wines was higher than that in other wines. The geographical origin of the wines influences the final metallic contents. Thus, univariate analysis showed that wines of the three denominations of origin could be differentiated using only the content of strontium. Wines of Condado de Huelva could be differentiated from those of Jerez and Montilla-Moriles by their contents of iron.

CA showed that Andalusian oloroso wines could be roughly grouped in accordance with the denomination of origin. Finally, LDA showed good recognition and prediction abilities using five elements: iron, sodium, magnesium, aluminium and strontium.

Acknowledgements

We express our gratitude to the Regulatories Councils of the Denominations of Origin which enabled us to use samples of precisely known origin, and to A.G. González for his critical review of the paper.

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Food Chemistry 117 (2009) 306–311

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Triglycerides constituted of short and medium chain fatty acids and dicarboxylic acids in *Momordica charantia*, as well as capric acid, inhibit PGE_2 production in RAW264.7 macrophages

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ARTICLE INFO

Article history:
Received 2 November 2008
Received in revised form 1 March 2009
Accepted 1 April 2009

Keywords:
Momordica charantia
Bitter gourd
Macrophages
Prostaglandin E_2
Capric acid

ABSTRACT

This study was aimed to identify compounds in bitter gourd (*Momordica charantia*) ethyl acetate extract (EAE), which inhibit lipopolysaccharide-induced prostaglandin E_2 (PGE_2) production in RAW264.7 cells. Bitter gourd EAE was partitioned between *n*-hexane and methanol/H_2O (90/10). The hexane fraction was further separated by repeated silica gel chromatographies, and a reverse phase (RP) C18 chromatography. Fraction RP-10 showed the highest inhibition effect on PGE_2 production (Max inhibition = 96%, IC_{50} = 2.3 μg/ml) and was identified to be triglycerides constituted of short and medium chain fatty acids by ^1H NMR, IR and H–HCOSY, and dicarboxylic acids by GC/MS. Fatty acids with 3–20 carbons were tested for the inhibitory activity, and capric acid exhibited the highest effect (Max inhibition = 99%, IC_{50} = 6.5 μM). In conclusion, triglycerides composed of short and medium chain fatty acids and dicarboxylic acids in bitter gourd inhibit PGE_2 production, and capric acid is the most potent inhibitor among the fatty acids.

1. Introduction

Prostaglandin E_2 (PGE_2) is an important pro-inflammatory mediator in many chronic inflammatory conditions, including rheumatoid arthritis (Robinson, McGuire, & Levine, 1975), inflammatory bowel diseases (Subbaramaiah et al., 2004), cancer (Sinha, Clements, Fulton, & Ostrand-Rosenberg, 2007), atherosclerosis (Gómez-Hernández et al., 2006), atherosclerotic plague rupture (Cipollone et al., 2004) and age-related degeneration (Wu & Meydani, 2004). In response to intrinsic cytokines (Arias-Negrete, Keller, & Chadee, 1995), or extrinsic factors, such as lipopolysaccharide (LPS) (Rhee & Hwang, 2000), macrophages release large amounts of PGE_2. PGE_2 also stimulates the production of proinflammatory cytokines (Williams & Shacter, 1997). Inhibition of the production of PGE_2 has been used to ameliorate inflammatory symptoms and suppress related diseases (Guadagni et al., 2007).

Bitter gourd (*Momordica charantia*) is a very common oriental vegetable in tropical and subtropical areas. It is traditionally regarded as a "cooling" or "fire-reducing" food. In our previous study, that characterised "heating" and "cooling" foods by their effects on PGE_2 production in LPS-stimulated RAW264.7 macrophages, we found that ethyl acetate extract (EAE) of bitter gourd inhibited PGE_2 production (Huang & Wu, 2002). This study was thus aimed to further investigate the active components in bitter gourd EAE that inhibit PGE_2 production.

2. Materials and methods

2.1. Materials

Wild bitter gourds (*M. charantia* L., strain Hualian No. 4), were kindly provided by Mr. Chong-Ho Chuan of Hualien District Agricultural Research & Extension Station. ^1H NMR and H–HCOSY spectra were obtained on Bruker AM-300 and Bruker-500 instruments, respectively. An HP 6890 series gas chromatograph (GC) equipped with a 30 m × 0.25 mm × 0.25 μm HP-5 MS column was used for the analysis of fatty acids. Mass spectral analysis was done on a JEOL JMS-HX300 mass spectrometer. Thin-layer chromatography (TLC) was performed on silica gel 60F254 TLC plates (Merck, Darmstadt, Germany). Silica gel (~230–400 μm) (Macherey-Nagel, Germany) and reverse phase C18 were used for column chromatography. Infrared spectra were obtained on a Bio-Rad FTS-40 FT-IR. The RAW 264.7 macrophage cell line (CCRC60001, originally from the American Type Culture Collection; designation, TIB-71)

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.04.004

was obtained from the cell bank of the Food Industry Research and Development Institute, Hsin Chu, Taiwan. Dulbecco's minimal essential medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, MD, USA). LPS (*Escherichia coli*, serotype 026: B6) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Fatty acids, propionic acid (3:0), butyric acid (4:0), caproic acid (6:0), caprylic acid (8:0), capric acid (10:0), lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0) and dicarboxylic acids, octanedioic acid (suberic acid), nonanedioic acid (azelainic acid) and decanedioic acid (sebacic acid), were purchased from Sigma–Aldrich; oleic acid (18:1), linoleic acid (18:2n6), arachidonic acid (20:4n6) and conjugated linoleic acid (CLA) were purchased from Cayman (Ann Arbor, MI, USA); medium chain triglycerides (MCT) containing 67% caprylic acid (8:0), and 23% capric acid (10:0) were purchased from Mead Johnson (Evansville, IL, USA). Conjugated linolenic acid (CLN) was isolated and purified from bitter gourd in our laboratory, previously (Chuang et al., 2006); it was composed of 77% 9c, 11t, 13t-CLN, 11% stearic acid and 12% palmitic acid, based on the mass spectral data.

2.2. Preparation of water-soluble and ethyl acetate-soluble fractions of bitter gourd

Fresh whole bitter guard was homogenised and filtered through cotton cloth. The juice was centrifuged and the supernatant was lyophilised to obtain the water extract (WE). Lyophilised whole bitter guard was extracted with ethyl acetate (EA) for 48 h. The extract was filtered through Whatman no. 1 filter paper, followed by removing solvent in a rotary vacuum evaporator (Buchi, Essen, Germany), and ethyl acetate extract (EAE) was obtained. WE and EAE were tested for their effects on LPS-stimulated PGE_2 production in RAW264.7 cells.

2.3. Fractionation of bitter gourd ethyl acetate extract (EAE) in large quantity

One hundred and thirteen kilograms of bitter gourd were lyophilised to produce 7.8 kg of powder. The powder was extracted with EA twice (2×80 l) at room temperature for two days. The pooled extracts were evaporated in a rotary evaporator to remove the solvent and yielded 250 g of EAE. EAE was suspended in 1 l of *n*-hexane, and partitioned with 1 l of methanol (MeOH)/H_2O (90/10) three times. The resulting two fractions were evaporated to yield 234 g of *n*-hexane extract and 6 g of MeOH/H_2O (90/10) extract. Hexane extract was subsequently chromatographed over silica gel with an EA/hexane gradient solvent system and finally an EA/MeOH (90/10) solvent. The eluted fractions showing similar thin-layer chromatography (TLC) and ^1H NMR patterns were collected, evaporated and weighed; the evaporated crude compounds were screened for their inhibition effects upon LPS-stimulated PGE_2 production in the RAW264.7 macrophages.

Active fractions F189–191 (8.3 g) eluted by EA/MeOH (90/10) were combined, dissolved in acetone, subjected to a second silica gel column chromatography, eluted first by chloroform/ethanol (6/1), to yield two subfractions, F1–6 (4.3 g) and F8–12 (0.4 g), and then eluted by chloroform/ethanol (5/1) to yield F13–21 (2.8 g). F1–6 and F8–12, but not F13–21, were found to have the PGE_2 inhibition effect in macrophages. F1–6 (1.7 g) were dissolved in ethyl acetate, subjected to a third silica gel column chromatography, and eluted by EA/hexane (60/40). Fractions with similar TLC patterns were combined to yield seven fractions, and tested for the PGE_2 inhibition effect in macrophages. The three active fractions F1–6-2 (247 mg), F1–6-3 (237 mg) and F1–6-4 (258 mg) were combined (742 mg) and subjected to a fourth silica gel column chromatography, successively eluted by an EA/hexane gradient, starting at 90% hexane with a 5% increase in EA for every 12 fractions (150 ml/fraction) collected. Fractions with similar TLC patterns were combined to yield 16 fractions. The active fractions p11–13 (220 mg) were combined and subjected to a reverse phase (RP) C18 chromatography, eluted by MeOH/H_2O (80/20), MeOH/H_2O (90/10) and 100% MeOH. Fraction RP-10 (19.7 mg), eluted by 100% MeOH, showed the highest PGE_2 inhibition effect. RP-10 was analysed by ^1H NMR and H-HCOSY for structural identification. Fraction F1–6 was subjected to GC/MS for fatty acid analysis after saponification and methylation by the diazomethane method (Mueller, 1996).

2.4. Cell culture

RAW 264.7 cells were seeded on 96 well plates at a concentration of 5×10^5 cells/ml, and incubated for 24 h at 37 °C in 5% CO_2. The unattached cells were washed away and the cells were treated with serum-free Dulbecco's modified Eagle's medium (DMEM) containing bitter guard extracts or fatty acids in the present or absence of 100 ng/ml of LPS for 18 h. Then, the medium was collected and analysed for PGE_2 using a commercial enzyme immunoassay kit (Cayman, Ann Arbor, Mich., USA). Cell viability and cell numbers after treatments were assayed using the MTT method (Denizot & Lang, 1986).

2.5. Statistical methods

Data were expressed as means ± SD. Experiments were repeated at least three times in triplicate. The significance of difference was analysed by one way analysis of variance (ANOVA) and a Duncan's multiple rank test, using SPSS software. To calculate the percentage inhibition of LPS-stimulated PGE_2 production, PGE_2 production in the presence of 100 ng/ml of LPS was taken as 0% inhibition and basal PGE_2 production as 100% inhibition; the percentage inhibition by a sample at a specific concentration was calculated as $[1 - (PGE_2$ sample with LPS$-PGE_2$ basal$)/(PGE_2$ LPS only $- PGE_2$ basal$)] \times 100\%$. The IC_{50} value, calculated from the curve of percentage inhibition versus concentration, is the concentration of sample that results in 50% inhibition.

3. Results

3.1. Culture conditions for measuring PGE_2

PGE_2 production induced by LPS (100 ng/ml) in RAW264.7 was tested for 0, 12, 18, and 24 h and the maximal production of PGE_2

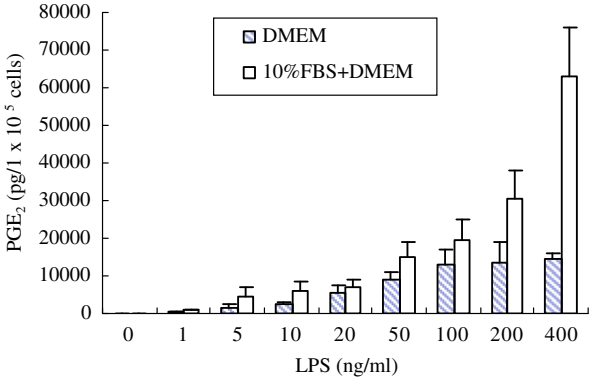

Fig. 1. PGE_2 production induced by various concentrations of LPS in RAW264.7 cells cultured with or without the addition of fetal bovine serum (FBS). The cells were treated with LPS at 0–400 ng/ml with or without 10% FBS for 18 h and medium was collected for PGE_2 analysis using an EIA. At least three batches of separate experiments were carried out with similar results. The values are means ± SD of triplicates in a representative experiment.

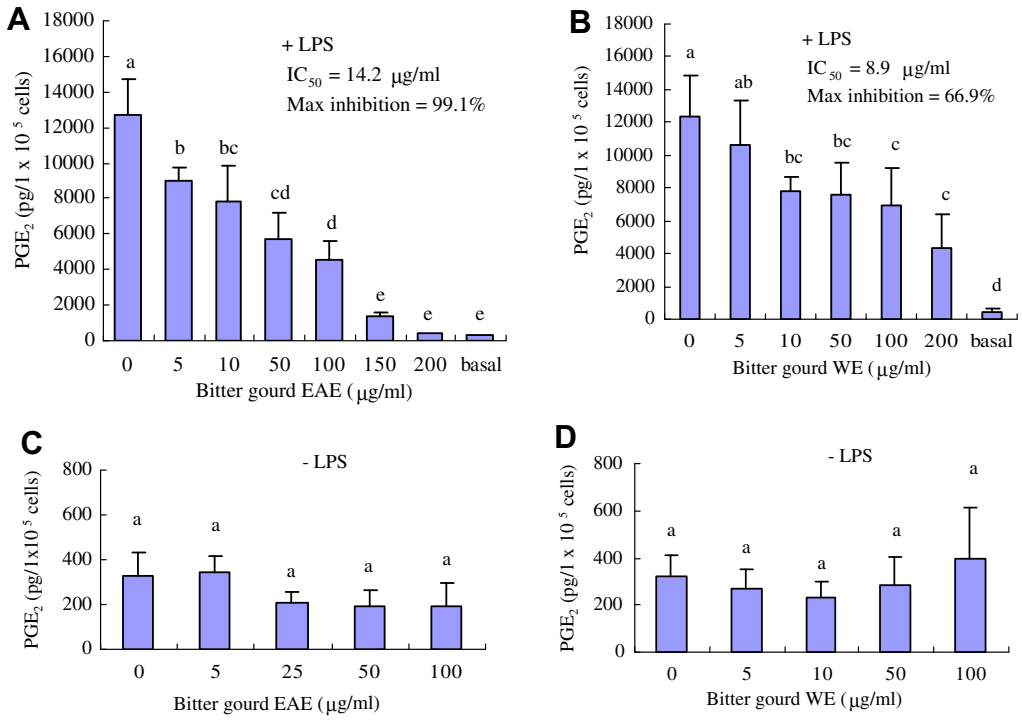

Fig. 2. Effects of ethyl acetate extract (EAE) (A and C) and water extract (WE) (B and D) of bitter gourd on PGE_2 production in RAW264.7 cells. The cells were treated with various concentrations of the extracts in the presence (+LPS) (A and B) or absence (-LPS) (C and D) of LPS (100 ng/ml) for 18 h and then medium was collected for PGE_2 analysis. The values are means ± SD of triplicates in a representative experiment. Values not sharing the same letter are significant different ($p < 0.05$). Basal: cells incubated with medium only. 0: cells treated with LPS only.

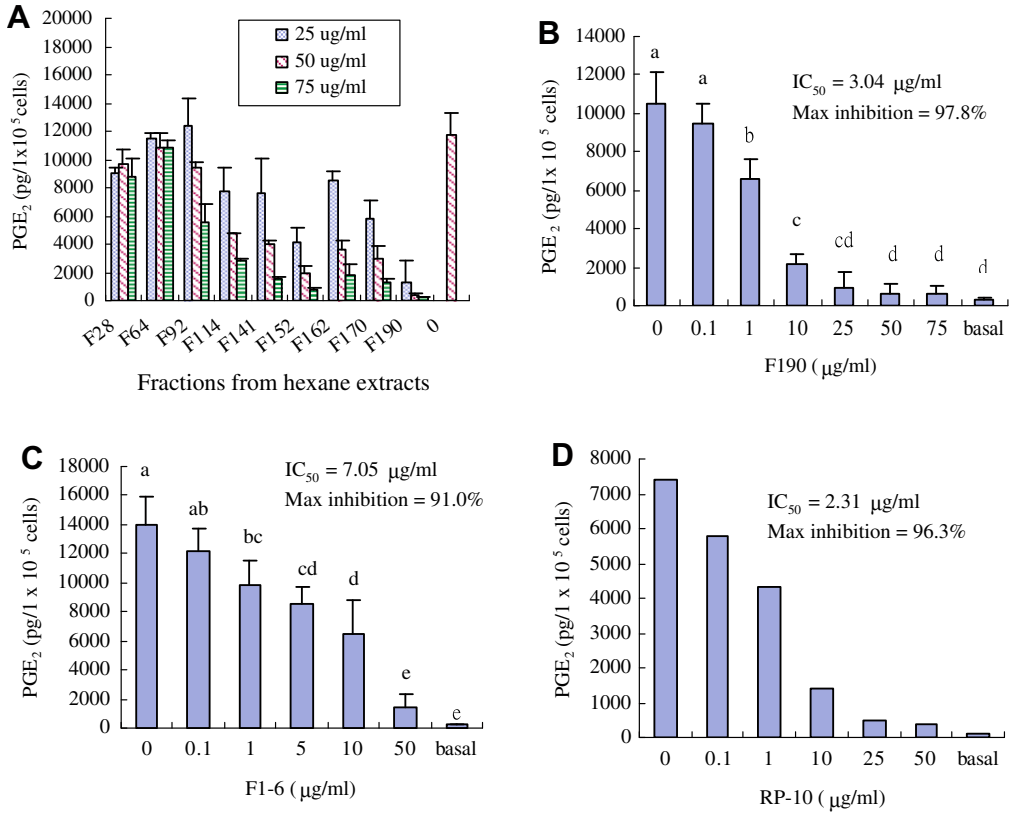

Fig. 3. Effects of different fractionations of bitter gourd EAE after chromatography on PGE_2 production in LPS-stimulated RAW264.7 cells. Bitter gourd hexane-partitioned fraction was subjected to a silica gel chromatography and eluted by EA/hexane. F28 from EA/hexane (v/v) 2/98, F64 from 5/95, F92 from 10/90, F114 from 20/80, F141 from 30/70, F152 from 40/60, F162 from 60/40, F170 from 80/20 and F190 from EA/MeOH (90/10) were tested for PGE_2 suppression effect in LPS-stimulated RAW264.7 cells (A). Dose–responses of bitter gourd active fractions F190 (B), F1–6 (C) and RP-10 (D) on PGE_2 suppression. The values are means ± SD of triplicates in a representative experiment. Values not sharing the same letter are significant different ($p < 0.05$). Basal: cells incubated with medium only. 0: cells treated with 100 ng/ml LPS only.

was found to occur at 18 h (data not shown). PGE_2 production induced by LPS at concentrations of 0, 1, 5, 10, 20, 50, 100, 200, 400 ng/ml in RAW264.7, incubated with or without 10% FBS for 18 h, was tested. At LPS concentration of 100 ng/ml, the production of PGE_2 in cells cultured without FBS reached peak level (Fig. 1) but, in cells cultured with FBS, PGE_2 production increased dramatically as the concentration of LPS increased. To avoid over stimulation, culture medium without FBS was used during LPS stimulation in the following experiments. The concentrations of extracts used to culture cells were those which did not cause cytotoxicity examined by the MTT assay.

3.2. Inhibition of PGE_2 production by bitter gourd extracts and the separated fractions

Both kinds of bitter gourd extracts, WE and EAE, decreased the secretion of PGE_2 in LPS-stimulated RAW264.7 (Fig. 2A and B), but the EAE had higher inhibition activity than had WE. Without LPS stimulation, the basal PGE_2 levels were low and not affected by either extract (Fig. 2C and D).

After partition of EAE, the hexane-soluble fraction showed higher inhibitory effect than did the MeOH/H_2O (90/10)-soluble fraction (data not shown). The hexane-soluble fraction was then separated by a silica gel chromatography. The fraction F190, eluted by EA/MeOH (90/10), showed the highest PGE_2 inhibitory activity (Fig. 3A), with maximal inhibition of 97.8% and IC_{50} of 3.04 μg/ml (Fig. 3B). Then, fractions F189–191, with similar patterns on TLC, were pooled and separated by a second silica gel chromatography. The obtained fraction F1–6, eluted by chloroform/ethanol (6/1), had the highest activity (maximal inhibition = 91.0% and IC_{50} = 7.05 μg/ml) (Fig. 3C). The fraction F1–6 was further separated by two successive silica gel chromatographies and finally a reverse phase chromatography. The fraction RP-10, eluted with methanol, showed the highest PGE_2 inhibition effect (maximal inhibition = 96.3% and IC_{50} = 2.31 μg/ml) (Fig. 3D).

3.3. Chemical structures of active components

Analysis of RP-10, using ^1H NMR and H–HCOSY, revealed that its major components were triglycerides (TGs) constituted of short and medium chain fatty acids. To identify the fatty acid composition, the F1–6 fraction was further saponified, methylated and analysed using GC/MS. As a result, common fatty acids, and three dicarboxylic acids, octanedioic, nonanedioic and decanedioic acids, were identified.

3.4. Inhibition of PGE_2 production by specific fatty acids

Of all long chain fatty acids tested, none inhibited PGE_2 production in LPS-stimulated RAW264.7, but linoleic and arachidonic acids induced dose-dependent increase in the production of PGE_2 (Fig. 4A). In contrast, among short and medium chain fatty acids, capric (10:0) and lauric (12:0) acids induced dose-dependent inhibition of PGE_2 production in LPS-stimulated RAW264.7, and capric

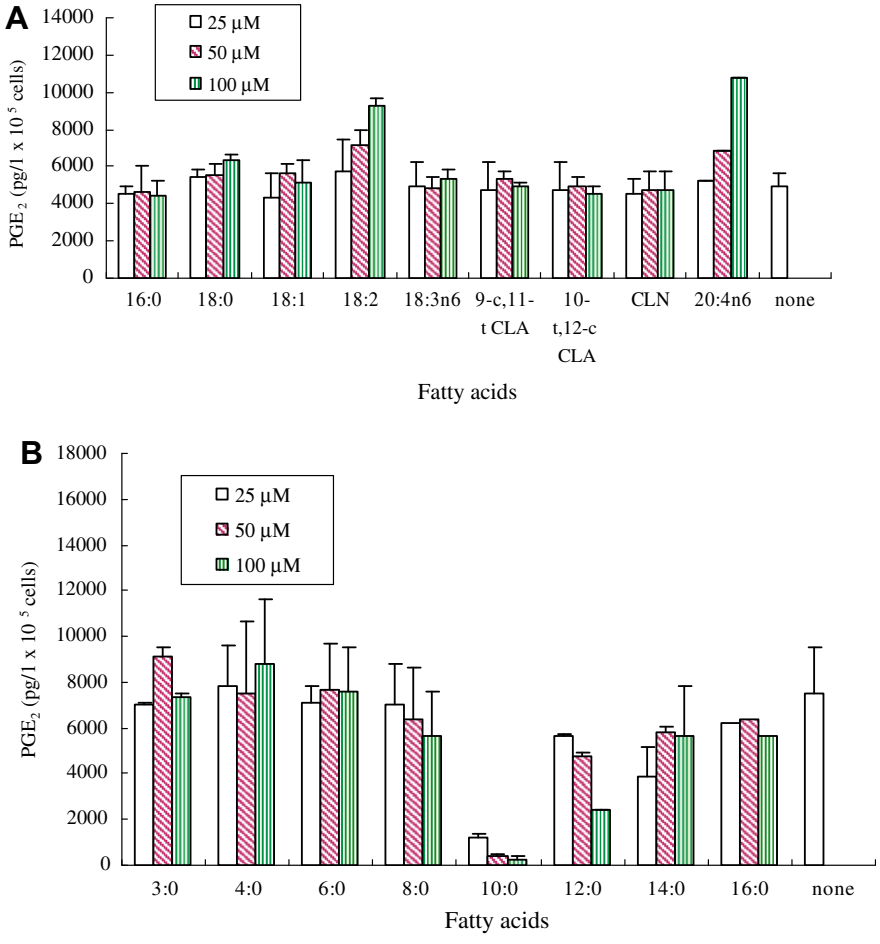

Fig. 4. Effects of different fatty acids on PGE_2 production in LPS-stimulated RAW264.7 cells. The cells were treated with long chain (A) and short and medium chain fatty acids (B) in the presence of LPS (100 ng/ml) for 18 h and then medium was collected for PGE_2 analysis. CLA, conjugated linoleic acid; CLN, 77% 9c, 11t, 13t-conjugated linolenic acid. none: cells treated with LPS only.

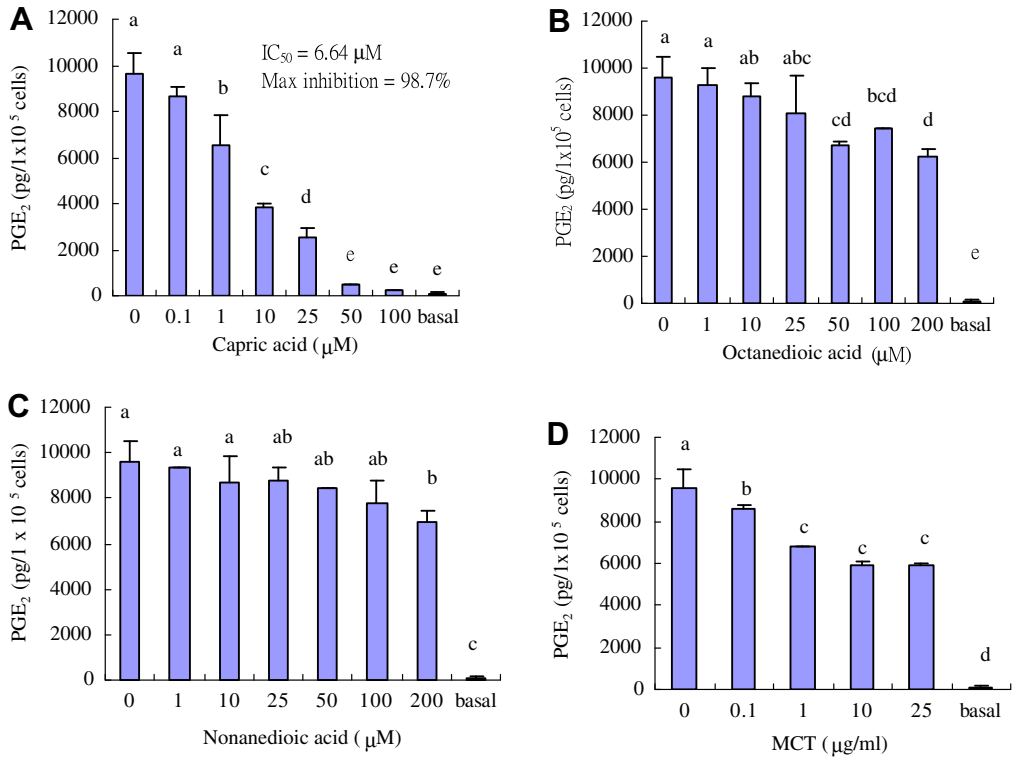

Fig. 5. Dose–response of capric acid (A), octanedioic acid (B), nonanedioic acid (C) and MCT (D) on PGE_2 suppression in LPS-stimulated RAW264.7 cells. The cells were treated with various concentrations of fatty acids or MCT in the presence of LPS (100 ng/ml) for 18 h and then medium was collected for PGE_2 analysis. The values are means ± SD of triplicates in a representative experiment. Values not sharing the same letter are significant different ($p < 0.05$). Basal: cells incubated with medium only. 0: cells treated with 100 ng/ml LPS only.

acid showed the highest inhibition activity (Fig. 4B). At a concentration of 50 μM, capric acid inhibited PGE_2 production almost completely with the IC_{50} of 6.46 μM (1.22 μg/ml) (Fig. 5A). Octanedioic and nonanedioic acids had mild PGE_2 inhibitory activities (Fig. 5B, C), but decanedioic acid failed to show any effect, even at concentration up to 200 μM (data not shown). MCT also showed significant inhibitory activity at rather low concentration (0.1 μg/ml) (Fig. 5D).

4. Discussion

Surprisingly, the present study demonstrated that the components identified in the subfraction of bitter gourd EAE with the highest suppression effect of PGE_2 production were TGs composed of short and medium chain fatty acids and dicarboxylic fatty acids. Due to similar polarities, it is difficult to further subfractionate these TG in terms of specific fatty acid composition and position. Noticeably, the active fractions were eluted by solvents with higher polarity than TGs with common long chain fatty acids, presumably because of their special fatty acid composition.

Recently, α-linolenoyl-lysophosphatidylcholine (LPC) and linoleoyl-LPC, identified in butanol extract of unripened bitter gourd placenta, where the seeds are attached, were found to contribute to the suppressive effect of bitter gourd on tumor necrosis factor-α (TNF-α) production in LPS-stimulated RAW264.7 cells (Kobori et al., 2008). Besides different parts of samples (ripened and whole vs unripened and placenta) used, our extraction procedures did not extract phospholipids or related compounds, but the effective concentration of RP-10 in this study was lower than that of purified α-linolenoyl- or linoleoyl-LPC reported by Kobori et al. (2008). Some fractions isolated in our study, also inhibiting PGE_2 production, albeit to a less extent, have not been further investigated. A predominant fatty acid, CLN, constituting 40% of bitter gourd EAE, is known to be a PPAR activator (Chuang et al., 2006), but did not change PGE_2 production in the present study (Fig. 4B).

This study was the first to find capric acid capable of inhibiting PGE_2 production in LPS-stimulated macrophages. The effective concentration of capric acid (IC_{50} = 6.46 μM) (Fig. 5A) is rather low and is likely to occur in the blood stream (Richieri & Kleinfeld, 1995). Capric acid has also been found to be the most effective among six fatty acids in killing Gram-negative bacteria by disrupting the outer membrane (Thormar, Hilmarsson, & Bergsson, 2006). The remaining fatty acids tested, including caprylic acid (8:0), lauric acid (12:0), mystic acid (14:0), palmitoleic acid (16:1) and oleic acid (18:1), had either no effect or only a low effect (Thormar et al., 2006). LPS is a major component of the outer membrane of Gram-negative bacteria, contributing greatly to the structural integrity of the bacteria. Capric acid might serve as a permeabiliser that induced the release of LPS from the outer membrane of Gram-negative bacteria as lactic acid did (Alakomi et al., 2000), and our observations indicated that it could simultaneously abolish the stimulation of LPS on PGE_2 production of macrophages.

Only few foods contain significant amounts of capric acid, of which bovine milk fat, coconut oil and MCT are major sources. Milk fat contains 2.4% capric acid, but also contains 11% myristic acid and 33% palmitic acid (Odongo et al., 2007). Coconut oil contains 6% capric acid, but also contains 49% lauric acid and 19% myristic acid (Laureles et al., 2002). The co-existence of atherogenic fatty acids may counteract the potential benefit of capric acid in these two oils. MCT oil, on the other hand, has a higher level of capric acid (23%) and is devoid of atherogenic fatty acid. Our study further showed that MCT inhibited the production of PGE_2 as well (Fig. 5D). Kono et al. (2003) compared the effects of MCT and corn oil on endotoxemia in rats, and found that MCT caused much less mortality and liver injury. Kupffer cells, isolated from rats given

MCT, showed reduced LPS-stimulated TNFα production, and CD14 expression (Kono et al., 2003), which is a cell membrane receptor of LPS (Lu, Yeh, & Ohashi, 2008).

The LPS-sensing machinery consists, primarily, of LPS-binding protein (LBP), CD14, and toll-like receptor 4 (TLR4), a signal-transducing integral membrane protein (Lu et al., 2008). Upon binding of LPS to cells, the sensing machinery then triggers intracellular signalling that leads to the expression of genes encoding proinflammatory cytokines or enzymes producing inflammatory molecules through the transcription factor nuclear factor κB (NFκB). The PGE_2 production of LPS-stimulated macrophage is known to be mediated by inducing COX-2 expression through the LPS-sensing machinery pathway (Lee & Hwang, 2006). The COX-2 expression and activity is considered the primary limiting factor for PG production in activated macrophages.

It has been demonstrated that saturated fatty acids, and especially lauric acid, which is the predominant fatty acid acylated in lipid A of LPS, could induce NFκB activation and expression of COX-2 in macrophages (Lee, Sohn, Rhee, & Hwang, 2001). The acylation and types of acylated fatty acids of lipid A determine the biological activities of LPS (Lee & Hwang, 2006). Conversely, unsaturated fatty acids inhibit lauric acid- or LPS-induced COX-2 expression (Lee et al., 2001). However, our results showed capric acid to be highly effective, and lauric acid (12:0) to a smaller extent, in inhibiting PGE_2 production, while linoleic acid (18:2n6) and arachidonic acid (20:4n6) enhanced PGE_2 production. We also found that the co-treatment with LPS and capric acid did not alter COX-2 protein expression in RAW 264.7 cells compared to treatment with LPS alone (data not shown). The discrepancy in the effect of saturated fatty acids on LPS-stimulated macrophages between our and Lee et al.'s (2003) studies might be partly explained by the differences in fatty acids tested (capric acid vs lauric acid) and experimental conditions (simultaneous treatment with fatty acids and LPS vs treatment with fatty acids prior to LPS) since lauric acid alone could have stimulated COX-2 expression (Lee et al., 2001). Besides the role of COX-2 expression, the activity of COX-2 and the availability of arachidonic acid, which is derived from the hydrolysis of phospholipids catalysed by phospholipase A_2, also affect the biosynthesis of PGE_2. The effects of capric acid on COX-2 activity and phospholipase A_2 expression and activity need further investigation.

In conclusion, the triglycerides composed of short and medium chain saturated fatty acids and dicarboxylic acids are the active components in bitter gourd EAE contributing to the inhibition of PGE_2 production, and capric acid is the most potent inhibitor among various fatty acids. Results reported here should prompt studies in other food products with similar triglyceride or fatty acid profiles. However, the potential benefits of the bioactive compounds in vegetables will need to be demonstrated in human trials.

Acknowledgement

This study was funded by a Grant, NSC 95-2317-B-003-001, from the National Science Council of the Republic of China, Taiwan.

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Food Chemistry 117 (2009) 312–317

Contents lists available at ScienceDirect

Food Chemistry

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Optimization of ethanol extraction and further purification of isoflavones from soybean sprout cotyledon

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ARTICLE INFO

Article history:
Received 11 September 2008
Received in revised form 16 March 2009
Accepted 1 April 2009

Keywords:
Isoflavone
RSM
Solid phase extraction (SPE)
Liquid–liquid extraction (LLE)

ABSTRACT

Isoflavones from cotyledons of soybean sprouts were extracted with aqueous ethanol and further concentrated to obtain a product with a high concentration of isoflavone. The ethanol concentration, extraction time and reaction temperature were optimized by using response surface methodology (RSM). Isoflavones in aqueous ethanol were concentrated by a three-step procedure comprised of solid phase extraction (SPE) with Diaion HP-20 and Amberlite-XAD-2 adsorption columns, acid hydrolysis, and liquid–liquid extraction. The maximum amount of isoflavone in aqueous ethanol extracts (11.6 mg/g solid) was obtained when isoflavones in cotyledons (2.18 mg/ g solid) were extracted with 80–90% (v/v) aqueous ethanol above 90 °C for more than 100 min. The isoflavone extracts, obtained by SPE with a Diaion HP-20 column contained 100 mg/g solid. The liquid–liquid extraction (LLE) with ethyl ether further concentrated the extracts up to 229 mg/g solid, retaining 63% of the initial isoflavones.

1. Introduction

Isoflavones are a class of flavonoids in the human diet that are mainly derived from soybean-based foods. The major dietary isoflavones, daidzein and genistein, have estrogen-like activities and are classified as phytoestrogens (Hodgson, Puddey, Beilin, Mori, & Croft, 1998) since they can bind to the estrogen receptor in place of estrogen due to their structural similarities to estrogen-β (Tikkanen & Adlercreutz, 2000). Thus, isoflavones in soybean-based foods are helpful in preventing certain cancers caused by hormone therapy, reducing the risk of cardiovascular disease and improving bone health (Anderson, Johnstone, & Cooknewell, 1995; Arjmandi et al., 1996).

The amounts of isoflavones in beans differ with the bean cultivars and culture environments, such as the region and length of culture (Wang & Murphy, 1994a). Development of isoflavone-based functional foods and dietary supplements requires screening of raw materials with high amounts of isoflavones and concentrating of the isoflavones to the level of efficacy. However, 100 g of bean contains only 0.1–0.5 g of isoflavones and this isoflavone level is too low for efficacious health benefits. Besides, the strong beany flavour of soybean powder is a major drawback for its utilization as a source for isoflavones (Macleod & Ames, 1988). Therefore, highly concentrated isoflavone-rich materials, with acceptable organoleptic properties, are needed for functional food materials and dietary supplements.

The soybean sprout (Kongnamool) is a low cost but highly nutritive traditional Korean vegetable food that can be produced relatively easily. Soybean sprouts have higher amounts of isoflavones than have soybeans (Kim, Hwang, & Lee, 2003; Kim, Lee, & Chee, 2004), and soybean sprout cotyledon has the highest amounts of isoflavones among the parts of the soybean sprout, including cotyledon, hypocotyls and root (Lee et al., 2007). Therefore, the use of soybean sprout cotyledon as a source of isoflavones is advantageous. In addition, the beany flavour of soybean is caused by lipoxygenase, and soybean germination decreases the lipoxygenase activity. Hence soybean sprouts have a less beany flavour. Soybean sprouts also have many functional ingredients, such as vitamins B1, B2, C, and carotene (Hofsten, 1979).

Extraction is a very important process for production of isoflavone concentrate from rich sources. Among the extraction methods, solid phase extraction (SPE) is one of the most widely used techniques for separating functional materials. Because of their excellent sorption characteristics, Amberlite-XAD-2 and Diaion HP-20 have been used to separate isoflavones (Choi & Kim, 2005; Fedeniuk & Shand, 1998; Hennion, 1999). Liquid–liquid extraction (LLE) also has been used to separate functional materials. In LLE, hydrophobic ingredients in the raw materials are extracted from aqueous samples with a water-immiscible organic phase. Various volatile organic solvents are used, including pentane, hexane, ethyl ether, ethyl acetate, chloroform and methylene chloride (Pedersen-Bjergaard, Rasmussen, & Halvorsen, 2000). The extraction pro-

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doi:10.1016/j.foodchem.2009.04.003

cesses are affected by process variables, such as solvent, extraction temperature, amount of solvent and extraction time.

Optimized process variables are required to efficiently produce highly concentrated isoflavone, and response surface methodology (RSM) is often used for optimization. RSM is a useful statistical technique that uses sequential experimental techniques to survey a domain of interest, focusing on the most important variables and their effects, to build an empirical model. Most RSM applications come from areas such as chemical or engineering processes, industrial research, and biological investigations, with emphasis on optimizing a process or system. The main advantage of RSM is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results (Hwang et al., 2002; Kim et al., 2002).

The use of soybean sprouts as an isoflavone rich source requires an established process for manufacturing the isoflavone-rich products. Therefore, the objective of the present study was to propose an optimized process for extraction and concentration of isoflavones from soybean sprout cotyledon, to produce highly concentrated isoflavone-rich materials.

2. Materials and methods

2.1. Reagents and materials

HPLC-grade water, ethanol and methanol were purchased from J.T. Baker Co. The standard isoflavones for quantification were daidzin (Waco Chemical Co., Japan), genistin (Fujicco Chemical Co, Japan), daidzein and genistein (Sigma Chemical Co., USA). Amberlite XAD-2 resin (surface area 300 m^2/g, pore diameter 90 Å and bead size 20–60 mesh) and Diaion HP-20 (surface area 500 m^2/g, pore diameter 260 Å and bead size 20–60 mesh) were obtained from Supelco Co. (USA). Soybean sprouts were purchased from Woo-Jung Food Co. (Korea).

2.2. Soybean sprout sample preparation

The soybean sprouts were separated into cotyledon, hypocotyl, and root. The separated cotyledon, hypocotyl and root were frozen at −70 °C and freeze-dried in a chamber Freeze-dryer (Ilshin Co., Korea). The freeze-dried parts of soybean sprout were ground in a cutting mill and passed through a 0.149 mm screen. The resulting cotyledon, hypocotyls and root powders were used for isoflavone analysis.

2.3. Isoflavone extraction

The freeze-dried cotyledons were ground in a cutting mill (Grindomix GM 200, Retsch GmbH, Germany), passed through a 0.149 mm screen, and defatted with a fivefold ratio of hexane to produce defatted soybean sprout cotyledon powder. During the defatting process, the cotyledons and hexane (1:5, w/w) were stirred with a vertical stirrer. 200 g of defatted soybean sprout cotyledon flour were suspended in 1 l of ethanol in a three neck flask reactor. Each reactor neck was attached to a thermocouple to control the temperature, a vertical stirrer for agitation and a Liebig condenser for reflux of ethanol. The concentrations of ethanol used were varied from 60% to 100%, according to the experimental design. The reactor was heated at 75–95 °C for 20–100 min in a hot water bath during the extraction process. After extraction, the suspensions were cooled to around 60 °C by immersing the reactor in a cold water bath (4 °C) immediately, and centrifuged (5000*g*) to obtain the supernatants. The floating materials in the supernatants were removed by filtering the supernatants through a nylon filter membrane (0.45 μm, Sigma Chemical Co., USA). The isoflavone-rich alcohol extract was obtained by removing the alcohols in the supernatants by vacuum evaporation with a rotary evaporator (Yamato Scientific Co., Japan).

2.4. Measurement of isoflavone

The amount of isoflavones in the extracts was analyzed using a HPLC (Waters Alliance 2690, Waters, USA) equipped with a photodiode array detector (Waters 996, Waters, USA) and Millennium^{32} chromatography manager software. A 10 μl sample was loaded onto a symmetry C18 column (Hypersil ODS, 250 × 4.6 mm, 5 μm particle size, Waters, Ireland) through an auto-sampler. The mobile phase was composed of 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B). The elution was performed with a linear gradient of A against B from 80:20 to 20:80 (A:B). The eluent flow rate was 1.0 ml/min and absorption was measured at 254 nm.

2.5. Experimental design for ethanol extraction

The process conditions for aqueous ethanol extraction of isoflavones from soybean sprout cotyledons were optimized by central composite design. Response surface methodology (RSM) was used to optimize the extraction process to yield the highest amount of total isoflavones (TI) by controlling the process variables: ethanol concentration (v/v, EC), extraction time (min, ET) and reaction temperature (°C, RT).

A five-level three-factor factorial design was adopted to optimize the extraction conditions, as shown in Table 1. Total isoflavones, including daidzein and genistein, were fitted to the quadratic response surface model as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_{13} + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (1)$$

where Y is the amount of total isoflavones, b_i are regression coefficients for linear effects, b_{ik} are regression coefficients for effects from interaction, b_{ii} are regression coefficients for quadratic effects, and X_i are coded experimental levels of the variables. Measurements were performed in duplicate and a Statistical Analysis System was used to fit the second-order polynomial equation to the experimental data.

2.6. Concentration of isoflavones by solid phase extraction and liquid–liquid extraction

The isoflavones in aqueous ethanol were concentrated by a three-step procedure comprised of solid phase extraction, acid hydrolysis, and liquid–liquid extraction. The first step was solid phase extraction, which concentrated the isoflavones by passing through Amberlite XAD-2 and Diaion HP-20 adsorbents. The adsorbents were used as adsorption resins to separate the isoflavones. Adsorbents were subsequently washed with 2 l of methanol and 2 l of distilled water and dried at 60 °C. The dried resins were swollen in methanol (1 l) overnight, and excess methanol was removed by rinsing the swollen adsorbent with distilled water. The resins were packed in a glass column (5 cm internal diameter and

Table 1
Coded and actual levels of independent variables for experimental design.

Variable	Coded level of variables				
	−1.68	−1	0	1	1.68
Ethanol concentration (%)	60.0	68.1	80.0	91.9	100.
Extraction time (min)	20.0	36.2	60.0	83.8	100
Reaction temperature (°C)	75.0	79.1	85.0	90.9	95.0

50 cm length). 500 ml of isoflavone-rich aqueous ethanol extracts were passed through the column at a flow rate of 1.2 ml/min and the column was rinsed with 2 l of distilled water to remove impurities. Isoflavones adsorbed by the column were eluted with 1.5 l of 80% aqueous ethanol at a flow rate of 1 ml/min. The glycosidic linkages were acid-hydrolyzed with 2.5 mol/l of hydrochloric acid at 85 °C for 1 h to obtain aglycones for further concentration of isoflavone. The acid-hydrolyzed isoflavones were mixed with diethyl ether in a separate funnel at a ratio of 8:2 for liquid–liquid extraction. The ether-extracted fractions were separated and dried with nitrogen gas to produce concentrated isoflavone.

3. Results and discussion

3.1. Amount of isoflavone in soybean sprout

The amounts of isoflavones in soybean sprout cotyledons, hypocotyls and roots are presented in Table 2 and are also compared with those from soybean. The isoflavone contents in soybean sprout ranged from 1.14–2.18 mg/g of dry matter and differed according to the part from which they were obtained. For example, soybean sprout cotyledon had 2.18 mg/g of dry matter, which was four times higher than the amount of isoflavones in hypocotyls. The amount of isoflavones has been reported to vary according to the type of product, as well as their cultivar, place of growth, cultivation conditions and harvest time (Kim et al., 2003; Kim et al., 2004; Wang & Murphy, 1994a; Wang & Murphy, 1994b). Likewise, the isoflavone content was higher in soybean sprouts than in soybean in the present study. This result is consistent with the report issued by Choi, Kwon, and Kim (1996), who reported an increase in total isoflavones during sprouting.

3.2. Optimization of extraction procedure

The concentration (%) of aqueous ethanol solution, the extraction time, and the reaction temperature were chosen as independent variables for optimization of the isoflavone extraction procedure. The total isoflavones (TI) in the aqueous ethanol extracts of cotyledon, prepared by controlling the independent variables at the predetermined combinations, are shown in Table 3. The combinations of coded variables were predetermined according to the central composite experimental design, and the specific coded values are shown in Table 1. The total amounts of isoflavones in ethanol extracts ranged from 9.14 to 11.6 mg/g of extracts with respect to variations in extraction conditions.

Table 4 summarizes the results of multiple regression analysis of three independent variables (X_1 = EC, X_2 = ET, and X_3 = RT) on TI in aqueous alcohol extracts, along with the results of analysis of variance (ANOVA). The regression analysis showed a significant probability of *F*-value ($p < 0.05$) in estimating TI values, which means that the three independent variables had significant effects on TI in aqueous ethanol extracts. At the 5% significance level, the significant independent variables in the TI model were the intercept ($p = 0.003$), EC ($p < 0.001$), ET ($p = 0.0408$) and RT ($p = 0.018$) in the linear term, EC^2 ($p < 0.001$) and RT^2 ($p = 0.0378$) in the quadratic term. The model could be expressed as:

$$TI = -59.76 + 0.6880X_1 + 0.1298X_2 + 0.8648X_3 - 0.003095X_1^2 - 0.004081X_3^2 \quad (2)$$

The proposed model had a sufficiently high *R*-square value ($R^2 = 0.9426$) to indicate that the TI data were adequately explained. Therefore, the model can be used as an estimate of tendency. The contour and three-dimensional plots that account for the effects of EC, RT and ET on TI were produced by the above regression equation and are presented in Fig. 1. The plots in Fig. 1 were produced for each pair of factors, whereas the third factor was taken as a constant

Table 2
Amounts of isoflavones in soybean and soybean sprouts.

Varieties	Isoflavone (mg/g)		
	Daidzein	Genistein	Total
Soybean	0.49 ± 0.02[a]	0.65 ± 0.16	1.14 ± 0.08
Soybean sprout – whole	0.68 ± 0.03	0.76 ± 0.02	1.44 ± 0.03
Cotyledon	0.80 ± 0.08	1.39 ± 0.17	2.18 ± 0.18
Hypocotyl	0.43 ± 0.13	0.10 ± 0.08	0.53 ± 0.16
Root	1.81 ± 0.15	0.20 ± 0.02	2.02 ± 0.13

[a] Experiments were performed in triplicate.

Table 3
Coded level combinations of three variables for central composite orthogonal and rotatable design.

Test run[a] no.	Coded level of variable[b]			TI[c] (mg/g)
	EC (%)	ET (min)	RT (°C)	
1	−1	−1	−1	9.14
2	−1	−1	1	10.0
3	−1	1	−1	10.5
4	−1	1	1	10.4
5	1	−1	−1	11.3
6	1	−1	1	11.2
7	1	1	−1	11.5
8	1	1	1	11.6
9	0	0	−1.68	10.8
10	0	0	1.68	11.1
11	0	−1.68	0	10.7
12	0	1.68	0	11.6
13	−1.68	0	0	9.46
14	1.68	0	0	10.7
15	0	0	0	11.5
16	0	0	0	11.4
17	0	0	0	11.3
18	0	0	0	11.5
19	0	0	0	11.2
20	0	0	0	11.2

[a] Test runs were performed in random order.
[b] EC: Ethanol concentration, ET: extraction time, RT: reaction temperature.
[c] TI: Total isoflavone.

Table 4
Regression coefficients of the quadratic regression model[a] for the determination of total isoflavones in the cotyledon of bean sprouts.

Coefficient	Regression coefficient	*T* value
b_0	−59.76	−3.88***[b]
Linear		
b_1	0.6880	5.73***
b_2	0.1298	2.35**
b_3	0.8648	2.82**
Quadratic		
b_{11}	−0.003095	−7.26***
b_{22}	−0.000139	−1.31
b_{33}	−0.004081	−2.39**
Two-factor cross		
b_{12}	−0.000490	−1.71
b_{13}	−0.001374	−1.20
b_{23}	−0.000733	−1.28
Regression		
R square	0.9426	
F value	18.24	$P < 0.0001$

[a] Model for regression analysis of total isoflavones (Y) used X_1 = Ethanol concentration of aqueous ethanol solution (EC,%, v/v), X_2 = Extraction time (ET, min), and X_3 = Reaction temperature (RT, °C), and $Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_{13} + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$.
[b] **Significant at 5% level ($p < 0.05$), and ***Significant at 1% level ($p < 0.01$).

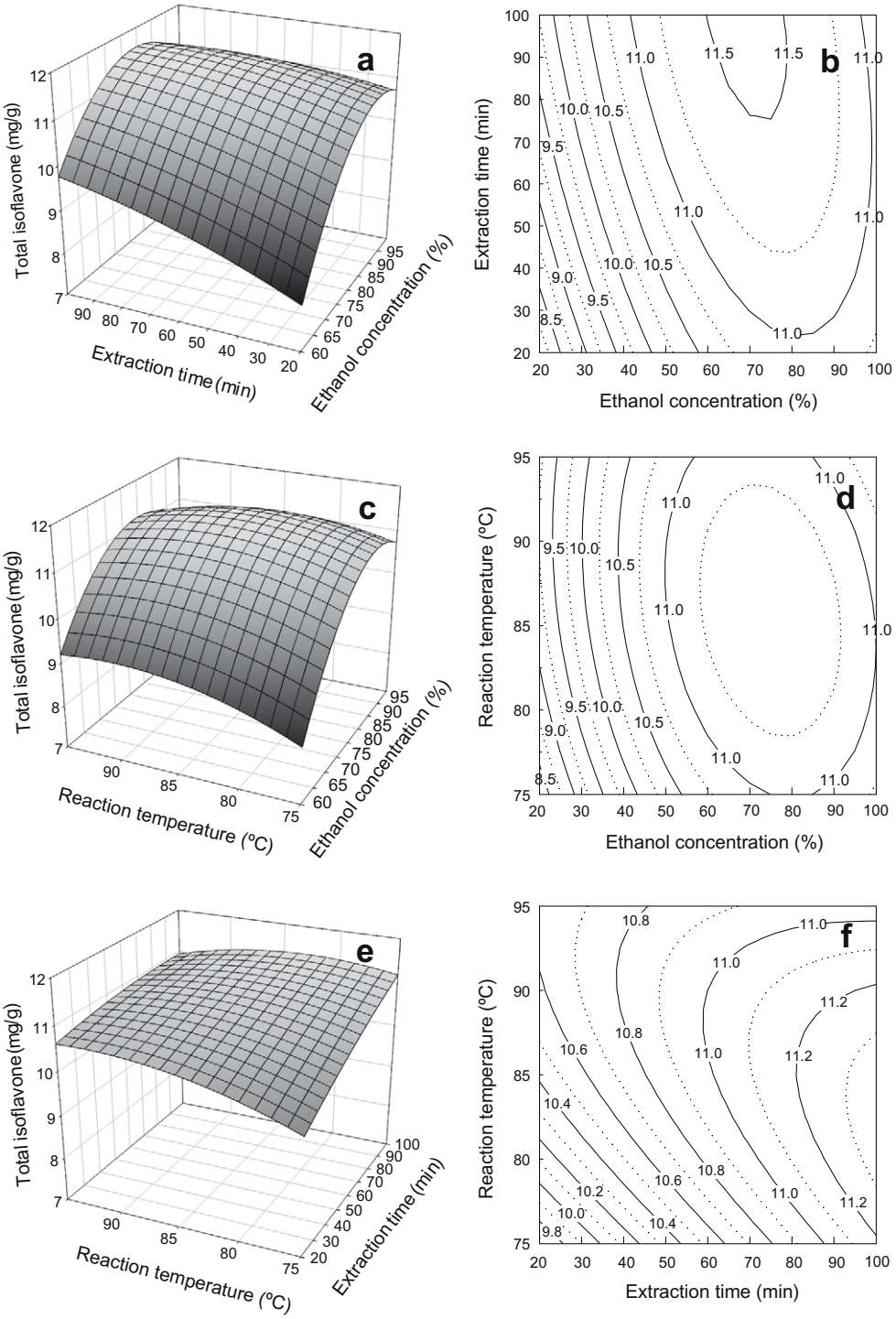

Fig. 1. Response surface and contour plots for the effects of variables on the total isoflavones (TI) in the ethanol extracts of soybean sprout cotyledon: (A and B) ethanol concentration (EC) and extraction time (ET); (C and D) ethanol concentration (EC) and reaction temperature (RT); (E and F) extraction time (ET) and reaction temperature (RT).

at its middle level. The plot a and b in Fig. 1 represent the effects of EC and ET on TI in the ethanol extracts of soybean sprout cotyledon. The maximum TI could be observed at optimized ethanol concentrations, and an increase of TI was observed with increased extraction time. The maximum TI was obtained with 80–90% aqueous ethanol solution and more than 90 min of extraction time. EC higher than 90% decreased TI due to the poor solubility of isoflavone in pure ethanol. Plots c and d in Fig. 1 illustrate the effects of EC and RT on the TI of aqueous ethanol extracts. The maximum TI was obtained with EC between 85% and 90% and RT around 85 °C. The decrease of TI with RT above 90 °C seemed be due to the unstable concentration of solvent mixture above the boiling temperature of ethanol. The effects of ET and RT on the TI of aqueous ethanol extracts are shown in plot e and f. The maximum TI could be obtained with RT around 85 °C, and an increase of TI was observed with increased extraction time. The maximum TI was obtained when the extraction procedure was operated at 85 °C for 95 min or more.

The amount of total isoflavone in extracts prepared with the above extraction conditions was 11.6 mg of isoflavone/g of extract (Table 5). The TI in aqueous ethanol extracts was 5.3 times higher

Table 5
The yield, retention and content of isoflavone in cotyledon, ethanol extracts and further concentrated isoflavone extracts.

	Isoflavone (mg/g)[a]			Yield[b] (%)	Retention[c] (%)	Content (g/100 g)
	Daidzein	Genistein	Total			
Extraction						
Cotyledon	0.80 ± 0.08	1.39 ± 0.17	2.18 ± 0.18	100	100	0.22
Ethanol extracts	3.93 ± 0.39	7.25 ± 0.95	11.6 ± 0.77	17.7	90.3	1.16
Purification						
Amberlite XAD-2	22.1 ± 3.22	59.6 ± 5.61	81.7 ± 4.18	2.42	86.7	8.17
Diaion HP-20	29.7 ± 1.47	70.1 ± 2.94	100 ± 3.91	1.95	88.5	10.0
Liquid–liquid xtraction	60.7 ± 4.28	170 ± 3.96	229 ± 4.86	0.60	63.0	22.9

[a] Experiments were performed in triplicate.
[b] Yield is the weight of dried extracts from 100 g of soybean sprout cotyledon feed (=$W_{extract} \times 100/W_{soybean\ sprout\ cotyledon\ feed}$).
[c] Retention is the weight of total isoflavone in dried extracts from 100 g of total isoflavones in soybean sprout cotyledon feed (=$(W_{TI})_{extract} \times 100/(W_{TI})_{soybean\ sprout\ cotyledon\ feed}$).

than the amount of isoflavones in cotyledon (2.18 mg/g of dry weight). Using this optimized extraction process, 90.3% of isoflavones in the soybean sprout cotyledon were retained in the aqueous ethanol extracts.

Several researchers have explored the optimal conditions for extraction of isoflavones from beans. Choi et al. (1996) compared the amounts of isoflavones in extracts according to extraction temperature, solvents and time. They reported that the optimal conditions for isoflavone extraction were 60% and 80% ethanol, 90 °C extraction temperature and 1 h extraction time. Rostagno, Palma, and Barroso (2003) optimized the solvent condition for extraction of soy isoflavones and developed an ultrasound-assisted extraction method for isoflavone determination. Their optimized condition for ultrasound-assisted isoflavone extraction was 50% ethanol concentration, 60 °C reaction temperature and 20 min of ultrasound-assisted extraction. The ultrasound-assisted extraction might be responsible for the alteration of optimal extraction conditions between their work and the present study. Zhang, Ng, and Luo (2007) optimized extraction and purification parameters for manufacturing soy isoflavone products on the basis of the yields of isoflavones. The optimum extraction conditions of their study were determined to be 80 °C for 8 h with a 96% ethanol over soybean flour ratio of 3:1 to achieve the best extraction of isoflavones, and the yield was about 0.87 mg of total isoflavones from 1 g of soybean flour. Their optimal conditions for soy isoflavone extraction are similar to those of the present study, except for extraction time. In this study, the extraction time over 90 min had only a small effect on extraction of isoflavone when isoflavone was extracted with 85% ethanol at 85 °C. Based on these studies, ethanol concentration and extraction temperature had the most profound effects on the amounts of isoflavones in aqueous ethanol extracts of soybean products.

3.3. Purification of isoflavones

The aqueous ethanol-extracted isoflavone solution was further purified by solid phase extraction (SPE) with adsorption columns packed with Diaion HP-20 and Amberlite XAD-2. The results are presented in Table 5. Solid phase adsorption with a Diaion HP-20 column performed better in separating isoflavones than did the Amberlite-XAD-2 resin column. The amount of isoflavone after SPE with Diaion HP-20 increased to 100 mg/g of sample. The isoflavone-rich fraction retained 88.5% of the soybean sprout cotyledon isoflavone with 1.95% yield. This result is in accordance with the previous work of Choi and Kim (2005) who reported the effectiveness of Diaion HP-20 in extracting phytochemicals.

After SPE, the isoflavone-rich sample was acid-hydrolyzed for 1 h at 95 °C to yield the aglycone forms of isoflavones, which had increased hydrophobicity. The presence of aglycone isoflavones is preferable, due to the ease of separation and their biological effects. The aglycones of isoflavones were further purified by ethyl ether extraction, and the results are presented in Table 5. The liquid–liquid extraction procedure increased the amount of isoflavone in extracts up to 229 mg/g of sample, and the extract retained 63% of the initial isoflavones.

The performance of liquid–liquid extraction in the present study was comparable to that in previous works on the concentration and purification of isoflavones (Chang, Cheng, & Chang, 2004; Choi et al., 2003). Chang et al. (2004) used Amberlite 16-HP resin for purification and obtained 24.7% of isoflavones when they scaled up the liquid–liquid extraction. They suggested that production of high purity total isoflavone might be significantly influenced by the feed concentration of total isoflavones. Thus, using the feed extracts obtained under optimum conditions is beneficial for purifying isoflavones (Chang et al., 2004).

4. Conclusion

The soy isoflavone concentrate was prepared by a series of procedures, comprised of ethanol extraction at optimized condition and followed by a three step concentration process, including solid phase extraction, acid hydrolysis and liquid–liquid extraction. 0.22 mg of isoflavone/g of soybean sprout cotyledon was concentrated to 229 mg/g of concentrate through a series of concentration procedures with 63% recovery of the total isoflavones. The production of highly concentrated isoflavone might allow its application as a biologically active food material since the trace amount of isoflavone in soybean sprout has limited its use in health food.

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Food Chemistry 117 (2009) 318–325

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Impact of maturity on the physicochemical and biochemical properties of chinook salmon roe

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ARTICLE INFO

Article history:
Received 28 October 2008
Received in revised form 2 April 2009
Accepted 2 April 2009

Keywords:
Fish roe
Chinook
Physicochemical properties
New Zealand

ABSTRACT

Various physicochemical and biochemical properties were compared between raw mature and immature chinook salmon (*Oncorhynchus tshawytscha*) roe. The pH, osmolality and moisture content were lower for mature salmon roe but egg size, viscosity, protein, crude lipid and ash content values were all higher. Maturity had an impact on the profile of amino acids but not on fatty acids. Maturity affected mineral and trace elements with higher Ca, Co, Mg, P, S and lower Cu, Fe, Mn, Na, Zn found in mature salmon roe. Immature roes had more α- and total tocopherols but less γ- and δ-tocopherols. Soluble proteins from mature and immature roes were similar, but the profiles of membrane-bound proteins differed. Thus maturity will affect functional and physical properties during processing and consumption of chinook roes.

1. Introduction

World production of salmon increased 2.6-fold from 1970 to 2005 as a result of increased aquaculture, with Norway, Chile, UK and Canada providing 85% of the world production of farmed salmon (Liu & Sumaila, 2008). Farmed salmon production in New Zealand increased rapidly from almost non-existing in 1983 to 9000 tons in 2002. This increase was fuelled by technological advances, consumers' perceptions of fish as healthy food option and the convenience of year-round production. This system has led to significant amounts of salmon roe of different sizes and maturity as poorly utilised by-products. Processed mature salmon roe has been used in many parts of the world. The most common salmon roe products are ikura (salted individual eggs) and suijko (salted cured whole skeins) which are produced mainly from chum salmon (*Oncorhynchus keta*) and pink salmon (*Oncorhynchus gorbuscha*) (Bledsoe, Bledsoe, & Rasco, 2003). In New Zealand, 80% of farmed salmon is chinook salmon (*Oncorhynchus tshawytscha*), which is about 50% of the world production of this species. Several studies have suggested the potential of using fish by-products, including immature fish roe, for the manufacturing of high quality protein powder (Bechtel, 2003; Bechtel, Chantarachoti, Oliveira, & Sathivel, 2007; Sathivel et al., 2004).

Farmed chinook salmon production is small compared with other salmon species and little is known of the technological properties of its components. Information on the nutritional and physicochemical properties is needed to evaluate the potential to utilise chinook salmon roes. Furthermore, apart from the expected difference in the size of eggs, there is no available information on the impact of the roe maturity on the physicochemical and nutritional properties of chinook roe.

The objective of the present study was to characterise the physicochemical properties (weight, length, grain size, pH, viscosity, osmolality, proximate analysis, fatty acid composition, amino acids profile, tocopherols content and soluble and urea soluble protein profiles) of chinook salmon roe and to investigate the impact of roe maturity on these parameters.

2. Materials and methods

2.1. Chinook roe samples

Chinook salmon (*O. tshawytscha*) roes (grade 1 for mature roe and there was no classification for immature roe; 25 kgs each) were obtained from commercial production establishment in New Zealand. The grades correspond to the classification scheme used in commercial plants and refer to the intactness. The mature salmon sample was received frozen in free flow form while the immature salmon sample was fresh. Samples were vacuum packed in about 1 kg portions, kept frozen at −20 °C and were thawed in a cool room (5 °C) overnight when required for analysis (over 6 weeks from purchase). The fish size and weight were

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doi:10.1016/j.foodchem.2009.04.009

unavailable and only whole (undamaged) roe skeins were used for analysis.

2.2. Physicochemical analysis

2.2.1. Weight and length

The weight and length of individual roes were determined on thawed roes.

2.2.2. Measurements of egg size using image analysis

The imaging system consisted of a digital camera (Sony Cybershot DSC-F505 V) mounted on a stand (RSX copy stand, Kaiser, Germany), lighting system (two sets of RB 5004 HF copy lighting units, Kaiser, Germany), personal computer, and image processing and analysis software (ImageJ 1.37v, available free from <http://rsb.info.nih.gov/ij/index.html>). Skeins were cut and three lots of 10 g of fish eggs ($n = 3$), were suspended in 0.4 M phosphate buffer (pH 6.1) in a petri dish and washed twice with the same buffer. For imaging, samples in petri dishes were placed on a non-glare black surface and illuminated with standard lighting. A standard scale was included in all the images taken for subsequent image processing. The same lighting conditions were used for all the images and the still images were transferred to the PC for storage and analysis. The measurements were performed in density slice mode using the particle analysis function in ImageJ. The images were adjusted using the threshold function manually and particles touching the edge were omitted from analysis. The area and maximum diameter of individual eggs was measured.

2.2.3. Viscosity

The viscosity of the roes was determined using Brookfield Viscometer (LVDV-II+, Brookfield Engineering Laboratories, MA, USA). Roe homogenates were prepared using a hand-held blender, cooled and maintained at 5 °C in a walk-in chiller before measurements. The viscosity measurements were carried out using spindle 4 and appropriate spindle speed (within 10–90% of the total torque according to the manufacturer instruction). The viscosity in (mPa.s) was read directly off the instrument digital panel. The viscosity measurements were not possible on individual skeins, therefore five replicates of homogenates ($n = 5$) for each maturity level were used for the analysis.

2.2.4. Osmolality

A vapour pressure osmometer (VAPRO 5520, Wescor Inc., Logan, UT, USA) was used to measure the osmolality of the fish roes. The machine was calibrated with Optimol™ osmolality standards (100, 290 and 1000 mmol/kg or mOsm/kg). A 10 μl aliquot of roe homogenate (1 g roe: four parts deionised distilled water to facilitate the pipetting) was placed on the sample disc and the measurements were carried according to the manufacturer's instructions (eight readings/skein and four skeins/maturity level). The osmolality of unit weight (1 g) was expressed as (mmol/kg).

2.2.5. Chemical analysis

All the results are presented as the mean ± SD of three individual skeins for each maturity level, unless otherwise indicated.

2.2.6. pH

Measurements were carried out using a combination puncture pH electrode (InLab 427, Mettler-Toledo Process Analytical Inc., Wilmington, MA) attached to a pH meter (Hanna HI 9025, Hanna Instruments, Woonsocket, RI). For the thawed raw material and, 4–5 skeins were homogenised separately using a hand-held blender (Miniprimer MR400, Braun, Spain) until smooth blend was achieved (90s of blending). The probe was inserted directly into the homogenised samples at three different locations and pH was measured.

2.2.7. Proximate analysis

Proximate analysis was carried out in duplicate for three samples (separate skeins) per maturity level. Moisture content was determined using gravimetric measurement of water content by freeze drying (AOAC, 1995). Crude lipid content was determined according to AOAC (1995) using a Tecator Soxtec System HT 1043 Extraction Unit™ (Tecator, Högänas, Sweden). Crude protein content was determined according to AOAC (1995) using a Tecator Kjeltec Auto Sampler System 1035 Analyser™ (FOSS Tecator AB, Högänas, Sweden). Ash content was determined on freeze–dried samples. The crucibles were placed in a muffle furnace which was set to run at an initial temperature of 200 °C for 1 h before being turned up to 550 °C for 4 h. We found that without the hour at the lower temperature the samples had a tendency to explode out of the crucible. After the crucibles had cooled sufficiently (in a desiccator) they were weighed and returned to the oven. This process was repeated until a constant weight was achieved. Carbohydrates were calculated by difference [100 – the sum (moisture + lipid + protein + ash)%].

2.2.8. Amino acid analysis

All the amino acids, except tryptophan, cysteine and methionine, were determined by HPLC analysis after acid hydrolysis as described by Carducci et al. (1996).

Cysteine and methionine were analysed as cysteic acid and methionine sulphone after oxidation of the sample with performic acid followed by hydrolysis with 6 M HCl, as described by McDonald, Krueger, and Keller (1985). Amino acid analysis was carried out in duplicate for three samples (separate skeins) per maturity level.

2.2.9. Fatty acids

Lipid was extracted from 10 g of roe samples (triplicates of different skeins) using Folch, Lees, and Stanley (1957) chloroform:methanol (2:1 v/v) procedure with the modifications of West, Bickerstaffe, Annison, and Linzell (1972) using phosphate buffer (pH 6) to replace water and aid clean bi-phase partitioning. The chloroform plus lipid extract was evaporated *in vacuo* at 40 °C. Lipid was transferred to pre-weighted vials and solvent evaporated using oxygen free nitrogen at 40 °C. The resulting oil was stored under oxygen free nitrogen at −30 °C prior to analysis.

Fatty acid analysis was carried out by transmethylating 50 mg of oil (in duplicate) using H_2SO_4 (1%) in methanol as per Christie (1989). The fatty acids methyl esters (FAME) were separated using a Hewlett Packard HP-6890 GLC with split/splitless injector (250 °C, split ratio 50:1), FID (250 °C) on a capillary HP-INNO Wax Column (30 m, 0.25 mm i.d. and 0.25 μm film thickness) using programmed temperature 50–205 °C and pressure 54–200 kPa ramps over 60 min. Tridecanoic acid (C13:0) (T0502-5G, Sigma Aldrich, Bellefonte, USA) and heneicosanoic acid (C21:0), Acros Organics, USA were used as internal standards (3 mg/ml). Fatty acids were identified by comparing the retention times of FAME with standards of mixed known compositions (Nu-Check GLC-68E, GLC-68F and GLC-411, Nu-Check, Elysian, USA, Supelco 37, USA and ME 61 from Larodan, UK). Two replicate GC analyses were performed for each oil sample and the results were expressed as a percentage of the total fatty acid methyl esters present as mean values ± standard deviation. Small and unidentified peaks are shown as a percentage of other fatty acids.

2.2.10. Analysis of tocopherols

Tocopherols were determined as described by Schwartz, Ollilainen, Piironen, and Lampi (2008) with modifications. A 0.1 g of oil

sample obtained for fatty acid analysis was dissolved in 10 mL of *n*-heptane, and the extracts were filtered through a 0.45 μm nylon filter. All the samples were run with parallel spiked samples. The contents of α-tocopherol, γ-tocopherol and δ-tocopherol, respectively, were determined by HPLC equipped with a fluorescence detector set at 294 nm for excitation and 320 nm for emission. The HPLC system consisted of degasser, HPLC quaternary pump, auto-sampler with thermostat, heated column compartment and a fluorescence detector, controlled by ChemStation (version A10.02). Tocopherols were separated isocratically using a mobile phase containing *n*-heptane:tert-butylmethylether:tetrahydrofuran:methanol [79:20:0.98:0.02, v/v/v/v] and a flow rate of 1.5 ml/min. The column was a Phenomenex LiChroCART (250 mm, 4 mm ID) packed with LiChrospher 100 NH2 (5 μm). The tocopherols were separated at 30 °C and the autosampler tray was kept at 4 °C. The total run time was 20 min and calibration curves were constructed with standard solutions of each of the three tocopherols and used for quantification. Results are given in mg of α-, γ-, δ- and total tocopherols per 100 g of oil. Each oil sample was analysed in duplicate.

2.2.11. Analysis of minerals in fish roes

A 2.5 g sample of fresh roe, was placed in 50 mL graduated polycarbonate tubes and 2.5 mL of nitric acid and 0.5 mL of hydrochloric acid were added. The tubes were capped and digested for 60 min in a 90 °C heating block. The digested samples were cooled and diluted to 50 mL with deionised water. The mineral contents in the diluted digests (Al, As, B, Ca, Co, Cu, Fe, K, Mg, Mn, Na, Ni, P, S and Zn) were analysed by Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Varian 720-ES). The calibrating standards were prepared as "multi-element" solutions using BDH Spectrosol standards and were diluted in a solution of 5% (v/v) nitric acid/1% (v/v) hydrochloric acid to match the acid matrix of the diluted sample digests. Caesium and Yttrium were used as internal standards. Certified reference material (NRCC-DORM-3 fish protein) and samples spiked with BDH Spectrosol standards were analysed for corresponding elements to validate the method for accuracy and precision. The recoveries of the measured elements were between 81% and 102%.

2.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE)

The proteins in fish roe were fractioned into soluble protein fraction as described by Geesink, Morton, Kent, and Bickerstaffe (2000) and membrane-bound protein fraction as described by Lee, Morton, Sanderson, Bickerstaffe, and Robertson (2008). A 2 g sample of frozen fish roe was homogenised in 6 mL cool (4 °C) extraction buffer (10 mM EDTA, 2 mM DTT in 100 mM Tris–HCl, pH 8.0) for 1 min at a speed of 12,000 rpm (Polytron PT3100, Littauk Switzerland). The homogenates were centrifuged at 45,000*g* for 30 min at 4 °C. The supernatant was collected and mixed with the protease inhibitors (Mini Complete™, Roche applied Sciences) resulting in the "soluble protein fraction". The pellet was washed three times by resuspension in cold extraction buffer and re-centrifuged to remove any remaining soluble protein. The washed pellet was re-suspended in three volumes of 6 M urea followed by homogenisation and centrifugation (45,000*g* for 30 min at 4 °C). The supernatant was collected resulting in the "membrane-bound protein fraction".

Protein concentration of each protein fraction was determined using BCA Assay Reagent Kit (Pierce, Rockford, USA). Each protein fraction was mixed with SDS–PAGE sample loading buffer [58 mM Tris–HCl (pH 6.8), 1.7% SDS (w/v), 5% (v/v) glycerol, 0.002% bromophenol blue] containing 100 mM DTT and denatured at 95 °C for 4 min. After cooling, 10 μg protein samples were loaded onto a SDS–PAGE gel (15% polyacrylamide mix) and protein separation was performed according to Laemmli (1970). After electrophoresis, gels were stained with Simply Blue™ Safe Stain (Invitrogen).

2.4. Statistical analyses

The data was analysed using MINITAB (Release 14.1). For pH, viscosity, osmolality and chemical composition, tocopherol contents, amino acid and fatty acid concentrations, the analysis of variance (one way ANOVA) was used to test for maturity effect on the mean values. The significance of the difference between means was determined by Tukey's test ($P < 0.05$).

3. Results and discussion

3.1. Weight and length of raw material

We used the gonad maturity scale for teleost fishes (Kesteven, 1960) to estimate the maturity stage of the fish roes. This eight stage scale describes the developments of gonads depending on the size, shape, colour, sac fullness and ease of flow of gonad material upon applying pressure. According to this scale roes from mature salmon are in stage (VI, spawning) and immature salmon at stage (IV, developing late).

Katselis et al. (2005) reported that flathead mullet fish roe that is classified as gravid and spawning stage are suitable for production of salted dried roe. Thus mature salmon samples would probably be suitable for this type of products as well as the well-known ikura and suijko. Although developing stage gonads (such as immature salmon in our study) are not traditionally regarded as a suitable raw material, they may still be nutritionally sound or able to be presented in a different form.

Although there was no difference between the length of mature and immature salmon roes, the mature salmon roes were three times heavier ($P < 0.05$), reflecting the expected storage of nutrients required for the ova and larvae development (Zhu, 1999).

3.2. Measurements of egg size

We used image analysis to measure the size of salmon eggs from mature and immature roes to evaluate their processing potential and compare their size with the commonly known caviar and caviar-like products. Mature salmon eggs were more than twice the diameter and had six times the surface area ($P < 0.05$) of immature eggs (Table 1). Oocyte and fish egg size measurements have been carried out manually. Manual measurements can be appropriate if the size distribution is narrow, in which case a small number of eggs can be accurately measured, as in Atlantic salmon (*Salmo salar* L.) and Atlantic herring (*Clupea harengus* L.) which spawn only one batch in a season (Thorsen & Kjesbu, 2001). However, when the eggs have broader size distribution such as in the case of immature eggs, manual measurements are time consuming and image analysis will be more efficient.

Grain size is of particular importance for a caviar-like product due to desired mouth feel characteristics (Bledsoe et al., 2003). Generally caviar and caviar-like products have a diameter of >2.0 mm (Bledsoe et al., 2003; Wirth et al., 2000). Both immature and mature salmon eggs would fit within this range, however immature salmon roe (diameter range 0.16–2.64 mm) will require size screening. Alternatively, other products such as salted dried roe (karasumi), fermented roe (karashi mentaiko), or protein powder will be more suitable products for the smaller salmon eggs.

Fish egg size measurement is important in marine biology since it is linked to the quality of oocyte and fecundity (Thorsen & Kjes-

Table 1
pH, viscosity (m Pa.s), osmolality (mmol/kg), weight and length (mean ± SD, range) of raw mature and immature Chinook salmon roes and plane egg area (mean ± SD, range) and egg diameter (mean ± SD, range) of raw mature and immature chinook salmon eggs.

Parameter		Mature	Immature
Weight (g)	Mean ± SD	266.36 ± 64.85[b]	75.00 ± 19.36[a]
	Range	200.2–400.9	50.0–110.5
Length (cm)	Mean ± SD	16.78 ± 1.63	15.17 ± 2.16
	Range	14.0–19.9	11.0–20.0
Egg area (mm^2)	Mean ± SD	25.35 ± 8.30[b]	3.79 ± 1.00[a]
	Range	14.00 – 57.00	0.02 – 5.48
Egg diameter (mm)	Mean ± SD	5.69 ± 0.78[b]	2.16 ± 0.41[a]
	Range	4.22 – 8.52	0.16 – 2.64
pH		5.62 ± 0.09[a]	6.13 ± 0.05[b]
Viscosity (mPa.s)		541.25 ± 157.01[b]	189.88 ± 12.13[a]
Osmolality (mmol/kg)		197.42 ± 10.69[a]	219.32 ± 13.40[b]
Colour	L^*	28.65 ± 2.55[a]	43.03 ± 13.85[b]
	a^*	20.22 ± 4.07[a]	29.51 ± 11.53[b]
	b^*	21.10 ± 4.49	25.91 ± 7.94
	C^*	29.25 ± 5.91[a]	39.59 ± 12.92[b]
	h^*	46.19 ± 2.90	44.63 ± 14.10
Proximate composition	Moisture	55.60 ± 0.36[a]	68.50 ± 0.46[b]
	Protein	26.16 ± 0.60[b]	17.69 ± 0.18[a]
	Crude lipid	10.59 ± 0.46[b]	8.45 ± 0.93[a]
	Total ash	1.42 ± 0.01[b]	1.11 ± 0.01[a]

[a,b]Values within raw with different superscripts are significantly different ($P < 0.05$).

bu, 2001) and can indicate the health and initial growth rates of produced larva (McDonough, Roumillat, & Wenner, 2003). As food, the size of fish egg can be very important since the nutrients available in fish eggs change with maturity and the consumer's perception could be influenced by size and maturity-linked pigments. From a technological point of view, the size of roes and eggs will have a great influence on the processing conditions (e.g. salting and drying times, salt/roe surface area ratio) and the suitability of the fish eggs for certain products (e.g. caviar-like products, dried products or fermented products).

3.3. pH

The average pH of mature salmon roe was 5.62 which was significantly less ($P < 0.05$) than the 6.13 for immature salmon roe (Table 1). During maturation biochemical and physical changes take place in the fish egg which lead to increased egg mass and lipid to moisture ratio (Bledsoe et al., 2003), increased protein levels (Fauvel, Omnès, Suquet, & Normant, 1993), and a decrease in ovarian fluid pH and moisture content (Fauvel et al., 1993). With over-ripening there are further changes in the composition of fish roe and reduction in ovarian fluid pH (Kaitaranta & Ackman, 1981; Vuorela, Kaitaranta, & Linko, 1979).

Our pH results for chinook salmon roe are lower than the 7.94 ± 0.04 reported for chinook salmon (Barnes, Sayler, & Cordes, 2003). This might be related to the sample preparation. Direct pH measurements on homogenised roes, as in the present study (Table 1) and other reports (Gagné & Adambounou, 1994; Katsiadaki, Taylor, & Smith, 1999) resulted in a lower pH range compared with other studies (Barnes et al., 2003; Fauvel et al., 1993; Lahnsteiner, Weismann, & Patzner, 1999) which measured the pH of the ovarian fluids after separation of solids by filtration. Thus Katsiadaki et al. (1999) reported a pH range between 5.68 and 6.96 for cod (*Gadus morhua*) roe while Fauvel et al. (1993) found the pH of ovarian fluids from turbot (*Scophthalmus maximus*) ranged from 7.0 to 8.2, and Lahnsteiner et al. (1999) reported that trout (*Salmo trutta lacustris*) roe pH ranged between 8 and 9. The pH variation within fish species due to maturity might be a specie-dependent phenomena since the pH of herring was not different across three maturity stages (Gagné & Adambounou, 1994).

It worth mentioning that the term "quality" and the relationship between the quality of fish eggs and the pH of the eggs differ in the context of food and biology studies. For Fauvel et al. (1993), Lahnsteiner et al. (1999), and Barnes et al. (2003) egg quality means the viability and fecundity of the eggs which probably increases with the increase in pH within a certain range depending on the fish species, whereas in the present work and in the study of Katsiadaki et al. (1999) fish egg quality refers to the potential eating and keeping quality of the eggs as food. The impact of pH on a food material is quite complex. On the one hand; lower pH lowers handling damage during processing, slows bacterial growth and provides a longer microbiological shelf-life. On the other hand; low pH makes food materials (especially material contains high level of lipids such as fish eggs) vulnerable to lipid oxidation (Thomsen, Jacobsen, & Skibsted, 2000).

3.4. Viscosity and osmolality

Mature salmon roe were more viscous ($P < 0.05$) than immature salmon roe (Table 1). The same factors that cause the decrease in pH with maturity can also contribute to the increased viscosity since differences in the types and amounts of proteins and lipids will affect the net charge and the ionisation of the molecules, and alter the overall viscosity of roe (Gagné & Adambounou, 1994). The viscosity of chum salmon eggs (*O. keta*) did not change with freezing (Craig & Powrie, 1988) and is similar to the viscosity range reported for immature and mature salmon in the present study.

The osmolality of mature salmon roe was lower ($P < 0.05$) than immature salmon roe. Osmolality seems to decrease with the maturity of salmon despite the lower moisture content of mature roe. This probably was due to the depletion of free amino acids during the maturation process as a mechanism to counteract the continuous reduction in yolk volume and water loss (Ronnestad, Groot, & Fyhn, 1993). The osmolality of immature and mature salmon in the present work were lower than the range (244.2–291.6 mmol/kg) reported for the ovarian fluid of rainbow trout (*Oncorhynchus mykiss*), charr (*Savelinus alpinus*), lake trout (*Salmo trutta lacustris*) and Danube salmon (*Hucho hucho*) (Lahnsteiner, Weismann, & Patzner, 1995; Lahnsteiner et al., 1999) which probably reflect maturity differences since within the same species maturity can cause wide variation in the moisture content of the roe (e.g. 66.3–90.1% moisture content range within four and five maturity stages) (Katsiadaki et al., 1999).

Osmolality is a measure of the number of moles of a solute per kilogram of solvent and thus it regulates the colligative properties of the material (e.g. freezing point depression; vapour pressure depression; osmotic pressure elevation) and consequently may affect further processing and handling processes (e.g. freezing and drying conditions of the material).

3.5. Chemical composition

The chemical composition of fish roe and caviar is affected by both intrinsic (species, maturity of the eggs, egg location within the skein) and extrinsic (diet, fish maturity, season, harvest area and processing conditions) factors (Bledsoe et al., 2003; Katsiadaki et al., 1999; Shirai, Higuchi, & Suzuki, 2006; Wirth et al., 2000). Bledsoe et al. (2003) compiled and presented comprehensive data on the chemical composition of fish roes that clearly demonstrate the inter- and intra-species variability. The data reported in the present study are within the range reported for chinook salmon by Bledsoe et al. (2003). Variation in the composition of eggs can be found in eggs produced by fish that grown under the same environmental conditions and fed identical diets (Peleitero, Lavens, Rodriguez-Ojea, & Iglesias, 1995) and even within eggs from the

same roe or the same female (Bledsoe et al., 2003; Kjorsvik, Mangor-Jensen, & Holmefjord, 1990). This variation in chemical composition of eggs/roes has been attributed mainly to diet and biological conditions (reviewed by Zhu (1999)).

Mature salmon roe had lower moisture content and higher protein, lipid and ash contents ($P < 0.05$) compared with immature salmon roe (Table 1). Katsiadaki et al. (1999) highlighted the importance of moisture content to the quality grade of raw cod roes and demonstrated the decrease in roe quality with the increase in the moisture content. The present study may support this contention since the nutritional value (represented by the contents of protein and lipid) is inversely related to the moisture content in mature and immature salmon roes.

3.6. Fatty acid composition

Palmitic acid (16:0) was the dominant saturated fatty acid in salmon roe (Table 2). Similarly, oleic acid (18:1ω9) was the most abundant monounstaturated fatty acid. Eicosapentaenoic acid [EPA; 20:5(ω3)] and docosahexaenoic acid [DHA; 22:6(ω3)] are the dominant polyunsaturated fatty acids. These are universal features for fish oils regardless of source; e.g. organ, product or by-product (Hughes, Czochanska, Pickston, & Hove, 1980; Bechtel et al., 2007; Wu & Bechtel, 2008), maturity (Kaitaranta & Ackman, 1981) and species (Hughes et al., 1980; Shirai, Suzuki, Tokairin, & Wada, 2001). Fish oils play an important role in providing the energy required by the eggs during development (for more information, see the excellent review by Tocher (2003)). Maturity had no effect on the percentage of the fatty acids. This is in agreement with earlier reports that demonstrated that diet rather than the maturation level affected the fatty acid composition (Kaitaranta & Ackman, 1981; and Pickova, Kiessling, Pettersson, & Dutta, 1999). The reported results demonstrate that the nutritional quality of the oil fraction from mature and immature salmon roes is not different (Table 2), although the contents per mass unit may be slightly different (Table 1).

Table 2
The effects of maturity on the fatty acid composition (%) of chinook salmon roe.

Fatty acid	Mature salmon	Immature salmon
12:0	0.02 ± 0.04	0.07 ± 0.00
14:0	1.54 ± 0.16	1.35 ± 0.0
15:0	0.35 ± 0.01	0.34 ± 0.01
16:0	11.65 ± 0.42	12.15 ± 0.23
17:0	0.28 ± 0.02	0.29 ± 0.02
18:0	4.32 ± 0.39	3.95 ± 0.17
22:0	0.17 ± 0.21	0.03 ± 0.02
24:0	ND[A]	0.01 ± 0.02
16:1 ω9	0.01 ± 0.01	0.02 ± 0.02
16:1 ω7	4.54 ± 1.05	3.20 ± 0.06
16:1 ω5	0.76 ± 0.07[b]	0.65 ± 0.01[a]
17:1 ω9	0.54 ± 0.07	0.46 ± 0.03
18:1 ω9	22.33 ± 2.03	19.68 ± 0.34
18:1 ω7	3.25 ± 0.46	2.66 ± 0.04
20:1	2.25 ± 0.19	2.51 ± 0.06
24:1(ω9)	0.14 ± 0.14	0.35 ± 0.05
18:2	3.42 ± 0.68	2.53 ± 0.02
18:3(ω3)	0.60 ± 0.08	0.50 ± 0.0
18:3(ω6)	0.08 ± 0.0	0.05 ± 0.04
18:4(ω3)	0.53 ± 0.01	0.53 ± 0.01
20:3 ω6	0.47 ± 0.11	0.34 ± 0.01
20:3 ω3	0.13 ± 0.01	0.13 ± 0.01
20:4(ω6)	2.40 ± 0.62	3.19 ± 0.05
20:5(ω3){EPA}	6.86 ± 0.23	7.15 ± 0.09
22:5(ω3){DPA}	3.85 ± 0.03	3.79 ± 0.04
22:6(ω3){DHA}	24.32 ± 3.70	29.06 ± 0.15
Others	5.06 ± 0.83	5.00 ± 0.13
SFA	18.33 ± 0.41	18.18 ± 0.39
MUFA	33.96 ± 3.34	29.55 ± 0.45
PUFA	42.65 ± 3.65	47.27 ± 0.20
SFA/PUFA	0.43 ± 0.012	0.38 ± 0.017
ω3	36.29 ± 3.82	41.16 ± 0.15
ω6	2.95 ± 0.51	3.58 ± 0.10
ω3/ω6	12.41 ± 0.76	11.50 ± 0.32

SFA = saturated fatty acids.
SMFA = monounsaturated fatty acids.
PUFA = polyunsaturated fatty acids.
[a,b]Values within raw with different superscripts are significantly different ($P < 0.05$)
[A] ND = not detected.

3.7. Mineral composition

The minerals P, S and K were the most abundant in salmon roe, followed by Na, Mg and Ca (Table 3). Small amounts of Fe, Zn, Mn, B, Cu and Al and traces of Ni, As and Co were present in salmon roe. A similar distribution of P, K, Na, Mg, Ca, Fe, Zn, Mn, Cu, Ni and As was found in immature Pollock roe (Bechtel et al., 2007). The concentrations of Ca, Mg, P, S and Co increased ($P < 0.05$) while the concentrations of Cu, Fe, Mn, Na and Zn decreased ($P < 0.05$) with the maturity of salmon roe (Table 3). The variation of the elements content between mature and immature salmon roes in the present study is in agreement with the variation range reported for cod roe at different maturation stages by Julshamn and Braekkan (1976; cited in Hellou, Warren, Payne, Blekhode, & Lobel, 1992). According to that study, the concentrations of minor (Fe, Mn, Zn, Cu, and Co) and major (K, Na, Ca and Mg) elements varied two fold within seven maturation stages of cod roe. Furthermore, the reduction of Co, Cu, Fe, Mn and Zn concentrations with the maturity of chinook salmon roe observed in the present study are similar to those found in coho salmon (Hardy, Shearer, & King, 1984).

The variation in the mineral composition with the roe maturity means that the degree of maturity of salmon roe will play an important role in determining the amount of essential mineral available in the final product and depending on the minerals of interest, either mature or immature roe may be desired as the raw material. However, it must be noted that processing may alter the mineral content as demonstrated by Bechtel et al. (2007), thus final products need to be evaluated individually.

3.8. Amino acid composition

The mean total amino acids, essential amino acids and total aromatic amino acids were about 12%, 16% and 29%, respectively,

Table 3
Effects of maturity on the concentrations of trace elements (mean ± SD, mg/kg wet weight) in chinook salmon roes.

Element	Mature salmon	Immature salmon	Limits of detection
Al	1.93 ± 0.08	1.90 ± 0.22	0.00077
As	0.15 ± 0.11	0.17 ± 0.14	0.00150
B	3.50 ± 0.10	3.22 ± 0.07	0.00122
Ca	428.3 ± 38.9[b]	288.16 ± 14.9[a]	0.00031
Co	<0.00062	0.059 ± 0.013	0.00062
Cs[c]	46.10 ± 0.65	46.17 ± 0.25	–
Cu	2.51 ± 0.42[a]	5.39 ± 0.78[b]	0.00096
Fe	36.04 ± 8.24[a]	74.83 ± 7.39[b]	0.00041
K	1698.7 ± 69.3	1811.7 ± 121.5	0.01224
Mg	584.7 ± 25.2[b]	362.4 ± 29.6[a]	0.00037
Mn	3.76 ± 0.90[a]	10.65 ± 1.51[b]	0.00012
Na	596.1 ± 40.5[a]	967.8 ± 138.3[b]	0.00155
Ni	0.17 ± 0.11	0.13 ± 0.01	0.00081
P	3373 ± 195[b]	2195 ± 359[a]	0.08478
S	2443 ± 184[b]	1647 ± 234[a]	0.00411
Y[c]	48.29 ± 0.62	48.22 ± 0.34	–
Zn	32.44 ± 4.2[a]	60.02 ± 8.13[b]	0.00015

[a,b]Values within raw with different superscripts are significantly different ($P < 0.05$)
[c] Internal standards.

Table 4
Effect of maturity on the amino acid profile in chinook salmon roes.

Amino acid (g/16 gN)	Mature salmon	Immature salmon
Ala	5.2 ± 1.00[a]	7.6 ± 0.42[b]
Arg	6.5 ± 0.99	5.6 ± 0.42
Asp	6.7 ± 1.89	7.2 ± 0.32
Cys	1.5 ± 0.08	1.5 ± 0.10
Glu	10.0 ± 0.20[b]	7.8 ± 0.36[a]
Gly	1.8 ± 0.44	2.3 ± 0.45
His	2.1 ± 0.20[a]	3.4 ± 0.60[b]
Ile	6.2 ± 1.17[b]	4.2 ± 0.21[a]
Leu	7.4 ± 0.24[b]	6.5 ± 0.49[a]
Lys	6.8 ± 0.01	6.6 ± 0.60
Met	2.8 ± 0.55	3.1 ± 0.28
Phe	5.8 ± 1.14	4.5 ± 0.31
Pro	8.7 ± 3.54	5.0 ± 0.31
Ser	5.4 ± 0.54	5.5 ± 0.31
Tau	0.2 ± 0.02[a]	0.63 ± 0.07[b]
Thr	4.9 ± 0.56	4.4 ± 0.22
Tyr	4.4 ± 0.88	3.4 ± 0.20
Val	6.4 ± 1.45[b]	3.8 ± 0.20[a]
TAA	92.8	83
EAA	42.4	36.5
TSAA	4.3	4.6
TAAA	10.2	7.9
EAA/NEAA	0.84	0.78

TAA = total amino acids.
EAA = essential amino acids.
NEAA = non essential amino acids.
TSAA = total sulphur amino acids.
TAAA = total aromatic amino acids.
[a,b]Values within raw with different superscripts are significantly different ($P < 0.05$)

higher in mature salmon compared with immature salmon roe (Table 4). The profile of dominating amino acids varied with maturity of salmon roe. In agreement with the literature, glutamic acid was the dominant amino acid in mature salmon (Bledsoe et al., 2003) followed by leucine, proline, lysine and aspartic acid. Immature salmon roes contained more or less similar amounts of glutamic acid, proline, alanine, aspartic acid, lysine, and leucine (Table 4). Alanine and taurine contents decreased ($P < 0.05$) while glutamic, isoleucine, leucine and valine were increased with the maturity of salmon roe. Earlier reports demonstrated that changes in amino acid concentrations with different maturity levels do occur without consistent trends (Vuorela et al., 1979).

The uncorrected scores of essential amino acids for mature and immature roes were higher than that required for preschoolers (FAO/WHO, 1991) apart from tryptophan, which was not determined. Even at 89% digestibility level (Folador et al., 2006), all the reported essential amino acids will provide 100% of the required essential amino with the exception of leucine in immature roe. Mature salmon roe had slightly higher ratio of essential amino acids to non-essential amino acids (EAA/NEAA) compared to immature salmon roe (Table 4). Therefore, the protein quality of mature salmon roe is better than immature salmon roe. Bledsoe et al. (2003) compiled the amino acid profiles for the roes of 21 aquatic animal species. The protein quality as indicated by EAA/NEAA ration was the highest for squid's roe (0.93) and was lowest for sea urchin roe (0.65). For fin fish, mullet's roe had the lowest EAA/NEAA ratio (0.67) and the roe of chum salmon had the highest ratio (0.82). The EAA/NEAA ratios for immature (0.78) and mature salmon (0.84) in the present study are similar to those reported for immature Pollock (0.77) and chum salmon (0.82), respectively (Bechtel et al., 2007; Bledsoe et al., 2003).

Immature salmon roe contained 0.63 g/16gN of taurine which decreased to 0.2 g/16 gN with maturity (Table 4). This is similar to the level of taurine in fresh lumpfish eggs reported by Basby, Jeppesen, and Huss (1998) of 0.6 ± 0.05 g/100 g protein.

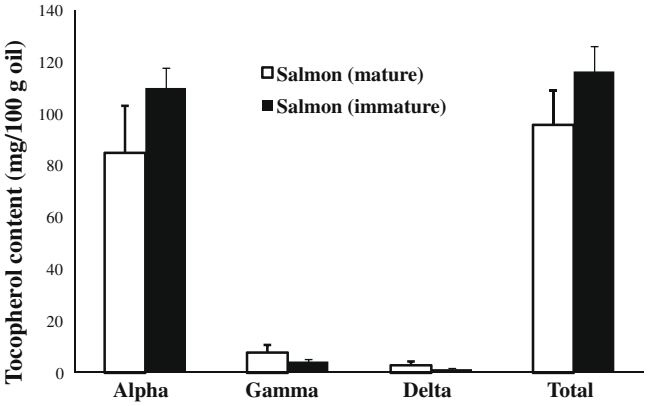

Fig. 1. Effect of maturity on the concentrations of tocopherols (α, γ, δ and total) in chinook salmon roes. The error bars represent the standard deviation of the means.

3.9. Tocopherols

The concentration of α-tocopherol numerically decreased whereas γ-tocopherol and δ-tocopherol increased with the maturity of salmon roe (Fig. 1). Overall, the total concentrations of the three tocopherols were higher in immature roe salmon (116.39 ± 9.56 mg/100 g oil) compared with mature roe salmon (96.04 ± 13.11 mg/100 g oil). The average α-tocopherol content in salmon roe (85 and 110 mg/100 g oil for mature and immature roes, respectively) is in agreement with the trend reported by Cowey, Bell, Knox, Fraser, and Youngson (1985) for *S. salar* where α-tocopherol concentration was decreased during the egg development period. The level of α-tocopherol in salmon roe is higher than that reported for vendace roe (61 mg/100 g oil) but less than that reported for Baltic herring and white fish roes (137 and 154 mg/100 g oil, respectively) (Syväoja et al., 1985).

Vitamin E (Vit E) plays a vital biochemical role in fish in addition to its function as an antioxidant (Mourente, Bell, & Tocher, 2007) and it is only obtained through diet as fish cannot synthesise Vit E. The different levels of tocopherols (γ and δ) between mature and immature salmon roes may be explained by the physical and biochemical activities of the fish during the spawning season. For example, the relative distribution of Vit E in different organs of Atlantic salmon was constant but the concentrations deposited in the organs were influenced by the dietary intake of Vit E (Hamre & Lie, 1995). Thus the amount of Vit E in fish organs will be mainly dependent on the dietary intake which may vary widely depending on environmental and physiological factors such as starving during spawning (for review see Palace & Werner, 2006). Also, the pathways regulating the transport of α, γ and δ tocopherols to different organs in Atlantic salmon may have different mechanisms (Hamre & Lie, 1997) leading to different levels of tocopherols in different organs. Interest in Vit E content in fish roe is not new with some reports on the subject are dated as early as 1949 indicating the concentration in fresh cod roe to be in the range of 5.25–7.6 mg Vit E/100 g fresh cod roe (equivalent to 350–500 mg Vit E/100 g oil based on the reported 1.5% lipid content) (Keringstad & Folkvord, 1949). However, Vit E in fish roe has not been reported widely in literature. The α-tocopherol concentration in the salmon roe in the present study is much higher than those reported for fish muscles where α-tocopherol content in the muscle of steelhead trout, rainbow trout and Atlantic salmon were 13, 19 and 18.1 mg/100 g oil, respectively (Lopéz, Satué, González, & Agramont, 1995; Zhong, Madhujith, Mahfouz, & Shahidi, 2007). This is consistent with the higher α-tocopherol contents in roe compared with the muscles in vendace, Baltic herring and white fish reported by Syväoja et al. (1985).

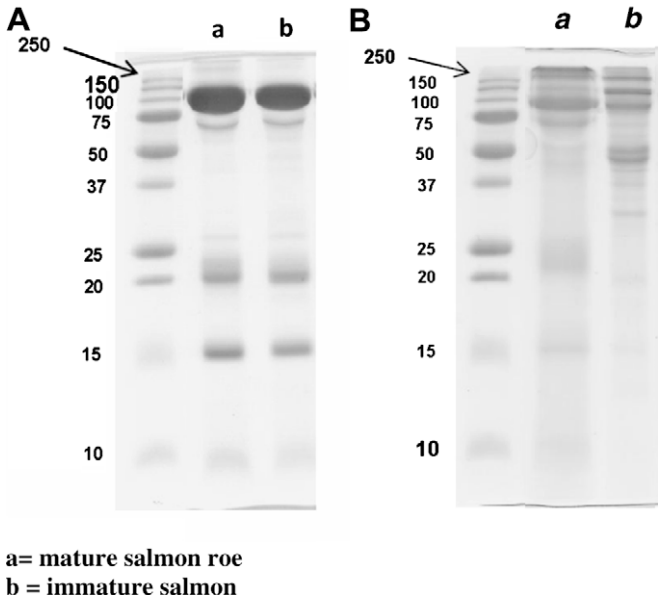

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) profiles of soluble proteins fraction (A) and membrane-bound proteins fraction (B).

3.10. Protein profiles in mature and immature fish roes

The major bands in the soluble protein fraction in mature and immature salmon roes were identical with the major band at about 100 kDa which probably reflect a vitellin-like protein that was found at 96 kDa in the roe of *S. salar* (Scobbie & Mackie, 1990) and *O. keta* (Al-Holy & Rasco, 2006). Smaller discreet bands were present at about 67–68, 20 and 15–16 kDa (Fig. 2A). The 67–68 and 15–16 kDa bands were reported in the roes of several species including *S. salar* (Lahnsteiner, 2007; Scobbie & Mackie, 1990). The 20 kDa band seems to be not available in *S. salar* (Scobbie & Mackie, 1990) and cannot be confirmed for *O. keta* (Al-Holy & Rasco, 2006). Trace band about 9 kDa was equally present in mature and immature salmon roes. Membrane-bound proteins had different bands pattern in mature and immature salmon roes (Fig. 2B). A common band was present at 80–90 kDa. Two bands above 250 kDa were available in the membrane-bound protein fraction of mature roe where the lower molecular band of the two was present in the immature roe. This likely to represent the collagen which have molecular of 360 kDa (Al-Holy & Rasco, 2006). Several discreet bands at about 130, 45–50, 37 and 30 kDa were present only in the immature salmon roe.

The membrane-bound proteins reflect the structural proteins which are related to the degree of roe maturity (Fig. 2). While the classification of the different proteins into lipo-, glycol- and phosphoproteins was reported for fish roes (Lahnsteiner, 2007), the present proteins bands cannot be confirmed with blue staining. However, the variation in the bands in terms of quantity and patterns of different proteins means that the nutritional impact from the consumption as well as the texture of the individual grains from mature and immature will be different.

In conclusion, the maturity variation in the physicochemical characteristics of roe from salmon might indicate that different functional and physical properties during processing and consumption will arise as a result of the maturity of the roe. Also, the maturity of salmon eggs will influence the nutritional impact of consuming the raw roes as well as any products may be produced from salmon roe.

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Food Chemistry 117 (2009) 326–331

Contents lists available at ScienceDirect

Food Chemistry

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Free radical scavenging and antibacterial activities of southern Serbian red wines

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ARTICLE INFO

Article history:
Received 11 January 2009
Received in revised form 20 February 2009
Accepted 2 April 2009

Keywords:
Red wines
Antibacterial activity
Free radical scavenging activity
Polyphenols
Anthocyanins

ABSTRACT

Free radical scavenging and antibacterial activities were determined for several red wines from different grape varieties from the southern Serbia wine region. The amount of polyphenols and anthocyanins in selected wines was investigated and the potential antibacterial activity of the wines against pathogenic strains of *Staphylococcus aureus* and *Escherichia coli* was also determined. The free radical scavenging and antibacterial activity of the wines was correlated with polyphenolic and anthocyanin content. The significant differences in the antioxidant and antibacterial activities between the wines from grape cultivars (*Cabernet Sauvingon* and *Pinot Noir*) and the indigenous varieties (*Vranac* and *Prokupac*) were not confirmed. The hypothesis of a protective effect of red wine is supported by our results that show that the red wines with higher amounts of polyphenols and anthocyanins had higher antioxidant and antibacterial properties.

1. Introduction

Recent studies indicate that consumption of a small amount of red wine on a regular basis reduces the risk of coronary heart disease and atherosclerosis, and this benefit is attributed to the antioxidant properties of the polyphenolic compounds (Gey, 1990; Renaud & de Lorgeril, 1992; Youdim, McDonald, Kalt, & Joseph, 2002). Several studies suggest that some phenolic compounds, when ingested at high concentrations, may exhibit certain roles in carcinogenicity, genotoxicity, thyroid toxicity and can be connected with oestrogenic activity (Lila, 2004; Meiers et al., 2001). Phenolic compounds represent one of the most important quality parameters of grapes and wines since they contribute to organoleptic characteristics such as colour, astringency and bitterness.

The phenolic composition of wine is determined initially by the phenolic composition of the grapes used for making the wine (Ribéreau-Gayon, Dubourdieu, Dončche, & Lonvaud, 1998). Exposure to sunlight and temperature are the main factors influencing the phenolic composition of grapes. These factors depend of the climate in the area, but can be modified by controlling the canopy microclimate. The other factor that influences wine phenolic composition is the extraction of phenolic compounds from the grape skins and seeds during maceration. Finally, the higher the concentration of polyphenols in wines, the higher their antioxidant capacity and physiological effects (De Beer, Joubert, Gelderblom, & Manley, 2003, 2005). Some studies suggest that wine possesses antimicrobial activity against various pathogens (Daglia et al., 2007; Daroch et al., 2001; Papadopoulou, Soulti, & Roussis, 2005; Sugita-Konishi, Hara-Kudo, Iwamoto, & Kondo, 2001; Weisse, Eberly, & Person, 1995). Phenols have an important role in the stabilization of lipid oxidation, antihypertensive and antithrombic effects, and reduce carcinostatic properties (Jang et al., 1997). Studies *in vitro* have shown that the viable count of *Enterobacteriaceae* is more rapidly reduced in wine compared to other alcohol-containing beverages (Just & Daeschel, 2003). The mechanism by which anthocyanins are absorbed and metabolised in the body is currently unclear (Munoz-Espada, Wood, Bordelon, & Watkins, 2004). The role of anthocyanins in protection from cardiovascular disease is strongly linked to oxidative stress protection (Meiers et al., 2001). Anthocyanins were found to be highly bioavailable in endothelial cells and have been correlated to their role in prevention of atherosclerosis and neurodegenerative disorders (Youdim et al., 2002). In both *in vitro* and *in vivo* research trials, anthocyanins have been shown to reduce the proliferation of cancer cells and to inhibit tumor formation (Hou, 2003). Recently, it has been reported that the anthocyanin pigments may play an important function in the prevention of carcinogenesis and in extending the life span of animals. It was also shown that dietary antioxidants may offer effective protection from peroxidation damage in living systems (Lila, 2004; Tsuda, Horio, & Osawa, 2000).

Wine consists of different phenolic compounds, so the antioxidant and the antibacterial activity of different wines are related to these compounds. Still, it is very important to determine which group of phenolic compounds is most influential in antioxidant and antibacterial properties of wine.

The south of Serbia has a longstanding tradition of viticulture and winemaking since the region's dominant soil types are very

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0308-8146/$ - see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2009.04.008

favourable for the cultivation of wines. The aim of this study was to determine the free radical scavenging and antibacterial activities of wines produced in southern Serbia vineyards and to correlate these with total polyphenolic content and the amount of anthocyanins.

2. Materials and methods

2.1. Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH·) in free radical form was obtained from Sigma Chemical Co. (St. Louis, MO). Folin–Ciocalteu reagent, gallic acid and methanol were purchased from Merck Co. (Germany). All reagents were analytical grade.

2.2. Bacterial cultures

The following microorganisms were used for the antibacterial tests: *Staphylococcus aureus* (strain ATCC 28053 gram-positive) and *Escherichia coli* (strain ATCC 25922 gram-negative). The bacteria were grown on Mueller–Hinton dextrose agar.

2.3. Wine samples

The wines were produced from different grape varieties grown in southwest Serbia. Six red wines were supplied by Rubin – Kruševac: Terra Lazarica – Cabernet Sauvingon (2006); Terra Lazarica – Pinot Noir (2006); Vranac (2007); Rubinovo Crno (2007); Medvedja krv (2007) and Car Lazar (2006).

2.4. Determination of total phenolic content

The total phenol content in selected wine samples was determined spectrophotometrically according to the Folin–Ciocalteu colorimetric method (Singleton & Rossi, 1965) using gallic acid as a standard polyphenol: 0.25 ml of wine was mixed with 12.5 ml distilled water and 1.25 ml of Folin–Ciocalteu reagent. After 3 min, 5 ml of 20% Na_2CO_3 was added. The absorbance was measured after 30 min at 750 nm. The concentration of the total phenolic compounds in the wines was expressed as gallic acid equivalents (mg/l). The results in every assay were obtained from three parallel determinations.

2.5. Determination of monomeric anthocyanins

The total monomeric anthocyanin content in the wine samples was determined using the pH-differential method described by Guisti and Wrolstad (2003). Anthocyanins have a maximum absorbance at a wavelength of 520 nm at a pH of 1.0. The coloured oxonium form predominates at pH 1.0 and the colourless hemiketal form at pH 4.5. The pH-differential method is based on this reaction and permits accurate and rapid measurement of the total monomeric anthocyanins. The result, considered as the monomeric anthocyanin pigment, was calculated as mg of cyanidin-3-glucoside, by using a molar absorptivity (ε) of 26,900 and a molecular weight of 449.2. Data presented are the average of three measurements.

2.6. Determination of indices for anthocyanin pigment degradation, polymeric colour, and browning

Indices for anthocyanin degradation in wine can be derived using the pH-differential method described by Guisti and Wrolstad (2003). The absorbance at 420 nm of the bisulfite treated sample serves as an index for browning. Colour density is defined as the sum of absorbances at the maximum absorbance and at 420 nm. The ratio between polymerisation colour and colour density is used to determine the percentage of the colour that contributed by polymerised material. The ratio between the amount of monomeric and total anthocyanins can be used to determine a degradation index. The date presented are the average of three measurements.

2.7. Free radical scavenging activity

The free radical scavenging activity of the wine samples was analysed by using the 2,2,-diphenyl-1-picrylhydrazyl (DPPH·) assay (Fuhrman, Volkova, Suraski, & Aviram, 2001; Lachman, Sulc, & Schilla, 2007; Sanchez-Moreno, Larrauri, & Saura-Calixto, 1999; Turkoglu, Duru, Mecan, Kivrak, & Gezer, 2007; Villano, Fernandez-Pachon, Troncoso, & Garcia-Parrilla, 2006). The antioxidant assay is based on the measurement of the loss of DPPH· colour by the change of absorbance at 517 nm caused by the reaction of DPPH· with the tested sample. The reaction was monitored by a UV/vis spectrophotometer. Wine diluted with water (1:25 v/v) (5 ml) and 5 ml of freshly prepared DPPH· in methanol 1×10^{-4} M were put into a cuvette at room temperature. After 20 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. The determinations were performed in triplicate. Inhibition of DPPH· in percent (I %) of each wine sample was calculated from the decrease of absorbance according to the relationship:

$$I\% = \left(A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}\right) \times 100$$

where A_{blank} is the absorbance of control reaction (methanol–water with DPPH·) and A_{sample} is the absorbance of the tested wine sample.

2.8. Antibacterial activity

The disc diffusion method was used for the determination of the antibacterial activity of the wine samples (Bauer, Kirby, Sherris, & Truck, 1966; Papadopoulou et al., 2005; Turkoglu et al., 2007). The microorganisms mentioned above were incubated at 37 °C for 24 h by inoculation into Mueller–Hinton broth. The number of cells in 1 ml of suspension for inoculation measured by the McFarland nephelometer was 1×10^7 cfu/ml. A volume of 1 ml of this suspension was homogenized with 9 ml of melted Mueller–Hillton agar and poured into petri dishes. For screening, sterile 4 mm discs were impregnated with 50 μl of wine sample. After incubation for 24 h at 37 °C, inhibition zone diameters, ZI (including disc), were measured and expressed in mm. The magnitude of the inhibition zone indicates the activity of the tested wines against bacteria. The tests were done in triplicate.

2.9. Statistical analysis

All the measurements were carried out in triplicate, and presented as mean ± SEM or standard deviations (*SD*). The direction and magnitude of the correlation between the variables was quantified by the correlation factor r^2. Correlations were considered statistically significant, if the p-value was less than 0.05.

3. Results and discussion

3.1. The content of polyphenols and anthocyanins in wine samples

The phenolic composition in wines is determined by several factors, such as the variety of the grapes and the conditions under which they were grown (soil, location, and weather). Winemaking techniques also play a very important role in the extraction of polyphenols from the grape: e.g. the time of maceration and fermentation in contact with the grape skins and seeds, pressure,

maturation and ageing conditions (Sato et al., 1996, Villano et al., 2006).

Serbia, with 82,000 ha of vine area and around 2 million hl of annual wine production, is one of the middle-weight wine producers in Europe. Many of the Serbian growing districts boast remarkably good ecological conditions for the production of high-quality wines and quality wines with geographically controlled designations of origin. Vineyards of southern Serbia are focused on the old autochthonous vine varieties: *Vranac* and *Prokupac*. *Vranac* originated in Balkan and is the most common type in Serbia and Macedonia. The grapes are ripened in mid-September, when their skins are a dark-blue colour. The juice turns the colour of blood during the first minutes of breaking the grapes. They have a harmonious taste, with an excellent aroma. The skin of *Prokupac* (*Nishevka*) is a dark-blue colour, with characteristic dots. The juice is light red, with a sugar level of 22–24% and acidity of 6–8 g/l. The wine is very suitable for coupling with other red wines. These indigenous varieties are often compared to the French vine varieties *Cabernet Sauvingon, Pinot Noir* and *Gamay*.

Some relevant analytical parameters of the wines produced by the Rubin – Kruševac winery, are given in Table 1. It is obvious that this wine differs in many analytical parameters such as content of alcohol, total extract, total and free SO_2, reducing sugar, total acidity (as tartaric acid), volatile acidity (as acetic acid) and specific weight.

The phenolic content of selected wines, obtained using the Folin–Ciocalteu reagent, is presented in Table 2.

The mean concentration of the phenolic content of the Serbian wines was 3442 mg of gallic acid equivalent. This is higher than the levels quoted in the literature for wines grown in different countries (see Table 3).

The differences between the Serbian wines and the other published results may be a result of the higher levels of phenolic compounds in the Serbian grape varieties. The numerous viticultural factors could affect the grape phenolic content. The phenolic composition has an important role in stabilizing lipid oxidation and is associated with antioxidant activity. The red wines (Medvedja krv, Rubinovo Crno and Vranac) coming from indigenous grape varieties *Vranac* and *Prokupac* have a mean phenolic content of 3606 versus 3603 mg/l for red wines from *Cabernet Sauvingon* and *Pinot Noir* varieties (Terra Lazarica – Cabernet Sauvingon and Terra Lazarica – Pinot Noir).

Table 1
Some relevant analytical parameters in the wines.

Wine[a]	Specific weight	Alcohol content (vol %)	Total extract (g/l)	Free SO_2 (mg/l)	Total SO_2 (mg/l)	Reducing sugar (g/l)	Total acidity (tartaric acid) (g/l)	Volatile acidity (acetic acid) (g/l)
Terra Lazarica – Cabernet S.	0.9946	12.0	28.1	28.2	98.6	2.2	5.4	0.7
Vranac	0.9954	11.5	30.1	25.6	93.4	–	5.5	0.7
Rubinovo Crno	0.9946	11.0	23.3	28.2	87.0	–	5.4	0.5
Medvedja krv	1.0029	11.5	47.0	32.0	99.8	21.0	5.5	0.6
Terra Lazarica – Pinot Noir	0.9948	11.5	25.9	29.4	83.2	–	5.5	0.6
Car Lazar	0.9969	12.0	33.0	32.7	76.8	8.1	5.5	0.6

[a] Analysis from Rubin – Kruševac.

Table 2
The phenolic content, anthocyanin content and antioxidant activity of red wines[a].

Wine and vintage	Grape verities	Polyphenols (mg GAE/l)	Monomeric anthocyanins (mg/l)	Polymeric colour (%)	DPPH[a] (%I)[b]
Terra Lazarica – Cabernet Sauvingon (2006)	*Cabernet Sauvingon*	3805.0 ± 1.4	369.9 ± 0.9	51.3 ± 0.7	95.6 ± 0.9
Vranac (2007)	*Vranac*	3505.5 ± 1.3	310.5 ± 1.0	48.2 ± 0.7	90.7 ± 0.3
Rubinovo Crno (2007)	Vranac Prokupac Burgundac B.	3664.6 ± 1.5	336.0 ± 1.5	49.3 ± 0.9	93.4 ± 0.3
Medvedja krv (2007)	*Vranac Prokupac*	3648.5 ± 1.2	330.0 ± 0.8	46.8 ± 1.2	92.6 ± 0.4
Terra Lazarica – Pinot Noir (2006)	*Burgundac B.*	3401.5 ± 1.5	298.0 ± 1.4	55.3 ± 0.8	89.0 ± 0.2
Car Lazar (2006)	*Vranac Prokupac Burgundac B. Gamay*	2625.2 ± 1.8	147.0 ± 1.2	42.2 ± 0.5	70.9 ± 0.1

[a] Data are the means ± standard deviation values ($n = 3$).
[b] I %, the inhibition determined by DPPH radical scavenging method (wines were diluted with water 1:25, v/v).

Table 3
Published values of phenolic content in different red wines.

Country	Total phenolics content (mg GAE/l)	Reference
Croatia	2809–3183	Katalinic, Milos, Modun, Music, and Boban (2004)
Croatia	4576–4989	Piljac, Martiney, Valek, Stipcevic, and Ganic (2005)
Czech Republic	874–2262	Stratil, Kuban, and Fojtova (2008)
France	1018–3545	Landroult, Poucheret, Ravel, Gasc, and Cros (2001)
Greece	1217–3722	Arnous, Markis, and Kefals (2002)
Greece	1710–2825	Roussis et al. (2005)
Italy	3314–4177	Minussi et al. (2003)
Japan	1810–2151	Sato et al. (1996)
Poland	2200–3200	Tarko, Duda-Chodan, Sraka, Satora, and Jurasz (2008)
Spain	1010–2446	Sanchez-Moreno et al. (1999)
Spain	1738–3292	Villano et al. (2006)
Slovenia	1637–2717	Košmerl and Cigić (2008)

Anthocyanin composition is an important quality parameter of the red grapes since the anthocyanins influence the final colour of the resulting wines (Munoz-Espada et al., 2004). Anthocyanins have several different ionic forms in the wine based on wine pH value. At typical wine pH, between 10% and 15% of the present monomeric anthocyanins are pigments. Anthocyanin pigments undergo reversible structural transformation with a change in pH manifested by strikingly different absorbance spectra. At a pH 1.0, anthocyanins are represented by the red coloured oxonium form (the flavilium cation), however, at pH 4.5 deprotonation results in a colourless carbinol pseudobase structure (hemiketal) (Guisti & Wrolstad, 2003). The anthocyanin content is obtained by the different values of the absorbance at these two pH values, and relies on the structural transformations of the anthocyanin chromophore as a function of pH, represented in Table 2.

The mean amount of monomeric anthocyanin content of the investigated wines was 299 mg/l. The highest concentration of the anthocyanin pigment has been found in the young wine. The concentrations of the anthocyanins in the local wines Rubinovo Crno, Medvedja krv and Vranac were 336, 330 and 311 mg/l, respectively. These anthocyanin are essential for the good colour of these wines produced from the *Vranac* and *Prokupac* grapes.

As expected, the significant linear correlation (Fig. 1) was confirmed between total phenols content and the amount of the monomeric anthocyanins ($r^2 = 0.9960$, $SD = 5.6$, $p < 0.0001$, $n = 6$).

Anthocyanin extraction and stability are affected by winery production practices. Monomeric anthocyanins are subject to hydrolysis, oxidation, and polymerisation in wines. Their concentration usually decreases during the fermentation and maceration, but that process may continue throughout the life of a wine. When coloured forms of anthocyanins are combined with grape or wine polymers, they become the significant contributor to the colour expression and the colour stability. These polymeric forms are less changed by the pH value and SO_2 presence than monomeric anthocyanin forms and do not usually decrease with wine age. With ageing, a greater proportion of the wine anthocyanins becomes polymerised.

Polymeric colour was 42.2–55.3%, depending on the age of the wine and the variety of grapes used.

3.2. The correlation between the antioxidant and antibacterial activities and phenolic composition of the wine samples

The antioxidant activity of the wine samples was estimated by the ability of the sample to scavenge the stable DPPH· free radicals (Roussis, Lambropoulos, & Soulti, 2005; Yen & chen, 1995). Wine is a complex mixture of polyphenols and the antioxidative capacity can be expressed as the amount of wine needed to quench a certain amount of DPPH·. The decrease in the absorbance at 517 nm is taken as a measure of the extent of radical-scavenging. All wine samples showed a higher DPPH· radical scavenging activity after incubation with a free radical solution.

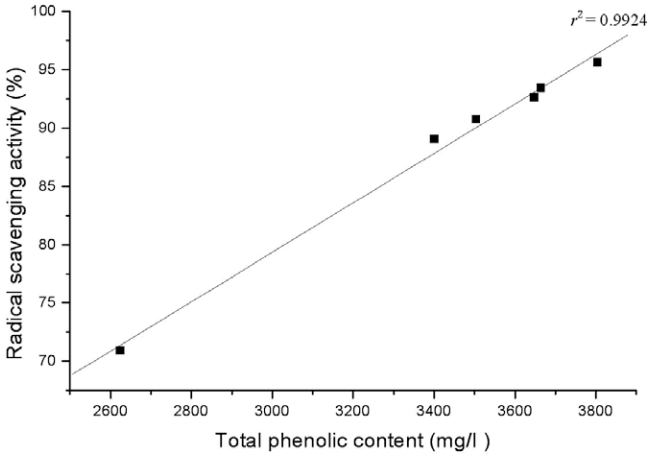

Fig. 2. Relationship between radical scavenging activity and total phenolic content of selected wine samples.

The high phenolic content in the investigated wines contributes to their increased antioxidant activity (Table 2). The investigated wines showed antioxidant behaviour in the range from 95.6% to 70%. Since structural features of phenolic compounds are responsible for the antioxidant activity, the antioxidant activities of the tested wines may be related to their total phenols content.

The percentage of DPPH· radical scavenging activity was plotted against total phenolic content of the wine samples in Fig. 2. For the investigated wines, the correlation between antioxidant activity and the total phenolic content was very good ($r^2 = 0.9924$, $SD = 0.9$, $p < 0.0001$, $n = 6$).

Also, the significant correlation between the anthocyanin content, and the DPPH· scavenging ability ($r^2 = 0.9936$, $SD = 0.8$, $p < 0.0001$, $n = 6$) of the tested wines was confirmed (Fig. 3).

Polyphenols have become a subject of much anti-infective research, due to the antibacterial, antifungal and antiviral activities (Daglia et al., 2007; Just & Daeschel, 2003; Papadopoulou et al.,

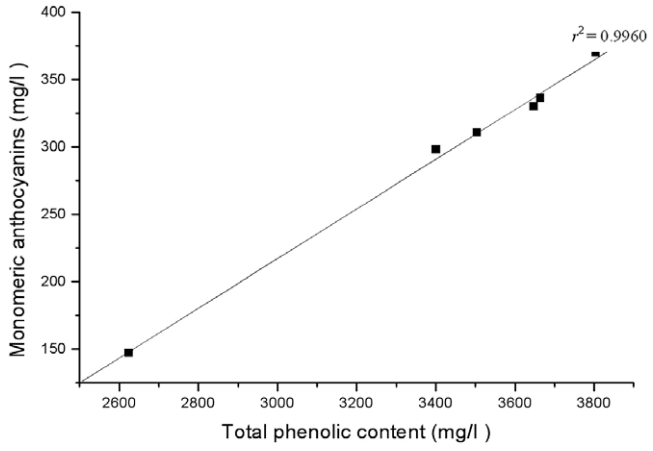

Fig. 1. Relationship between total phenolic content and monomeric anthocyanins of selected wine samples.

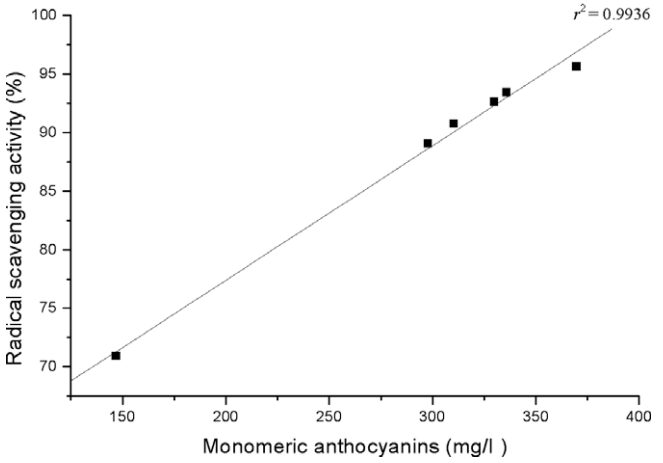

Fig. 3. Relationship between radical scavenging activity and monomeric anthocyanins of selected wine samples

Table 4
Antibacterial activities of red wines.

Wine[a]	*Staphylococcus aureus* ZI (mm)[b]	*Escherichia coli* ZI (mm)[b]
Terra Lazarica – Cabernet Sauvingon (2006)	22.0 ± 1.0	20.0 ± 0.5
Vranac (2007)	19.0 ± 0.5	18.0 ± 0.8
Rubin Crno (2007)	21.0 ± 1.2	18.5.0 ± 0.3
Medvedja krv (2007)	20.0 ± 0.5	19 ± 0.4
Terra Lazarica – Pinot Noir (2006)	17.8 ± 1.3	15 ± 1.2
Car Lazar (2006)	16.0 ± 1.2	12 ± 0.7
Penicillin (10 IU/disc)	30.3 ± 2.2	10 ± 0.0
Amoxicillin (25 μl/disc)	27.3 ± 1.2	21.3 ± 1.0

[a] 50 μl/disc.
[b] The value shown for the diameter of the inhibition zone are the means ± standard deviation values ($n = 3$).

2005). They can have an effect on the growth and metabolism of bacteria, activating or inhibiting microbial growth according to their constitution and concentration (NCCL (National Committee for Clinical Laboratory Standard), 1999). We have analysed the antibacterial activity of selected wine samples against *S. aureus* and *E. coli* strains. The diameter of inhibition zone (mm) obtained from the wine samples, reported in Table 4, shows that antibacterial activity against *S. Aureus* strains was in range from 16.0 to 22.0 mm, and against *E. coli* strains was in range from 12.0 to 20.0 mm.

It is clear, that the wines have different activities and that the different antibacterial activity is dependent on the grape varieties of tested wines.

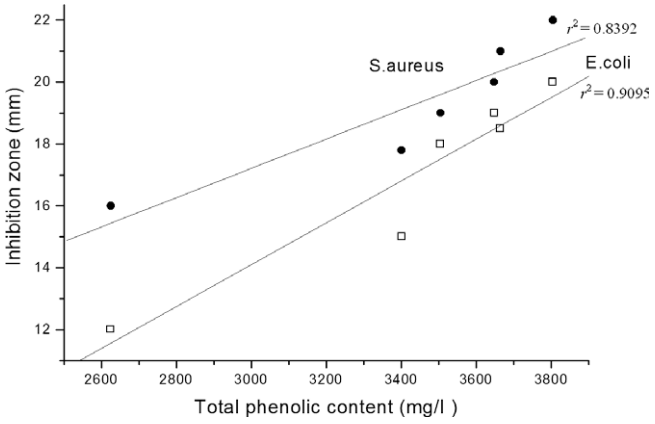

Fig. 4. Relationship between antibacterial activity against *S. aureus* and *E. coli* bacteria and total phenolic content ns of selected wine samples.

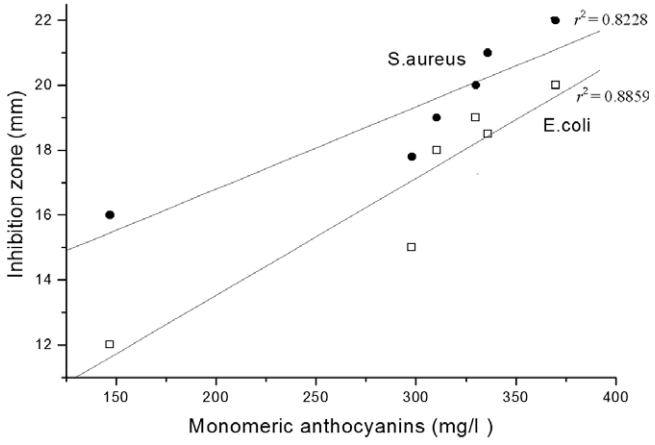

Fig. 5. Relationship between antibacterial activity against *S. aureus* and *E. coli* bacteria and monomeric anthocyanins of selected wine samples.

In all wines, the diameter of the inhibition zone for *S. aureus* was greater than the zone for *E. coli* strain, indicating that the gram-positive strain was more sensitive than the gram-negative one.

These data were used to derive a relationship between polyphenol content, anthocyanin content and antibacterial effect. The significant linear correlation was confirmed between total phenolic content (Fig. 4) and antibacterial activity against *S. Aureus* ($r^2 = 0.8392$, $SD = 190$, $p < 0.010$, $n = 6$) and against *E. coli* strains ($r = 0.9095$, $SD = 142$, $p < 0.003$, $n = 6$) (see Fig. 5).

There also was a significant linear correlation, between monomeric anthocyanins and antibacterial activity against *S. Aureus* ($r^2 = 0.8228$, $SD = 36.8$, $p < 0.012$, $n = 6$) and *E. coli* strains ($r^2 = 0.8859$, $SD = 29.5$, $p < 0.005$, $n = 6$).

It is important to determine which group of phenolic compounds is most significant in antioxidant and antibacterial activities of wines. Our results suggest that the amount of anthocyanins is very important for understanding of antioxidant and antibacterial potency of tested wines.

4. Conclusions

According to the obtained results, the free radical scavenging ability of Serbian wines is strongly correlated with phenolic and anthocyanin content. High amounts of polyphenols and anthocyanins and significant antioxidant activity were observed in the red wines produced from autochthonous vine varieties from the southern Serbia regions. Our results also show the significant correlation between the amounts of polyphenols and anthocyanins in Serbian wines with the antibacterial activity against *S. aureus* and *E. coli strains*. It can be concluded that Serbian red wines, produced from indigenous varieties *Prokupac* and *Vranac*, may serve as a very good source of potential antioxidant and antibacterial agents.

Acknowledgements

The research was supported by the Europen Union, FP7 – REGPOT – 2007–3-01, KBBE: Food, Agriculture, and Biotechnology, Project «CHROMLAB-ANTIOXIDANT», No. 204756. We thank B. Simić (“Rubin” – Kruševac) for analysis of analytical parameters of the wines.

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Food Chemistry 117 (2009) 332–341

Contents lists available at ScienceDirect

Food Chemistry

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Antioxidant and tyrosinase inhibitory activity of mango seed kernel by product

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ARTICLE INFO

Article history:
Received 9 July 2008
Received in revised form 9 March 2009
Accepted 6 April 2009

Keywords:
Antioxidant
Tyrosinase
Inhibition
Mango
Seed

ABSTRACT

The antioxidant and tyrosinase inhibitory properties of extracts of mango seed kernel (*Mangifera indica* L.), which is normally discarded when the fruit is processed, were studied. Extracts contained phenolic components by a high antioxidant activity, which was assessed in homogeneous solution by the 2,2-diphenyl-1-picrylhydrazyl radical and 2,2′-azinobis (3-ethylbenzothialozinesulfonic acid) radical cation-scavenging assays and in an emulsion with the ferric thiocyanate test. The extracts also possessed tyrosinase inhibitory activity. Drying conditions and extraction solvent were varied, and optimum conditions for preparation of mango seed kernel extract were found to be sun-drying with ethanol extraction at room temperature. Refluxing in acidified ethanol gave an increase in yield and the obtained extract had the highest content of total phenolics, and also was the most effective antioxidant with the highest radical-scavenging, metal-chelating and tyrosinase inhibitory activity. The extracts did not cause acute irritation of rabbit skins. Our study for the first time reveals the high total phenol content, radical-scavenging, metal-chelating and tyrosinase inhibitory activities of the extract from mango seed kernel. This extract may be suitable for use in food, cosmetic, nutraceutical and pharmaceutical applications.

1. Introduction

The importance of natural antioxidants for cosmetic and food applications has been underlined by numerous works, as reported by Spigno and De Faveri (2007). The limitations of using a plant extract as a natural antioxidant are availability and cost. Conventionally, plants are harvested in a particular season, and hence the costs of plant sources vary, depending on the season. Moreover, synthetic antioxidants, such as butylated hydroxy anisole and butylated hydroxy toluene, are cheaper than are natural ones. Solid agri-food waste materials cause serious environmental problems, such as water pollution, unpleasant odours, explosions and combustion, asphyxiation, vegetation damage and greenhouse gas emissions (Zamorano, Pérez, Pavés, & Ridao, 2007). Consequently, there is considerable emphasis on the recovery, recycling and upgrading of agricultural wastes as a low-cost source of antioxidants. Many investigations on the reuse of agricultural wastes have been aimed at converting the waste materials into food ingredients, bio-fuels, and other value-added applications. Some agricultural wastes, such as grape seed and olive waste extracts, have been applied successfully for industrial scale isolation of natural antioxidants from the large quantities of plant residues (Peschel, Dieckmann, Sonnenschein, & Plescher, 2007). Solid wastes, generated from the processing of fruits and vegetables, are favourable raw materials for obtaining extracts rich in phenolic compounds with good antioxidant properties because of their low cost and the possibility of reducing environmental problems caused by waste disposal. In addition, the external fractions of vegetables (such as skins, peels, hulls, or seeds) are generally discarded, although they often have a greater concentration of phenolic compounds, potentially useful as antioxidants, than have their corresponding inner fractions (Soong & Barlow, 2004).

Mango (*Mangifera indica* L.), which belongs to the family Anacardiaceae, is the most cultivated fruit in Thailand. Processed mango products are among the major goods exported from Thailand. Therefore, several million tons of mango seed wastes are produced annually from factories. There are several varieties of mango grown in Thailand; the better known cultivars are Nam-Dok-Mai, Kaew and Chok-Anan. The Chok-Anan cultivar is popular for processing in factories and is available in all seasons. A preliminary study showed that the seed represents from 20% to 60% of the whole fruit weight, depending on the mango variety (Kaew, Nam-Dok-Mai, Khiew-Sawoey, Pimsaen, Chok-Anan, Rad, Phalun, Hua Chang, Mun Duan Kao, Ok-Rong and Maha-Chanok) and the kernel inside the seed represents from 45% to 75% of the whole seed. The Chok-Anan cultivar showed the highest seed weight of all the varieties studied.

Several studies have shown that mango seed kernels contain various phenolic compounds and can be a good source of natural antioxidants (Abdalla, Darwish, Ayad, & El-Hamahmy, 2007; Puravankara, Bohgra, & Sharma, 2000). Gallotannins and condensed

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doi:10.1016/j.foodchem.2009.04.010

tannin-related polyphenols were reported to be present in mango kernels (Arogba, 1997). In addition, polyphenols from dry mango kernel meal were found to contain tannic acid, gallic acid, and epicatechin in the ratio 17:10:1, respectively (Arogba, 2000). Abdalla et al. (2007) have recently characterized the phenolic compounds in Egyptian mango seed kernels. The components included tannins, gallic acid, coumarin, ellagic acid, vanillin, mangiferin, ferulic acid, cinammic acid and unknown compounds.

Some phenolic compounds, such as ellagic acid, tannic acid and quercetin, act as potent tyrosinase inhibitors (Rompel et al., 1999; Shimogaki, Tanaka, Tamai, & Masuda, 2000). Tyrosinase is responsible for enzymatic browning in plants, and it may cause undesirable changes in colour, flavour and nutritive value of plant-derived foods and beverages (Kubo et al., 2000). This enzyme is a copper-containing enzyme which is mainly involved in melanin biosynthesis. Tyrosinase catalyses melanin biosynthesis in human skin and epidermal hyperpigmentation may cause various dermatological disorders, such as melasma, freckles and age spots (Karioti, Protopappa, Megoulas, & Skaltsa, 2007). Recently, safe and effective tyrosinase inhibitors have become important for their potential applications in improving food quality and preventing pigmentation disorders and other melanin-related health problems in humans (Miyazawa, Oshima, Koshio, Itsuzaki, & Anzai, 2003). Furthermore, tyrosinase inhibitors are also important in cosmetic applications for skin-whitening effects, because many men and women prefer a lighter skin colour. Since plants are a rich source of bioactive chemicals, and are mostly free of harmful side effects, there is an increasing interest in using them as a source of natural tyrosinase inhibitors.

The properties (e.g. antioxidant activity) of extracts from plants depend on the extraction solvent and extraction techniques used (Durling et al., 2007; Lim & Murtijaya, 2007; Spigno & De Faveri, 2007). Water and ethanol are commonly used to extract phytochemicals from plants due to absence of toxicity. Extraction with ethanol and water was investigated for isolation of possible antioxidants in the present study. A further study of the effect of the conditions for drying seed wastes on the shelf life of the raw material was also included. Since mango seeds are perishable in their fresh state, preservation of this raw material in the factory by sun-drying or hot air oven-drying, in order to preserve their desirable qualities, reduce storage volume and to extend their shelf life, is important. Moreover, most of the bound forms of phenolic compounds, after release by hydrolysis, were reported to show slightly lower antioxidant activity than do the free forms (Kumar, Nayaka, Dharmesh, & Salimath, 2006). Hence, extraction processes, which were employed to liberate free phenolics from their bound forms, were investigated. Selection of the preparation method, solvent and extraction conditions, on the basis of the free radical-scavenging capacity, metal-chelating activity, antioxidant potential, total phenolic content and tyrosinase inhibitory properties of the extract, was considered.

2. Materials and methods

2.1. Chemicals

Folin Ciocalteu's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH·), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS·), ferrous sulfate, ferrozine, linoleic acid were purchased from Sigma Chemical Co., Ltd (St. Louise, USA). Gallic acid and ammonium thiocyanate were purchased from Acros Organics (New Jersey, USA). Dimethyl sulfoxide (DMSO), methanol and water (HPLC grade) were obtained from Merck (Darmstadt, Germany). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), ferrous chloride and mushroom tyrosinase were purchased from Fluka Co. (Buchs, Switzerland). Sodium bicarbonate, L-tyrosine, arbutin and the other chemicals and solvents used in this experiment were of analytical grade, purchased from Sigma-Aldrich Co., Ltd (Steinheim, Germany).

2.2. Preparation of plant sample

Three batches of sun-dried mango (*Mangifera indica* L. cultivar Chok-Anan) seeds were donated by Woraporn Co., Ltd., a mango processing manufacturer in Thailand from March to June, in 2007, as by-products. The seeds were washed and sun-dried in the greenhouse for three days and the kernels were removed manually from the seeds. The mango seed kernels (MSK) thus obtained were also oven-dried at 60°C for 72 h. The moisture content of sample materials was measured at 1, 3, 5, 10, 24, 48 and 72 h for calculation of the drying curve on a dry weight basis according to AOAC (1990). All determinations were performed in triplicate.

2.3. Preparation of mango seed kernel (MSK) extract

Each sun-dried or oven-dried sample (200 g) was extracted according to Maisuthisakul, Pongsawatmanit, and Gordon (2006). Briefly, a sample was ground using a CIM 24 SS pin mill (New York, USA) and blended with 95% ethanol (600 ml) in a blender for 1 min and shaken for 4.5 h (method 1). The supernatant was passed through Whatman filter paper (no. 4). All filtrates were evaporated under reduced pressure using a rotary evaporator at 50°C and the remaining water was removed by freeze-drying and weighed to determine the yield of soluble components. The freeze-dried extract was flushed with nitrogen and stored in aluminium foil at −20 °C until it was analysed. Extraction was repeated on each batch of plant material until at least 50 g of extract was collected for each plant material. The dried extracts and reference samples (arbutin, coumarin ellagic acid, gallic acid and tannic acid) were used to estimate the total phenolic content, chelating activity, and antioxidant capacity through various chemical assays and also the tyrosinase inhibition assay.

In order to investigate the effect of extraction and hydrolysis conditions, Dried MSK from the selected drying method was divided into three portions. Each portion (ca. 200 g) was accurately weighed. The first sample was extracted as described above (method 1). The other samples were refluxed with ethanol for 3 h (method 2) and refluxed with 1.2 M hydrochloric acid in ethanol for 3 h, after which the acid solution was neutralized with 1% sodium carbonate (method 3). The supernatant was passed through Whatman filter paper (no. 4). All filtrates were dried as described above. Extractions were performed in triplicate.

2.4. Determination of total phenolic content

The total phenolic content of extracts was determined using Folin–Ciocalteu's phenol reagent (modified from Kähkonen et al., 1999). Briefly, each extract (200 l) was mixed with one ml of Folin–Ciocalteu's phenol reagent (thoroughly). After mixing for 3 min, 0.8 ml of 7.5% (w/v) sodium carbonate was added. The mixtures were agitated and allowed to stand for a further 30 min in the dark. The absorbance of plant extracts and a prepared blank were measured at 765 nm using a spectrophotometer (UV–Vis model 1601, Shimadzu, Japan). The concentration of total phenolic compounds in all plant extracts was expressed as mg of tannic acid equivalents per g dry weight of MSK using a linear equation. All determinations were performed in triplicate.

2.5. Determination of chelating activity

The ferrous ion-chelating activity of studied samples was estimated, based on the decrease in the maximal absorbance of the

iron (Fe^{2+})–ferrozine complex according to previously reported methods (Dinis, Madeira, & Almeida, 1994), with some modifications. Briefly, 1 ml of a solution of a test compound at various concentrations dissolved in ethanol was incubated with 0.5 ml of $FeCl_2 \cdot 4H_2O$ (1.0 mM). The reaction was initiated by the addition of 1 ml of ferrozine (5.0 mM), and then the total reaction volume was adjusted to 4 ml with ethanol. After the mixture had reached equilibrium (10 min), the absorbance at 562 nm was read. The control was prepared without the test compound. Fe^{2+}-chelating activity of the test compound was calculated from the following formula: chelating activity (%) = $[(A_{control} - A_{sample})/A_{sample}] \times 100$. The chelating activity (%) was plotted against the plant extract concentration (g/ml) to determine the concentration of extract necessary to reduce chelation by 50% (CA_{50}). Samples with a lower CA_{50} had a stronger chelating activity. All tests and analyses were carried out in triplicate and averaged.

To check the ability of MSK or reference samples to chelate copper, the UV–visible spectra (200–400 nm) of the MSK extracts and their Cu-complexed counterparts were measured. The mixture, consisting of 1.0 ml of methanol, 1.0 ml of sample (16.5 mg ml^{-1}) solution and 1.0 ml of the aqueous solution of $CuSO_4$ (5–50 μM) or water (control) was incubated at 25° C for 10 min before UV–Vis spectral analysis.

2.6. Determination of antioxidant activity

The total free radical-scavenging capacity of MSK or reference samples was determined by using the DPPH· and ABTS· methods. The antioxidant activity in a linoleic acid emulsion system was also determined.

The free radical-scavenging activity of MSK or reference samples was evaluated using the stable radical, DPPH· according to the method of Masuda et al. (1999). DPPH radical in methanol (5 mM) was prepared and this solution (100 l) was added to the extract or antioxidant sample solutions in methanol (4.9 ml) at various concentrations. After 30 min, absorbance was measured at 517 nm. The percentage of DPPH radical-scavenging activity of each plant extract was calculated from: $[A_o - (A_1 - A_s)]/A_o \times 100$. A_o is the absorbance of the control solution (containing only DPPH); A_1 is the absorbance of the DPPH· solution containing plant extract; and A_s is the absorbance of the sample extract solution without DPPH·. The DPPH· activity (EC_{50}) was calculated using a plot of per cent radical-scavenging activity against concentration, as described for CA_{50}. These values were changed to antiradical activity (A_{AR}), defined as $1/EC_{50}$, since this parameter increases with antioxidant activity. All determinations were performed in triplicate.

The ABTS radical cation-scavenging activity was determined according to Re et al. (1999). The ABTS radical cation was prepared by reacting an aqueous solution of ABTS (7 mM) with potassium persulfate (2.45 mM, final concentration), which was kept in the dark at 25°C for 12–16 h. The solution was diluted in ethanol to an absorbance of 0.70 (0.20) at 734 nm before use. Aliquots of trolox or sample in water (20 l) were added to 2.0 ml of this diluted solution, and the absorbance at 734 nm was determined at 30°C exactly 6 min after initial mixing. The antioxidant solution reduces the radical cation to ABTS which reduces the colour. The extent of decolorization is calculated as percentage reduction of absorbance, and this is determined as a function of concentration and calculated relative to the equivalent trolox concentration. The activity of each antioxidant was determined at three concentrations, within the range of the dose–response curve of trolox, and the radical-scavenging activity was expressed as the trolox equivalent antioxidant capacity (TEAC), defined as mMol of trolox per gramme of sample. All determinations were performed in triplicate.

The antioxidant activity in a linoleic acid emulsion system of MSK or reference samples was determined using the thiocyanate method (Hu, Xu, Chen, & Yang, 2004), with some modifications. Each sample, at various concentrations, in 0.5 ml of absolute ethanol, was mixed with 0.5 ml of 5.21% linoleic acid in absolute ethanol, 1 ml of 0.05 M phosphate buffer (pH 7), and 0.5 ml of distilled water and placed in a screw-capped tube. The reaction mixture was incubated in the dark at 40 °C in an oven. Aliquots of 0.1 ml were removed every 24 h during incubation and the degree of oxidation was measured by sequentially adding ethanol (9.7 ml, 75%), ammonium thiocyanate (0.1 ml, 30%) and ferrous chloride (0.1 ml, 0.02 M in 3.5% HCl). After the mixture was rested for 3 min, the peroxide value was determined by monitoring absorbance at 500 nm until the absorbance of the control reached the maximum. The antioxidant activity was calculated as percentage of inhibition relative to the control. The degree of linoleic acid peroxidation was calculated using the following formula: antioxidant activity = $[(A_{control} - A_{sample})/A_{control}] \times 100$. The antioxidant activity was plotted against sample concentration in order to determine the concentration required to achieve a 50% inhibition of linoleic acid oxidation [AA_{50}]. All tests and analyses were carried out in triplicate and averaged.

2.7. Determination of tyrosinase inhibition activity

Mushroom tyrosinase (EC 1.14.18.1) was used for the bioassay because it is readily available. Since the mode of inhibition depends on the structure of both the substrate and inhibitor, L-DOPA was used as the substrate in this experiment, unless otherwise specified. Therefore, inhibitors discussed in this paper are inhibitors of diphenolase activity of mushroom tyrosinase, and their effect on the enzyme was determined by spectrophotometry, based on dopachrome formation at 475 nm. All the samples were first dissolved in dimethyl sulfoxide (DMSO) and used for the experiment at 30 times dilution. The assay was performed as previously described (Fu, Li, Wang, Lee, & Cui, 2005), with some modifications. L-DOPA solution (0.87 ml, 4.5 mM) was mixed with 0.9 ml of 0.1 M phosphate buffer (pH 6.8), and incubated at 30 °C for 5 min. Then, 0.9 ml of various concentrations of sample solutions followed by 0.03 ml of the aqueous solution of mushroom tyrosinase (4000 units) was added to the mixture and the enzyme reaction was monitored by measuring the change in absorbance at 475 nm (at 30°C), corresponding to the formation of dopachrome, for 25 min at 1 min intervals. Controls, without inhibitor but containing 3.3%DMSO, were routinely determined. The per cent inhibition of the enzyme by the active compounds was calculated as follows: inhibition (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100$. The inhibitory effect (%) of the compounds was expressed as the inhibitor concentration causing 50% loss of enzyme activity (ID_{50}). All the studies were carried out at least in triplicate. The type of inhibition by MSK extracts and some reference compounds towards the enzyme was assayed by a Lineweaver–Burk plot. The inhibition constant was determined by plots of the apparent 1/Vm or km/Vm versus the concentration of the inhibitor, as described by Chen et al. (2005).

2.8. Determination of acute skin irritation

The acute skin irritation caused by the MSK extract was investigated in rabbit skin by the Thailand Institute of Scientific and Technological Research, Ministry of Science and Technology, Thailand according to the Test Guideline No. 404: Acute Dermal Irritation/Corrosion of OECD Guidelines for Testing of Chemicals (Organization for Economic Co-operation, 2002). Briefly, an area of skin approximately 10 × 10 cm on the dorso-lumbar region of each rabbit was clipped free of hairs. Two areas of the shaven skin approximately 2.5 × 2.5 cm were selected. Extract (0.5 g) was dissolved in 0.5 ml of distilled water and introduced onto a 2.5 × 2.5 cm gauze

patch, which served as the treated patch while 0.5 ml of distilled water, on another patch, served as a control patch. Both were applied to the selected skin sites on each rabbit. The patches were then secured to the skin by transpore adhesive tape. The entire trunk of the rabbit was wrapped with elastic cloth to avoid dislocation of the patch for 4 h. The scores of skin reaction were evaluated at 1, 24, 48 and 72 h after removal of the patches. Further observation was made, as necessary, to establish whether reversibility of the irritations occurred. In addition, to the observation of irritation, any lesions and other toxic effects were recorded.

2.9. Statistical analysis

Results are presented as mean values ± standard deviation (at least three replicate experiments). Analysis of variance and significant differences among means were determined by one-way ANOVA using SPSS (Version 10, SPSS Inc., Chicago, USA.). Significant differences were declared at $P < 0.05$.

3. Results and discussion

3.1. General investigations

A systematic investigation of the drying procedure, solvent type and extraction process was carried out on a laboratory scale to determine the conditions for preparation of the optimal MSK extract to use as an antioxidant or whitening agent. The effects of each process parameter on the extract yield, total phenolic content (TP), chelating activity (CA), antioxidant capacity (AC) and also tyrosinase inhibition (TIA) are discussed below.

3.2. Effects of drying procedure and solvent type on moisture content, yield, phenolic components, chelating activity, antioxidant activity and tyrosinase inhibition activity

Polyphenols are heat-sensitive (Spigno & De Faveri, 2007), therefore 60°C was chosen for drying in the hot air oven, since higher temperatures may cause excessive degradation.

Curves of moisture content versus drying time of MSK samples are shown in Fig. 1. The final moisture content of sun-dried MSK (SDMSK) was 9.81 ± 0.34% (dry weight basis). The average temperature in a greenhouse was 53°C. The moisture contents of MSK at 10 and 24 h were not different because it was night time. Usually, the final moisture content of dried natural plant products ranges from 5% to 15% (Nijhuis et al., 1998). It was found that, with hot air oven-drying of MSK (ODMSK), 4.38 + 0.47% moisture content could be reached within 48 h at 60°C. The shelf life for storage of SDMSK and ODMSK at 30°C without mould growth was more than 3 months.

The extract yield and colour, including TP, as a function of drying method and solvent type are shown in Table 1. In this study, ethanol and water were used as solvents because they are non-toxic and are accepted as ingredients in food and cosmetics. Statistical analysis indicated that drying method and extraction solvent

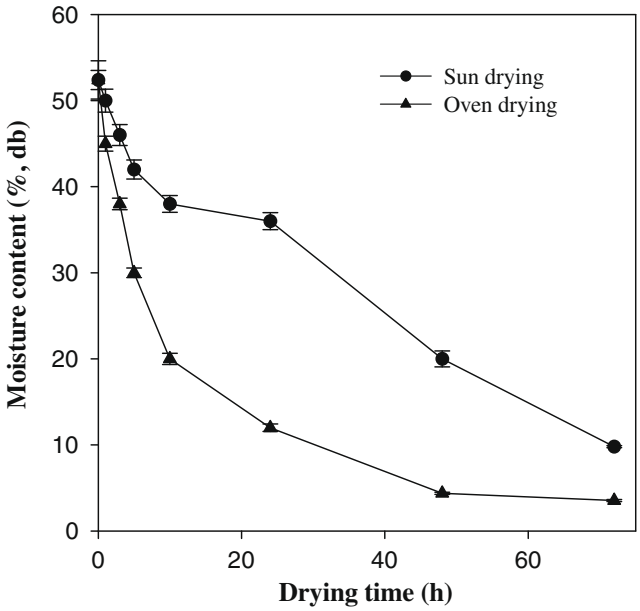

Fig. 1. Drying curves of MSK using sun-drying and oven-drying.

influenced yield for all the measured phenolic compounds ($P < 0.05$). The colour and moisture content of the extracts varied, depending on the drying conditions and solvent type (Table 1). The colour of SDMSK extracts, both with ethanol [SDMSK (EtOH)] and with water [SDMSK (H_2O)], was darker than that of both ODMSK (EtOH) and ODMSK (H_2O), while the moisture content of the SDMSK extract was higher than that of the ODMSK extract. The determination of total phenolic compounds by the Folin–Ciocalteu method was included in this study since many individual phenolic compounds, which provide antioxidant activity in fruits, cannot be identified and measured by HPLC methods (Ferreira et al., 2002). The content by the HPLC method was only 50–60% of the level analysed by the Folin–Ciocalteu method (Ferreira et al., 2002). The TP of MSK, expressed as tannic acid equivalents, varied from 44.5 ± 0.36 mg/g to 89.92 ± 0.23 mg/g. The content of phenolic compounds in our samples was slightly lower than those found in extracts of MSK extracted with methanol and water (50:50) by Soong and Barlow (2004) which were expressed as gallic acid equivalents.

Table 2 shows the CA and antioxidant activity in the linoleic acid emulsion system of extracts obtained using different drying methods and extraction solvents. In this study, the CA results were expressed as $1/CA_{50}$ values for comparison. A moderate metal-chelating effect, with ferrous ions (Fe^{2+}), of samples was observed in comparison to the standard gallic acid (Table 2). Measurements showed that the SDMSK (EtOH) extract had the highest total phenolic content (Table 1) and the highest chelating activity (Table 2).

The inhibition of lipid oxidation in the emulsion system by the standards (Table 2) followed the order: tannic acid > ellagic

Table 1
Effects of preparation conditions and solvents on the yield, colour, moisture content and total phenolic content of mango kernel extracts.[A]

Samples	Yield (%)	Colour	Moisture content (%, db)	Total phenolic content (mg of TAE/g)
SDMSK (EtOH)	2.01 + 0.06^a	Yellow	4.13 + 0.23^b	89.9 ± 0.23^d
SDMSK (H_2O)	2.56 + 0.12^b	Dark brown	4.23 + 0.11^b	68.1 ± 0.13^c
ODMSK (EtOH)	3.19 + 0.22^c	Pale yellow	3.15 + 0.06^a	53.7 ± 0.54^b
ODMSK (H_2O)	3.83 + 0.21^d	Dark brown	3.11 + 0.21^a	44.5 ± 0.36^a

In each column, different superscript letters mean significant differences ($P < 0.05$) between conditions.
Means of three replicates ± SD (standard deviation).
[A] Dry weight basis of the original sample of plant parts.

Table 2
The metal-chelating activity and antioxidant activity toward DPPH· of mango seed kernel obtained from different preparation conditions and solvents.[A]

Samples	Chelating activity (CA_{50}, μg/ml)	Chelating efficiency ($1/CA_{50}$)	Antioxidant activity (AA_{50})	Antioxidant efficiency ($1/AA_{50}$)
Samples				
SDMSK (EtOH)	52.5 + 0.20^{c}	0.019 + 0.000^{d}	71.0 ± 6.35^{d}	0.014 ± 0.001^{c}
SDMSK (H_2O)	78.6 + 0.22^{f}	0.013 + 0.000^{b}	84.1 ± 1.16^{e}	0.012 ± 0.000^{b}
ODMSK (EtOH)	62.3 + 0.05^{d}	0.016 + 0.000^{c}	84.8 ± 7.28^{e}	0.012 ± 0.001^{b}
ODMSK (H_2O)	80.3 + 0.03^{g}	0.012 + 0.000^{a}	105 ± 3.87^{g}	0.010 ± 0.000^{a}
References				
Arbutin	ND	ND	26.3 ± 0.29^{c}	0.038 ± 0.001^{d}
Coumarin	ND	ND	94.9 ± 0.06^{f}	0.011 ± 0.000^{a}
Ellagic acid	23.2 ± 0.22^{a}	0.043 ± 0.000^{f}	19.3 ± 0.57^{b}	0.052 ± 0.002^{e}
Gallic acid	65.5 ± 0.23^{e}	0.015 ± 0.000^{c}	19.2 ± 0.07^{b}	0.052 ± 0.000^{e}
Tannic acid	26.7 ± 0.15^{b}	0.038 ± 0.003^{e}	18.4 ± 0.08^{a}	0.054 ± 0.000^{f}

Means of three replicates ± SD (standard deviation).
In each column, different superscripts mean significant differences ($P < 0.05$) between conditions.
ND means not determined.
[A] Dry weight basis of the original sample of plant parts.

Table 3
The DPPH· and ABTS radical cation-scavenging activities of mango seed kernel obtained from different preparation conditions and solvents.[A]

Samples	DPPH activity (EC_{50}, μg/ml)	Antiradical activity (A_{AR}, $1/EC_{50}$)	ABTS activity (mmol of trolox/g)
Samples			
SDMSK (EtOH)	0.56 + 0.06^{b}	1.80 + 0.17^{f}	1.03 ± 0.13^{e}
SDMSK (H_2O)	2.61 + 0.32^{d}	0.39 + 0.05^{d}	0.78 ± 0.05^{d}
ODMSK (EtOH)	4.45 + 0.31^{e}	0.23 + 0.02^{c}	0.77 ± 0.05^{d}
ODMSK (H_2O)	14.0 + 1.32^{f}	0.07 + 0.01^{b}	0.44 ± 0.03^{b}
References			
Arbutin	64.3 ± 0.05^{g}	0.02 ± 0.00^{a}	0.52 ± 0.01^{c}
Coumarin	71.1 ± 0.17^{h}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}
Ellagic acid	0.48 ± 0.02^{a}	2.08 ± 0.07^{g}	3.76 ± 0.01^{h}
Gallic acid	0.53 ± 0.01^{b}	1.89 ± 0.03^{f}	3.49 ± 0.03^{g}
Tannic acid	1.06 ± 0.01^{c}	0.95 ± 0.01^{e}	3.10 ± 0.01^{f}

Means of three replicates ± SD (standard deviation).
In each column, different superscripts mean significant differences ($P < 0.05$) between conditions.
[A] Dry weight basis of the original sample of plant parts.

acid = gallic acid > arbutin > coumarin. Antioxidant activity is generally higher in polyaromatic polyphenol compounds, such as tannic acid; but there are other factors: O–H bond dissociation energy, resonance delocalisation of the phenoxy radical and steric hindrance derived from a bulky substituent in the ortho position on the aromatic ring that also can influence this property (Rao & Gianfreda, 2000). Sánchez-Moreno, Larrauri, and Saura-Calixto (1999) reported that tannic acid was more effective than was gallic acid as an antioxidant in the linoleic acid emulsion system which was consistent with this result. The ethanolic extracts of both SDMSK and ODMSK exhibited higher antioxidant efficiency and chelating efficiency than did the extracts obtained by water extraction (Table 2). Typically, proteins and polysaccharides are also extracted when using water extraction (Shi et al., 2003). This may be the reason why yields of water extracts were higher than that of ethanolic extracts (Table 1) in contrast with the results for antioxidant efficiency and chelating efficiency (Table 2).

The scavenging of DPPH· and $ABTS^{\cdot+}$ were determined and compared to reference compounds (Table 3). Among the reference compounds studied, the order of DPPH·-scavenging activity was: ellagic acid > gallic acid > tannic acid > arbutin = coumarin, which is consistent with the DPPH·-scavenging activity of some polyphenols reported elsewhere (Kumar et al., 2006). The order of scavenging of $ABTS^{\cdot+}$ by the standards was slightly different from that of DPPH·. Arbutin showed higher $ABTS^{\cdot+}$-scavenging activity than did coumarin. This may be due to the stronger attractive interactions between the more polar arbutin and the more polar $ABTS^{\cdot+}$. Dried aqueous extracts were less effective in scavenging both DPPH· and $ABTS^{\cdot+}$ than were the ethanolic extracts. In the present study, the highest DPPH·- and $ABTS^{\cdot+}$-scavenging activities of the

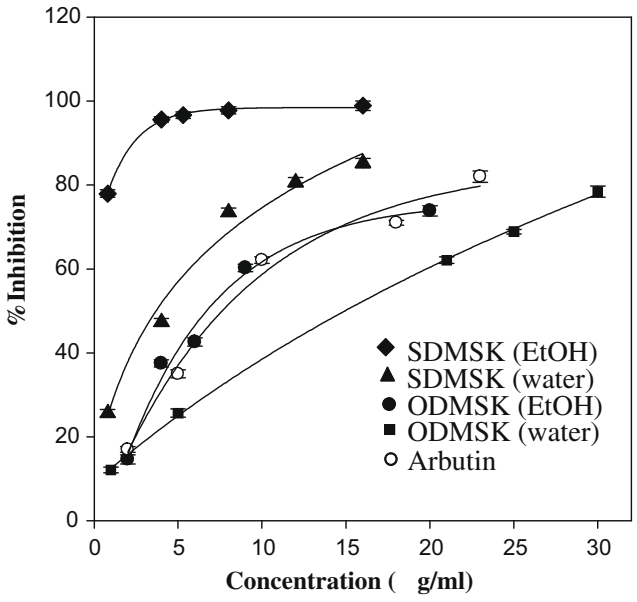

Fig. 2. Tyrosinase activities of MSK prepared with different drying conditions and extraction solvent compared to arbutin.

sample extracts were shown by SDMSK (EtOH). It is known that the solvent used in the extraction may affect the antioxidant activity of the extract, by its effect on the content and composition of the phenolic extract. The radical-scavenging activity of extracts is related to the content and structures of the phenolic components (such as gallic acid, ellagic acid, coumarin (Abdalla et al., 2007) and tannic acid (Arogba, 2000)), which differ in their electron transfer/hydrogen-donating abilities.

Fig. 2 shows the effect of concentration of MSK extracts, prepared by different drying and solvent extraction procedures, on inhibition of tyrosinase activity. This is the first report of the tyrosinase inhibitory activity of MSK. Tyrosinase catalyses two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to o-quinones (diphenolase activity), both using molecular oxygen (Karioti et al., 2007). The tested compounds were assayed for the diphenolase inhibitory activity which demands L-DOPA as substrate. In comparison with arbutin, a naturally occurring cosmetic vehicle and whitening agent with tyrosinase inhibitory activity (Barsoom, Abdelsamad, & Adib, 2006), MSK extracts exhibited potent inhibitory activity (Fig. 2). Masuda, Kirikihira, and Takeda (2005) observed that seashore plant species, which are exposed to full sunlight, possess strong antioxidant activity and high tyrosinase inhibition ability. Findings from this study are consistent with this observation. In the comparison of extracts prepared with the different drying methods and extraction solvents at room temperature, SDMSK (EtOH) had the highest TP and AC values in the three methods, the DPPH·- and $ABTS^{\cdot+}$-scavenging activities and the emulsion method (Tables 1–3). SDMSK (EtOH) also showed a high metal-chelating activity. This extract thus has good tyrosinase inhibition ability, high antioxidant activity and good metal-chelating properties due to the content and structures of phenolic compounds present in the extract. This is consistent with previous reports that a good correlation exists between TP and tyrosinase inhibition ability (Madani, Kermasha, & Bisakowski, 1999).

The selected optimum drying and solvent extraction conditions for extraction of phenolics from raw material (MSK) were sun-drying for 3 days with ethanol extraction, which gave an extract with

Table 4
Effects of extraction and hydrolysis conditions on the yield, colour, moisture content, total phenolic content and metal-chelating efficiency of mango kernel extracts.[A]

Conditions	Yield (%)	Colour	Moisture content (%, db)	Total phenolic content (mg of TAE/g)	Chelating efficiency ($1/CA_{50}$)
Shaking (method 1)	3.31 ± 0.19^{a}	Yellow	4.12 ± 0.06^{b}	90.0 ± 0.06^{a}	0.019 ± 0.000^{a}
Refluxing (method 2)	11.9 ± 0.04^{c}	Yellow	4.07 ± 0.05^{ab}	212 ± 0.07^{b}	0.025 ± 0.000^{b}
Acid hydrolysis (method 3)	10.8 ± 0.07^{b}	Black	4.03 ± 0.06^{a}	286 ± 0.28^{c}	0.031 ± 0.001^{c}

Means of three replications ± SD (standard deviation).
In each column, different superscripts mean significant differences ($P < 0.05$) between conditions.
[A] Dry weight basis of the original sample of plant parts.

Table 5
The antioxidant capacities of mango seed kernel obtained from different extraction and hydrolysis conditions.[A]

Conditions	Antioxidant efficiency Ferric thiocyanate method ($1/AA_{50}$)	Antiradical activity DPPH· (A_{AR}, $1/EC_{50}$)	ABTS activity (mmol of trolox/g)
Shaking (method 1)	0.014 ± 0.000^{a}	1.75 ± 0.15^{a}	1.03 ± 0.02^{a}
Refluxing (method 2)	0.016 ± 0.000^{b}	2.60 ± 0.24^{b}	1.14 ± 0.02^{b}
Acid hydrolysis (method 3)	0.019 ± 0.000^{c}	4.16 ± 0.54^{c}	1.41 ± 0.01^{c}

Means of three replicates ± SD (standard deviation).
In each column, different superscripts mean significant differences ($P < 0.05$) between conditions.
[A] Dry weight basis of the original sample of plant parts.

Table 6
Tyrosinase inhibition activity of mango seed kernel extracts obtained under different conditions compared to some references.[A]

Samples	ID_{50} (mg/ml)	K_m (mg/ml)	V_{max} (mg/ml min) [NS]	Mode of inhibition
Samples				
No inhibitor	–	7.10 ± 0.02^{a}	0.017 ± 0.001	-
SMSK	7.45 ± 0.06^{g}	9.39 ± 0.04^{b}	0.016 ± 0.002	Competitive
RMSK	6.94 ± 0.04^{f}	12.1 ± 0.02^{c}	0.018 ± 0.001	Competitive
AMSK	4.13 ± 0.03^{d}	19.2 ± 0.03^{d}	0.017 ± 0.000	Competitive
References				
Arbutin	6.52 ± 0.04^{e}	ND	ND	Competitive[B]
Coumarin	20.9 ± 0.04^{h}	ND	ND	[C]
Ellagic acid	1.04 ± 0.01^{a}	ND	ND	[C]
Gallic acid	1.93 ± 0.05^{c}	ND	ND	Competitive[D]
Tannic acid	1.16 ± 0.03^{b}	ND	ND	[C]

In each column, different superscripts mean significant differences ($P < 0.05$) between conditions.
Means of three replications ± SD (standard deviation).
NS means non-significant.
ND means not determined.
[A] Dry weight basis of the original sample of plant parts.
[B] According to Nihei and Kubo (2003).
[C] Unable to establish.
[D] According to No et al. (1999).

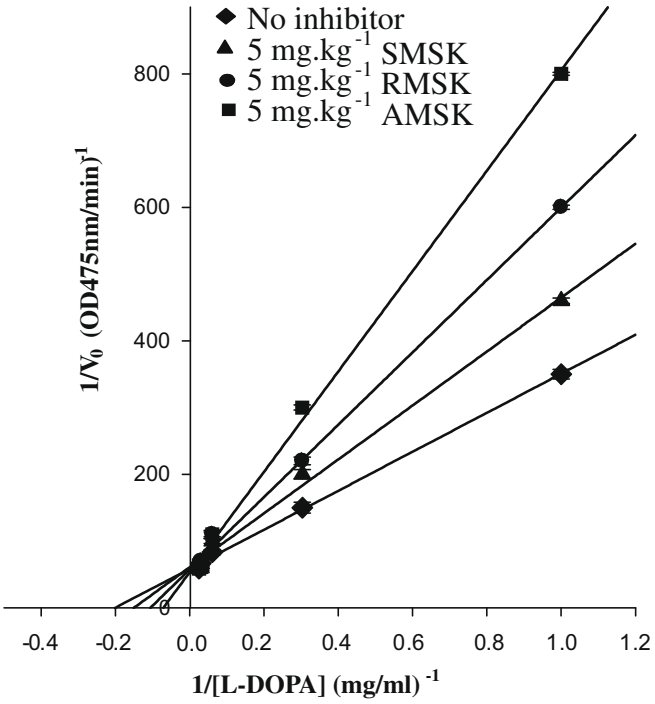

Fig. 3. Lineweaver–Burk plots for the determination of kinetic constants (km and Vm) for mushroom tyrosinase inhibited by MSK from different extraction conditions: shaking method (SMSK), refluxing method (RMSK) and acid hydrolysis (AMSK).

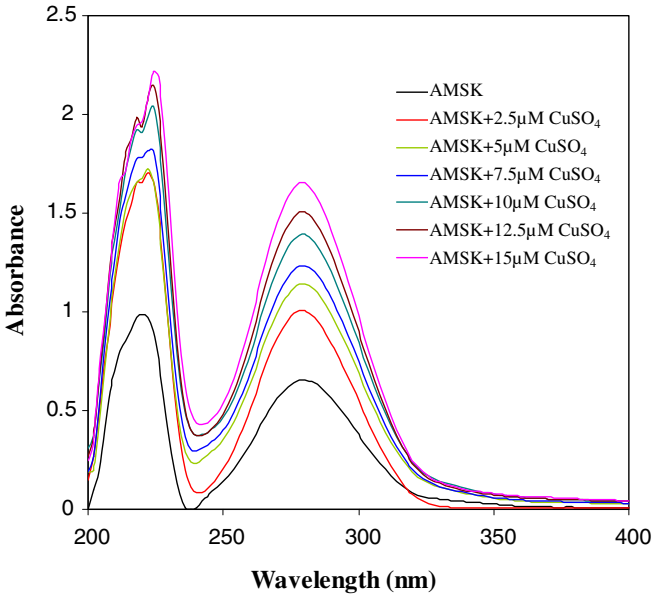

Fig. 5. Spectrum of the complex formed between mango seed kernel extract and several concentrations of Cu (II) ion.

the highest total phenolic content, chelating efficiency and antioxidant efficiency.

3.3. Effect of extraction and hydrolysis conditions on moisture content, yield, phenolic components, chelating activity, antioxidant activity and tyrosinase inhibition activity

Acid hydrolysis with heating was reported to give an extract with the highest antioxidant activity from mango kernel, and these conditions released free gallic acid and ellagic acid (Soong & Barlow, 2004). In order to achieve the best conditions, TP, CA, AC and TIA were evaluated for the SDMSK obtained using three different conditions (shaking, refluxing and acid hydrolysis). As expected, the TP and CA of MSK extracted after acid hydrolysis (AMSK) were higher than the values for samples of MSK extracted using shaking (SMSK) and refluxing (RMSK) conditions (Table 4). The TP of MSK extract in our study ranged from 90.0 mg of tannic acid equivalents per gramme (TAE/g) to 286 mg of TAE/g. The level of TP was slightly higher than that reported by Arogba (1997). This work showed that the extraction and hydrolysis procedure has a substantial effect on the content of phenolic compounds present in MSK. Acid hydrolysis released and degraded some phenolic compounds which is consistent with the work of Krygier, Sosulski, and Hogge (1982) and Chiang, Shih, and Chu (2001). Most phenolic

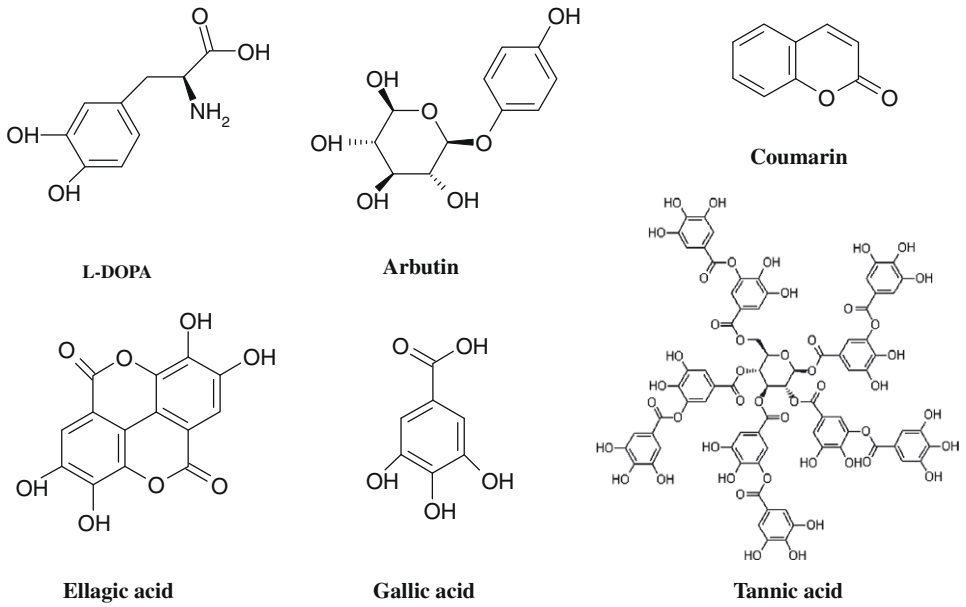

Fig. 4. Chemical structures of studied compounds.

compounds in plant seeds primarily occur in the bound form as conjugates with sugars, fatty acids, or proteins (Krygier et al., 1982). Therefore, it is important that a hydrolysis process is adopted in order to obtain maximum yield of the phenolic content of mango seed kernel. Moreover, the yield from the acid hydrolysis method was greater than that from the extraction methods without hydrolysis. The result was consistent with that of Troszynska and Ciska (2002). They found that acid hydrolysis released a greater concentration of phenolic compounds, such as protocatechuic acid, from the seed. The colour and moisture content of the extracts varied, depending on the extraction conditions (Table 4). The colour of MSK extracts from acid hydrolysis (method 3) was darker than that of both methods (methods 1 and 2). The moisture contents of all extracts from the different procedures was not sig-

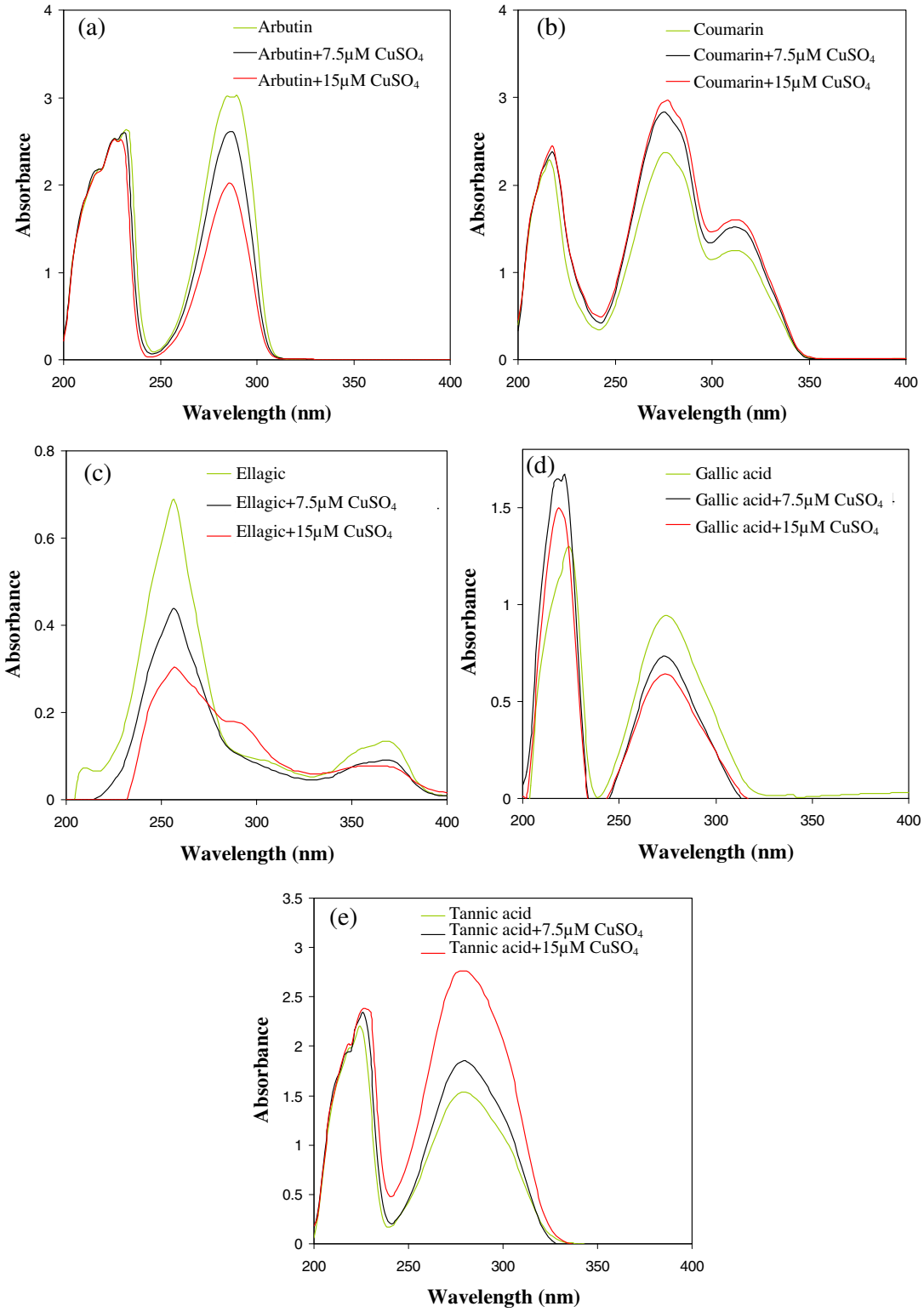

Fig. 6. Spectra of the complex formed between (a) arbutin, (b) coumarin, (c) ellagic acid, (d) gallic acid, and (e) tannic acid and several concentrations of Cu (II) ion.

nificantly different ($P \geqslant 0.05$) and ranged from 4.03 to 4.12% (dry weight basis).

There are few studies comparing the antioxidant properties of MSK extracts prepared with different extraction and hydrolysis conditions. According to the results shown in Table 4, The amount of TP showed the same order as the metal-chelating efficiency (Table 4) and the antioxidant capacity (Table 5). The DPPH radical-scavenging activity of SMSK, RMSK and AMSK extracts was strong, since the average EC_{50} values were 0.57 ± 0.05, 0.39 ± 0.04 and 0.24 ± 0.03 μg/ml, respectively, and these values were similar to those of gallic acid and ellagic acid. A similar order of radical-scavenging activity was seen in the ABTS radical cation assay. It can be seen that AMSK showed the highest antioxidant activity of the isolated extracts. This can be attributed to the higher phenolic content in AMSK (286 mg TAE/g) compared to that in the other extracts (212 and 90.0 mg TAE/g, respectively). The results suggested that some phenolic conjugates might be released by acid hydrolysis and the free form might provide more potent antioxidant activity. The good correlation between antioxidant activity and phenolic content of extracts obtained from various natural sources has been demonstrated by many workers (Jung, Heo, & Wang, 2008; Liu et al., 2008). Pérez-Jiménez and Saura-Calixto (2006) found that some compounds which have $ABTS^{\cdot+}$-scavenging activity did not show DPPH radical-scavenging activity. This is not the case in this study, as the plant extracts were able to quench both radicals. This showed the ability of the extracts to scavenge free radicals in different systems, indicating that they could be investigated as possible therapeutic agents for reducing radical-related pathological damage. All MSK samples obtained from methods 1–3, exhibited antioxidant activity, using the ferric thiocyanate method (FTC), similar to the DPPH and ABTS assays (Table 5). These findings showed that the phenolic compounds found in MSK were capable of both radical-scavenging activity inhibition of lipid oxidation.

The tyrosinase inhibitory activity (ID_{50}) of the MSK extracts was established using tyrosine as a substrate. The ID_{50} value for the most active MSK extract (AMSK) was lower than that of arbutin, and several components of the extract, including ellagic acid, gallic acid and tannic acid, contributed to the tyrosinase inhibitory activity, since they were more active than was the reference inhibitor used (arbutin) with a lower ID_{50} value (Table 6). Coumarin, however, was not an active component. Currently available tyrosinase inhibitors, such as arbutin, suffer from toxicity and/or a lack of efficacy. For these reasons, this is a good opportunity to use MSK as an alternative tyrosinase inhibitor.

To get an insight into the mode of inhibition of tyrosinase by MSK extracts, the effect of increased L-DOPA concentration on the kinetics of inhibition of mushroom tyrosinase by the studied compounds was further investigated by a Lineweaver–Burk plot. The results (Fig. 3) show that the plots of $1/V_o$ versus $1/[S]$ give a family of straight lines with different slopes that intersect one another in the Y-axis. The value of V_{max} remained the same (Table 6) and the value of km differed with different extraction procedures used for isolation of the MSK. The same value of V_{max} indicates that MSK extract is a competitive inhibitor of the enzyme. These inhibitors bind at the same site as the substrate and bind only to the free enzyme. As stated earlier, most competitive inhibitors closely resemble the molecular structure of the substrate. The phenolic compounds present in the MSK extract (including ellagic acid, gallic acid and tannic acid) are ortho-dihydroxyphenols, and they share this structural feature with L-DOPA (Fig. 4). Gallic acid also acted as a competitive inhibitor (Table 6). The km value of AMSK was the lowest one. A competitive inhibitor increases the apparent km value for a particular substrate, as shown by the three MSK extracts. Typically, the stronger the inhibitory activity, the higher the km value obtained. This indicates that the tyrosinase inhibitory effect of AMSK was stronger than that of RMSK and SMSK (Table 6).

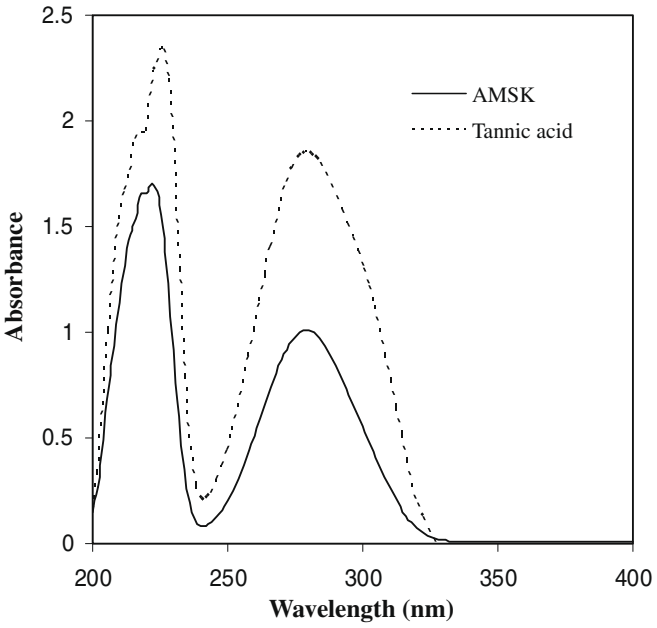

Fig. 7. Spectra of AMSK and tannic acid.

The inhibitory effects of MSK and studied reference compounds might be due to binding with copper, which is the metal at the centre of the active site of tyrosinase. The copper-chelating properties of the MSK extract and its component phenolics were investigated. Spectrophotometric analyses were conducted to establish the ability of polyphenols to bind copper. The UV–Vis spectra of polyphenol mixtures extracted from SMSK, RMSK and AMSK were not different (data not shown). As shown in Fig. 5, the $_{max}$ value was 220 nm with a shoulder at 212 nm for the AMSK sample. The increase in ratio of Cu (II) to AMSK caused increased absorbance at 224 and 280 nm, with the disappearance of the absorbance at 220 nm, showing that binding to Cu (II) occurred. The reference compounds, coumarin and tannic acid, showed similar binding to Cu (II) (Fig. 6b, d and e). Coumarin showed increasing absorbance at 217, 276 and 312 nm with increasing Cu (II) concentration. The UV–Vis spectrum of gallic acid increased in absorbance at 220 nm with increasing Cu (II) concentration, but the absorbance at 280 nm was reduced. Tannic acid showed changes in the absorbance pattern similar to AMSK. Interestingly, the UV–Vis spectrum of the AMSK extract was similar to that of tannic acid (Fig. 7). However, arbutin showed a decrease in the maximum absorbance at 288 nm (Fig. 6a) after the addition of Cu (II) ion, while ellagic acid showed a characteristic shift in $_{max}$ to shorter wavelength (Fig. 6c). There are few reports on changes in the UV–Vis spectra of plant extracts with Cu (II) addition. Wu, Chen, Ho, and Yang (2003) reported that adding Cu (II) ion to red koji extract caused an increase in absorbance at 250 nm.

Safety is a primary consideration for tyrosinase inhibitors, especially for cosmetic products. Therefore, the acute skin irritation values of SDMSK (EtOH) and AMSK were investigated. The results showed that all treated rabbits did not exhibit dermal irritation when treated with either extract.

4. Conclusions

It can be concluded that sun-drying, followed by ethanol extraction, is suitable for the preparation of an MSK extract. The optimum extract prepared by sun-drying and ethanol extraction contained a higher phenolic content, and its antioxidant capacity,

metal-chelating activity and ability to inhibit tyrosinase were higher than those of extracts prepared with oven-drying or water extraction. Acid hydrolysis of MSK extracts gave an increased yield of total phenolics and the extract had higher activity in terms of CA with Fe^{2+}, AC and TIA, compared to shaking and heating solvent extraction conditions. The kinetics of inhibition of mushroom tyrosinase showed that the MSK extracts acted as a competitive inhibitor of the enzyme. MSK extracts were also shown to be good chelators of copper.

Acknowledgements

This research was supported by the research fund of the University of the Thai Chamber of Commerce. We also thank Dr. Sirikarn Phasuk from Valaya Alongkorn Rajabhat University under Royal Patronage, Thailand, for making equipment available for the experiment.

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Food Chemistry 117 (2009) 342–348

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Purification and characterisation of polyphenol oxidase from red Swiss chard (*Beta vulgaris* subspecies *cicla*) leaves

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ARTICLE INFO

Article history:
Received 28 October 2008
Received in revised form 3 March 2009
Accepted 7 April 2009

Keywords:
Polyphenol oxidase
Red Swiss chard
Purification
Characterisation
Diphenolase

ABSTRACT

We report purification and characterisation of a polyphenol oxidase from red Swiss chard (rcPPO). Our purification procedure resulted in a 39-fold enrichment in specific activity and 17% recovery of total enzyme activity. The purified rcPPO appeared as a monomeric protein of 41 kDa, with a specific conformation conserved in the Cu^{2+} combining region. It was optimally active at pH 7.5 and 45 °C. It had a diphenolase substrate preference towards L-DOPA, catechol and chlorogenic acid, but also exhibited weak monophenolase one toward 4-methoxyphenol and L-tyrosine. We also found that the enzyme was activated by K^+, Na^+, SDS and laurouyl sarcosine, but inhibited by divalent cations including Ca^{2+}, Cu^{2+}. Its activity was completely inhibited by ascorbic acid, cysteine, 1,4-dithiothreitol, β-mercaptoethanol, sodium diethyldithiocarbamate, sodium metabisulphite, sodium sulphite and thiourea. This first report on the purification and characterisation of red Swiss chard PPO provides a basis for understanding and use of this enzyme.

1. Introduction

Polyphenol oxidase (PPO) is a copper-containing enzyme that is capable of catalysing both molecular oxygen-dependent hydroxylation of monophenols to their corresponding *o*-diphenols (hydroxylation or monophenolase activity, E.C. 1.14.18.1) exemplified by the oxidation of tyrosine to L-(3,4-dihydroxyphenyl)-alanine (L-DOPA), and oxidation of *o*-diphenols such as L-DOPA to their cognate *o*-quinones, dopaquinone (diphenolase activity, E.C. 1.10.3.1) (Sánchez-Ferrer, Rodríguez-López, García-Cánovas, & García-Carmona, 1995). This enzyme is widely distributed in microorganisms, animals and higher plants. In plants, a significant body of literature on the catalytic properties of PPO has been reported. Research to date is mainly focused on the following three aspects: (1) its antiviral and antioxidant properties, as well as the protective effect against damage from ultraviolet radiation (Mayer, 2006), (2) its roles in darkening damaged tissue and in fruit and vegetable browning during storage and processing (Friedman, 1996), (3) its potential involvement in the betalain biosynthetic pathway (Steiner, Schliemann, Böhm, & Strack1, 1999; Gandía-Herrero, Escribano, & Garcia-Carmona, 2005), in which the PPO catalyses two different reactions: hydroxylation of tyrosine to form L-DOPA and oxidation of the DOPA to produce dopaquinone.

Red Swiss chard (*Beta vulgaris* subspecies *cicla*) is a herbaceous biennial leafy vegetable cultivated in many parts of the world for its year round availability, low cost and wide use in many traditional dishes. Different from its sister variety garden beet, red chard is rich in red–violet betacyanins in its leaf (Kugler, Stintzing, & Carle, 2004), not the root. The betacyanins in garden beet have been investigated widely for its physiological functions, such as scavenging powerful radical (Pavlov, Kovatcheva, Georgiev, Koleva, & Ilieva, 2002), inhibiting the proliferation of tumour cells in vitro (Reddy, Alexander-Lindo, & Nair, 2005), antimicrobial properties (Abdou, Abou-Zeid, & El-Sherberny, 1972), protective UV-filter and fungal resistance (Sepúlveda-Jiméneza, Rueda-Beníteza, Portaa, & Rocha-Sosa, 2004). The same physiological functions were demonstrated for the betacyanins of red chard. In addition to betacyanins, red chard contains in the leaf also a substantial amount of phenolic acids with antioxidant activity (Pyo, Lee, Logendra, & Rosen, 2004) and flavonoids with antimitotic activity on human breast cancer cells (Ninfali et al., 2007). Red chard has been used as an ingredient in folk medicine for hypoglycaemia (Bolkent, Yanardag, Tabakoglu-Oguz, & Özsoy-Sacan, 2000), inflammatory and haemorrhage. All of these physiological effects make red chard very valuable in field of medicine, in addition to a good alternative plant material for betacyanin pigment production. As leaf vegetables, red chard not only has a vivid colour in the leaf vein and petiole but also has some unique nutritional properties, for example, rich in vitamins A, C and B, calcium, iron, and phosphorus (Pyo et al., 2004). The attractive appearance of red chard it was even explored as a decoration item in many occasions. However, the appearance, flavour, taste and nutritional value of the red chard can be significantly reduced by enzymatic blackening or browning during harvest, processing or prolonged cold storage. The browning also interferes with extraction of betalain pigments and other

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doi:10.1016/j.foodchem.2009.04.013

beneficial bioactive compounds, leading to a destructive colour change from bright charming red to dark-brown red, especially in the production of the red chard vegetable juice. Understanding red chard PPO characteristics is therefore needed to control enzymatic browning of red chard plant and its products.

To the best of our knowledge, red chard PPO has not been investigated although PPOs from garden beet (Gandía-Herrero, Garcia-Carmona, & Escribano, 2004) and a number of other plant species have been characterised. In this study, red Swiss chard PPO was purified and characterised, and its substrate specificity, pH and thermal stability of the PPO and the influence of surfactants, metal ions and inhibitors on the PPO's activity were determined. The information reported here provides a basis for understanding and potential use or inhibition of red chard PPO.

2. Materials and methods

2.1. Plant materials

Red chard plants were grown in a greenhouse with natural light and temperature fluctuated from 20–25 °C during the day to 15–20 °C at night. Plants were watered every 5 d. Fresh leaves harvested from the plants were washed, cut and processed for enzyme extraction immediately.

2.2. Reagents and chemicals

DEAE-Sepharose and Sephadex G-75 were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), substrates and molecular weight markers from Sigma Chemical Co. (St. Louis, MO, USA), the Bradford protein assay reagent, bovine serum albumin and electrophoresis reagents from Bio-Rad (Bio-Rad Laboratories, Hercules, CA). Other reagents were of analytical grade.

2.3. Enzyme extraction and purification

Fresh red chard leaves were homogenised with ice-cold 20 mM Tris–HCl (pH 7.5). The crude extract samples were filtered through four layers of cheesecloth and then centrifuged at 12,000*g* for 20 min. After centrifugation, several precipitations with solid ammonium sulphate, between 0–20%, 20–40%, 40–60%, 60–70%, and 70–80%, were examined to find the proper saturation point. The precipitate was dissolved in a small quantity of 20 mM Tris–HCl buffer (pH 7.5) and dialysed at 4 °C in the same buffer.

For further purification of PPO, the dialysed enzyme solution was loaded onto a Sephadex G-75 column (1.2 × 90 cm) equilibrated with 20 mM Tris–HCl buffer (pH 7.5). The dialysed enzyme solution was passed through the column, washed with the same buffer containing 25 mM NaCl. The elution rate was adjusted to 15–20 ml/h. Fractions (3 ml, each) were collected and the active fractions were pooled.

The final purification was done by DEAE-Sepharose Fast Flow ion-exchange column chromatography. The column was equilibrated with 20 mM Tris–HCl buffer (pH 7.5). The enzyme solution from the gel filtration was applied to the column and washed with the same buffer without any NaCl. Then the proteins bound to the column were eluted with a linear gradient of 0–1.0 M NaCl in Tris–HCl buffer (pH 7.5) at the same flow rate. Fractions were collected with 1.0 ml/min and were monitored at 280 nm. The enzymatic activities of the fractions were detected using 15 mM L-DOPA as substrate and the fractions with higher enzymatic activity were collected and pooled together for further characterisation studies.

In the elution processes of the column chromatograph, PPO activity was measured in the eluates showing absorbance at 280 nm. Thus, values obtained were plotted against the tube number. The final active elute was used for bioassay, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and other biochemical studies. Protein content was determined by the dye-binding method of Bradford (1976) with bovine serum protein as the standard.

2.4. Electrophoretic procedures

To determine the homogeneity and apparent molecular mass of the purified PPO, SDS–PAGE was performed on 12% acrylamide gels according to Laemmli (1970). In-gel staining of PPO activity was carried out using the reported procedures (Wang & Constabel, 2003).

2.5. Western blot

Immunodetection by Western blot was performed following the method described by Bollag and Edelstein (1991). Polyclonal anti-PPO antiserum was raised against recombinant 31-kDa polypeptide of *Amaranthus* sp. PPO protein, which was produced in our lab (Han et al., unpublished data). The purified red chard PPO was subjected to denaturing SDS–PAGE or Non-denaturing SDS–PAGE, transferred to nitrocellulose membrane and probed with anti-*Amaranthus* PPO polyclonal antibodies. Antibody binding was visualised using goat anti-rabbit IgG (H + L) alkaline phosphatase conjugate in a dilution of 1:4000 and alkaline phosphatase colour development reagents 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium chloride (NBT).

2.6. Enzyme assays and analysis of protein

PPO activity was determined by measuring the initial rate of quinone formation, as indicated by an increase in absorbance at 475 nm using L-DOPA as a substrate. Increment of 0.001 min^{-1} in absorbance was taken as one unit of enzyme activity (Ho, 1999). The increase in absorbance was linear with time for the first 120 s. The sample cuvette contained 2.95 ml of substrate solution in 20 mM Tris–HCl (pH 8.0) and 0.05 ml of the enzyme solution. The blank sample contained 2.95 ml of substrate solution and 0.05 ml of Tris–HCl buffer. Unless otherwise stated, experiments were repeated in triplicate. The results were expressed as absorbance increment/min.

2.7. Effect of pH and temperature

Optimum pH for rcPPO activity was estimated by monitoring its activity at pH range of 3.0–10.0 with buffers at concentration of 20 mM. The buffer systems used were citrate buffer for pH 2.0–3.5; acetate buffer for pH 4.0–5.5; phosphate buffer for pH 6.0–7.5; Tris–HCl buffer for pH 7.5–9.0; carbonate–bicarbonate buffer for pH 9.5–10.0. For determining its pH stability, the enzyme solution was incubated for 1 h at the given pH at 35 °C and then, its residual PPO activity was determined under the standard assay conditions.

For determining the optimum temperature of PPO reaction, the enzyme activity was measured at temperature ranging from 10 to 70 °C. The effect of temperature on PPO stability was determined by analysing PPO residual activity after the enzyme was incubated in a 20 mM Tris–HCl buffer (pH 7.5) for pre-determined time periods (10–60 min) at temperatures ranging from 30 to 80 °C.

2.8. Effect of inhibitors on enzyme activity

The effects of several potential inhibitors were evaluated, using L-DOPA as substrate. The reaction mixture was incubated at 35 °C and the change in absorbance was measured spectrophotometri-

cally at 475 nm. A control test was run in parallel in the absence of the inhibitor.

For determining the effect of metal ions on enzyme activity, reactions were performed by incubating the reaction mixture containing 1 ml of enzyme and 1 or 10 mM of metal ion, such as Na^+, K^+, Zn^{2+}, Co^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+} or Cu^{2+}. After incubation for 30 min at 35 °C, the remaining enzyme activity was assayed.

2.9. Substrate specificity

The oxidation of substrates by rcPPO was determined spectrophotometrically at the specific wavelength of each substrate in a 20 mM Tris–HCl buffer (pH 7.5). The PPO concentration used for the oxidation of each substrate was the same. Substrate specificity was examined by using 10 mM L-DOPA, catechol, *p*-dimethylphenol, *m*-dimethylphenol, chlorogenic acid, L-tyrosine, 4-methoxyphenol or gallic acid.

3. Results and discussion

3.1. Purification of red chard PPO

The purification steps and results were summarised in Table 1. Purification of rcPPO began with the extraction of PPO from fresh leaves of red chard in 20 mM Tris–HCl (pH 7.5) buffer containing ascorbic acid, which was used to reduce quinones to phenolic substrates during extraction. The precipitate obtained with 40–80% of $(NH_4)_2SO_4$ displayed maximum enzymatic activity on a protein content basis. This active fraction was dialysed using 10 kDa cut-off range dialysis membrane and applied to Sephadex G-75 column. Peak fractions with high enzymatic activities on 15 mM L-DOPA were collected in fractions 64–80 (Fig. 1a) and three protein peaks were eluted. This chromatography step removed most unwanted proteins of high and low molecular weight (Fig. 1a) but with a loss of only 30–40% of the total enzyme activity. The most active fractions (65–75) were combined for further purification. Up to this step, the betalain pigments influencing the measurement of PPO activity were completely removed. The last step of purification was performed with DEAE-Sepharose anion exchange chromatography. The enzyme was finally eluted at a salt concentration of 0.35 M NaCl along with a protein peak, corresponding to fractions of 35–60 (Fig. 1b).

Our purification steps resulted in a 39-fold purification. The overall activity yield of the purified PPO was 17%, with specific PPO activity of 18,084 U/mg. The obtained enzyme was used for further studies.

3.2. Molecular weight and purity

The molecular weight of the purified enzyme, as determined by SDS–PAGE under reducing conditions, was 41 kDa in average (Fig. 2a), which was lower than that of butter lettuce PPO (60 kDa; Gawlik-Dziki, Złotek, & Świeca, 2008), potato PPO (57–60 kDa), tomato PPO (57–62 kDa) and broccoli florets PPO (53.1 kDa) (Gawlik-Dziki, Szymanowska, & Baraniak, 2007), but higher than that of *Hevea brasiliensis* PPO (32 kDa and 34 kDa; Wititsuwannakul, Chareonthiphakornb, Pacec, & Wititsuwannakul, 2002). It was also much less than that of the PPO from beet root (54 kDa), a sister variety of red chard (Gandía-Herrero et al., 2004). Under non-denaturing SDS–PAGE, a single protein band was detected for PPO activity (Fig. 2b). On the same gel, a single protein band was also visualised and the band was at the same migration distance as that of the active staining, which confirmed that the purified homogeneous protein was PPO and suggested that the red chard PPO was a monomer.

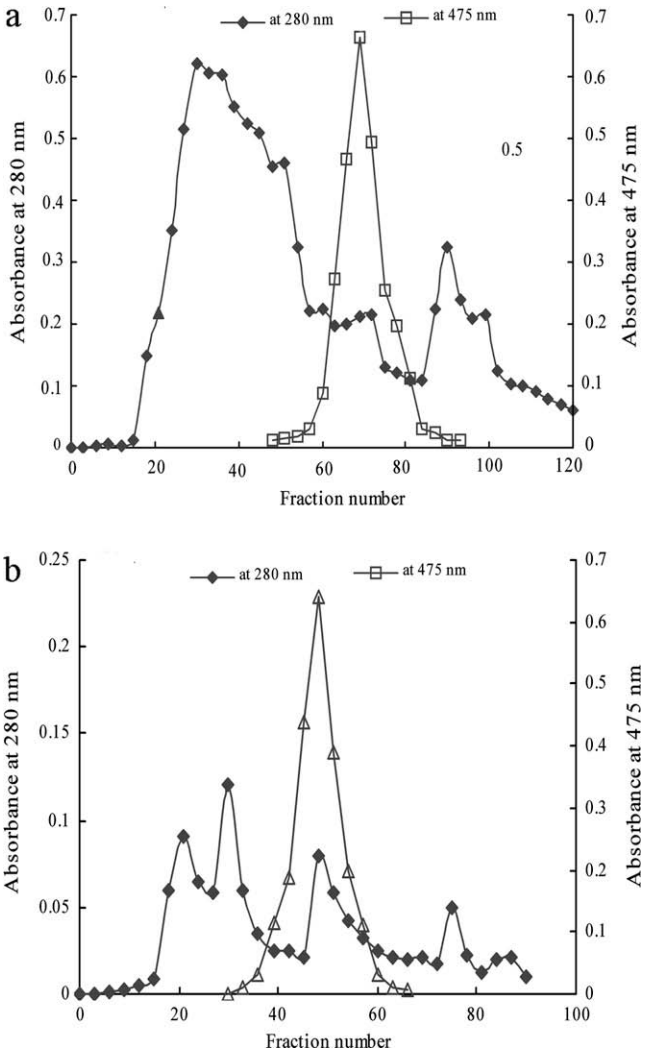

Fig. 1. Chromatographic elution profiles of PPO from red Swiss chard (a) Sephadex G-75 gel filtration chromatography. (b) Separation on DEAE-Sepharose Fast Flow anion exchange chromatography. Protein was monitored by UV absorbance at $\lambda = 280$ nm and the PPO activity, at $\lambda = 475$ nm as described in Section 2.

3.3. Western blot

To study the structural features of purified rcPPO, Western blotting was conducted following fully and non-denaturing SDS–PAGE

Table 1
Purification procedure of PPO from red Swiss chard leaves.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purification (*n*-fold)
Crude extract	57216.75	124.00	461.42	100.00	1.00
Ammonium sulphate precipitation	38675.26	41.96	921.81	67.59	2.00
Sephadex G-75	19251.12	5.80	3318.75	33.65	7.19
Sepharose FF	9567.48	0.53	18084.21	16.72	39.19

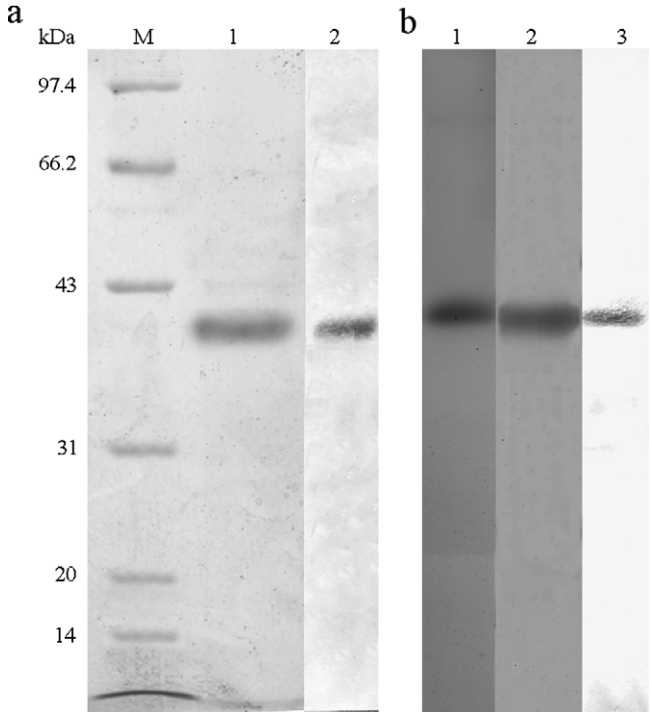

Fig. 2. Denaturing and non-denaturing SDS–PAGE separation and verification of purified red Swiss chard PPO. (a) SDS–PAGE of purified red chard Swiss PPO: lane 1, low molecular weight marker of protein standard; lane 2, purified red Swiss chard PPO; lane 3, Western blotting. (b) Non-denaturing SDS–PAGE of purified red chard Swiss PPO: lane 1, active staining of purified red Swiss chard PPO; lane 2, Coomassie brilliant blue staining; lane 3, Western blotting.

with Anti-*Amaranthus* PPO antiserum. The purified rcPPO cross-reacted with the antiserum, and showed a single immunoreactive band corresponding to the single protein band visualised with Coomassie brilliant blue under fully and non denaturing conditions (Fig. 2). These features suggested that the purified rcPPO share the specific conformation conserved in the Cu^{2+} combining region of PPOs from many sources.

3.4. Effects of pH and temperature on PPO activity

Activity profiles of rcPPO were investigated at 45 °C between pH 3.0 and 10.0. As shown in Fig. 3a, the PPO was rapidly inactivated by the pH below 5.0. The activity of rcPPO gradually increased with pH value in the range of 5–7. Below and above pH 7.5, the activity decreased rapidly. Hence, the optimum pH value of rcPPO was 7.5. Furthermore, the enzyme appeared to prefer alkaline conditions since more than 70% of its optimal activity was kept between pH 7.5 and 10.0. The optimal pH of rcPPO is similar to that of *Allium* PPO (Arslan, Temur, & Tozlu, 1997), but higher than that of *Ferula* sp. (6.0) (Erat, Sakiroglu, & Kufrevioglu, 2006), and medlar fruit (6.5) (Dincer, Colak, Aydin, Kadioglu, & Güner, 2002).

For assessment on pH stability of the purified rcPPO, the enzyme was preincubated for 1 h at 35 °C using citrate buffer (pH range from 2 to 3.5), acetate buffer (pH 4.0–5.5), phosphate buffer (pH 6.0–7.5), Tris–HCl buffer (pH 7.5–9.0) or carbonate–bicarbonate buffer (pH 9.5–11). The enzyme was stable within pH 4–10 (Fig. 3b), retaining more than 80% of its activity in the pH range of 5–9, and the maximum in the pH 7.0–8.0. In general, the rcPPO showed more stable under the alkaline conditions. After incubating at pH 11.0 for 1 h the rcPPO still retained 74% of its activity whereas at pH 3.0, it was left with only 39.4% (Fig. 3b).

To determine temperature effect on enzyme activity, the standard reaction mixture was incubated at different temperatures

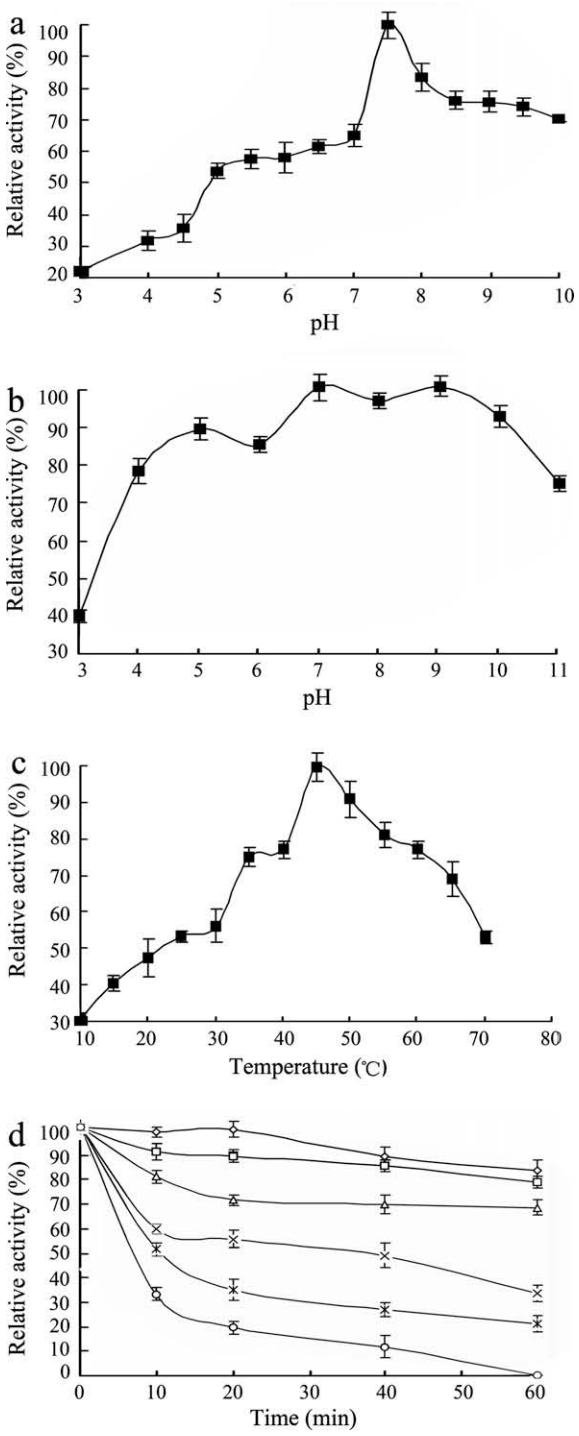

Fig. 3. Effect of pH and temperature on the activity of purified red Swiss chard PPO. (a) Optimal pH of rcPPO activity. (b) pH stability of rcPPO. (c) Optimal temperature of rcPPO activity. (d) Thermal stability of rcPPO. The rcPPO activity was measured with L-DOPA as substrate. For determination of the optimal temperature, the purified rcPPO was preincubated for 2 min at different temperatures, and then its activity was assayed at 45 °C. For determination of the thermal stability, the activity of purified rcPPO was tested after preincubation for 80 min at a given time at different temperatures: 30 °C (◇), 40 °C (□), 50 °C (Δ), and 60 °C (×), 70 °C (+) and 80 °C (○). The experiment was performed in triplicate and each value is expressed as the mean value ± standard deviation.

(10–70 °C). As shown in Fig. 3c, the activity of rcPPO gradually increased with temperature ranging from 10 to 45 °C, but followed by a decrease at higher temperatures. Thus, the temperature for the maximum PPO activity was estimated to be 45 °C for L-DOPA,

which was consistent with that of the mulberry PPO (Arslan, Erzengin, Sinan, & Ozensoy, 2004), but higher than that of some reported plant PPOs, such as peach, grape, plum, thymus and aubergine (Dincer et al., 2002; Dogan, Arslan, & Dogan, 2002; Dogan & Dogan, 2004).

The effect of temperature on rcPPO stability was also investigated by incubating the sample at 30–80 °C at definite intervals for a total of 80 min. The residual soluble PPO activity (Fig. 3d) remained above 60% after 40 min at 30, 40 and 50 °C and then decreased significantly when temperature reached at 60 °C, with only 55% activity in just 10 min. When the temperature was raised to 80 °C, the enzyme retained less than 18% of its original activity after 20 min and even less after prolonged incubation. A number of PPOs purified from different plants were stable in the 10–50 °C range. For example, butter lettuce PPO was stable at 40 °C for 60 min (Gawlik-Dziki et al., 2008), *Allium* sp. PPO at 40 °C (Arslan et al., 1997) and peppermint PPO at 30 °C for 30 min (Kavrayan & Aydemir, 2001).

3.5. Effects of surfactants on PPO activity

For determining the effect of different surfactants on the rcPPO activity, the purified rcPPO was incubated with surfactants at 35 °C for 1 h and the enzymatic activity was determined under normal assaying conditions. The PPO activity without any surfactants (control) was taken as 100%. As displayed in Table 2, the enzyme activity was drastically inhibited by CTAB (cationic), slightly by Tween-20 (nonionic), but not by Triton X-100, with 51%, 16% and 0% enzyme activity lost, respectively. In contrast to cationic (CTAB), anionic surfactants activated rcPPO, and the enzyme activity with 0.2% Laurouyl Sarcosine or SDS treatment was 124% and 133% of the control, respectively. The activation effect of SDS on the rcPPO was also reported in beet root (Gandía-Herreroi et al., 2004). SDS activation of PPO is a well-known phenomenon for many plants PPO (Moore & Flurkey, 1990), and this effect was attributed to the SDS-causing alterations of the enzymatic and physical characteristics, and a limited conformational change due to binding of small amounts of SDS (Moore & Flurkey, 1990).

Table 2
Effects of metal ions, common PPO inhibitors and surfactants on purified red chard PPO activity.

Chemicals	Residual activity[a] (%)	
	1 mM[b]	10 mM
Metal ions		
Na^+	127 ± 2.2	114 ± 1.5
K^+	107 ± 1.3	114 ± 0.7
Mg^{2+}	82 ± 1.6	65 ± 0.5
Ca^{2+}	81 ± 0.9	65 ± 0.4
Mn^{2+}	81 ± 0.5	75 ± 1.6
Co^{2+}	81 ± 0.9	75 ± 0.2
Cu^{2+}	53 ± 0.2	50 ± 0.9
Zn^{2+}	80 ± 0.7	77 ± 0.5
Inhibitors		
Ascorbic acid	12 ± 0.6	0
Citric acid	75 ± 1.6	65 ± 1.9
Cysteine	7 ± 0.7	0
1,4-Dithiothreitol (DTT)	12 ± 0.8	0
EDTA	95 ± 1.7	81 ± 1.1
β-Mercaptoethanol	10 ± 0.6	0
Sodium azide	90 ± 1.8	83 ± 1.5
Sodium diethyldithiocarbamate	8 ± 0.4	0
Sodium metabisulphite	11 ± 0.6	0
Sodium sulphite	8 ± 0.6	0
Thiourea	9 ± 0.4	0
Surfactants	0.1% (w/v)[a]	0.2%
CTAB	49 ± 0.9	46 ± 1.1
Laurouyl sarcosine	112 ± 0.5	124 ± 1.3
SDS	129 ± 2.4	133 ± 1.6
Triton X-100	100 ± 1.6	100 ± 0.8
Tween-20	92 ± 1.8	84 ± 0.9

[a] The experiment was performed in triplicate and each value is expressed as the mean value ± standard deviation.
[b] Final concentration of the chemicals.

3.6. Effect of metal ions and possible inhibitors on PPO activity

In order to determine the effect of metal ions and possible inhibitors on rcPPO activity, the rcPPO was incubated with 1 mM or 10 mM of different metal ions or some common PPO inhibitors (Table 2) for 30 min at 35 °C or pH 7.5, 45 °C, respectively, and then the residual activity was measured using standard protocol. The purified rcPPO activity in the absence of tested metal ions or inhibitors under optimal conditions (pH 7.5, 45 °C) was set as 100%.

As presented in Table 2, K^+ and Na^+, at 1 mM and 10 mM, had a positive effect, whilst divalent cations including Mg^{2+}, Ca^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} generally had a negative one. Among the divalent cations tested, Cu^{2+} at 10 mM showed the strongest inhibitory effect, and 50% of the enzymatic activity was inhibited. This is not in agreement with what was reported by Aydemir in 2004 in artichoke heads.

Several common PPO inhibitors had an inhibitory effect on the rcPPO (Table 2). Purified rcPPO was extremely sensitive to sodium diethyldithiocarbamate (SDDC), a copper chelator, and more than 90% of its activity was inhibited by 1 mM of SDDC. However, the rcPPO was insensitive to EDTA and sodium azide, and more than 80% of its activity was retained even treated with higher concentration (10 mM). The tolerance of PPO to EDTA was also observed in the Barbados cherry (Anil Kumar, Kishor Mohan, & Murugan, 2008). Another metal ion chelator, citric acid, could inhibit the rcPPO activity by 25% and 35% at 1 mM and 10 mM, respectively. Strong reducing agents, sodium metabisulphite, sodium sulphite and ascorbic acid, inhibited completely rcPPO activity at the concentration of 10 mM. Sulphur containing compounds are known to be specific potent inhibitors of PPO by removing quinones and in turn preventing them from participating in melanizing reactions and/or reacting directly with the enzyme (Kong, Hong, Choi, & Kim, 2000). With regard to ascorbic acid, it may act more as an antioxidant than as an enzyme inhibitor, because it reduces the initial quinone formed by the enzyme to the original diphenol before it undergoes secondary reactions leading to browning (Gawlik-Dzik et al., 2008), although a irreversible inhibition of PPO was also observed (Golan-Goldhirsh & Whitaker, 1984). SH-blocking reagents, L-cysteine, β-mercaptoethanol and DTT, when used at 10 mM, resulted in a complete inhibition of rcPPO activity, suggesting that Cys residue(s) may be significant on the structure and conformation of the enzyme molecule. Thiourea had also a significantly inhibitory effect on rcPPO activity, and even at concentration of 1 mM, it inhibited more than 90% of the PPO activity (Table 2). These results suggest that cysteine, ascorbic acid, sodium sulphite and sodium metabisulphite may be used as inhibitor of enzymatic browning in red chard and its products.

3.7. Substrate specificity

The substrate specificity of purified rcPPO was studied by using 15 mM of monohydroxy-, dihydroxy- and trihydroxyphenol as substrates and at the optimal pH and temperature conditions, and results, presented as enzymatic activity relative to the oxidation of L-DOPA, are summarised in Table 3. The purified rcPPO showed enzymatic activity of monophenolase, diphenolase and even triphenolase, with the highest diphenolase activity detected with L-DOPA, followed by catechol, chlorogenic acid, *m*-dimethylphenol and *p*-dimethylphenol. The monophenolase activity of the

Table 3
Substrate specificity of purified red chard PPO.

Substrate	Wavelength	Relative activity[a] (%)
Diphenols		
L-DOPA	475	100
Catechol	420	86
p-Dimethylphenol	420	10.0
m-Dimethylphenol	420	11
Chlorogenic acid	400	80
Monophenols		
L-Tyrosine	400	2
4-Methoxyphenol	420	40
Trihydroxyphenols		
Gallic acid	420	73

[a] The relative activity is based on the enzymatic activity of rcPPO to substrate L-DOPA.

rcPPO was week when L-tyrosine and 4-methoxyphenol were used as monophenol substrates, and the relative activity was 5% and 40%, respectively. The triphenolase activity of the rcPPO indicated with gallic acid as substrate was moderate, between its monophenolase and diphenolase activity. These results revealed that the purified red chard PPO was a diphenolase with some low monophenolase activity, which is similar to PPOs purified from peppermint (Kavrayan & Aydemir, 2001), butter lettuce (Gawlik-Dziki et al., 2008) and dormant saffron PPO (Saiedian, Keyhani, & Keyhani, 2007).

Determined from Lineweaver–Burk plot, the K_m and V_{max} of rcPPO for L-DOPA, the most favourite substrate tested, were 3.17 mM and 1667 U/min/ml, respectively. Beet root PPO was reported to have the K_m of 6.16 mM (Gandía-Herrero et al., 2004), which is much higher than that of red chard PPO.

In summary, a polyphenol oxidase from red Swiss chard leaf was extracted, purified and characterised. The purified red Swiss chard PPO was as a monomeric protein of 41 kDa, with a specific conformation conserved in the Cu^{2+} combining region indicated by Western blot. It displayed as diphenolase with a weak monophenolase activity, and had much higher activity in the oxidation of L-DOPA than in hydroxylation of L-tyrosine. Its optimal active pH was at 7.5, and temperature at 45 °C. This enzyme was sensitive to some common PPO inhibitors, especially to sulphur-containing compounds, reducing agents and SH-blocking reagents. It could be activated by K^+, Na^+, SDS and laurouyl sarcosine, but inhibited by divalent cations, such as Mg^{2+}, Ca^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, and Zn^{2+}. Purification and characterisation of the PPO provide a basis for understanding the enzyme in red Swiss chard, an excellent plant source for betacyanin pigment, bioactive compounds and nutritive ones. However, this is the first report on red Swiss chard PPO, and more studies are warranted to understand its isoenzymes if there are, its inhibition in relation to the browning reaction and its role in biosynthesis of betalain pigments.

Acknowledgements

This research was supported by grant from Chinese High-Tech R&D Program "863" (Grant No. 2008AA10Z103) to XGX. We thank Dr. J Zou (PBI, NRC, Canada) for critical reading and language improvement.

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Food Chemistry 117 (2009) 349–351

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Short communication

Influence of α-, γ-, and δ-tocopherol on the radiation induced formation of peroxides in rapeseed oil triacylglycerols

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ARTICLE INFO

Article history:
Received 7 October 2008
Received in revised form 13 March 2009
Accepted 30 March 2009

Keywords:
Antioxidant
Tocopherol
Lipid oxidation
Irradiation
Rapeseed oil

ABSTRACT

The effect of α-, γ-, and δ-tocopherols (enrichment: 1000 ppm) on the peroxide formation in rapeseed oil triacylglycerols (RSOTG) was evaluated. The oxidation process was initiated by gamma-irradiation with doses of 4 and 10 kGy. Whereas a pronounced antioxidant effect was observed for γ- and δ-tocopherol (sequence: δ- > γ-tocopherol), the inhibition extent of α-tocopherol was insignificant.

1. Introduction

An increase in hydroperoxide formation with increasing radiation dose was found in various plant oils such as sunflower, soybean, palm and black cumin oils (Arici, Colak, & Gecgel, 2007; Zeb & Lutfullah, 2005; Lutfullah, Zeb, Ahmad, Atta, & Bangash, 2003). Tocopherols (TOH), especially α- and γ-TOH, naturally occur in a large number of mainly plant-derived tissues. Their antioxidative effect is based on an H-transfer from their hydroxyl group to lipid peroxyl radicals ($LOO^{\cdot}$), leading to the formation of lipid hydroperoxides (LOOH) and tocopheroxyl radicals ($TO^{\cdot}$). Although, the hydrogen-donating power is in the order α > γ > δ, a reverse order in antioxidant potency has been obtained in most experiments with fats, oils and lipoproteins (Kamal-Eldin & Appelquist, 1996; Yanishlieva, Kamal-Eldin, Marinova, & Toneva, 2002). Also prooxidant effects of tocopherols have been reported (Isnardy, Wagner, & Elmadfa, 2003). For α- and γ-TOH a concentration dependent antioxidant effectiveness in rapeseed oil triacylglyceroles was reported (Lampi, Kataja, Kamal-Eldin, & Vieno, 1999). At levels of 500 ppm a lower activity, combined with a faster disappearance, was found for α-TOH. However, both, α- and γ-TOH, showed a pronounced antioxidant activity.

The aim of this study was directed to the evaluation of the efficacy of α-, γ-, and δ-tocopherols to prevent radiation induced formation of peroxides in purified rapeseed oil triacylglycerols (RSOTG).

2. Materials and methods

2.1. Materials

Rapeseed oil was purchased at a local supermarket, the chemicals were from Sigma–Aldrich, Steinheim, Germany, and all gases were obtained from Air Liquide Austria GmbH, Schwechat, Austria.

Rapeseed oil purification was carried out by adsorption chromatography and it was verified by normal phase HPLC; no residual tocopherols could be detected (<1 ppm). The oil enrichment procedure (1000 ppm α-, γ- and δ-TOH) followed the method described by Isnardy et al. (2003). The irradiation treatment was performed immediately after oil purification and enrichment.

2.2. Irradiation and storage

Irradiation was carried out at room temperature in 15 ml glass bottles using a ^{60}Co gamma radiation source "Gammacell 220" (MDS Nordion International Inc., Kanata, ON, Canada) with doses of 4 and 10 kGy. The dose rate was 35.2 Gy/min. Irradiated and non irradiated oil samples were stored at −20 °C in closed plastic tubes containing approximately 2 ml each.

2.3. Tocopherol content

Tocopherol content was determined by normal phase high pressure liquid chromatography (HPLC) as described by Kamal-Eldin, Görgen, Pettersson, and Lampi (2000). HPLC system, Merck Hitachi, Vienna, Austria: L-7110 isocratic pump, L-7200 autosampler,

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doi:10.1016/j.foodchem.2009.03.104

250 × 4.0 mm LiChrospher NH_2 100 5 μm column, and L-4250 UV–VIS detector, detection wavelength: 295 nm. The mobile phase consisted of 89% hexane, 9.9% tertiary-butylmethyl ether, 1% tetrahydrofuran, and 0.1% methanol.

2.4. Peroxide value (PV)

The lipid peroxides were measured using the PV-acetic acid/chloroform method (AOCS, 1998, Cd 8–53). The PV is expressed in milliequivalents peroxide per 1000 g of sample:

$$PV = [(S - B)\ N\ 1000]/m$$

with "*S*" and "*B*" being the volume (ml) of titrant used for the sample and blank, respectively, "*N*" being the normality of sodium thiosulphate solution and "*m*" the mass of sample (g).

3. Results and discussion

The major reactions immediately after radiation absorption in lipids (presence of oxygen and TOH) are summarised in Eqs. (1)–(12).

Initiation (ionisation, excitation):

$$LH \overset{gamma}{\rightarrow} [LH^{\bullet +}, e^-]\ (\text{geminate ions}) \rightarrow LH^* \quad (1a)$$

$$\overset{gamma}{\rightarrow} LH^* \quad (1b)$$

$$LH^* \rightarrow L^\bullet + H^\bullet,\ R_1^\bullet + R_2^\bullet\ (\text{C—C bond scission}) \quad (2)$$

Competition reactions:

$$L^\bullet + O_2 \rightarrow LOO^\bullet \quad (3)$$

$$L^\bullet + TOH \rightarrow LH + TO^\bullet\ (\text{repair}) \quad (4)$$

$$L^\bullet + LH \rightarrow LH + L^\bullet\ (\text{chain}) \quad (5)$$

$$H^\bullet + O_2 \rightarrow HO_2^\bullet \quad (6)$$

$$H^\bullet + TOH \rightarrow H_2 + TO^\bullet \quad (7)$$

$$H^\bullet + LH \rightarrow H_2 + L^\bullet\ (\text{chain}) \quad (8)$$

$$LOO^\bullet + TOH \rightarrow LOOH + TO^\bullet \quad (9)$$

$$LOO^\bullet + LH \rightarrow LOOH + L^\bullet\ (\text{autoxidation chain}) \quad (10)$$

$$HO_2^\bullet + TOH \rightarrow TO^\bullet + H_2O_2 \quad (11)$$

$$HO_2^\bullet + LH \rightarrow L^\bullet + H_2O_2\ (\text{chain}) \quad (12)$$

In the first step lipid radicals ($L^\cdot$) and $H^\cdot$ radicals are generated. The initial yield of free radicals in e.g. cyclohexane is (5–5.7) × 10^{-7} mol/L J^{-1}, the radical concentration per kGy is (5–5.7) × 10^{-4} mol/L (Swallow, 1987). The radicals originating from C–C bond scissions ($R_1^\bullet$, $R_1^\bullet$, etc.) are not discussed, but it should be mentioned that the formation of hydrocarbons is one method for detection of irradiated, fat containing food (Delincee, 2002). The radical-molecule reactions (Eqs. (3)–(12)) are competition reactions. Although, the reaction with oxygen (Eq. (3)) is very fast, at sufficient TOH concentration a repair of the lipid molecule (Eq. (4)) could take place. The solubility of O_2 in edible oils is high. For soybean oil a value of 55 mg/kg (1.7 × 10^{-3} mol/kg) was reported (Buchner, 1999). To possibly reduce an initial peroxyl radical formation (Eq. (3)) by scavenging of $L^\cdot$ radicals (Eq. (4)), scavenger concentrations comparable to that of oxygen are required. In this report 1000 ppm TOH, corresponding to 2.3 × 10^{-3} mol/kg α-TOH, 2.4 × 10^{-3} mol/kg γ-TOH, 2.45 × 10^{-3} mol/kg δ-TOH, were used. For δ-TOH this concentration is in the range of high effectiveness, whereas for α-TOH low antioxidant and even prooxidant effects might be expected. In general optimum concentrations for tocopherols were found to be for α-TOH ~ 100 ppm, for γ-TOH ~ 500 ppm and for δ-TOH an inhibition of hydroperoxide formation was observed up to 2000 ppm. For α-TOH also prooxidative activity were observed at high concentrations (Huang, Frankel, & German, 1994, 1995). Besides concentration the antioxidant action of TOHs is highly dependent on the lipid system, temperature and oxidation time. This study compared the antioxidant action of equal concentrations of TOHs using irradiation as oxidising system instead of storage and temperature.

RSOTG was chosen because of its high part of unsaturated fatty acids which are more sensitive to oxidation than saturated fatty acids. Since the oil samples were immediately frozen after irradiation to prevent autoxidation, no secondary oxidation products were to be expected. The experiments were focused on the determination of the primarily formed lipid peroxides.

For RSOTG and tocopherols the following investigations have been presented: In the range 5–500 μg/g α- and γ-TOH it was found that at concentrations above 100 mg/g γ-TOH was more effective than α-TOH (Lampi et al., 1999). Using 0.025–1.5 × 10^{-3} mol/kg α-TOH, the best effect was obtained up to 0.1 × 10^{-3} mol/kg (Ohm, Stöckmann, & Schwarz, 2005). At higher concentration a decrease of antioxidant efficiency was observed. Yet, both groups did not observe prooxidant effects. Authors from our group (Isnardy et al., 2003) applying α-, γ- and δ-TOH between 0.01% and 0.25% (100–2500 ppm) reported that at concentrations > 0.05% (500 ppm) especially α-TOH can act as prooxidant, whereas δ-TOH was practically not influenced in its activity, even at high concentrations.

The peroxide values (PVs) of the RSOTG with and without addition of 1000 ppm α-, γ-, and δ-TOH depending on dose are summarised in Table 1. For the non-enriched oil PVs showed a significant increase as a function of irradiation dose (correlation coefficient 0.898, $p < 0.01$). This is in accordance with the results from previous irradiation studies on various plant oils (Arici et al., 2007; Zeb & Lutfullah, 2005; Lutfullah et al., 2003). In presence of α-TOH there was no reduction of PVs, both, at 4 kGy and at 10 kGy. For γ- and δ-TOH a significant decrease of PVs was obtained, the reduction at 10 kGy was 35% and 44%, respectively (Table 1). The order of the antioxidant efficiency (δ > γ > α) is in conformity with literature data (Huang et al., 1995; Kamal-Eldin & Appelquist, 1996; Yanishlieva et al., 2002; Isnardy et al., 2003).

The results for the TOH-decrease are compiled in Table 2. The remaining concentrations are similar for all TOHs. At 4 kGy the degradation was 15–20% and at 10 kGy it was 22–28%. Since α-TOH did not show any peroxide inhibition effect, this compound must be consumed by other reactions, like Eqs. 7, 9, 11 and side reactions (e.g. reactions with peroxides, TOH + LOOH, and tocopherol mediated peroxidation, $TO^\cdot$ + LH). Side reactions of α-TOH have been extensively studied by various authors (Bowry & Ingold, 1995; Kamal-Eldin & Appelquist, 1996; Lampi et al., 1999; Yanishlieva et al., 2002). The consumption of γ-TOH at 4 kGy (20%)

Table 1
PVs ($n = 3$) of RSOTG (and their percentage decrease) without and with 0.1% (1000 ppm) α-, γ-, and δ-tocopherol depending on dose.

Dose	Mean PV [meq/kg]						
kGy	No TOH	α-TOH (0.1%)	Increase/decrease %	γ-TOH (0.1%)	Decrease %	δ-TOH (0.1%)	Decrease %
0	1.3 ± 0.3	1.3 ± 0.3		1.3 ± 0.3		1.3 ± 0.3	
4	6.5 ± 0.2	6.9 ± 0.2	+6	5.1 ± 0.3	−22	4.0 ± 0.2	−38
10	8.0 ± 0.1	7.9 ± 0.3	−1.25	5.2 ± 0.2	−35	4.5 ± 0.4	−44

Table 2
Remaining tocopherol concentration (%) in irradiated RSOTG (initial enrichment: 1000 ppm, corresponding to 100%).

Dose	Remaining tocopherol concentration in%		
kGy	α-tocopherol	γ-tocopherol	δ-tocopherol
4	85 ± 3	80 ± 2	82 ± 3
10	78 ± 3	78 ± 2	72 ± 10

equals the PV decrease, at 10 kGy (22%) it was less than the corresponding PV decrease. For δ-TOH the consumption at 4 kGy and 10 kGy (18% and 28%) was significant less than the PV decrease (38% and 44%). This might be referred to the formation of antioxidative products in the decay process of TO·, e.g. to the formation of δ-TOH-dimers (Eq. (13)), which have at least one free phenolic hydroxyl group for an antioxidant action.

$$TO^{\bullet} + TO^{\bullet} \rightarrow \text{products (dimerisation and disproportionation)} \quad (13)$$

Dimerisation was reported as preferred pathway for γ-TO· radicals (Choe & Min, 2006).

4. Conclusions

The radiation induced formation of peroxides/hydroperoxides in RSOTG was significantly reduced by δ- and γ-TOHs, whereas α-TOH did not affect the PV values. These findings demonstrate the different reaction pathways of the α-TOH/α-TO·, having two CH_3-groups vicinal to OH–/O·–, compared to γ-TOH and δ-TOH, having H–/CH_3– and H–/H–, respectively. Summarising it can be stated the main difference in using irradiation instead of temperature and storage time for oxidation of lipids is the time scale of initial radical production. It is fast and determined by the dose rate of the radiation source. Oxidation reactions (presence of O_2) and oxidation prevention (presence of TOH) showed a similar course as the conventional methods, with a sequence of antioxidant activity δ-TOH > γ-TOH > α-TOH.

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Food Chemistry 117 (2009) 352–355

Contents lists available at ScienceDirect

Food Chemistry

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Short communication

Inhibition of aldehyde dehydrogenase enzyme by Durian (*Durio zibethinus* Murray) fruit extract

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ARTICLE INFO

Article history:
Received 24 January 2009
Received in revised form 24 March 2009
Accepted 31 March 2009

Keywords:
Alcohol
ALDH inhibition
Disulfiram–ethanol reaction
Durian
Durian–alcohol reaction

ABSTRACT

The scientific basis of the adverse, or at times lethal, effect of ingesting durian (*Durio zibethinus* Murray) while imbibing alcohol has not been established. Symptoms are reminiscent of the disulfiram–ethanol reaction (DER) arising from the inhibition of aldehyde dehydrogenase (ALDH). Cognizant of the inhibitory effect of sulphur compounds like disulfiram on ALDH and the rich sulphur content of durian, the influence of durian fruit extract on the ALDH-mediated oxidative metabolism of acetaldehyde was investigated. We report a dose-dependent inhibition of yeast ALDH (yALDH), at most 70% at 0.33 ppm (mg extract/l assay mix), by dichloromethane:pentane extracts. Sulphur-rich TLC fruit extract fractions that eluted farthest from the origin effected the greatest inhibitory action. yALDH assay using diethyl disulfide as internal standard further supports the role of durian's sulfury constituents in the fruit's ALDH-inhibiting property. Insight into the etiology of DER-like symptoms felt upon simultaneous durian and alcohol consumption is hereby presented.

1. Introduction

Compelling scientific evidence support the beneficial effects of fruit and vegetable consumption (WHO, 2003), and moderate alcohol intake (Hines & Rimm, 2001) on cardiovascular health. Thus, dietary policies in countries like the US support the inclusion of these food items as part of disease preventive diets (USDA/HHS, 2005). However, risky interactions with ethanol were observed in some vegetables (Desager, Golnez, De Buck, & Horsmans, 2002; Lindros, Badger, Ronis, Ingelman-Sundberg, & Koivusalo, 1995).

Durian (*Durio zibethinus* Murray) is an important nutritional resource for Southeast Asian diets (FAO, 2003a). Its increasing popularity even in distant markets as the US and EC ensued the intensification of research to inform and familiarise consumers on the characteristics of the fruit (FAO, 2003b). Novel findings on its bioactive composition and health benefits (Charoensiri, Kongkachuichai, Suknicom, & Sungpuag, 2009; Mahattanatawee et al., 2006) point to its potential use in disease preventive diets (Leontowicz et al., 2008). However, anecdotal accounts on the interaction of durian with ethanol suggest an unsafe combination.

Reports on the adverse effects, which include cardiac episodes or deaths, in patients drinking alcohol with durian persists (Croft, 1981; Fuller, 2007; Singh, 1941). The symptoms have been described as being very unpleasant with clinical manifestations such as facial flushing, palpitation, drowsiness, vomiting and nausea. Similar effects have been associated with alcohol aversion therapy using disulfiram (tetraethylthiuram disulfide), a sulphur-containing drug (Lipsky, Shen, & Naylor, 2001; Veverka, Johnson, Mays, Lipsky, & Naylor, 1997). Disulfiram is known to inhibit aldehyde dehydrogenase (ALDH), causing the accumulation of alcohol-derived acetaldehyde. Although the possible contribution of alcohol itself to the unpleasant physiological effects has been discussed in several studies (Duranceaux et al., 2006; Jensen & Faiman, 1986), acetaldehyde appears to have a central role in mediating the adverse reactions referred to as the disulfiram–ethanol reaction or DER (Brien & Loomis, 1985; IARC, 1999; Inoue, Fukunaga, Kriyama, & Komura, 1984).

The inhibitory effects of sulphur-containing compounds from natural products on ALDH have been proposed by several groups (Brien & Loomis, 1985; Kitson & Weiner, 1996; Lindros et al., 1995; Veverka et al., 1997). Durian is rich in sulphur compounds (Baldry, Dougan, & Howard, 1972; Moser, Duvel, & Greve, 1980; Voon, Hamid, Rusul, Osman, & Quek, 2007a, 2007b; Weenen, Koolhaas, & Apriyantono, 1996; Wong & Tie, 1995), but there is yet no study demonstrating the inhibitory effects of durian or its sulphur constituents on ALDH. This study provides information on the possible link between some components of durian and DER-like effects.

Abbreviations: ALDH, aldehyde dehydrogenase; DE, durian extract; DER, disulfiram–ethanol reaction; K_m, Michaelis constant; β-NAD or NAD, nicotinamide–adenine dinucleotide; *Rf*, retardation factor; V_0, initial velocity; V_{max}, maximum velocity.

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doi:10.1016/j.foodchem.2009.03.106

2. Materials and methods

2.1. Chemicals

Inhibition studies were conducted using potassium-activated ALDH enzyme from baker's yeast (*Saccharomyces cerevisiae*) in lyophilised powder form (Sigma–Aldrich, St. Louis, Missouri, USA) with β-NAD^+ (Oriental Yeast Corporation, Osaka, Japan) as cofactor. Dichloromethane, pentane, and authentic diethyl disulfide were procured from Wako Pure Chemical Industries, Ltd. (Osaka City, Japan). All chemicals and solvents used were of analytical grade and purity.

2.2. Durian fruit extract preparation

The pulp of table-ripe durian fruit (*Durio zibethinus* Murray *cv.* Monthong) purchased from a supermarket in Tsukuba City, Japan was mixed with dichloromethane:pentane (1:1 v/v) (Weenen, Koolhaas, & Apriyantono, 1996) in a 1:2 proportion (1 kg:2 L solvent mix) for 48 h at 4 °C. The clear liquid was decanted and the solvent was evaporated under vacuum (27.5 ± 2.5 in Hg) at 50 °C and 50 rpm in an RE52 Rotavaporator set-up (Yamamoto, Tokyo, Japan). The fruit extract (1.7 ml/kg pulp) was sealed in a glass container and kept at −20 °C.

2.3. Fractionation by TLC

TLC fractions of durian extract (DE) and durian extract spiked with authentic diethyl disulfide (55 mM) (DE + DD) were developed in 0.33 mm Chromato sheets (2 cm × 7 cm) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) using water, 2-butanol and 28% ammonia (1:1:1) as solvent. Eight sheets each for DE and DE + DD were spotted with 10 μl sample (1 cm above the base) and developed in a chamber at 25 °C for 45 min until the solvent front reached 1 cm below the tip of the sheets. Chromatograms were dried in a fumehood at 25 °C, trimmed (0.5 cm base and top), cut into six equal parts (where *Rf* 0 encloses the spot; *Rf* 1 the solvent front). Each part was soaked with 150 μl 1 M Tris–HCl buffer, and centrifuged in Beckman GS-15R Centrifuge (Beckman Inst. Inc., Palo Alto, California) at 5000 rpm for 5 min, 10 °C. Fractions from the same *Rf* values of DE and DE + DD were pooled (≅1.2 ml/*Rf*), placed in ice bath and immediately used in inhibition assay.

2.4. yALDH assay

Enzymatic assay was monitored by following the rate of NADH accumulation indicated by the increase in A_{340} using a thermostat-equipped JASCO V-550 spectrophotometer (Tokyo, Japan) at 25 °C for 3 min (Sigma., 1996). A standard 3 ml final reaction mixture contained: 103 mM Tris–HCl buffer, pH 8.0; 0.67 mM β-NAD^+; 0.25–0.5 units yALDH solution (diluted in 100 mM Tris–HCl buffer with 0.02%(w/v) BSA); 100 mM-KCl; 10 mM 2-mercaptoethanol; 10 mM acetaldehyde; filled to volume with deionized water. Reactions were commenced by adding 0.10 ml β-NAD^+ to an otherwise complete reaction mixture.

Assay in test samples: authentic diethyl disulfide (1.1 ppm, final concentration); and filtered (PTFE 0.2 μm Millipore Millex-LG syringe-driven filter) durian extract (0.03, 0.07, 0.16, 0.33, 0.65, and 1.63 ppm, mg extract/l assay mix) and TLC fractions of DE and DE + DD (55 mM) followed the conditions described above except that reactions were commenced by adding a mix of the test sample and β-NAD^+.

Initial velocity studies in 0.33 ppm durian extract (mg/l assay mix) at 1, 2, 5, 10 and 50 mM acetaldehyde followed the reaction conditions described above. Computed kinetic parameters (i.e. K_m and V_{max}) were compared to that obtained without the extract at 2, 5, 7, 10, 30, and 50 mM acetaldehyde.

Determinations were done in triplicate *vis-à-vis* an almost identical solution (blank) that contains the enzyme diluent in *lieu* of the yALDH solution. Order of enzyme, substrate, cofactor and test sample addition was maintained in all enzymatic assay conducted. Results were subjected to a *t*-test ($P > 0.05$).

2.5. Sulphur test

Collected TLC fractions (300 μl) from the durian extract were mixed with 600 μl 20% (w/v) sodium formate solution and heated to liberate bound sulphur as hydrogen sulphide that manifests as dark discolorations in the lead acetate paper upon contact with tainted fumes (adopted from FAO (1996)).

3. Results and discussion

The durian–alcohol anecdote has been sheltered with over 250 years of belief. Few attempts to validate the interaction have vaguely disproved the alleged antagonism between durian and ethanol (Nilveses & Seangsirinavin, 1986; Ogle & Teh, 1969, 1971; Techapaitoon & Sim, 1973). None of these *in vivo* studies, however, considered the possible interference of some components in durian on the enzymatic system for detoxifying alcohol. To our knowledge, this is the first attempt to explore the molecular basis of the durian–alcohol anecdote and no other literature has yet linked a fruit to adverse reactions that may arise upon interaction with alcohol.

We used yALDH as a model protein because its active region is 95–100% homologous to that of the mammalian ALDHs (Saigal, Cunningham, Farres, & Weinar, 1991). Veverka et al. (1997) also used yALDH for studies on the mechanism of inhibition by disulfiram for the same reason.

yALDH significantly lost ($P < 0.05$) enzymatic activity in the presence of durian fruit extract at all concentrations used (Fig. 1, line graph). The intensity of inhibition increases with fruit extract concentration to a maximum of 70% at 0.33 ppm extract (final concentration). At higher concentrations (0.67 ppm and 1.67 ppm), the inhibitory effect weakened. Several studies reported the potent inhibitory action of sulphur compounds on ALDH (Brien & Loomis, 1985; Desager et al., 2002; Lindros et al., 1995; Park, Chung, Lee, Lee, & Kim, 2003). Similarly, the non-polar organic constituents (*Rf* 0.6, 0.8 and 1.0) of the durian fruit extract that also registered positive results in the sulphur-test elicited significant inhibitory effects on yALDH (Fig. 1, shaded bar graph). The degree of inhibition progressively declined from *Rf* 1.0 to *Rf* 0.4 with increasing polarity of these fractions. Veverka et al. (1997) reported a complete inhibition of yALDH activity upon treatment with 20 μM disulfiram by oxidising proximal cysteines in the active site to form a disulfide bridge. With this mechanism, they suggested that sterically suitable compounds containing a disulfide bridge may potentially inhibit ALDH. In durian, diethyl disulfide is the most abundant sulphur compound that contains a disulfide bridge (Laohakunjit, Kerdchoechuen, Matta, Silva, & Holmes, 2007; Voon et al., 2007a). Assay in 1.1 ppm authentic diethyl disulfide (final concentration) lowered the activity of yALDH by 81.5%. The contribution of diethyl disulfide to the durian extract-elicited inhibition of yALDH activity is hereby supported.

Authentic diethyl disulfide added to the durian fruit extract (55 mM) and subjected to planar TLC fractionation enhanced the inhibition elicited by the fraction with a calculated *Rf* value of 0.8 (Fig. 1, unshaded bar graph). The possible involvement of other non-polar sulfury constituents of durian to the observed inhibition, as our results imply, merits further investigation since the result-

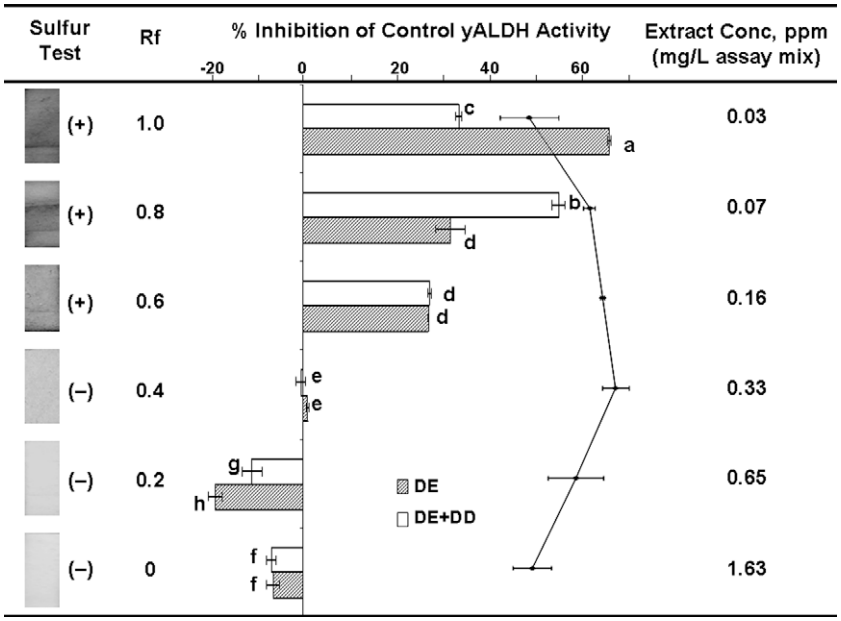

Fig. 1. yALDH inhibition by durian extracts (line graph), non-polar TLC fractions of durian extracts (DE, shaded bar graph) that yielded positive on the sulphur test (*Rf* 0.6, 0.8, and 1.0), and non-polar fractions of durian extract added with diethyl disulfide (DE + DD, unshaded bar graph). Values are reported as% inhibition of "control" yALDH activity, which was determined in the absence of any inhibitor. Data are means ± sd of three replicates. Graphs with similar letters indicate insignificant differences ($P > 0.05$).

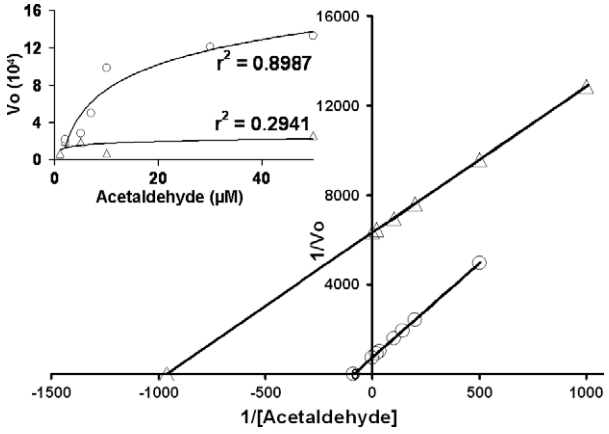

Fig. 2. Double reciprocal plot of V_0 vs. [Acetaldehyde] in 0.33 ppm durian fruit extract (Δ) and control (○) (regressed values). *Insert*, plot of V_0 as a function of [Acetaldehyde].

ing fractions from TLC may be broad and the contents of *Rf* 0.8 may have spread out to adjoining fractions.

Addition of the contents of *Rf* 0 and 0.2 enhanced yALDH activity (Fig. 1). Consistent with the fading inhibitory effect of durian extract at concentrations beyond 0.33 ppm earlier observed, enhanced yALDH activity in these polar fractions may have resulted from the increase in substrate concentration contributed by the endogenous aldehyde content of the durian fruit (Voon et al., 2007a). Fractions obtained from *Rf* 0.4 elicited insignificant alteration ($P > 0.05$) of the enzyme's activity.

Assay in 0.33 ppm crude extract lowered both the K_m and V_{max} values of yALDH (Fig. 2). Increasing the substrate concentration did not alter the activity of the enzyme as suggested by the low coefficient of determination ($r^2 = 0.2941$) (Fig. 2 insert). Based on the kinetic parameters, components of the durian fruit extract appear to elicit a mixed type of inhibitory action on yALDH. However, as this study involved the use of crude extract, further investigation of the inhibitory constituents is required to provide a conclusive characterisation of the mechanism of inhibition.

4. Conclusion

This study provides evidence that some component(s) of durian fruit inhibit(s) yALDH *in vitro*. Inhibitory action of the non-polar sulfury constituents of the fruit like diethyl disulfide on yALDH, with further investigation, offers a novel rationale to the etiology of the durian–alcohol anecdote. As such, work on this theme is currently undertaken to confirm the bioactive component(s) in the durian fruit, and validate the consistency of the findings hereby reported in mammalian models *in vivo*.

An important implication of studies along this line lies in strengthening science-based food safety precautions serving as impetus for issuing reliable toxicological disclaimer on the durian–alcohol interaction in a similar fashion to that of the watercress–alcohol. Such information should be useful to health care professionals or even the general consumer to avoid risky food–beverage interactions.

Acknowledgements

Gratitude is hereby extended to Dr. Alex Buchanan for his seminal contribution in developing the perspective of this research; Dr. Norie Anne B. Nolasco, Department of Food Science and Nutrition, College of Home Economics, University of the Philippines, for her masterful critiquing; Dr. Custer C. Deocaris, Japan National Institute for Advanced Industrial Science and Technology, for insights on the paper.

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Food Chemistry 117 (2009) 356–363

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Development of a reversed-phase high performance liquid chromatography (RP-HPLC) procedure for the simultaneous determination of phenolic compounds in peanut skin extracts

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ARTICLE INFO

Article history:
Received 1 August 2008
Received in revised form 18 March 2009
Accepted 29 March 2009

Keywords:
Reverse-phase
HPLC
Peanut skin
Phenolic acids
Stilbenes
Flavonoids

ABSTRACT

A reversed-phase high performance liquid chromatography (RP-HPLC) procedure was developed for simultaneous determination of five phenolic acids, two stilbenes and eight flavonoids in peanut skin extract. A C_{18} column fitted with diode array detection at 250 and 320, 280 and 370, and 306 nm for phenolic acids, flavonoids and stilbenes, respectively, with mobile phase consisting of 0.1% formic acid in water and 0.1% formic acid in 100% acetonitrile. Phenolic compounds were eluted with good resolution (Rs > 1.5) within 95 min as follows: gallic, protocatechuic, epigallocatechin, catechin, β-resorcylic (internal standard), caffeic, procyanidin B_2, epicatechin, epigallocatechin gallate, *p*-coumaric, ferulic, piceid, epicatechin gallate, catechin gallate, resveratrol and quercetin. The variation in recovery and reproducibility in peak area was <11 and <2.5%, respectively. The correlation coefficients, *r*, of calibration curves of the 15 compounds were >0.999. The method was used to quantify phenolic compounds in peanut skin extracts from Runner, Virginia and Spanish peanuts.

1. Introduction

A report identified peanut skins as one of the important sources of antioxidants with healthcare application that food manufacturer's can tap into (http://www.kaloramainformation.com/Healthcare-Opportunities-Food-1468551/). Peanut skins have a pink-red colour and an astringent mouth feel when consumed. They are typically removed before peanut consumption or inclusion in confectionary and snack products. In the early 1940's, these were initially thought to be toxic. But after a thorough examination by Dr. Jack Masquelier, then a doctoral candidate at the Faculty of Medicine and Pharmacy, University of Bordeaux, in France, peanut skin was found nontoxic, and protects and strengthens blood vessels (Louis, 1999). The colourless extract obtained was named OPC, oligomer proanthocyanidins. Peanut skins are rich in phenolic compounds and potentially other health-promoting compounds. Seventeen percent by weight of peanut skins are proanthocyanidins, consisting of low and high molecular weight oligomers (Karchesy & Hemingway, 1986). Lou et al. (1999) made a comprehensive analysis of the water-soluble phenolic extract from peanut skins, resulting in six A-type proanthocyanidins, including procyanidins A_1 and A_2, and three newly found epicatechin oligomers. They isolated ten compounds from the water-soluble fraction of peanut skins, including eight flavonoids and two novel indole alkaloids, reported for the first time from a natural source (Lou, Yuan, Yamazaki, Sasaki, & Oka, 2001). In 2004, they isolated five oligomeric proanthocyanidins, B_2, B_3 and B_4 from the water-soluble fraction and two new polyphenols, epicatechin-(2-β-*O*-7,4-β-6)-[epicatechin-(4-β-8)]-catechin and epicatechin-(2-β-*O*-7,4-β-8)-[epicatechin-(4-β-8)]-catechin-(4-α)-epicatechin, based on their spectral data (Lou et al., 2004). Huang, Yen, Chang, Yen, and Duh (2003) isolated and identified the ethanolic extract fraction from peanut seed testa that showed the highest yield and marked antioxidant activity. Thin layer chromatographic separation of this fraction allowed the isolation of the antioxidant component in peanut seed testa which was identified as ethyl protocatechuate (3,4-dihydroxybenzoic acid ethyl ester). Other types of compounds isolated and identified in peanut skins include phenolic acids, flavonoids and stilbenes (Yu, Ahmedna, & Goktepe, 2005). Yu, Ahmedna, Goktepe, and Dai (2006) identified and quantified catechins, procyanidin dimers, trimers and tetramers using reversed-phase high performance liquid chromatography (RP-HPLC). These compounds were detected in chemically purified peanut skin aqueous and ethanol extracts. Furthermore, higher concentrations of compounds mentioned were observed in raw peanut skins than roasted peanut skins. Caffeic acid, chlorogenic acid, ellagic acid and resveratrol were identified but not quantified due to very small

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doi:10.1016/j.foodchem.2009.03.110

peaks, as a result of suppression by major procyanidin peaks. Resveratrol was found to be present at higher levels in the seedcoat than the peanut kernel. Seed coats from Runner and Virginia types contained approximately 0.65 μg/g of seed coat, which is equivalent to <0.04 μg/seed (Sanders, McMichael, & Hendrix, 2000). Ethanolic extract prepared from defatted peanut skins even showed higher resveratrol contents. The ethanolic extract contained 91.4 μg/g while the dry peanut skins contain 9.07 μg/g (Nepote, Grosso, & Guzman, 2004).

Reverse-phase chromatography is the most popular mode of analytical liquid chromatography for phenolic compounds. A RP-HPLC method for food phenolics requires optimising a wide variety of mobile-phase conditions (ionic strength, pH, ion pair, organic modifier, etc.) and column parameters (Lee, 2000). Columns range from 100 to 300 mm in length and usually with a 4.6 mm internal diameter. The most often used columns have been packed with C_{18} column material. Elution systems are usually binary with an aqueous acidified polar solvent such as aqueous acetic acid, perchloric acid, phosphoric acid, or formic acid (solvent A) and a less polar organic solvent such as methanol or acetonitrile, possibly acidified (solvent B). The greatest alteration observed in the mobile phase was the type of acid used as the modifier to minimise peak tailing (Merken & Beecher, 2000). The pH range most often used for RP-HPLC for phenolics is low, between 2 and 4. The pH and ionic strength of the mobile phase are known to influence the retention of phenolics on the column, depending on whether there is protonation, dissociation, or a partial dissociation (Lee, 2000). HPLC runs are generally an hour maximum, with equilibration between runs (Merken & Beecher, 2000).

Phenols absorb in the ultraviolet (UV) region. Most of the phenolic acids displayed their maxima at 246–262 nm with a shoulder at 290–315 nm (Lee, 2000). Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyrane rings A, B, and C. Two absorption bands are characteristic of flavonoids. Band II, with a maximum in the 240–285 nm range, is believed to arise from the A ring. Band I with a maximum in the 300–550 nm range, presumably arises from the B-ring (Merken & Beecher, 2000). Food phenolics are commonly detected using UV–vis and photodiode array (DAD) detectors.

Several hundred papers on the HPLC of phenolic compounds have been published in the past 20 years, yet HPLC of phenolic compounds can detect across one, two or perhaps three classes or subclasses in one analysis. Foods may contain several classes of phenolic compounds (Merken & Beecher, 2000). Phenolic extracts are always a mixture of different classes of phenolics that are soluble in the solvent system used (Naczk & Shahidi, 2004). Due to the large number, and the structural variations in closely related food phenolic compounds, analytical procedures for the analysis of individual phenolic compounds have been relatively difficult and complicated (Lee, 2000). The ideal profiling method should be as simple as possible, should detect all the compounds present, should provide as much information as possible for each peak in the chromatogram for the purpose of identification, structural evaluation, and quantification, and should accomplish all this in a single chromatographic run (Harnly, Bhagwat, & Lin, 2007). A review of some examples of profiling methods for the last 15 years is discussed by Harnly et al. (2007). With the increasing interest in peanut skins as a rich source of antioxidants, it is imperative to accurately determine and quantify as many phenolic compounds to answer the demand for timely information on health-promoting compounds present in peanut skins. The objective of this study was to develop a RP-HPLC method using DAD for the identification and quantification of phenolic compounds in peanut skin extracts.

2. Materials and methods

2.1. Chemicals and materials

Standards including gallic acid, quercetin, catechin, (−)-catechin gallate, (−)-epicatechin gallate, (−)-epigallocatechin, procyanidin B_2, and β-resorcylic acid (internal standard, IS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Resveratrol, (−)-epigallocatechin gallate, caffeic acid, *p*-coumaric acid, protocatechuic acid, ferulic acid, and (−)-epicatechin were purchased from Fisher Scientific (Atlanta, GA, USA). Methanol, acetonitrile and formic acid were purchased from VWR (West Chester, PA, USA).

2.1.1. Standard solutions for quantifying phenolic compounds

Stock solutions of gallic acid (1000 ppm), quercetin (1000 ppm), catechin (1000 ppm), (−)-catechin gallate (2000 ppm), (−)-epicatechin gallate (2000 ppm), (−)-epigallocatechin (2000 ppm), procyanidin B_2 (2000 ppm), β-resorcylic acid (1000 ppm), resveratrol (1000 ppm), (−)-epigallocatechin gallate (1000 ppm), caffeic acid (1000 ppm), *p*-coumaric acid (1000 ppm), protocatechuic acid (1000 ppm), ferulic acid (1000 ppm) and (−)-epicatechin (1000 ppm) were prepared by dissolving the compounds separately in methanol, and stored in 2 oz. amber bottles (VWR West Chester, PA, USA) at −15 °C.

2.2. Preparation of peanut skin extract

Raw peanuts (2004 crop year) were purchased from Golden Peanut Company (GA). Runner medium (Georgia Green) were harvested in Dawson, GA; Virginia medium in Aulander, NC; and No. 1 Spanish in Anadarko, TX. All peanut bags were stored for 1 month under refrigerated storage in a cold room at 4 °C prior to analysis. Raw, peanuts were manually sorted to remove foreign material or damaged kernels. Kernels were manually peeled and skins were packaged in 12.7 × 16.5 cm polyethylene bags (Koch Supplies, N. Kansas City, MO, USA) flushed with nitrogen gas (medical grade, Air Products and Chemicals, Inc., Allentown, PA, USA), immediately heat-sealed (Thermal Impulse Heat Sealer, Model 14C/CAB, Vertrod Corp., USA) then stored in a walk-in freezer at −15 °C until used in HPLC analyses.

The extraction of phenolic compounds in peanut skin was carried out according to the method published by Nepote, Grosso, and Guzman (2005). Briefly, 20 mL of 70% ethanol was added to a flask containing 1 g of peanut skins. The flasks were shaken for 10 min using a Wrist Action Shaker (Model 75, Burrell Corp., PA, USA) at ambient temperature. The extract was filtered through a Whatman no. 1 filter paper and the residue was extracted again under the same conditions, for a total of three extractions.

All flasks were wrapped with aluminium foil to prevent light degradation during extraction as done by Yu et al. (2005). The combined filtrate was transferred into an evaporating flask and placed in a water bath (HB4 basic, IKA, Fisher Scientific Co., Fair Lawn, NJ, USA) set at 40 °C according to Duh and Yen (1997) for drying extracts from peanut hulls. The extract was evaporated under vacuum for 15 min in a rotary evaporator (RV05 Basic 1B, IKA, Fisher Scientific Co., Fair Lawn, NJ, USA) to a concentrated extract of about 5 mL. The extract was transferred to a plastic cup (No. P325, Solo Cup Co., Urbana, IL, USA) and the evaporating flask was washed with approximately 2 mL of deionised water which was combined with the extract in the cup. Sample cups were sealed with aluminium foil and frozen overnight in a walk-in freezer at −15 °C. The frozen mixture was then freeze-dried (Virtis Genesis 25ES, SP Industries Inc., Gardiner, NY, USA) at <100 mTorr vacuum for 24 h. Dried extracts were scraped from the cups and packaged in

8-mL vials wrapped with aluminium foil, and stored in a freezer at −15 °C until used.

2.3. HPLC analysis

Analysis was performed using a ProStar HPLC system (Varian Inc., Walnut Creek, CA, USA) comprised of a 210 Binary Pump, 410 AutoSampler and 335 DAD. The Star Work Station Software version 6.41 was used to control the autosampler, gradient settings, DAD and data acquisition. A C_{18} reverse-phase column, 4.6 × 250 mm, 5 μm particle size (Eclipse Plus, Agilent Technologies, Wilmington, DE, USA) was used. Preceding the analytical column was a C_{18} guard column (4.6 × 12.5 mm, 5 μm particle size, Eclipse Plus, Agilent Technologies, Wilmington, DE, USA), used to prevent any non-soluble residues from samples from contaminating the column. Peak areas were determined at 250 nm for the benzoic acid derivatives; 280 nm for the flavanols; 306 nm for stilbenes; 320 nm for the cinnamic acid derivatives; and 370 nm for flavonols.

2.3.1. Determination of solvent composition

The mobile phase consisted of 0.1% formic acid in filtered deionised water as solvent A and 0.1% formic acid in 100% acetonitrile as solvent B (Yu et al., 2006). To determine the gradient conditions the method of Snyder, Kirkland, and Glajch (1997) was used. Initial and final composition of A and B solvents were determined using exploratory gradient elution.

2.3.1.1. Exploratory gradient elution. The first phase involved increasing solvent B linearly from 5% to 100% over 60 min (gradient steepness, GS = 1.6%) with a flow rate of 2.0 mL/min, at room temperature (Snyder et al., 1997). Experimental peanut skin extracts from Runner, Virginia and Spanish skins were dissolved each in aqueous solvent at 300 ppm and injected at 20 μL. All analyses were conducted in duplicate. The retention times (t_R) of the first and last peaks observed in the chromatogram from the peanut skin samples were used to calculate the initial and final concentrations of solvent B using a tabular value on estimating the initial and final % B for gradient elution, based on retention time for first and last band in the initial gradient run, published by Snyder et al. (1997). In the second phase, the same conditions were used except for the initial and final gradient composition of solvent B which was adjusted to reflect the concentrations determined in the first phase. Total run time was 60 min with a flow rate of 1.5 mL/min.

2.3.1.2. Determination of the effect of gradient steepness and flow rate. The second experiment was carried out by examining the effect of different gradient steepness (GS) and flow rates on band spacing. A total of nine conditions, including three GS (1.6%, 1.3% and 1.0%) and three flow rates (1.5, 1.0 and 0.8 mL/min), using Runner skin extracts injected at 20 μL, were tested to determine the effect on resolution of peaks. Resulting chromatograms were evaluated for adequate resolution of peaks.

2.3.1.3. Improvement by segmentation of the gradient elution. The chromatograms from peanut skin extract obtained were rather complex in nature, thus a series of segmented gradients were conducted to enable the separation of the unresolved peaks. A solution of standards at 20 ppm was injected at 20 μL and resolutions and retention times were determined. Three segments were incorporated into the gradient elution to obtain a calculated resolution (Rs) >1.5 for all 16 compounds. When all compounds were eluted with sufficient resolution, a Runner peanut skin extract was spiked with the compounds to check that proper identification of the standards can be obtained. If standard peaks were being suppressed by peaks from peanut skins, adjustments were made in the gradient elution until all 16 compounds were resolved in the sample extract. Absorption spectra of the 16 standards were also obtained for reference when identifying phenolic compounds.

2.4. HPLC sample preparation

Preparation of peanut skin extracts was modified to remove sugar, protein and lipid from the matrix as suggested by Yu et al. (2005). The sample preparation of Nepote et al. (2004) employing liquid–liquid partitioning was used in the preparation of the purified extract. An internal standard, β-resorcylic acid, was used as recommended for HPLC samples requiring significant pretreatment or preparation before HPLC analysis (Snyder et al., 1997). This compound was not reported to be present in peanut skins. The freeze-dried peanut skin extract (20–25 mg) was weighed and 3 mL of dichloromethane (DCM), 2 mL of filtered deionised water and 1 mL of internal standard solution (20 ppm) were added. After mixing, partitioning and phase separation, the organic phase (DCM) was discarded. Water layer was then mixed with 5 mL ethyl acetate. After phase separation, the ethyl acetate layer was collected and evaporated in a rotary evaporator (RV05 Basic 1B, IKA, Fisher Scientific Co., Fair Lawn, NJ, USA) at 35 °C under reduced pressure. The dried phenolics were re-dissolved in 2 mL methanol and evaporated to dryness under nitrogen gas, re-dissolved in 1 mL 15% methanol before HPLC injection at 20 μL. The entire sample procedure was conducted under yellow light to prevent degradation.

2.5. HPLC method validation

After the RP-HPLC gradient conditions were determined, validation tests were performed for accuracy, precision, linearity, range and limit of detection and quantification.

2.5.1. Determination of accuracy

A stock standard solution containing 20 ppm of epigallocatechin, catechin and IS; 10 ppm of procyanidin B_2, epicatechin, epigallocatechin gallate, epicatechin gallate and catechin gallate; and 4 ppm of gallic acid, protocatechuic acid, caffeic acid, *p*-coumaric acid, ferulic acid, piceid, resveratrol and quercetin was prepared. Determination of accuracy was done by recovery method (Snyder et al., 1997), where peanut skin extracts (Runner type) were spiked by adding 50%, 75% and 100% of stock standard solution containing the 16 compounds, which where compared with peanut skin extracts without standards. Calculations were adjusted for protocatechuic acid, epigallocatechin, catechin, epicatechin, resveratrol, *p*-coumaric acid and quercetin, to account for its natural concentration, determined in peanut skin extracts not containing added phenolic compounds. Peak area was used for quantification purposes, using internal standard calibration using the equation of Macrae (1988)

$$\text{Amount of } i = \frac{\text{abs. RF}(i) \times A(i)}{\text{abs. RF(IS)} \times A(\text{IS})} \times M(\text{IS})$$

where abs. RF is the absolute response factor; *M* is the mass of component; *A* is the area under its peak; *i* is the phenolic compound; IS is the internal standard. Peanut skin extracts spiked with standards were injected at a volume of 20 μL in triplicate. Recovery of the 15 compounds was calculated using the equation

$$\text{Accuracy} = \% \text{ recovery} = \frac{\text{Actual concentration of analyte}}{\text{Theoretical concentration of analyte}} \times 100$$

For each level of concentration three replications were performed resulting in 12 analyses including control extract.

2.5.2. Determination of precision

To evaluate precision, a solution of the standards containing 20 ppm of epigallocatechin, catechin and IS; 10 ppm of procyanidin B_2, epicatechin, epigallocatechin gallate, epicatechin gallate and catechin gallate; and 4 ppm of gallic acid, protocatechuic acid, caffeic acid, *p*-coumaric acid, ferulic acid, piceid, resveratrol and quercetin was injected at 20 μL for a total of eight times. The precision of the method was expressed by coefficient of variation (% CV) of the data set. The mean peak area and t_R values for each compound were determined.

2.5.3. Determination of linearity and range

An intermediary mixed standard solution was prepared by dilution in filtered deionised water of the stock standard solution to give a concentration of 20 ppm for epigallocatechin, catechin and IS; 10 ppm for procyanidin B_2, epicatechin, epigallocatechin gallate, epicatechin gallate and catechin gallate; and 4 ppm for gallic acid, protocatechuic acid, caffeic acid, *p*-coumaric acid, ferulic acid, piceid, resveratrol and quercetin; from which a calibration working solutions with concentrations in the range of 4–20, 2–10 and 0.8–4 ppm, respectively, were prepared in triplicate and evaluated by injecting 20 μL. Calibration curve for each compound was constructed separately by plotting peak areas (*y*-axis) versus concentrations (*x*-axis). Using statistical analysis software (version 8.1, SAS Institute Inc., Cary, NC, USA) regression analysis (PROC REG) was used to relate each standard concentration as the dependent variable with peak area as the independent variable. Pearson's product correlation coefficients (*r*) between peak area and the concentration of each compound were calculated.

2.5.4. Determination of limit of detection and quantification

Limits of detection and quantification were calculated based on the standard deviation of the response and the slope (ICH, 1998). Calculated amounts per compound were prepared and standard mixture was injected in duplicate to verify the LOD and LOQ of each compound. The detection limit was expressed as

$$\mathrm{LOD} = \frac{3.3\sigma}{S}$$

where σ is the standard deviation of the response, S is the slope of the calibration curve.

The quantification limit was expressed as

$$\mathrm{LOQ} = \frac{10\sigma}{S}$$

2.6. Identification and quantification of phenolic compounds in peanut skin extracts

Phenolic compounds in Runner, Virginia and Spanish skins (raw) were identified on the basis of the retention times and UV–vis spectra of phenolic compounds analysed. Peak area was used for quantification purposes, using internal standard calibration. Peanut skin extracts were injected at a volume of 20 μL in duplicate. Values were reported as μg/g dry peanut skin.

3. Results and discussion

3.1. Determination of solvent composition

3.1.1. Exploratory gradient elution

From the five wavelengths monitored, the earliest and last peaks were observed at 280 nm as shown in Fig. 1a. The t_R of the first and last peaks observed in the chromatograms slightly differed for each peanut skin type as shown in Fig. 1a. For all peanut skin types, the t_R of the initial and final peaks were approximately

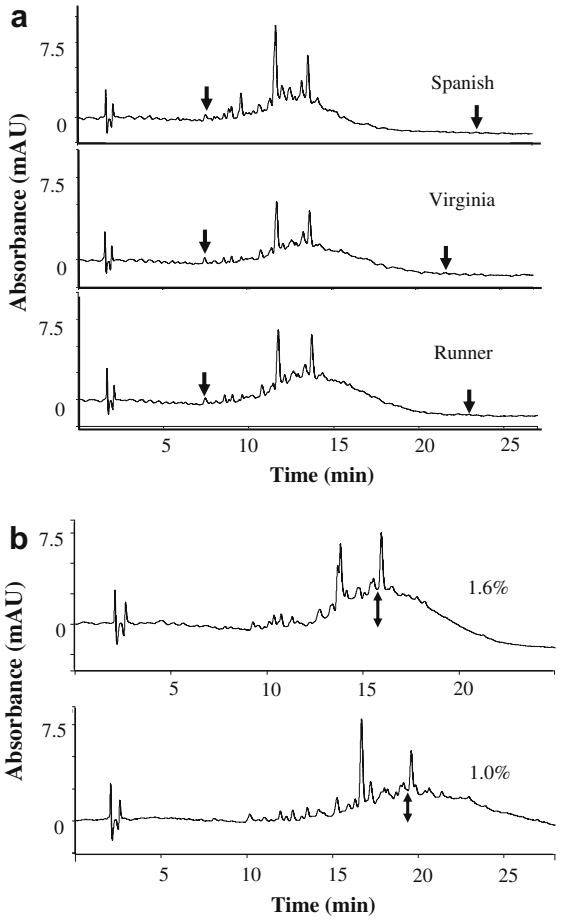

Fig. 1. (a) Determination of a gradient solvent composition for RP-HPLC analysis of phenolic compounds in peanut skin extract at 280 nm, with a linear gradient increasing solvent B (0.1% formic acid in acetonitrile) in solvent A (0.1% formic acid) at a flow rate of 2.0 mL/min, and analysis time of 60 min. Arrows show the first and last peak detected in Runner, Virginia and Spanish skin extracts. (b) Effect of RP-HPLC gradient steepness (GS) of 1.6% and 1%, on analysis of phenolic compounds in Runner peanut skin extract at 280 nm. Elution of peaks from analysis with a linear gradient increasing solvent B (0.1% formic acid in acetonitrile) from 5% to 43% at 1.5 mL/min. Arrows show the difference in baseline drifts from the unresolved 'hump' detected in the sample.

7 and 23 min, respectively. Between the first and last peaks, a non-separated 'hump' (detected as an elevation in the baseline) was present for all types. The 'hump' is a characteristic chromatogram of flavanoids, specifically procyanidins (Lazarus, Adamson, Hammerstone, & Schmitz, 1999). Peanut skins contain a complex series of procyanidin oligomers. Mass spectral data of peanut skins obtained by Lazarus et al. (1999) indicated presence of several singly and doubly-linked procyanidin oligomers through octamers in peanut skins. Lazarus et al. (1999) observed that although RP-HPLC has the ability to separate oligomers, analysis is not feasible due to retention time overlap of isomers from procyanidins causing the oligomers to co-elute as a large unresolved peak, as initially observed in this study. In other foods containing procyanidins, Adamson et al. (1999), Karonen et al. (2007), Salminen et al. (2005), and Ndhlala et al. (2007) obtained the typical unresolved peaks for procyanidin polymers, resulting in a recommendation to use normal phase HPLC to analyse these compounds.

Snyder et al. (1997) suggested that the initial composition of water (solvent A) should start at 95% or higher in order to minimise exposure of the column to high water conditions. Thus, the recommended initial solvent B composition was maintained at 5%. The fi-

nal composition of 43% solvent B was calculated from a t_R of 23 min, based on the tabular value of Snyder et al. (1997).

3.1.2. Determination of the effect of gradient steepness and flow rate

The unresolved 'hump' discussed previously was observed in all gradient steepness and flow rate combination tested. The 'hump' was observed to occur at solvent B composition of 7–17%, regardless of GS and flow rate conditions. Significant decrease in the elevation of the 'hump' was observed as GS decreased from 1.6% to 1% at a high flow rate of 1.5 mL/min as shown in Fig. 1b. Decrease in GS likewise resulted in increased resolution of peaks within the 'hump'. Adequate separation of peaks can therefore be obtained at reduced GS. Since the 'hump' occurs at a specific gradient range of 7–17% solvent B, use of nonlinear gradients as a means to improve the separation of peaks is suggested by Snyder et al. (1997).

3.1.3. Improvement by segmentation of the gradient elution

Table 1 lists the optimum instrumental parameter values for the chromatographic determination of phenolic acids, flavonoids and stilbenes. Three segments of the gradient elutions were incorporated into the final method to resolve the peaks. The first segment had a GS of 0.28%, with solvent B increasing from 5% to 7% in 7 min. This segment allowed the elution of the more polar compounds, the benzoic acid derivatives. The second segment, which were mostly flavanols had a 0.15% GS, with solvent B increasing from 7% to 17% in 68 min. At this GS, the peaks within the previously observed 'hump' were now adequately resolved and exhibited a normal baseline. The third segment had a GS of 0.86% to elute the less polar compounds. All compounds were eluted in 95 min. The use of the three segments allows quantification of polymeric procyanidins simultaneously with other phenolic compounds using RP-HPLC. Whereas previously, these compounds had to be run separately using normal HPLC.

During consecutive RP-HPLC runs, changes in retention time occurred after every sample. To minimise this, an additional run time of 7 min was added to the end of the analysis to flush the system by increasing solvent B to 100%. Another 7 min was incorporated to recondition the column and bring down solvent B to 5%. This new HPLC method enables detection of all phenolic compounds as distinct peaks with good resolution (Rs > 1.5). Fig. 2 shows the profile of 15 compounds and internal standard (IS) used for identifying and quantifying phenolic compounds in peanut skin extracts. Phenolic acids (compounds no. 1, gallic acid; no. 2, protocatechuic acid; no. 12, caffeic acid; no. 13, *p*-coumaric acid; no. 14, ferulic acid) were eluted with retention times between 4 and 45 min; flavonoids (compounds no. 3, epigallocatechin; no. 4, catechin; no. 5, procyanidin B2; no. 6, epicatechin; no. 7, epigallocatechin gallate; no. 8, epicatechin gallate; no. 9, catechin gallate) between 15 and 92 min; and stilbenes (compounds no. 10, piceid; no. 11, resveratrol) between 50 and 84 min. The specific retention times and the maximum UV band based on spectral data using diode array detector for the 16 compounds is shown in Table 2.

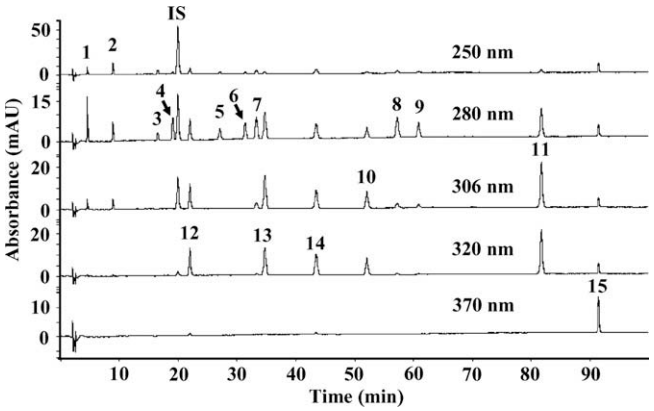

Fig. 2. HPLC profile for phenolic compounds (mAU vs. time) at different wavelengths. Number shows the following standard chemicals: 1 = gallic acid; 2 = protocatechuic acid; 3 = epigallocatechin; 4 = (+)-catechin; 5 = procyanidin B_2; 6 = (−)-epicatechin; 7 = (−)-epigallocatechin gallate; 8 = (−)-epicatechin gallate; 9 = (−)-catechin gallate; 10 = piceid; 11 = trans-resveratrol; 12 = caffeic acid; 13 = *p*-coumaric acid; 14 = ferulic acid; and 15 = quercetin.

3.2. HPLC method validation

3.2.1. Determination of accuracy

Recovery of peanut skin extracts containing all 15 compounds ranged from 58% to 119% as shown in Table 2. Recommended average recovery of an analyte for an active drug substance in a pharmaceutical formulation ranges from 99% to 101% (Snyder et al., 1997). Since the phenolic compounds were added to peanut skin extracts, other components in the sample matrix may have interfered with the accurate quantification of the analytes, thus, giving <99% recovery values for some of the compounds assayed in this

Table 1
Chromatographic conditions for the determination of phenolic compounds in peanut skin extracts.

Chromatographic conditions	
Injection volume	20 μL
Guard column	C_{18} guard column, 4.6 × 12.5 mm, 5 μm particle size (Eclipse Plus, Agilent Technologies, Wilmington, DE)
Analytical column	C_{18} reverse-phase column, 4.6 × 250 mm, 5 μm particle size (Eclipse Plus, Agilent Technologies, Wilmington, DE)
Mobile phase	A (formic acid in water, 0.1% v/v) B (formic acid in acetonitrile, 0.1% v/v) *First segment:* 0–7 min: 5–7% B *Second segment:* 7–75 min: 7–17% B *Third segment:* 75–110 min: 17–45% B *Flushing step:* 110–117 min: 45–100% B *Conditioning step:* 117–124 min: 100–5% B
Flow rate	1.5 mL/min
Temperature	Ambient temperature
Detection conditions	
Scanning	200–400 nm
Scan rate	10 Hz
Detection wavelength	Benzoic acid derivatives: 250 nm Cinnamic acid derivatives: 320 nm Flavanols: 280 nm Flavonols: 370 nm Stilbenes: 306 nm

Table 2
Validation parameters for the determination of phenolic compounds in peanut skin extracts.

Peak no.	Phenolic compounds	Retention time (min)	Maximum UV band (nm)	Recovery[A] (%)	Precision CV[B] (%)	LOD[C] (ppm)	LOQ[D] (ppm)	r^{E}	Linear range (ppm)
1	Gallic acid	4.4	270	58 ± 5	1.98	0.2	0.5	0.9995	0.8–4
2	Protocatechuic acid	8.6	260; 293	98 ± 6	1.85	0.2	0.5	0.9991	0.8–4
3	Epigallocatechin	15.8	271	86 ± 9	2.11	0.6	1.8	0.9993	4–20
4	Catechin	18.3	279	86 ± 8	1.13	0.9	2.6	0.9991	4–20
5	Caffeic acid	21.3	323	119 ± 2	0.79	0.2	0.5	0.9991	0.8–4
6	Procyanidin B_2	26.1	279	93 ± 7	2.14	0.3	1.0	0.9991	2–10
7	Epicatechin	30.4	278	118 ± 7	1.98	0.2	0.6	0.9994	2–10
8	Epigallocatechin gallate	32.2	274	121 ± 8	1.91	0.4	1.2	0.9993	2–10
9	*p*-Coumaric acid	33.6	309	102 ± 2	0.69	0.1	0.2	0.9996	0.8–4
10	Ferulic acid	42.3	323	109 ± 3	0.68	0.1	0.4	0.9993	0.8–4
11	Piceid	50.8	320	116 ± 7	1.82	0.1	0.4	0.9991	0.8–4
12	Epicatechin gallate	56.0	277	119 ± 10	1.53	0.3	0.8	0.9995	2–10
13	Catechin gallate	59.6	278	114 ± 11	1.96	0.4	1.2	0.9994	2–10
14	Resveratrol	80.6	318	104 ± 4	0.97	0.1	0.2	0.9993	0.8–4
15	Quercetin	90.9	371	112 ± 3	1.59	0.2	0.5	0.9990	0.8–4

[A] % Recovery, $n = 9$.
[B] Coefficient of variation, $n = 8$.
[C] Limit of detection, LOD = (3.3 * standard error of estimate)/slope.
[D] Limit of quantification, LOQ = (10 * standard error of estimate)/slope.
[E] r, correlation coefficient $= \sqrt{R^2}$.

study. The lowest recovery of 58% was obtained for gallic acid. This value was lower than those found by Sakakibara, Honda, Nakagawa, Ashida, and Kanazawa (2003) and Garcia-Falcon, Peres-Lamela, Marinez-Carballo, and Simal-Gandara (2007) of 87% and 79%, respectively, on the recovery of polyphenol standards in radish root and wines, respectively. The extraction procedure used in this study involved removal of lipids and lipid soluble compounds from the peanut skin extracts before extraction of polyphenolic compounds. Sakakibara et al. (2003) directly extracted the polyphenols in radish root using methanol as extracting solvent while Garcia-Falcon et al. (2007) employed acidic fractionation of wines to obtain hydroxybenzoic acids (including gallic acid). The differences in recovery values therefore may be attributed to the extraction procedures employed in these studies.

Resveratrol recovery in this study was 104% after accounting for its natural concentration in peanut skins. This value was higher than those found by Rudolf, Resurreccion, Saalia, and Phillips (2005) in peanuts, where a recovery range of 71–96% was obtained. Rudolf et al. (2005) used a clean-up column to remove interfering compounds that co-elute with resveratrol, which may have resulted in lower recovery. The obtained value in this study however, was comparable with those found by Sobolev and Cole (1999) where they obtained recoveries of 100–117% in fresh peanuts.

Recoveries for protocatechuic acid, quercetin and caffeic acid were 98%, 112% and 119% respectively. These values were higher than those found by Sakakibara et al. (2003) with obtained recoveries of 75%, 83% and 77% for these compounds using chalcone as internal standard. Their extraction procedure, as mentioned, was entirely different from those used in this study, thus, differences in values can be expected. The recovery calculation for catechin gallate was made at 306 nm instead of the recommended 280 nm (Sakakibara et al., 2003). Analysis of peak purity for catechin gallate when detected at 280 nm showed two different spectra, suggesting that another peak is partially co-eluting with it, resulting in an overestimated recovery value for this compound. But when measured throughout this study at 306 nm, only the peak for catechin gallate was detected.

3.2.2. Determination of precision

Calculated coefficient of variation (% CV) for the 15 compounds ($n = 8$) are presented in Table 2. Acceptable limits of 1–2% for compounds of low-level concentrations were suggested by Snyder et al. (1997). The low variance (0.68–2.14%) obtained in this study was considered to be sufficient to determine phenolic compounds in foods quantitatively. The CV range found for this paper was better compared to those reported in literature, 1.19–13.49% (Betes-Saura, Andres-Lacueva, & Lamuela-Raventos, 1996) and 1–9% (Garcia-Falcon et al., 2007) for simultaneous determination of 19 and 28 phenolic compounds, respectively, in wine.

3.2.3. Determination of linearity and range

Five-point standard curves were generated for each phenolic compound. The calibration plots of peak area vs. concentration of phenolic compound for each compound exhibited a straight line. Results of regression analysis are presented in Table 2. Calibration curves were linear over the concentration range studied. All Pearson's product correlation coefficients, r, were >0.999, indicating positive linear relationship between peak area and concentration. All r values reported were within the acceptable limits of $r > 0.999$ (Snyder et al., 1997).

3.2.4. Determination of limit of detection and quantification

Calculated limit of detection and limit of quantification are presented in Table 2. LOD ranged from 0.1 to 0.9, while LOQ varied from 0.2 to 2.6 ppm ratio. Values were comparable to the LOQs established for 28 phenolic compounds in wine ranging from 0.2 to 1 ppm, with LOD values of 3× lower than the LOQ (Garcia-Falcon et al., 2007).

3.3. Identification and quantification of phenolic compounds in peanut skin extracts

The developed RP-HPLC method was applied to raw peanut skin extracts and typical chromatograms showing compounds detected at 250, 280, 306, 320 and 370 nm are presented in Fig. 3. The three peanut types varied differently and significantly in terms of composition based on the compounds identified. Yu et al. (2005) identified three classes of phenolic compounds in raw peanut skins (type not identified): (1) phenolic acids including chlorogenic acid, caffeic acid and ferulic acid; (2) flavonoids including epigallocatechin, epicatechin, catechin gallate, epicatechin gallate; and (3) stilbenes (resveratrol). In their follow-up study, caffeic acid, chlor-

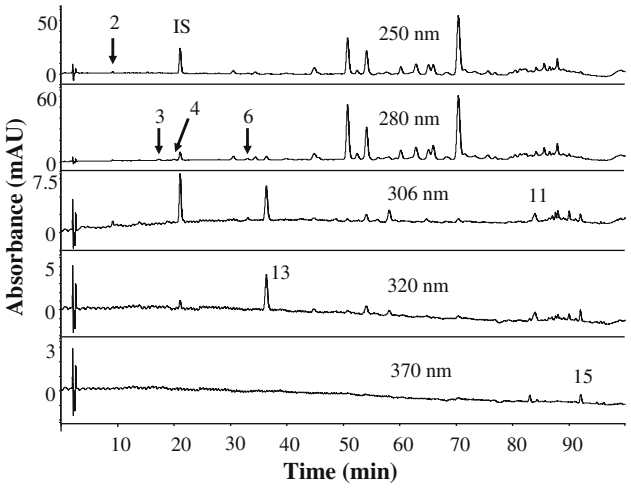

Fig. 3. HPLC profile of peanut skin extract from raw Runner peanuts (mAU vs. time) at different wavelengths. Peaks: 2 = protocatechuic acid; 3 = epigallocatechin; 4 = (+)-catechin; 6 = (−)-epicatechin; 11 = trans-resveratrol; 13 = *p*-coumaric acid; and 15 = quercetin.

ogenic acid, ellagic acid, resveratrol and its glycoside were identified but not quantified due to very low concentration and peaks were suppressed by major procyanidins.

3.3.1. Phenolic acids

Three phenolic acids namely: protocatechuic acid, caffeic acid and *p*-coumaric acid were identified and quantified from the peanut skin extracts (Table 3). Protocatechuic acid was significantly higher in Virginia (34.03 μg/g) followed by Spanish (15.45 μg/g) and Runner skins (7.62 μg/g). Previous studies showed that ethyl protocatechuate, a protocatechuic acid ethyl ester, was identified through fractionation in Spanish peanut skin ethanolic extracts (Huang et al., 2003; Yen, Chang, & Duh, 2005), but no amount was provided by the authors. Protocatechuic acid was identified and quantified using the HPLC method developed in this study with no further need for fractionation.

Caffeic acid was detected only in Spanish skins. This compound was detected by Yu et al. (2005) in peanut skins (peanut type not specified), but amount was not reported. *p*-Coumaric acid was highest in Runners (23.34 μg/g), followed by Spanish (12.31 μg/g) then Virginia (4.98 μg/g). *p*-Coumaric acid was not found by Yu et al. (2006) in peanut skins but it is predominantly present in peanut kernels as reported by Duncan, Gorbet, and Talcott (2006). One of the most prevalent antioxidant polyphenolics isolated from the water-soluble components of peanuts obtained by Duncan et al. (2006) was *p*-coumaric acid. Insignificant changes in *p*-coumaric concentration were observed after 8 weeks storage at 35 °C, but no amount was provided in their report.

Table 3
Mean values for phenolic compounds, calculated as μg/g dry skin, as measured by RP-HPLC, in raw peanut skin ethanol extracts.[A]

Compound	Runner	Virginia	Spanish
Protocatechuic acid	7.62c ± 1.34	34.03a ± 1.95	15.45b ± 0.87
Caffeic acid	nd	nd	3.49 ± 0.36
p-Coumaric acid	23.35a ± 0.91	4.98c ± 0.63	12.31b ± 1.42
Epigallocatechin	440.05b ± 16.70	1275.92a ± 77.10	1274.72a ± 67.50
Catechin	74.35c ± 13.14	535.03a ± 41.72	448.30b ± 36.47
Procyanidin B2	20.67b ± 5.63	17.69b ± 1.61	107.00a ± 18.99
Epicatechin	60.06c ± 11.44	144.75b ± 1.42	238.55a ± 9.20
Quercetin	20.14b ± 1.49	22.88b ± 2.92	27.99a ± 2.10
Resveratrol	4.30b ± 0.10	3.66b ± 0.44	15.04a ± 1.57

[A] Mean values for each compound in a row followed by the same letter are not significantly different ($\alpha = 0.05$) as determined by Fisher's least significant difference (LSD) mean separation test. Amounts were calculated as means of four analyses ± standard deviation. nd = not detected.

3.3.2. Flavonoids

Several peaks were observed at 280 nm (Fig. 3), indicating presence of flavanols, as shown by their spectral data (data not shown) having characteristic maximum wavelengths between 275 and 280 nm. Thirty to forty peaks were detected from the three peanut skin types at 280 nm. Similar findings were observed by Yu et al. (2006) where two monomer peaks and 39 other polymer peaks (dimers, trimers, tetramers) at 280 nm where detected by LC–MS. A small hump is still present at 75–90 min of runtime for all three peanut skin types. However, the compounds of interest are not found within this region.

Virginia and Spanish peanut skins had significantly more epigallocatechin than Runner skins (Table 3). In terms of total flavanol content (calculated as the sum of epigallocatechin, catechin and epicatechin), Spanish skins had 1962 μg/g; Virginia had 1956 μg/g and only 574 μg/g for Runners. All values were higher than those found by Yu et al. (2006) of 161 μg/g dry skin (peanut type not specified) for total procyanidin monomers. Differences in peanut skin type, sample extraction, preparation and analytical method may have contributed to the large discrepancy in values. Lazarus et al. (1999) also identified procyanidins (monomers to octamers) in peanut skin extracts, but these were not quantified. The dimer procyanidin B_2 was present at very low levels in Runner and Virginia skins compared to Spanish skins. As observed by Yu et al. (2006), directly peeled peanut skin contained mostly procyanidin trimers and tetramers. Higher forms of procyanidins may be present in raw peanut skins. As peanut skins were roasted, a considerable amount of trimers and tetramers were lost due to degradation (Yu et al., 2006).

The flavonol quercetin was highest in Spanish peanut skin. Other flavonols with peaks exhibiting characteristic absorption spectra similar to those of quercetin were also detected in Spanish skins. Quercetin was not found by Yu et al. (2006) in peanut skin extracts, while quercetin derivatives were identified by Daigle, Conkerton, Hammons, and Branch (1975) on selected white testa peanuts.

3.3.3. Stilbenes

Resveratrol but no piceid was detected in all peanut skin types. Resveratrol content was highest in Spanish skins (15.04 μg/g) followed by Runners and Virginia skins (4.30 and 3.66 μg/g, respectively). The amount present in Runners was lower compared to those reported by Nepote et al. (2004) of 9 μg/g peanut skins (Florunners), obtained from a blanching process and extracted with ethanol. The authors however had to fractionate the ethanol extract due to very low concentration of resveratrol. Sanders et al. (2000) also reported that seed coats from Runner and Virginia types (raw) contained ~0.65 μg/g of seed coat.

4. Conclusions

A RP-HPLC method for identification and quantification of 15 compounds, including phenolic acids, flavonoids and stilbenes from peanut skin extracts was developed and validated in this study. The gradient verified in this paper can be successfully applied to a reverse-phase HPLC system with a C_{18} column (4.6 × 250 i.d. mm, 5 μm particle size), with DAD detection and mobile phase consisting of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. A maximum of nine of 15 phenolic compounds were identified and quantified in raw peanut skins. These were successfully quantified using β-resorcylic acid as an internal

standard using the HPLC reverse-phase gradient elution established in this study. The method detected the natural forms in peanut skin extracts directly without need for hydrolysis. Thus, the present method can determine quantitatively individual classes of flavonoids, phenolic acids and stilbenes.

Previous study by Francisco and Resurreccion (2009) showed that heat-treated peanut skins had higher total phenolics and antioxidant capacity than raw peanut skins. Similar observation was reported by Yu et al. (2005) when roasted peanut skins were compared to raw and water-blanched skins. An investigation on the quality and quantity of phenolic compounds in heat-treated skins is currently being conducted using this new method to determine and quantify health-promoting compounds present in peanut skins.

Acknowledgements

This project was supported in part by the Peanut Collaborative Research Project Grant No. LAG-G-00-96-00013-00 and the National Research Initiative Grant No. 2006-3550-316989 from the USDA Cooperative State Research, Education, and Extension Service NRI 71.1 Improving Quality and Value programme.

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Food Chemistry 117 (2009) 364–370

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

A monoclonal antibody-based sensitive enzyme-linked immunosorbent assay (ELISA) for the analysis of the organophosphorous pesticides chlorpyrifos-methyl in real samples

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ARTICLE INFO

Article history:
Received 2 June 2008
Received in revised form 3 February 2009
Accepted 29 March 2009

Keywords:
Chlopyrifos-methyl
Monoclonal antibody
ELISA

ABSTRACT

Chlorpyrifos-methyl hapten, *O*-methyl-*O*-(3,5,6-trichloro-2-pyridinyl)-*N*-(2-carboxyethyl)-phosphoramidothionte (H1), was synthesized and conjugated with bovine serum albumin (BSA) and ovalbumin (OVA) by the active ester method. Then H1–OVA conjugate was used as coating antigen, while H1–BSA conjugate was used as immunogen for producing monoclonal antibody. After optimisation, a monoclonal antibody-based effective competitive indirect enzyme-linked immunsorbent assay (ELISA) was developed and applied for determination of chlorpyrifos-methyl with a novel combination of antibody/antigen, I_{50} of which was 75.22 ng/ml, limit detection (LD) was 0.32 ng/ml, and there was relative high cross-reactivity (CR) only with chlorpyrifos (1.4%), and CRs with other tested pesticides were all below 1% and regarded as negligible. The recoveries obtained by standard chlorpyrifos-methyl addition to real samples, including grape, Chinese cabbages, water and soil were all from 82.4% to 110.2%. Therefore, the optimised ELISA might become a convenient and satisfied analytical tool for monitoring chlorpyrifos-methyl residues in agriculture ecosystem.

1. Introduction

There is increasing concern over food and environmental contamination caused by the widespread use of pesticides (Kim, Lee, Chung, & Lee, 2003). Among them, organophosphorous pesticides (OPs) are described as a group of highly toxic compounds (Costa, 1988; Zhang et al., 2007) and widely used in agriculture for protecting plants. It is reported that OP toxicants could elicit their effects by inhibition of acetylcholinesterase, which leads to the accumulation of the neurotransmitter acetylcholine (Ach) in synapses, over stimulates the post synaptic cholinergic receptors with consequent signs of neurotoxicity (Ecobichon, 1996; Gallo & Lawryk, 1991). Therefore, more and more non-target organisms, including human, fish and been are threatened by OPs.

Chlorpyrifos-methyl (*O*,*O*-dimethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphorthioate) is a widely used nonsystemic OP, which is effective against a wide range of insects. The most sensitive and toxicologically relevant effect after administration of chlorpyrifos-methyl is inhibition of acetylcholinesterase activity in erythrocytes and brain. Because of its toxicity and extensive application to crops and cattle, chlorpyrifos-methyl was classified as a restricted pesticide with a strict maximum residue limited (MRLs) standard for use in farming in many countries, including China Government.

Current analytical methods, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) are sensitive and reliable for the detection of pesticide residues (Kim, Lee, et al., 2003). However, they require a high cost and skilled analysts and involve time-consuming sample preparation steps. Therefore, there is a growing demand for more rapid and economical methods for determining pesticide residues. Immunoassays have recently emerged as an alternative to the traditional methods that can meet such demands. Immunochemical techniques that have been used extensively in clinical laboratories began recently to gain acceptance as a fast, sensitive and cost-effective tool for detecting trace amounts of chemicals such as pesticides (Hammock et al., 1982). It is reported that several monoclonal antibodies of pesticides, including parathion-methyl and fenitrothion were successfully prepared and applied for determination in samples (Kim, Kim, & Lee, 2007; Kolosova, Park, & Sergei, 2004). In our previous work, fenthion polyclonal antibody is prepared and applied for fenthion detection in fruit samples (Zhang et al., 2008). To date, several reports describe immunoassay for chlorpyrifos-methyl detection (Edward, Skerritt, Hill, & McAdam, 1983; Skoczenski, Fat, Matt, Pitts, & Zettler, 1996; Sullivan, Chen, & Goh, 2007). However, it is clearly that, on one hand, these reports as well as the commercial kit for chlorpyrifos (Strategic Diagnostics Inc.) described a

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.03.097

polyclonal antibody-based immunoassay for the detection of chlorpyrifos-methyl, on the other hand, the detection samples are limited to grain or water. To the best of our knowledge, chlorpyrifos-methyl is a widely used pesticide to control pest insects in farm as well as in storage grain. Therefore, it was necessary to detect chlorpyrifos-methyl from various samples, such as vegetables, fruits and soil. Together, the main objective of this work was to establish a monoclonal antibody-based ELISA assay for the detection of chlorpyrifos-methyl from vegetables, fruits, soil and water. In the present study, we reported that: (i) a specific monoclonal antibody of chlorpyrifos-methyl was prepared; (ii) a monoclonal antibody-based sensitive ELISA for chlorpyrifos-methyl detection was developed; and (iii) the chlorpyrifos-methyl from different real samples, including grape, Chinese cabbages, water and soil was detected by using the established ELISA protocol.

2. Materials and methods

2.1. Chemicals and instruments

Chemical reagents for hapten synthesis and pesticide standards used for cross-reactivity studies were supplied by Jiangsu Pesticide Research Institute (Nanjing, China). Analytical grade solvents were from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM), HAT medium (hypoxantin, aminopterin, thymidin), HT medium (hypoxantin, aminopterin) were purchased from Gibco (USA). Tween 20, *N*-hydroxysuccinimide (NHS), N,*N*-dicyclohexylcarbodiimide (DCC), bovine serum albumin (BSA), ovalbumin (OVA), complete or incomplete Freund's adjuvant and tetramethylbenzidine (TMB) were all purchased from Sigma (St. Louis, USA). Faetal bovine serum (FBS) was purchased from Hangzhou 'Sijiqing' Company (Hangzhou, China). Horseradish peroxidase (HRP)-labelled goat anti-mouse IgG was purchased from Sino-American Biotechnology Co. (Dalian, China). The Mouse Monoclonal Antibody Isotyping Kit was purchased from Roche Co. (Mannheim, Germany).

Thin-layer chromatography (TLC) was performed on 0.25 mm, precoated silica gel 60 F254 on aluminium sheets (Merck, Darmstat, Germany). Column chromatographic purifications were carried out with silica gel (60–230 mesh), from Qingdao Haiyang Chemical Co., Ltd. (Qingdao, China). UV–vis spectra were recorded on a Bechman 640 spectrophotometer. Polystyrene 96-well microtitre plates were from Costar (Corning, Massachusetts, USA). A microplate washer from Prolong New Technology Co. (Beijing, China) was used to wash enzyme-linked immunosorbent assay (ELISA) plates. Absorbance was measured using a microtitre plate reader (Thermo Electron Co., USA).

SP2/0 cells were stored in the Immunology and Quarantine Lab of Nanjing Agricultural University (Nanjing, China), and BALB/c mice were purchased from the Center of Comparative Medicine of Yangzhou University (Yangzhou, China). All animal studies were performed in accordance with institutional guidelines.

2.2. Synthesis of hapten

The hapten synthesis assay was performed as described previously, with some modifications (Brun, Garcés-García, Puchades, & Maquieira, 2004; Kim, Cho, Lee, & Lee, 2003; Kim et al., 2003; Zhang et al., 2007). The synthetic routes for hapten are illustrated in Fig. 1. In brief, a solution of 3,5,6-trichloro-2-pyridinol (4.17 g, 21.1 mmol) in acetonitrile was added dropwise to a stirred mixture of 3.83 g (23.2 mmol) of methyl dichlorothiphosphate, 8.7 g (63 mmol) of finely ground K_2CO_3 and 20 ml of acetonitrile. After stirring for 1 h at room temperature, the mixture was filtered through celite, and the solvent was removed under reduced pressure. The residue was subjected to column chromatography (silica gel, petroleum ether:methylenechloride (15:1)) to give 6.88 g (72.6%) of a straw yellow oil, named as product A (*O*-methyl-*O*-(3,5,6-trichloro-2-pyridinyl)-phosphorochloridothioate).

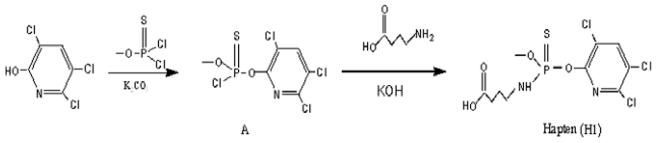


Fig. 1. Synthetic route for hapten (H1). A: *O*-methyl-*O*-(3,5,6-trichloro-2-pyridinyl)phosphorochloridothioate; hapten (H1): *O*-methyl-*O*-(3,5,6-trichloro-2-pyridinyl)-*N*-(2-carboxyethyl)phosphoramidothionte.

To a stirred solution of 1.37 g (4.2 mmol) of product A in 6 ml of methyl alcohol cooled in an ice-water bath was added dropwise a solution of 0.78 g (11.4 mmol) of KOH and 0.54 g (5.2 mmol) of 4-aminobutyic acid in 3.4 ml of methyl alcohol. After stirring for 5 min, the reaction mixture was filtered and adjusted pH to 4.0 with 1 mol/l (M) hydrochloric acid, then extracted with ethyl acetate and the solvent was evaporated, the residue gave 1.28 g (78%) of a straw yellow solid, termed as hapten H1 (*O*-methyl-*O*-(3,5,6-trichloro-2-pyridinyl)-*N*-(2-carboxyethyl)-phosphoramidothionte). The structure of H1 was confirmed by Mass Spectrometry (MS) and Proton Nuclear Magnetic Resonance (^{1}HNMR).

2.3. Preparation of hapten–protein conjugate

In order to generate immunogens and coating antigen, the hapten of chloropyrifos-methyl (H1) was coupled to protein BSA and OVA, respectively, according to the method as described previously (Langone & van Vunakis, 1982) The immunogen (H1–BSA) and coating antigen (H1–OVA) were purified by dialysis in phosphate buffer (PB: 0.02 M, pH 6.8). The structures of conjugates were confirmed by UV–vis spectral, and the final conjugates (H1–BSA and H1–OVA) were stored at −20 °C for further study. Assuming that the molar absorptivity of haptens was the same for the free and conjugated forms, the hapten densities (the number of hapten molecules per molecule of protein) of the conjugates were estimated directly by the mole absorbance: εHapten density = (εconjugation − εprotein)/εhapten as described previously (Kishiro, Kagawa, Naito, & Sado, 1995; Zhang et al., 2006).

2.4. Production of monoclonal antibody to chlorpyrifos-methyl

2.4.1. Immunisation and screening of sera

The immunisation of five BALB/c female mice was carried out according to the methods described (Kishiro et al., 1995; Zhu, Xu, Lou, Blake, & Liu, 2007). The immunogen (H1–BSA) was diluted into PBS (phosphate buffered saline), mixed with an equal volume of Freund's complete adjuvant, and stirred to prepare a water-in-oil emulsion. The emulsion (200 μg conjugate per mouse) was injected into the peritoneal cavity of 6-week-old BALB/c female mice. The same injection with incomplete Freund's adjuvant was repeated 3 weeks later, and at 3-weeks intervals thereafter. One week after the fifth immunisation, five antiserums for chlorpyrifos-methyl named Ab01, Ab02, Ab03, Ab04 and Ab05, respectively, were obtained from the tail vein of each mouse, and the sera were tested for antibody titres and for analyte recognition by indirect ELISA. The titres of the antisera from the different bleeds of mice were determined by measuring the binding of serial dilutions of the antisera to the corresponding coating antigen (H1–OVA) using noncompetitive ELISA protocol as described previously (Zhang et al., 2006). Flat-bottomed polystyrene microtitre plates were coated with H1–OVA (5 μg/ml, 50 μl per well) in 50 mM carbonate–bicarbonate buffer (pH 9.6) by overnight incubation at 4 °C. The following day, the coated plates were washed five times with

PBST (PBS containing 0.05% (vol/vol) Tween 20, pH 7.4) and blocked by incubation with 1% gelatin in PBS (100 μl per well) for 1.5 h at 37 °C. After another washing step, 50 μl per well of antiserum diluted with PBS (1:1600–1:512,000) were added to the plate, and the plates were incubated for 1 h at 37 °C. After another washing step, 50 μl of a GAM–HRP conjugate diluted 1:25,000 with PBST were added to each well and incubated for 1 h at 37 °C. Next, the plates were washed again, then, 50 μl of substrate solution (3.3 μl of 30% H_2O_2 and 400 μl of 0.6% TMB in DMSO (dimethyl sulfoxide) per 25 ml of acetate buffer, pH 5.5) were added to each well. Colour development was stopped after 15–30 min with 2 M H_2SO_4 (25 μl per well). The absorbance was measured by the single-wavelength mode at 450 nm.

2.4.2. Cell fusion and hybridoma selection

One mouse showing the highest serum reactivity was given peritoneal cavity injections of 200 μg H1–BSA conjugate in PBS at 1 week intervals. After four days of the last injection, the donor mouse was sacrificed. SP2/0 myeloma cells were cultured in high-glucose DMEM supplemented with 20% faetal bovine serum (referred to as s-DMEM). Cell fusion procedures were performed essentially as described previously (Nowinski, Lostrom, Tam, Stone, & Burnette, 1979). Mouse spleen lymphocytes were fused with SP2/0 myeloma cells at a 5:1 ratio, with PEG 1500 as the fusing agent. The fused cells were distributed in 96-well culture plates at an approximate density of 4×10^4 cells/μl of s-DMEM per well. Twenty-four hours after plating, 100 μl of HAT selection medium (s-DMEM supplemented with 100 μl hypoxanthine, 0.4 μM aminopterine, 16 μM thymidine) was added to each well. Half the medium of the wells was replaced with fresh HAT medium after cell fusion on forth and seventh day, respectively. Cells were grown in HAT medium for 2 weeks, then HAT was substituted by HT medium.

Eight to ten days after cell fusion, culture supernatants were screened for the presence of antibodies that recognised chlorpyrifos-methyl. Supernatants from these clones were collected and initially screened for antibodies by the indirect ELISA method as described above. Culture supernatants that passed the initial screen were subsequently tested for antibody specificity by competitive ELISA. Selected positive hybridoma cell lines were subsequently subcloned by limiting dilution.

2.4.3. Production, purification and isotyping of monoclonal antibodies

Ascites fluid was produced in BalB/c mice primed with incomplete Freund's adjuvant by intraperitoneal (Ip) injection of 2×10^7 hybridoma cells. Ascites fluid was harvested after 10–14 days of the hybridoma cell injection. Antibodies were purified on a small scale directly from ascites fluid by affinity chromatography on protein L-agarose as described previously (Schuler & Reinacher, 1991). The immunoglobulin subclass and light chain isotype of the antibodies were determined using a Mouse Monoclonal Antibody Isotyping kit.

2.5. Competitive indirect ELISA (CI-ELISA)

Microplates were coated overnight at 4 °C with 50 μl per well of the appropriate coating antigen concentration (3 μg/ml) in 0.05 M carbonate–bicarbonate buffer (pH 9.6). After washing with PBST (PBS containing Tween 20 (0.05%, vol/vol), pH 7.4), the wells were blocked with 100 μl of reagent in PBS (1% gelatin, or 1% OVA, or 1% polyvinyl alcohol, or 3% skimmed milk powder) for 1 h at 37 °C. After another washing step, 25 μl per well of culture supernatants diluted in PBS and 25 μl per well of analyte solution were added, and incubated for 1 h. Following a washing step, goat-anti-mouse–HRP conjugate (1:25,000 in PBST, 50 μl per well) was added and incubated for 1 h at 37 °C. The plates were washed again, and 50 μl per well of substrate solution was added. The colour development was stopped after 10–15 min with 2 M H_2SO_4 (25 μl per well). The absorbance was measured at 450 nm. Sigmoidal curves were fitted to a logistic equation (Raab, 1983) from which I_{50} values (concentration at which binding of the antibody to the coating antigen is inhibited by 50%) were determined.

2.6. Optimisation of a CI-ELISA

The screening of the variables to set up competitive ELISA procedures was performed in antibody- and conjugate-coated formats, following the protocols as described previously (Tijssen, 1987). Optimised assay was performed using chlorpyrifos-methyl as the competitor analyte. A set of experimental parameters, including ionic strength, pH and blocking agents were studied sequentially to improve the sensitivity of the immunoassay. The main criteria used to evaluate immunoassay performance were I_{50} and R^2 of their linear equation. In this work, the selection of optimised parameters of ELISA protocol was obtained according to two standards: "$R^2 > 0.9$" and "I_{50}/A_{max} was lowest" (described by Lee et al. (2006)). The effect of pH was evaluated using different PBS solutions, ranging from pH 5.0 to 8.0. To estimate the influence of salt (Na^+) concentration, PBS at 0, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml was tested. Finally, the influence of blocking reagent (1% OVA, or 1% gelatin, or 1% polyvinyl alcohol, or 3% skimmed milk powder) was investigated.

2.7. Evaluation of the optimised CI-ELISA

2.7.1. Cross-reactivity (CR) studies

The specificity of the optimised ELISA procedures was determined against several organophosphorus insecticides, including chlopyrifos-methyl, chloryrifos, fenitrothion, prarthion-methyl, triazophos, 3,5,6-trichloro-2-pyridinol and dichlorvos. The CR was calculated as follows: CR (%) = (I_{50}(methyl-chlopyrifos)/I_{50}(interferent)) × 100. Here, CR (%) of chlopyrifos-methyl was defined as 100%.

2.7.2. Accuracy (analysis of spiked samples)

The accuracy was evaluated by spiked samples experiment. To study spike recovery, the materials, including grape, Chinese cabbages, water and soil were selected for study. Samples were spiked with different concentrations of chlopyrifos-methyl and analysed in a blind fashion by the ELISA protocol. The grape samples were purchased from a local supermarket, the Chinese cabbages and soil samples were taken from farm of Nanjing Agricultural University and water is tap water. For the spiked-and-recovery test, five solutions (0, 5, 10, 25 and 50 μg/ml) of chlopyrifos-methyl in methanol were prepared. Then, 1 ml of each solution was added to 1 g of the samples. Here, the samples were water (1 ml), soil, grape and Chinese cabbages (the peel of grape and the leaves of Chinese cabbages were chopped into fine pieces before the solution was added). In the following steps, all samples were shaken (60 rpm/min) for 2 h. After stability of several minutes (the pieces of grape peel or cabbage leaves, and soil were deposited), the sample supernatant was diluted with PBS–methanol buffer for 10-times (the final concentrations of chlopyrifos-methyl were 0, 0.5, 1, 2.5 and 5 μg/ml) and analysed by the optimised ELISA protocol.

3. Results

3.1. Verification of hapten (H1) and the coupled identification of artificial antigen

The hapten (H1) was synthesized with only two steps and the structure of H1 was clarified by MS and ^{1}HNMR. The results of

MS and ^{1}HNMR were described as follows: ^{1}HNMR (CD_3–CN) δ: 1.77 (m, 2H, CH_2CH_2COOH), 2.35 (m, 2H, CH_2COOH), 2.93 (t, 2H, CH_2NH), 3.12 (s, 3H, CH_3–OP), 7.76 (s, 1H, Ar). MS (EI^+) m/z (%): 167.1 (53.66), 102.1 (26.16), 86.1 (100), 84.1 (72.13). These results showed the structure of compound was in accordance with that of hapten (H1) designed.

In the UV–vis spectra obtained from continuous wavelength scanning, there were a lot of obvious changes between the spectra of the conjugate and that of its carrier protein. The conjugate of up taking peak shape is the result of lapping between the pristine carrier protein and hapten that have been coupled. The conjugation molar ratio of H1–BSA and H1–OVA is 22 and 21, respectively, which was assessed by spectrophotometric method. Finally, the conjugate H1–BSA was used as immunogen to produce monoclonal antibody, while H1–OVA was used as coating antigen.

3.2. Production and characterisation of monoclonal antibody

After fifth injection, the titres of Ab01, Ab02, Ab03, Ab04 and Ab05 were $1{:}1.28 \times 10^6$, $1{:}0.64 \times 10^6$, $1{:}2.56 \times 10^6$, $1{:}1.28 \times 10^6$ and $1{:}1.28 \times 10^6$, respectively. The titres of antisera from these five mice did not rise further after the sixth immunity. Therefore, the mouse, produced Ab03, was selected for subsequent hybridoma production because of its highest relative reactivity with H1–OVA. Hybridoma clones were initially screened by indirect ELISA for their reactivities with H1–OVA, and a total of 56 hybridoma cell lines were obtained. Then, one hybridoma cell line (3E12/B6) was further selected by competitive ELISA, and its produced stable monoclonal antibody (MAb) for chlopyrifos-methyl was used for further study.

The hybridoma cell line (3E12/B6) was found to secrete antibodies of the IgG1, kappa type. The MAb was ascites fluid in mouse (7.91 mg/ml), purified and stored at −20 °C for following characterisation studies.

3.3. Optimisation of a CI-ELISA

In order to get a optimised protocol for CI-ELISA, the effects of pH, blocking agents and ionic strength on ELISA performance were evaluated in this study. In the system MAb (3E12/B6)/H1–OVA, a lower salt concentration (<0.4 M) in the assay system resulted in higher I_{50}, whereas a higher salt concentration (3.2 M) in the assay system resulted in lower R^2 value of its linear equation owing to lower optical densities (OD). As shown in Table 1, 0.8 M of salt concentration was selected in CI-ELISA system because the $R^2 > 0.9$ and the I_{50}/A_{max} was lowest. Furthermore, when analyte was dissolved in buffer at different pH values ranging from 5.0 to 8.0, no significant effect on the I_{50} was detected, but, interestingly, both strong acid and alkali matrix resulted in low sensitivity, indicating that the assay could effectively detect chlorpyrifos-methyl at pH values ranging from 6.0 to 7.5. In addition, it was showed that ionic strength had a strong influence on ELISA performance. As for the effect of blocking reagents, the common blocking reagents, such as OVA, gelatin, polyvinyl alcohol and skimmed milk powder were tested. Finally, 1% polyvinyl alcohol was selected based on two strands (the lowest I_{50}/A_{max} and $R^2 > 0.9$). At last, the optimised parameters of ELISA procedure were determined as follows: concentration of coating antigen H1–OVA was 3 μg/ml, dilution of culture supernatants of MAb 3E12/B6 was 1:16, the blocking reagent was 1% polyvinyl alcohol, pH was 6–7.5, and ionic strength was 0.8 M.

Table 1
Effect of ionic strengths, pH values and blocking agents on the ELISA sensitivity (I_{50}).

Factors	I_{50}[d] (μg/ml)	A_{max}	R^2 of linear equation
Ionic strengths (M)[a]			
0	>300	0.767	–
0.2	9.71	0.792	0.93
0.4	0.113	0.852	0.95
0.8	0.078	0.876	0.99
1.6	0.037	0.654	0.83
3.2	0.009	0.532	0.78
pH			
5	0.240	0.677	0.89
6	0.090	0.718	0.92
6.5	0.093	0.803	0.98
7	0.081	0.834	0.99
7.5	0.066	0.819	0.99
8	0.192	0.581	0.98
Blocking agents			
1% PA[b]	0.074	0.891	0.98
1% OVA	0.050	0.471	0.97
1% gelatin	0.079	0.865	0.95
3% SMP[c]	0.042	0.079	0.80

[a] mol/l.
[b] Polyvinyl alcohol.
[c] Skimmed milk powder.
[d] The concentration of chlorpyrifos-metyl that inhibited the colour development in the competitive immunoassay by 50%.

3.4. Evaluation of the optimised CI-ELISA

3.4.1. Evaluation of sensitivity

Under the optimised conditions mentioned above, the indirect competitive ELISA procedures were conducted in quintuplicate with a series of standard concentration of chlorpyrifos-methyl at different times. Then a competitive curve representing the average was obtained (Fig. 2a). We used logit(B/B_0) as the lateral coordinates (y), logarithm of concentration of chlorpyrifos-methyl (ng/ml) as the longitudinal coordinates (x). After conversion of Fig. 2a, it was observed that in the range of 0.01–100 μg/ml, the

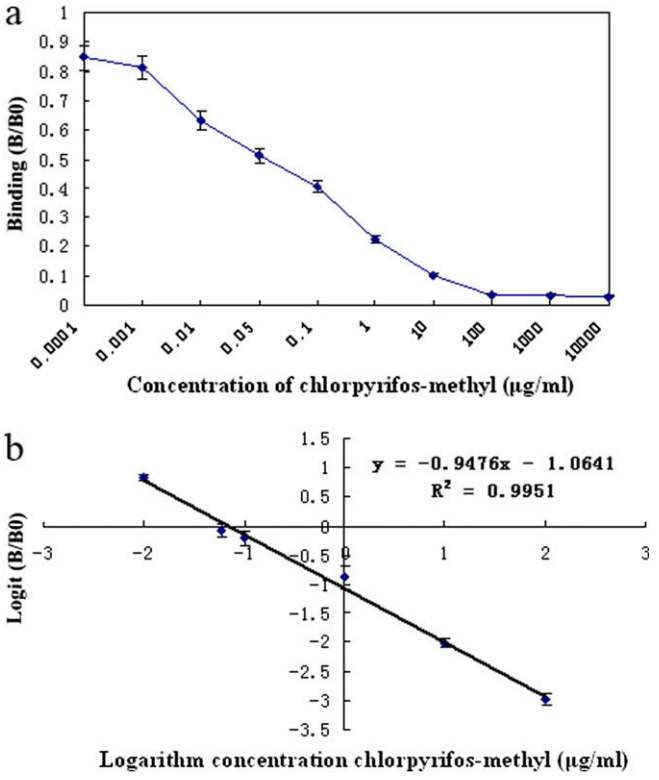
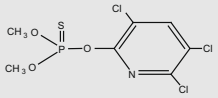
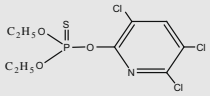
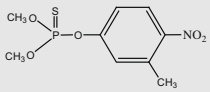
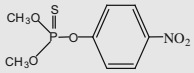
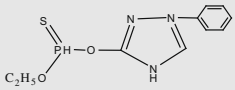
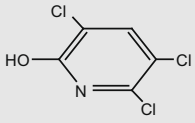
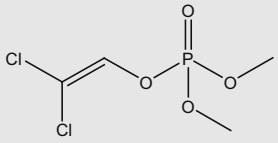


Fig. 2. Indirect competitive enzyme-linked immunsorbent assay (CI-ELISA) curve for chlorpyrifos-methyl. (a) Binding curve of the CI-ELISA, and "B/B_0" is binding ratio of antibody/coating antigen in wells; (b) the detection line conversed from (a), and "logit(B/B_0)"equals to logistic value of "B/B_0".

Table 2
Cross-reaction of monoclonal antibody 3E12/B6 with chlorpyrifos-methyl and its analogues.

Analogues	Structures	I_{50}[a] (μg/ml)	Cross-reactivity (%)
Chlopyrifos-methyl		0.075	100.0
Chloryrifos		5.2	1.4
Fenitrothion		11.52	0.64
Prarthion-methyl		300	<0.03
Triazophos		>300	<0.03
3,5,6-Trichloro-2-pyridinol		>300	<0.03
Dichloryos		>300	<0.03

[a] The concentration of chlorpyrifos-metyl that inhibited the colour development in the competitive immunoassay by 50%.

graph between logit(B/B_0) and logarithm of concentration of chlorpyrifos-methyl (μg/ml) was linear (Fig. 2b), and the regression equation was obtained ($y = -0.9476x - 1.0641$, $R^2 = 0.9951$). In this optimised ELISA, I_{50} value was 75.22 ng/ml and the limit detection (LD) was 0.32 ng/ml by the extrapolation of $B_0 - 3SD$ extrapolation.

3.4.2. Cross-reactivity

To determine the specificity of the optimised CI-ELISA, several organophosphorus pesticides were tested for cross-reactivity. As shown in Table 2, the highest cross-reactivity was 1.4%, obtained for chlorpyrifos. Meanwhile, the interferences of other tested similar compounds and organophosphorus pesticides were lower (<1%) and regarded as negligible. These results indicated that the optimised CI-ELISA had a high specificity and might be applied for determination of chlorpyrifos-methyl in samples.

3.4.3. Accuracy (analysis of spiked samples)

The spiked recoveries were used to confirm the accuracy of optimised CI-ELISA. As shown in Table 3, the average recoveries of spiked Chinese cabbages were from 88.5% to 92.6%, and those of spiked grape were from 82.4% to 94.6%, and those of spiked water were from 96.4% to 110.2%, those of spiked soil were from 82.2% to 87.2%. Overall, the CI-ELISA developed in this study could not only avoid complicated sample disposal protocol, but only accurately determine chlorpyrifos-methyl residues in samples of grape, Chinese cabbage, water and soil.

4. Discussion

The organophosphorous pesticides (OPs), including chlorpyrifos-methyl, have a thiophosphate group in common and differ only in the structure of their aromatic rings. Therefore, to achieve a high sensitivity ELISA for chlorpyrifos-methyl, it was desirable to synthesize hapten that has a bridge at the thiophosphate group in order to preserve the aromatic ring unique to chlorpyrifos-methyl. By this way, in this work, chlorpyrifos-methyl hapten (H1) was successfully synthesized. Compared to the previous works (Edward et al., 1983; Skoczenski et al., 1996; Sullivan et al., 2007), our work displayed three novelties: (i) a specific monoclonal antibody was prepared and applied for the detection of chlorpyrifos-methyl; (ii) various samples (especially for vegetables, fruits and soil) were selected to evaluate the efficiency of the optimised monoclonal antibody-based ELISA assay for chlorpyrifos-methyl; (iii) it was convenient to obtain stable and high-quality monoclonal antibodies from isolated positive hybridoma, whereas polyclonal antibody must be prepared each time by immunisation of rabbit. In this work, we have first developed an effective ELISA for chlorpyrifos-methyl based on specific monoclonal antibody using a novel combination of immunizing/coating hapten. Here, the "efficiency" of ELISA is mainly displayed on three aspects: (i) its high sensitivity. The limit detection (LD) of the optimised competitive indirect ELISA (CI-ELISA) was 0.32 μg/l, and its I_{50} was 75.22 μg/l, which made it possible to detect chlorpyrifos-methyl which are below the threshold level (0.1 mg/l) regulated by World Health Organization (WHO) for chlorpyrifos-methyl in vegetables. (ii) Its simplicity. Sample extraction is of great speediness and simplicity because of its high sensitivity mentioned above. In our work, it does not need to be concentrated after the pesticide (chlorpyrifos-methyl) in sample dissolves enough in organic solvent. Therefore, the ELISA could avoid the complicated former disposal procedure needed by chromatography (the sample extraction protocol of chromatography include sample dissolution, extraction and purification) (Arrebola, Martınez Vidal, Gonzaalez-Rodrıguez, Garrido-Frenich, &

Table 3
Recovery test of chlorpyrifos-methyl in real samples (soil, water, Chinese cabbages and grape).

Samples	Spiked (μg/ml)	Theoretical (μg/ml)	Found (μg/ml)	Average recovery ± SD (%)
Soil	0	0	0.000 ± 0.001	–
	5	0.500	0.411 ± 0.030	82.2 ± 7.1
	10	1.000	0.872 ± 0.042	87.2 ± 4.3
	25	2.500	2.116 ± 0.033	84.6 ± 5.1
	50	5.000	4.312 ± 0.027	86.2 ± 4.1
Water	0	0	0.002 ± 0.002	–
	5	0.500	0.551 ± 0.006	110.2 ± 5.0
	10	1.000	1.043 ± 0.007	104.3 ± 6.1
	25	2.500	2.412 ± 0.016	96.4 ± 2.5
	50	5.000	5.420 ± 0.063	108.4 ± 12.7
Chinese cabbages	0	0	0.001 ± 0.001	–
	5	0.500	0.443 ± 0.003	88.7 ± 5.5
	10	1.000	0.926 ± 0.009	92.6 ± 0.9
	25	2.500	2.281 ± 0.014	91.2 ± 5.7
	50	5.000	4.426 ± 0.019	88.5 ± 4.2
	0	0	0.001 ± 0.002	–
	5	0.500	0.412 ± 0.005	82.4 ± 5.0
Grape	10	1.000	0.946 ± 0.006	94.6 ± 4.5
	25	2.500	2.071 ± 0.013	82.8 ± 11.3
	50	5.000	4.168 ± 0.017	83.3 ± 6.0

Sanchez Morito, 2003; Zhang et al., 2006). (iii) Its utility. In our work, four independent samples, including fruit sample (grape), crop sample (Chinese cabbages), water and soil were chosen as the materials for studying spike recovery. As shown in Table 3, the recoveries obtained by standard chlorpyrifos-methyl addition to the four independent sample were all from 82.4% to 110.2%, indicating that optimised ELISA could detect chlorpyrifos-methyl in different samples with easy operation procedures. In addition, there was little or no cross-reactivity to similar compounds tested except for the insecticide chlorpyrifos, which showed a cross-reactivity of 1.4%. The high cross-reactivity of the antibody for chlorpyrifos is understandable, because it has the same thiophosphate structure as chlorpyrifos-methyl and its aromatic structure is very similar to chlorpyrifos-methyl. Together, the optimised ELISA might become a convenient and effective tool for monitoring chlorpyrifos-methyl in environment and agricultural samples.

Currently, work to amplify single chain variable fragment (scFv) from hybridoma cell line (3E12/B6) is in progress in our laboratory in order to produce recombinant antibody in *Escherichia coli*.

Acknowledgements

We appreciated Dr. Tiejun Li for critical revision of this manuscript. This work was supported by the National High Technology Research and Development Program of China (No. 2006AA10Z447).

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Food Chemistry 117 (2009) 371–374

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

A novel method using immuno-affinity chromatography for isolating β-conglycinin from soybean proteins

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ARTICLE INFO

Article history:
Received 29 November 2007
Received in revised form 17 March 2009
Accepted 29 March 2009

Keywords:
β-Conglycinin
Monoclonal antibody (Mab)
Immuno-affinity chromatography
Purification

ABSTRACT

A monoclonal antibody (Mab) $6G_4$ against soybean β-conglycinin has been prepared using a conjugate of chicken ovalbumin and a synthetic peptide that corresponded to one of the epitope sequences of β-conglycinin as the immunogen. An ELISA method for the quantification of β-conglycinin has also been developed. In the present study, we report a novel method for the purification of β-conglycinin by Mab $6G_4$-based immuno-affinity chromatography. β-Conglycinin with a purity of 92.9% was successfully isolated from soybean proteins. Western blot assay was used to further identify its characteristics and the results demonstrated that the purified β-conglycinin maintains its biological activities. Therefore, the Mab-based immuno-affinity chromatography is an available method for purification of β-conglycinin. It also provides a new opportunity for future study on the mechanism of food allergy responses using high purity β-conglycinin as the experimental material.

1. Introduction

Soybean is widely used as food ingredients because of its nutritional and functional properties (Astadi, Astuti, Santoso, & Nugraheni, 2008; Friedman & Brandon, 2001; Liu et al., 2008; Saito, Kohno, Tsumura, Kugimiya, & Kito, 2001). However, soybean is also considered as a major source of food allergies especially in industrialised countries (Guo, Piao, Cao, Ou, & Li, 2008; Herman, Helm, Jung, & Kinney, 2003; Huisman & Jansman, 1991). Soybean allergies are prevalently becoming "health hacker" in the world recently (Chandra, 2002; Song, Frias, Martinez-Villaluenga, Vidal-Valdeverde, & de Mejia, 2008; Zeiger, 2000).

As a major soybean allergen, β-conglycinin with α, α′ and β subunits plays important roles in hypersensitive responses for children (Xiang, Beardslee, Zeece, Marwell, & Sarath, 2002) and young animals such as piglets (Li et al., 1990), calves (Lallès & Dreau, 1996) and mice (Guo et al., 2008). However, study on β-conglycinin has been limited due to the difficulty of separating β-conglycinin from other soybean proteins. Most previous studies have mainly used soybeans or their extracts as the experimental materials but not pure β-conglycinin because of the lack of an available isolation approach. Although some methods such as ammonium sulphate precipitation (Thanh & Shibasaki, 1976), pH adjustment isolation (Lovati et al., 1992), ultrafiltration membrane separation (Wu, Murphy, Reuber, & Fratzke, 2000) and phytase treatment (Saito et al., 2001) can be conducted to isolate β-conglycinin from soybean proteins, it is difficult to obtain enough pure β-conglycinin for research purposes. Since monoclonal antibodies (Mabs) are powerful tools for many molecular immunology investigations (Nelson et al., 2000), they have frequently been applied in practical studies on recognising antigens and identifying new cell surface molecules. These binding concepts of allergen and antibody also provide a possibility for us to prepare an immuno-affinity column mediated by Mab and develop a new approach for purification of β-conglycinin which can be used for future research purposes.

2. Materials and methods

2.1. Preparation of monoclonal antibody against soybean β-conglycinin

The preparation of a stable murine-derived hybridoma cell line, named as $6G_4$, has been described previously (You et al., 2008). The Mab against soybean β-conglycinin produced by $6G_4$ is an IgG1 isotype. It shows high affinity for β-conglycinin with an association constant of $6.9 \times 10^9\ M^{-1}$ and can specifically bind to the α and α′ subunits of β-conglycinin (You et al., 2008).

2.2. Production and purification of antibodies

A large amount of Mab was produced *in vivo*. The hybridoma cells from $6G_4$ (1×10^6 cells/mouse) were injected

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doi:10.1016/j.foodchem.2009.03.098

intraperitoneally into BLAB/c mice pre-treated 7 days before with 0.5 mL mineral oil. About two weeks later, the ascitic fluids from mice inoculated by hybridoma cells were collected and centrifuged for 15 min at 10000×g to eliminate cells and dregs. The ascite supernatant was diluted with equal volume of 0.04 M barbital buffer (pH 7.0). An appropriate amount of SiO_2 powder was added into ascites dilution and gently stirred for 30 min. After centrifuging for 20 min at 2000×g, Mab $6G_4$ was primarily isolated from supernatant by ammonium sulphate, and a commercial Protein G Affinity Column (Amersham Biosciences, Uppsala, Sweden) chromatography was then performed to further purify IgG1 from ammonium sulfate precipitation.

2.3. Preparation of immuno-affinity column

The CNBr-activated Sepharose™ 4B (Amersham Biosciences, Uppsala, Sweden) medium was prepared according to its production instructions. Briefly, 2.3 g of CNBr-activated Sepharose™ 4B freeze-dried powder was suspended in 1 mM HCl (pH 2.5). After swelling, the medium was washed for 15 min with 1 mM HCl on a sintered glass filter to remove all additives.

Fifty-six mg of Mab $6G_4$ (IgG1) as ligand was dissolved in 8 mL of coupling buffer (0.1 M $NaHCO_3$, pH 8.3 containing 0.5 M NaCl) and mixed with prepared Sepharose 4B gel in a 10 mL test tube fitted with a stopper. Then, the tube was tied on the orbital shaker and the mixture was gently shaken for 1 h at 20–25°C. Excessive ligand was flushed away with at least five volumes of coupling buffer. Tris–HCl buffer (0.1 M, pH 8.0) was added to block any remaining active groups for 2 h. The gel was washed for at least three cycles of alternating pH using five volumes of each buffer. Each cycle consisted of a wash with 0.1 M acetate buffer (pH 4.0 containing 0.5 M NaCl) followed by a wash with 0.1 M Tris–HCl (pH 8.0 containing 0.5 M NaCl).

The gel coupled with IgG1 was loaded into a column (10 × 100 mm) which was washed with 20 mM sodium phosphate buffer (pH 7.0) at 4 °C. The residual space of the column was immediately filled with buffer, and the top of the column was connected to a pump. The pump was set to run at a flow rate of 1.3 mL/min. The elution was maintained for 10 bed volumes after a constant bed height was reached.

2.4. Extraction of total soybean proteins

Soybean seeds were ground with a Cyclotec Sample Mill (Model 1093, Tecator Inc., Herndon, VA, USA) to pass through a 60-mesh sieve. The soybean flour was defatted by *n*-hexane. The fat-free flour was then suspended in 0.03 M Tris–HCl buffer (pH 8.0 containing 0.01 M β-mercaptoethanol) (100 g flour per litre buffer). Proteins were extracted from the flour for 1.5 h by vortexing. Extracts were centrifuged at 12,000×g for 20 min at 4 °C using an Optima™ L-80 XP Ultracentrifuge (Beckman Coulter Inc, Palo Alto, CA, USA). After being filtered through a 0.45 μm Millex GP filter (Millipore, Cork, Ireland), the supernatant was used as test sample solution and stored at −20 °C until analysis.

2.5. Immuno-affinity chromatography of β-conglycinin

The total proteins was loaded onto the immuno-affinity column. The outlet tubing of the column was connected to an HD21-1 detector and a protein auto-collector (Huxi instrument Co., Shanghai, China). Flow rate was maintained for 1.0 mL/min. The medium was washed with binding buffer until the base line was stable. Glycine–HCl elution buffer (0.1 M, pH 2.7) was used to elute the sample and β-conglycinin was collected using the auto-collector according to absorbance profile. The pH of collection (β-conglycinin) was regulated immediately to neutral with 100 μL of 1 M Tris–HCl (pH 9.0).

2.6. Identification of β-conglycinin by Western blot

The proteins isolated from the immuno-affinity column were analysed by 12.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the standard procedure (Laemmli, 1970). The separated proteins were then transferred electrophoretically from gel to a 0.45 μm nitrocellulose membrane in a mini Trans-Blot Cell Apparatus (BioRad) for 1.5 h at 100 V. The membrane was incubated in 3% bovine serum albumin (BSA) for 2 h at 37 °C. After a stringent wash with Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST), the membrane was incubated with 0.1 μg/mL Mab $6G_4$ for 2 h at 37 °C. The unbound primary antibody was removed by washing with TBST. The horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG-HRP) was then added and the substrate 3,3′-diaminobenzidine (DAB) was used to show the specific protein bands.

The concentration of isolated β-conglycinin was determined with a Mab-based competitive ELISA method described previously (You et al., 2008) and the concentration of total proteins was tested by Bicinchoninic Acid Kit for Protein Determination (BCA™ Kit, Pierce, Rockford, IL, USA). The purity of β-conglycinin is expressed as a percentage as the content of β-conglycinin compared with that of total proteins.

2.7. Evaluation of chromatography efficiency in the case of different Mab/gel ratio

There is a close relationship between the ligand (Mab)/gel ratio and the chromatography efficiency. In order to enhance the chromatography efficiency, it is necessary to obtain an optimal ligand (Mab)/gel ratio. In this study, five grade concentrations of Mab, 2.5, 5.0, 7.5, 10.0, 12.5 mg/mL gel, were conducted to prepare five immuno-affinity columns with the same size. The efficiency of the purification of β-conglycinin under identical conditions, including soybean protein extraction and isolation procedures, was then evaluated.

The concentrations and purities of isolated β-conglycinin were determined and the results are reported as mean ± SEM. All data were the average values from five replicate determinations. Statistical analysis was done using the Stat-View program (SAS Institute, Cary, NC, USA). Differences between the concentrations (or purities) of β-conglycinin obtained from five immuno-affinity columns were examined for statistical significance using the Dunnett test. A difference was considered as significant at $P < 0.05$.

3. Results and discussion

Fig. 1 shows the SDS–PAGE profile (Left) and Gary Density profile (Right) of β-conglycinin purified from soybean proteins. Lane b represents total soybean proteins before purification and lane c represents β-conglycinin purified from total proteins. The purified β-conglycinin fraction contained mainly α, α′, and β subunits of β-conglycinin. Lane d is a grey density profile obtained from track c of electrophoresis. Direct observation clearly indicated that β-conglycinin was successfully isolated from soybean proteins. A Mab-based competitive ELISA method was used to further test its content. The result indicated that the purity of β-conglycinin was 92.9% (Data not shown).

In previous reports, the purity of β-conglycinin acquired from soybeans were 68% by ultrafiltration membrane separation method (Wu et al., 2000) and no more than 79% by ethanol extraction method (Rickert, Johnson, & Murphy, 2004). In the present study,

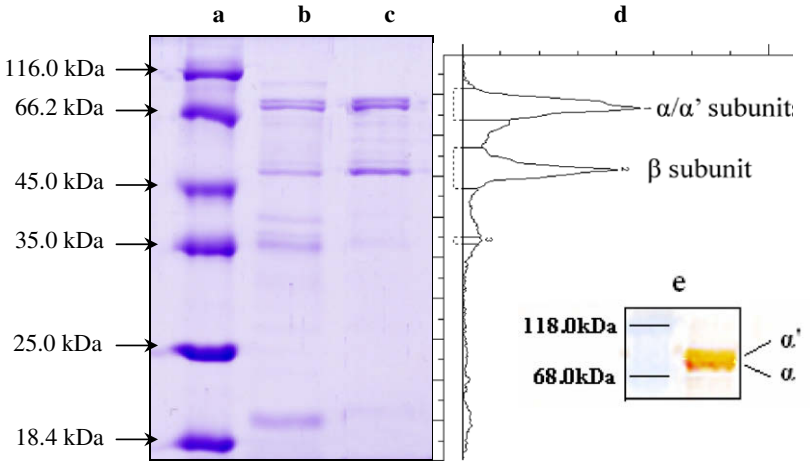

Fig. 1. SDS–PAGE profile (Left), grey density profile and Western blot assay (Right) of β-conglycinin purified by Mab-based immuno-affinity chromatography method. Proteins were separated by a 12.5% SDS–PAGE and stained with Coomassie Blue. Lane: a. Protein molecular weight markers; b. Total protein before purification; c. Goal protein (β-conglycinin) separated from soybean proteins through the immuno-affinity column; d. Gray density profile obtained from track c of electrophoresis using Syngene Analysis Solfware (Version 3.03.03); e. The proteins separated by SDS–PAGE were transferred electrophoretically to a 0.45 μm nitrocellulose membrane in a mini Trans-Blot Cell Apparatus. Wet blotting was performed at 100 V for 1.5 h. The blots were probed with 0.1 μg/mL of primary antibody (Mab). The goat anti-mouse IgG-HRP was used as the second antibody and KC™ General Western Blot Detection Kit (Roche Diagnostics Ltd., Lewes, UK) was used to detect the bound antibody.

a higher purity (92.9%) β-conglycinin was obtained by a Mab-based immuno-affinity chromatography method. We attribute the purity improvement to two factors. The first one is the use of immunochemical approach based on a highly specific Mab with moderate affinity. Using the prepared immuno-affinity column, the target protein was captured by the specific antibody molecule that immobilized on Sepharose 4B gel whilst other unwanted proteins were flushed from the column. The second factor is the convenient and rapid isolation procedure. It is well known that a complex and time consuming isolation procedure has an increased probability of degradation or denaturation of the target protein. Our purification procedure is very convenient and the time consumed per processing cycle is 5–10 min, which is less than about 30 min by methods of Thanh and Shibasaki (1976), Wu et al. (2000), and Rickert et al. (2004). The method developed by Thanh and Shibasaki (1976) and Wu et al. (2000) can separate two main soybean protein fractions, glycinin and β-conglycinin, by pH adjustment. However, it is difficult to avoid the present of an intermediate protein mixture (Wu et al., 2000). Also, other proteins of which the isoelectric point close to that of β-conglycinin are easy to precipitate in β-conglycinin samples. For the method described by Rickert et al. (2004), extraction environments (pH, ethanol concentration, temperature, and water-to-flake ratio) are each varied during bench-scale optimisation. Optimised conditions may yield more β-conglycinin with higher isoflavone and saponin concentrations, but fraction purity was diminished by glycinin contamination. Therefore, the probability that the target protein is degraded or denatured can be greatly reduced.

In addition to purity, the bioactivity of β-conglycinin is also an important index to evaluate the feasibility of the immuno-affinity column. The bioactivity of isolated β-conglycinin was assessed by the specific binding of β-conglycinin to Mab using Western blot assay. Fig. 1 (Lane e.) demonstrated that the Mab $6G_4$ could recognise the α′ and α subunits of the β-conglycinin molecule. This result indicated that the purified β-conglycinin maintained the natural bioactivity of protein molecule.

During the preparation of the immuno-affinity column, the coupling ratio of Mab and gel is one major factor impacting the isolation efficiency of β-conglycinin. The effects of Mab/gel ratio on isolation efficiency are presented in Table 1. No significant difference in terms of purity of isolated β-conglycinin was observed when the concentration of Mab coupled in Sepharose gel ranged from 2.5 to 12.5 mg/mL. However, different concentrations of Mab induced marked changes in yield of β-conglycinin. The concentrations of β-conglycinin in the 5.0, 7.5, and 10.0 mg/mL treatments were significantly higher than those in the 2.5 and 12.5 mg/mL treatments ($P < 0.05$). Interestedly, differences between 5.0, 7.5, and 10.0 mg/mL treatments were not significant ($P > 0.05$). Using this method, the yield of β-conglycinin separated from soybean proteins was 27.3–33.1 g/kg soybean. No differences were presented between 5.0, 7.5, and 10.0 mg/mL treatments ($P > 0.05$). Nevertheless, the yields in the 5.0, 7.5, and 10.0 mg/mL treatments were statistically superior to those in the 2.5 and 12.5 mg/mL treatments ($P < 0.05$). These results suggested that a low concentration of ligand (Mab) can reduce the isolation efficiency of β-conglycinin. Meanwhile, a very high concentration of ligand can have adverse effects on affinity chromatography. This may be because the less Mab couples in gel, the less likely goal protein in soybean solution binds to Mab. Namely, the more Mab couples in gel the more opportunity there is for goal protein to bind to Mab. However, too much Mab increases steric hindrance between the active sites and reduces the binding efficiency of the adsorbent. In

Table 1
Effects of Mab/gel ratio on efficiency of immuno-affinity chromatography for the isolation of soybean β-conglycinin.

	Concentration of Mab coupled in Sepharose 4B gel (mg/mL)				
	2.5	5.0	7.5	10.0	12.5
Concentration of isolated β-conglycinin (mg/mL)	0.48 ± 0.013^{b}	0.55 ± 0.010^{a}	0.56 ± 0.011^{a}	0.54 ± 0.008^{a}	0.50 ± 0.009^{b}
Yield in soybean (g/kg)	27.3 ± 1.9^{b}	31.6 ± 2.4^{a}	33.1 ± 2.1^{a}	32.4 ± 1.8^{a}	29.3 ± 1.5^{b}
Purity of isolated β-conglycinin (%)	93.2 ± 0.122	92.8 ± 0.292	93.1 ± 0.250	92.9 ± 0.203	92.7 ± 0.240

All data are means ± SEM ($n = 5$). Means within the same row that have no common superscript letters are significantly different ($P < 0.05$).

addition, substances bind more strongly to the immobilized ligand, which may result in difficult elution. On the other hands, the extent of non-specific binding increases at high ligand concentrations. For an efficient adsorbent, 5.0–10.0 mg ligand (Mab) per mL gel is suitable. In view of the above information, we considered that 92.7–93.2% purity of β-conglycinin can be successfully isolated from soybean proteins using the immuno-affinity column packed with 5.0–10.0 mg Mab per mL gel.

4. Conclusions

The Mab-based immuno-affinity chromatography developed in our study provides a novel method for the isolation of β-conglycinin. Under the optimized conditions, this technique can be used for collection of high purity β-conglycinin. In addition, the developed Mab-based immuno-affinity chromatography method also opens a new opportunity for the future study of the mechanism of food allergy responses using high purity β-conglycinin as an allergen protein material.

Acknowledgements

We thank the former research members, Dr. He P. L., Ou D. Y., & Dong B. (National Key Laboratory of Animal Nutrition, China Agricultural University), for the earlier stage works in the preparation of monoclonal antibody against soybean β-conglycinin and the development of competitive ELISA method for quantification of β-conglycinin. This work was supported in part by Grant from the National Natural Science Foundation of China (No. 30430520).

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Food Chemistry 117 (2009) 375–380

Contents lists available at ScienceDirect

Food Chemistry

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Analytical Methods

Isolation and purification of nootkatone from the essential oil of fruits of *Alpinia oxyphylla* Miquel by high-speed counter-current chromatography

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ARTICLE INFO

Article history:
Received 16 May 2008
Received in revised form 7 March 2009
Accepted 5 April 2009

Keywords:
Counter-current chromatography
Alpinia oxyphylla Miquel
Sesquiterpenoid
Nootkatone
Flavor
Bioactive component

ABSTRACT

HSCCC technique in a semi-preparative scale was successfully applied in isolation and purification of nootkatone from the essential oil of fruits of *Alpinia oxyphylla* Miquel. Twelve kinds of two-phase solvent systems, consisting of seven non-aqueous and five organic-aqueous solvent systems, were selected with not only suitable partition coefficients of nootkatone but also suitable separation factors between nootkatone and valencene, the dominant impurity in the essential oil. Further on HSCCC, n-hexane–chloroform–acetonitrile (10:1:10, v/v) amongst the non-aqueous solvent systems and n-hexane–methanol–water (5:4:1, v/v) amongst the organic-aqueous solvent systems were separately screened out. However, n-hexane–methanol–water (5:4:1, v/v) was thought optimal due to quite shorter elution time and better HSCCC peak form. By eluting the lower phase of this solvent system in head–tail mode, 3.1 mg of nootkatone was obtained at a purity of 92.30% by GC–MS from 80 mg of crude essential oil in one step operation in less than 4 h. The chemical structure of nootkatone fraction was confirmed by EI-MS and ^{1}H NMR.

1. Introduction

Alpinia oxyphylla Miquel (Zingiberaceae), "Yizhi" in Chinese, cultivated widely in South China, has been used as a traditional medicine for intestinal and urethral disorders in Chinese pharmacopeia. The essential oil from fruits of *A. oxyphylla* Miq. mainly consists of various sesquiterpenoids (Liu, Guo, Xiao, Cai, & Han, 2006; Luo et al., 2001; Yi, Xiao, & Liang, 2004), amongst which the two eremophilanes, i.d., valencene and nootkatone (shown in Fig. 1), are often present in a considerable amount. Nootkatone is a flavorant (FEMA 3166) used for flavouring the food and tobacco (Chen, Xie, Liu, Kong, & Li, 2006). More importantly, it is an antiulcer agent (Yamahara, Yu, & Tamai, 1990) and has an insecticidal activity against *Drosophila melanogaster* (Miyazawa, Nakamura, & Ishikawa, 2000). However, due to its chiral stereostructure, the preparation of nootkatone via organic synthesis is difficult.

High-speed counter-current chromatography (HSCCC) has been widely applied for purification of functional components from traditional Chinese herbs and other natural products (Frighetto, Welendorf, Nigro, Frighetto, & Siani, 2008; Ma, Li, Zhang, Liu, & Fan, 2005; Shi, Zhang, Huang, Liu, & Zhao, 2008; Yan et al., 2005). It is a support-free liquid–liquid partition chromatography technique so that the irreversible adsorption onto the solid stationary phase and denaturation of the compounds can be eliminated. What is more, HSCCC offers many other advantages such as choice of a wide range of the solvent systems, short separation time, high-purity of fractions, quantitative sample recovery and ease of scaling up.

In this paper, HSCCC is applied in isolation and purification of nootkatone from the essential oil of *A. oxyphylla* fruits. The composition of the two-phase solvent system is optimised in terms of partition coefficients (K) of nootkatone and proper separation factors (α) between nootkatone and valencene by testing seven non-aqueous and five organic-aqueous solvent systems. Finally, the two-phase solvent system composed of n-hexane–methanol–water (5:4:1, v/v) was selected and utilised for the isolation and purification of nootkatone from the essential oil.

2. Experimental

2.1. Apparatus

The present study employed a model TBE 300A high-speed counter-current chromatograph (Shanghai Tauto Biotech, Shanghai, China) with three polytetrafluoroethylene coils (tubing I.D. 2.6 mm, total volume 300 ml) and a 20 ml manual injection sample loop. The evolution radius (R) is 5 cm, and the β values of the multilayer coil vary from 0.5 at the internal terminal to 0.8 at the

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doi:10.1016/j.foodchem.2009.04.011

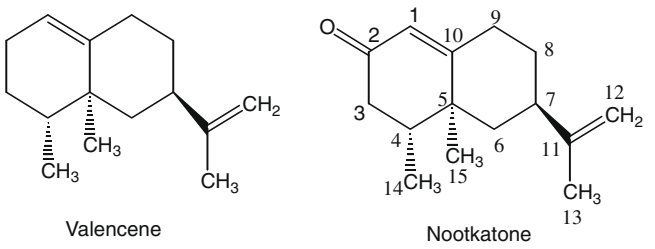

Fig. 1. Chemical structures of valencene and the target compound nootkatone.

external terminal ($\beta = r/R$, where r is the distance between the coil and the holder shaft). The rotary speed of the apparatus can be regulated at 700–1000 rpm with a speed controller. The elution of the solvent and the UV detection were performed by one ÄKTA prime system (Amersham Pharmacia Biotechnique Group, Sweden). The column temperature was controlled by an HX 1050 water-circulating constant temperature implement (Beijing Tianyou Science Development Co. Ltd., Beijing, China). The chromatogram was recorded by an N2010 workstation (Zhejiang University, Hangzhou, China).

An Agilent 6890N/5973i gas chromatograph and mass spectrometer (GC–MS) and an Agilent 6890 gas chromatograph coupled with a flame ionisation detector (GC-FID) (Agilent Technologies, USA) were used for analysis. ^{1}H NMR was performed on a Brucker Avance 400 MHz nuclear magnetic resonance (NMR) spectrometer.

2.2. Materials and reagents

The fruits of *A. oxyphylla* Miq. were purchased from Tongrentang drugstore (Qianmen, Beijing). Before use, they were ground into powder. The organic solvents, methanol, acetonitrile, ethyl acetate, ethanol, light petroleum (boiling point 30–60 °C), n-hexane, chloroform and dichloromethane, all in analytical grade, were from Beijing Chemical Reagent Company. The C_6–C_{23} n-alkanes used to analyse retention indices (RI) of the components in the essential oil, in chromatographic grade, were from Dikma Technologies in Beijing.

2.3. Preparation of essential oil by simultaneous distillation and solvent extraction (SDE)

Attached to a modified Likens-Nickerson apparatus were a 2000 ml round bottom flask and a 500 ml round bottom flask. In the 2000 ml round bottom flask, 500 g of pulverised sample was suspended in 1300 ml of water. In the 500 ml round bottom flask, 300 ml dichloromethane (purified in advance) was added. The sample and the solvent were heated by an oil bath and a water bath separately. After boiled and refluxed for 12 h, the dichloromethane fractions in both the solvent flask and the solvent loop were combined, dehydrated over anhydrous Na_2SO_4 and concentrated mildly by N_2 blowing. The essential oil obtained was pale yellow at a yield of 1.61%.

2.4. Determination of partition coefficients (K)

The partition coefficients (K) were determined as follows: a small amount of essential oil was dropped into a 10 ml test tube to which 2.0 ml of each phase of the equilibrated two-phase solvent system was added. The tube was shaken vigorously for 2 min to thoroughly equilibrate the sample between the two phases. Then an aliquot of each phase was analysed by GC. The K values were calculated by the peak areas in GC chromatograms.

2.5. Preparation of two-phase solvent systems and sample solutions

The solvents were mixed in a separatory funnel according to the selected volume ratios and thoroughly equilibrated by vigorous shaking at room temperature (ca. 22 °C). Prior to use, the upper phase and the lower phase were separated and degassed by sonication for 25 min.

For selection of two-phase solvent system the sample solution was prepared by dissolving 150 mg of essential oil into 18 ml two-phase solvent system, whilst for the isolation and purification of nootkatone the sample solution was prepared by dissolving 80 mg of essential oil into 15 ml two-phase solvent system.

2.6. HSCCC separation

Both head–tail and tail–head elution modes were carried out. When tail–head elution was used, the polytetrafluoroethylene tubes pertaining to entrance and exit on the injection loop were reversed. In each separation the multilayer-coiled column was first entirely filled with the stationary phase at a flow rate of 30 ml min^{-1}. Then the mobile phase was pumped through the column at a flow rate of 1.5 ml min^{-1} whilst the HSCCC apparatus was rotated at a speed of 850 rpm. After hydrodynamic equilibrium was established throughout the coil, the sample solution was injected into the separation column. During the separation the column temperature was controlled at 22 °C. The UV detector was set at 254 nm. The fractions were manually collected according to the chromatogram, and concentrated by N_2 blowing. The residue liquids were analysed by GC–MS. The purity of nootkatone fraction was expressed as the percentage of its peak area relative to the total peak area in GC–MS.

2.7. GC–MS and GC analysis

The DB-5 ms 30 m × 0.25 mm × 0.25 μm capillary column (Agilent Technologies, USA) was used in GC–MS analysis. The carrier gas was helium at 1 ml min^{-1}. The initial oven temperature was 100 °C held for 2 min; then raised to 165 °C at 10 °C min^{-1}; further raised to 170 °C at 1.5 °C min^{-1} kept for 2 min; and again raised to 183 °C at 1.5 °C min^{-1}. Finally it was raised to 280 °C at 30 °C min^{-1} and kept for 2 min. The sample of 2.0 μl was injected at 300 °C in a split mode (20:1). The mass detector was operated at 150 °C in an electron impact mode at 70 eV. The ion source temperature was kept at 230 °C whilst the transfer line temperature was at 250 °C. The chromatograms were recorded by monitoring the total ion currents in 40–450 mass range.

The HP-5 30 m × 0.32 mm × 0.25 μm capillary column (Agilent Technologies, USA) was used in GC analysis. The carrier gas was nitrogen at 1 ml min^{-1}. Other chromatographic conditions utilised were identical to those in GC–MS analysis above.

2.8. NMR analysis

In addition to GC–MS, the structure of nootkatone fraction was further confirmed by ^{1}H NMR. ^{1}H NMR spectra were recorded on a Brucker Avance 400 MHz spectrometer with TMS (tetramethylsilane) as internal standard and $CDCl_3$ as the solvent.

3. Results and discussion

3.1. Analysis of the essential oil

The total ion current chromatogram of the essential oil in GC–MS was shown in Fig. 2a. It could be seen that most of the peaks were distributed in the retention region over 8 min. According to NIST 02 mass spectra library as well as retention indices (RI), they

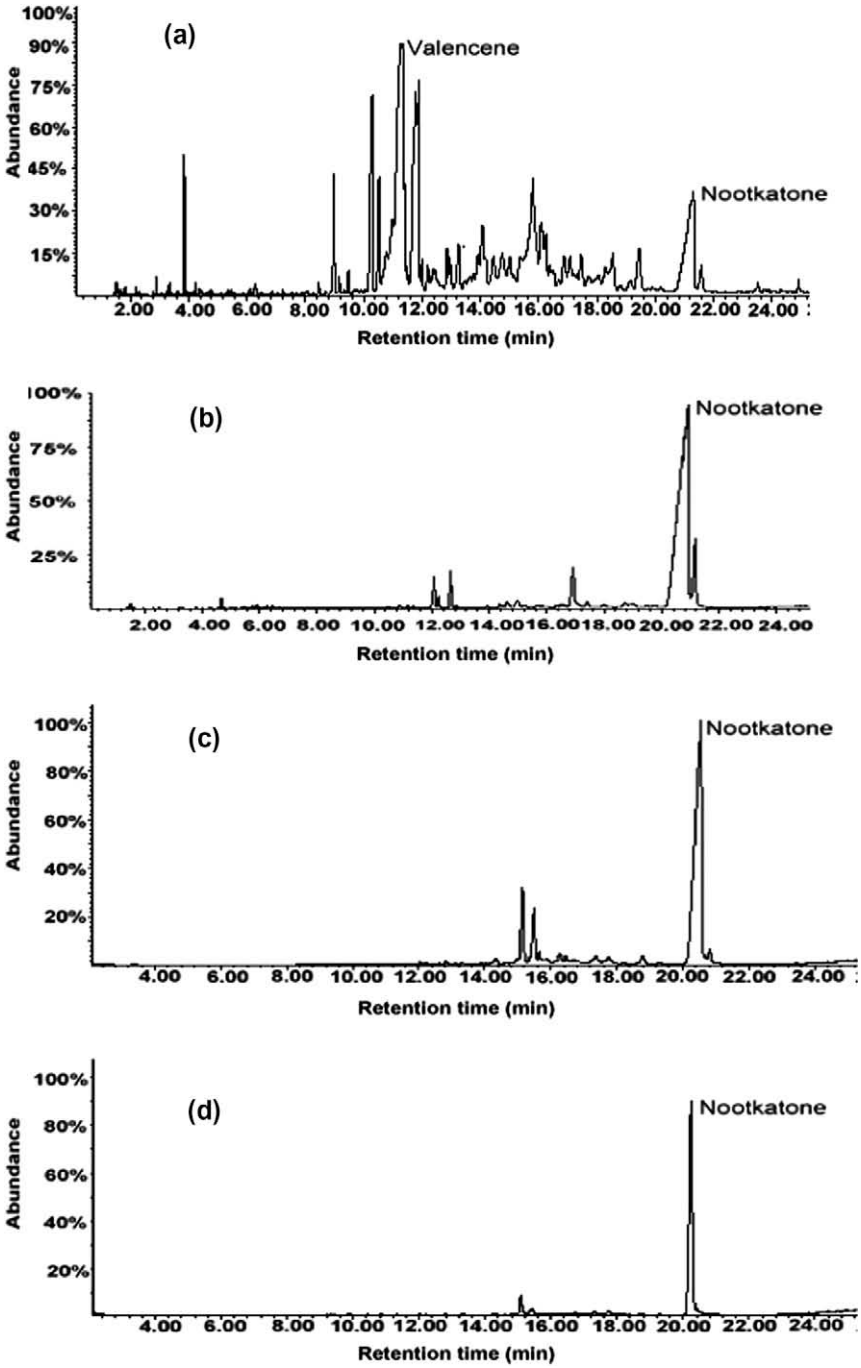

Fig. 2. Total ion current chromatograms in GC–MS analysis of the crude essential oil from fruits of *Alpinia oxyphylla* Miquel and the nootkatone fractions collected in HSCCC experiments. (a) The crude essential oil, valencene (RI 1477) in 33.68% and nootkatone (RI 1834) in 7.64%. (b) The nootkatone fraction corresponding to HSCCC separation in Fig. 3a, nootkatone in 80.55 %. (c) The nootkatone fraction corresponding to HSCCC separation in Fig. 3b, nootkatone in 77.39%. (d) The nootkatone fraction corresponding to HSCCC separation in Fig. 3c, nootakone in 92.30%. Operation conditions of GC–MS: DB-5 ms 30 m × 0.25 mm × 0.25 μm capillary column; carries gas, helium in 1 ml min^{-1}; sample, 2.0 μl injected at 300 °C in a split mode (20:1); oven temperature: initial 100 °C held for 2 min; then raised to 165 °C at 10 °C min^{-1}; further raised to 170 °C at 1.5 °C min^{-1} kept for 2 min; and again raised to 183 °C at 1.5 °C min^{-1}; finally raised to 280 °C at 30 °C min^{-1} and kept for 2 min. Mass detector, 150 °C in an electron impact mode at 70 eV; ion source temperature 230 °C; transfer line temperature 250 °C; mass detection range (*m*/*z*) 40–450.

mainly belonged to sesquiterpenes (9.00–12.00 min) and oxygenous sesquiterpenes (12.31–24.00 min). Valencene (RI 1477) was the most abundant in the essential oil, representing 33.68% of the total peak areas, whereas nootkatone (RI 1834) was 7.64%.

3.2. Selection and optimisation of two-phase solvent systems

Successful separation by HSCCC mainly depends upon the selection of a suitable two-phase solvent system. Essentially, the non-aqueous solvent system is favourable for the isolation of non-polar target compounds like squalene (Lu, Jiang, & Chen, 2003), whereas the organic-aqueous solvent system is favourable for the polar compounds such as amygdalin (Yan, Tong, & Li, 2006) and inflacoumarin A (Wang, Lee, & Wang, 2004). Since nootkatone is an oxygenous sesquiterpene of weak polarity, both non-aqueous and organic-aqueous two-phase solvent systems were examined in the present work.

In order to find the solvent systems with suitable partition coefficients (*K*) (generally within the range of 0.5–2), the partition pat-

tern of nootkatone was investigated in a series of selected two-phase solvent systems, all of which satisfied the following requirements: nootkatone was stable and soluble in the solvent system; the solvent system could form two phases with acceptable volume ratios to avoid wastage; and the settling time was less than 20 s (Ito, 2005). In addition, the K value of valencene was also determined since it dominates in the essential oil and bears a chemical structure similar to nootkatone. To resolve nootkanone from valencene, which is a prerequisite to pure nootkatone from the essential oil, the ratio of K values between nootkatone and valencene or the separation factor ($\alpha = K_1/K_2$, where $K_1 > K_2$) ought to be greater than 1.5 in the semi-preparative multilayer separation column used in the present study (Ito, 2005). Otherwise, the α value could give an indication of resolving nootkanone from other sesquiterpene impurities of similar polarities to valencene in the essential oil.

In Table 1, the non-aqueous and organic-aqueous two-phase solvent systems examined in proper K values of nootkatone as well as proper α values between nootkatone and valencene were shown. Besides, several items involved in HSCCC separation for these two-phase solvent systems were listed in Table 1, which included purity of nootkatone fraction collected (percentage of total peak areas in GC–MS), % retention of the stationary phase, the elution time and the calculational retention time of nootkatone in both head–tail and tail–head elution modes. For head–tail elution in which the lower phase was used as the mobile phase, K was expressed as $K_{U/L} = A_U/A_L$, where A_U and A_L was the peak area of the compound in the upper phase and the lower phase by GC, respectively. Whilst for tail–head elution the upper phase was used as the mobile phase, K is the reciprocal of $K_{U/L}$, that is $1/K_{U/L}$.

As we know, when the K value enlarged, compound discrimination on HSCCC can be better whilst the elution time becomes longer. It could be seen from Table 1 that for the same two-phase solvent system employed, higher purity of nootkatone fraction mainly pertained to the elution mode whose K value of nootkatone was larger than its opposite elution. However, the extended hours in those larger K value elution modes were still acceptable. In Table 1, the highest purity (80.55%) of nootkatone fraction amongst the non-aqueous solvent systems and that (77.39%) amongst the organic-aqueous solvent systems were achieved by n-hexane–chloroform–acetonitrile (10:1:10, v/v) in tail–head elution and n-hexane–methanol–water (5:4:1, v/v) in head–tail elution, separately. The HSCCC chromatograms in these solvent systems were shown in Fig. 3a and b, whilst the resulting GC–MS chromatograms on nootkatone fractions collected by HSCCC were shown in Fig. 2b and c. In comparison, the organic-aqueous solvent system composed of n-hexane–methanol–water (5:4:1, v/v) in head–tail elution was more efficient than the non-aqueous solvent system composed of n-hexane–chloroform–acetonitrile (10:1:10, v/v) in tail–head elution since similar purity was achieved in rather shorter elution hours when the former used. Otherwise, it could be further seen from Fig. 3b and a that the HSCCC peak corresponding to nootkatone fraction given by n-hexane–methanol–water (5:4:1, v/v) in head–tail elution was suitable, whereas that by n-hexane–chloroform–acetonitrile (10:1:10, v/v) in tail–head elution was quite lower and relatively broader, which was unfavourable for the collection and recovery of nootkatone. In fact, the efficiency for n-hexane–methanol–water (5:4:1, v/v) probably originated from stronger hydrophobicity present for valencene as well as other sesquiterpene impurities in the organic-aqueous solvent system, which could render much higher α value shown in Table 1 and bring better resolution of nootkatone from the essential oil. Conclusively, to isolate nootkatone from the essential oil, the organic-aqueous solvent system composed of n-hexane–methanol–water (5:4:1, v/v) in head–tail elution was considered the most appropriate.

3.3. Isolation of nootkatone from the essential oil by HSCCC

In fact, the crude essential oil may be more complex than those samples reported on preparative separation by HSCCC (Cao et al., 2006; Lu et al., 2003; Yan et al., 2005, 2006), since various sesquiterpenoids having similar polarities to nootkatone are likely present in the essential oil and even the presence of the target nootkatone is not so prominent as shown in Fig. 2a. Thus, after the two-phase solvent system had been selected and optimised for the above work, the purity of nootkatone obtained was still unsatisfactory, as shown in Table 1 and Fig. 2. It is well known that sample size often affects the separation resolution (Wang et al., 2004). Therefore, in order to improve the purity of nootkatone in one step

Table 1
Composition of seven non-aqueous and five organic-aqueous solvent systems and summary of experimental results on separation of nootkatone in crude essential oil by HSCCC.

Solvent system (v/v)[a]	$K_{U/L}$ (Valencene)[b]	$K_{U/L}$ (Nootkatone)[b]	α[c]	Purity[d] (%)		S_f (%)[e]		Elution time (min)[f]		t_R (min)[i]	
				$h-t$[g]	$t-h$[h]	$h-t$[g]	$t-h$[h]	$h-t$[g]	$t-h$[h]	$h-t$[g]	$t-h$[h]
Hex–$CHCl_3$–ACN (6:2:5)	1.65	0.70	2.33	16.47	18.46	31.1	61.1	160–195	280–300	181	251
Hex–$CHCl_3$–ACN (6:1:4)	4.46	0.63	7.22	53.93	64.98	82.3	83.0	105–135	305–350	139	298
Hex–$CHCl_3$–ACN (10:1:10)	5.04	0.45	11.00	63.73	80.55	81.7	83.0	85–120	370–420	110	399
Hex–CH_2Cl_2–ACN (10:3:7)	1.97	0.65	3.00	53.87	64.82	75.2	70.0	230–270	275–315	147	274
Hex–CH_2Cl_2–ACN (10:1:10)	6.46	0.49	13.53	45.66	76.90	82.0	84.1	85–120	410–470	116	373
Hex–CH_2Cl_2–EtOAc–ACN (6:1:1:4)	2.48	0.72	3.45	29.02	70.45	45.2	66.4	150–180	255–300	175	250
Hex–CH_2Cl_2–EtOAc–ACN (10:1:1:10)	4.49	0.50	9.00	51.77	75.74	80.6	85.2	90–120	390–440	119	367
LtPet–$(Et)_2O$–EtOH–H_2O (5:0.5:4:0.5)	7.28	0.69	10.55	56.25	64.23	72.4	84.1	130–155	310–370	155	274
LtPet–EtOH–H_2O (5:4:1)	28.64	0.95	30.17	75.87	61.85	79.2	86.9	160–185	180–230	192	209
LtPet–$(Et)_2O$–EtOH–H_2O (5:0.5:4:1)	31.50	1.14	27.63	66.36	55.44	76.7	87.6	300–380	180–240	221	177
Hex– EtOAc–MeOH–H_2O (5:1:4:1)	28.68	1.11	25.84	75.51	55.26	82.2	91.2	185–210	145–170	218	182
Hex–MeOH–H_2O (5:4:1)	93.56	1.25	74.85	77.39	29.55	81.3	92.6	190–220	140–200	241	163

[a] Hex, n-hexane; ACN, acetonitrile; EtOAc, ethyl acetate; LtPet, light petroleum (30–60 °C); $(Et)_2O$, diethyl ether; EtOH, ethanol; MeOH, methanol.
[b] The partition coefficients (K): in head–tail elution using the lower phase as the mobile phase, the K value was expressed as $K_{U/L} = A_U/A_L$, where A_U and A_L was the peak area of the compound in the upper phase and the lower phase by GC, respectively; whilst in tail–head elution using the upper phase as the mobile phase, the K value is the reciprocal of $K_{U/L}$, that is $1/K_{U/L}$.
[c] α is the separation factor obtained by the ratio of K values between valencene and nootkatone, e.g. $\alpha = K_{U/L}$ (Valencene)/$K_{U/L}$ (Nootkatone).
[d] Percentage of peak areas of nootkatone analysed by GC–MS.
[e] S_f, retention of the stationary phase, $S_f = V_s/V_C$, where V_s is the volume of the retained stationary phase, V_C is the total column volume.
[f] The time range of nootakone fraction being eluted out.
[g] Head–tail elution mode, using the lower phase as the mobile phase.
[h] Tail–head elution mode, using the upper phase as the mobile phase.
[i] t_R, the theoretical retention time of nootkatone, calculated by $t_R = V_R/F$ and $V_R = V_C[1 + (K - 1)S_f]$, where V_R was the retention volume of nootkatone, F was the flow rate of the mobile phase and K was the partition coefficient of nootkatone.

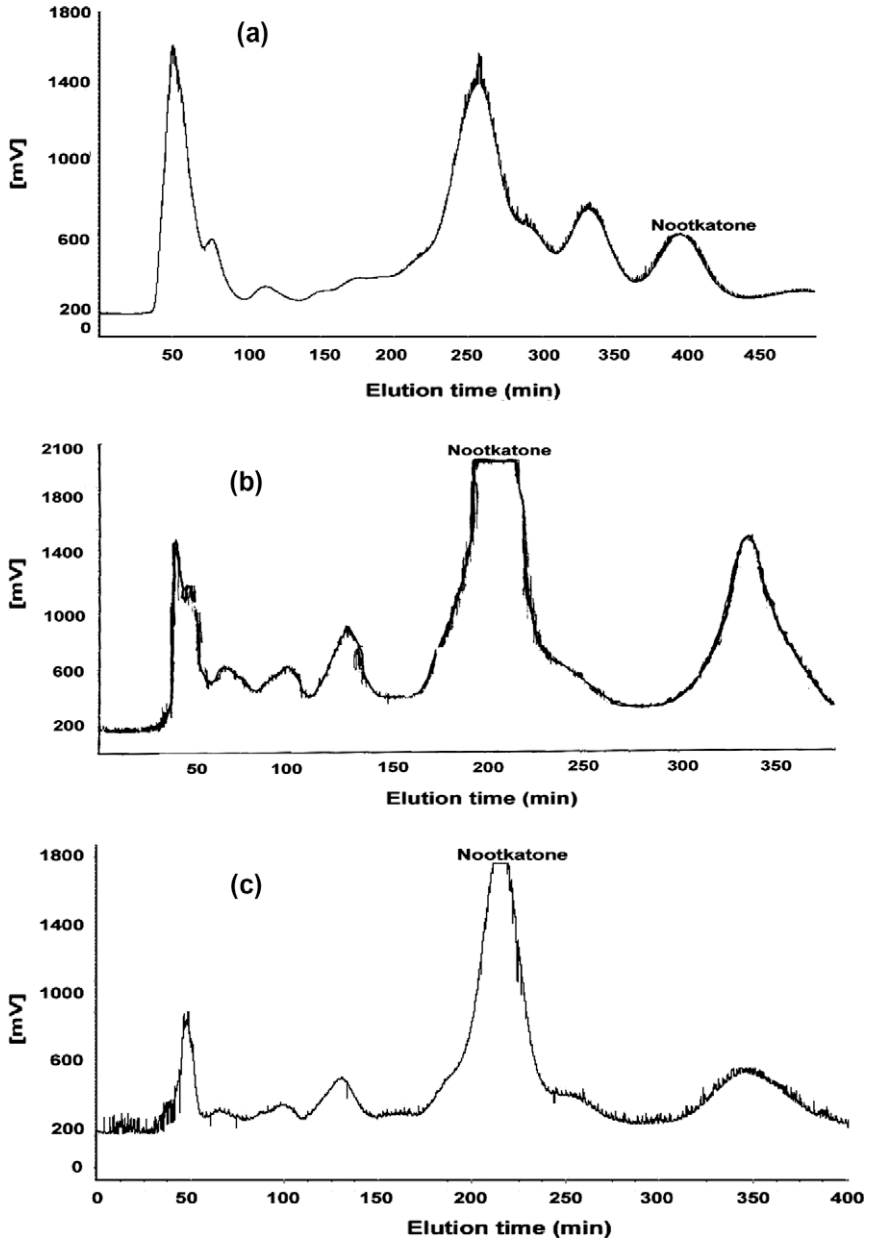

Fig. 3. HSCCC chromatograms on the separation of nootkatone from the crude essential oil of fruits of *Alpinia oxyphylla* Miquel. Revolution speed: 850 rpm; separation temperature: 22 °C, flow rate: 1.5 ml min^{-1}; and UV detection wavelength 254 nm. (a) Two-phase solvent system: hexane–chloroform–acetonitrile (10:1:10, v/v); mobile phase: the upper phase; sample size: 150 mg of essential oil dissolved into 18 ml two-phase solvent system. (b) Two-phase solvent system: n-hexane–methanol–water (5:4:1, v/v); mobile phase: the lower phase; sample size: 150 mg of essential oil dissolved into 18 ml two-phase solvent system. (c) Two-phase solvent system: n-hexane–methanol–water (5:4:1, v/v); mobile phase: the lower phase; sample size: 80 mg of essential oil dissolved into 15 ml two-phase solvent system.

HSCCC separation, the separation was similarly carried out with the solvent system composed of n-hexane–methanol–water (5:4:1, v/v) in head–tail elution mode by reducing the sample load (shown in Fig. 3c). It turned out that 3.1 mg of nootkatone, with a purity at 92.30% by GC–MS (shown in Fig. 2d), was obtained from 80 mg of crude essential oil.

However, when less sample loaded, separation on the non-aqueous solvent system composed of hexane–chloroform–acetonitrile (10:1:10,v/v) in tail–head elution mode was still not improved, and the HSCCC peak corresponding to nootkatone fraction often became too flat to be identified.

3.4. The structural identification

The nootkatone fraction obtained in purity of 92.30% was an pale oil with sweet, woody and orange-like odours. Its chemical structure was identified by EI-MS (in GC–MS) and ^{1}H NMR as follows:

MS (70 eV, *m*/*z*): 218 [M^+] (23); 147(100); 121(72); 91(69); 41(64). ^{1}H NMR ($CDCl_3$) δ(ppm): 5.80 (1H, s, H-1); 4.78 (2H, d, H-12); 1.77 (3H, s, H-13); 1.15 (3H, s, H-15); 0.99 (3H, d, H-14). The MS and ^{1}H NMR spectra data were in agreement with those reported (Luo et al., 2000; Miyazawa et al., 2000).

4. Conclusions

The HSCCC method in a semi-preparative scale was established and applied successfully in the isolation and purification of nootkatone from the essential oil of fruits of *A. oxyphylla* Miq. Five organic-aqueous and seven non-aqueous two-phase solvent systems were selected in terms of the partition coefficients of nootkatone

and also the separation factors between nootkatone and valencene. When subjected to HSCCC, the non-aqueous solvent system composed of n-hexane–chloroform–acetonitrile (10:1:10, v/v) in tail–head elution mode and the organic-aqueous solvent system composed of n-hexane–methanol–water (5:4:1, v/v) in head–tail elution mode proved potential to purify nootkatone from the essential oil. Nevertheless, because of rather less elution time and better HSCCC peak form of nootkatone, n-hexane–methanol–water (5:4:1, v/v) was determined optimal and finally employed in the preparative separation of nootkatone. By eluting the lower phase of n-hexane–methanol–water (5:4:1, v/v) at a flow rate of 1.5 ml min^{-1} under 850 rpm of revolution speed, 80 mg of crude essential oil was separated yielding 3.1 mg of nootkatone at a purity of 92.3% in less than 4 h. The chemical structure of nootkatone fraction obtained was confirmed by EI-MS and ^1H NMR.

Acknowledgements

The present work was supported by Beijing Municipal Talent Development Organization (No. 20051D0500310) and National Natural Science Foundation of China (NSFC20676003).

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Food Chemistry 117 (2009) 381–386

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Biological evaluations of novel vitamin C esters as mushroom tyrosinase inhibitors and antioxidants

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ARTICLE INFO

Article history:
Received 19 December 2008
Received in revised form 9 March 2009
Accepted 31 March 2009

Keywords:
Vitamin C esters
Mushroom tyrosinase
Inhibitory effect
Antioxidant activity

ABSTRACT

The inhibitory effects of vitamin C esters **1** and **2** on the diphenolase activity of mushroom tyrosinase have been studied. The results showed that compounds **1** and **2** inhibited tyrosinase with IC_{50} values of 0.58 and 0.16 mM, respectively. The dose–response curves demonstrated that compounds **1** and **2** not only lengthened the lag time, but also decreased the steady-state rate. The kinetic analyses showed that the inhibition by compound **2** was reversible and its mechanism was mixed type, which was different from compound **1** (irreversible inhibitor). Furthermore, the antioxidant activities of these compounds against hydroxyl radical scavenging, superoxide anion radical scavenging, and DPPH radical scavenging were also investigated. Compounds **1** and **2** exhibited potential antioxidant activities. In particular, compound **2** was found to be the most effective antioxidant, more potent than the well-known antioxidants vitamin C and TBHQ.

1. Introduction

Tyrosinase (EC 1.14.18.1; polyphenol oxidase, PPO), a multifunctional copper-containing enzyme, is widely distributed in fungi, plants and animals (Huang, Lin, Qiu, Shi, & Chen, 2006; Huang et al., 2006). It catalyses two distinct reactions of melanin synthesis, the hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones, both depending on molecular oxygen (Khan et al., 2006; Martynez & Whitaker, 1995). The produced quinone is a highly reactive compound and can polymerise spontaneously to form melanin, which determines the colour of mammalian skin and hair. However, recently investigations demonstrated that some dermatological disorders, such as age spots and freckles, were caused by the accumulation of an excessive level of epidermal pigmentation (Mercedes & Francisco, 1997). Moreover, this highly active quinone can also react with amino acids and proteins in vegetables and fruits, and thus enhance the development of brown colour, which contributes to both quality loss and undesirable changes in colour, flavour and softening (Nerya, Musa, Khatib, Tamir, & Vaya, 2004; Shi, Chen, Wang, Song, & Qiu, 2005). In addition, tyrosinase is known to be involved in the moulting process of insects and adhesion of marine organisms (Liu, Yi, Wan, Ma, & Song, 2008).

The control of tyrosinase activity is of great importance in preventing the browning of vegetables and fruits and the accumulation of an excessive level of epidermal pigmentation in animals. So far, many efforts have been spent in the search for effective and safe tyrosinase inhibitors, and a large number of naturally-occurring and synthetic tyrosinase inhibitors have already been reported (Cho, Roh, Sun, Kim, & Park, 2006; Ley & Bertram, 2001; Um et al., 2003). However, some of them are either not potent enough to be considered of practical use or not compatible with safety regulations for food and cosmetic additives. So an urgent effort to discover and develop novel and potent tyrosinase inhibitors has become increasingly important in the food industry (Qiu et al., 2009) as well as in medicinal and cosmetic products (Maeda & Fukuda, 1991).

Vitamin C, also known as ascorbic acid, is widely distributed in plants, such as rose hips, blackcurrants and citrus fruits. It is well known that vitamin C has been considered by some scientists as a "universal panacea" due to its broad biochemical and pharmacological functions. For example, it could prevent the occurrence and development of some chronic diseases, such as cardiovascular disease, cancer and cataracts; acting as a highly effective antioxidant it can protect indispensable molecules in the body from damage by free radicals and reactive oxygen species. More recently, vitamin C as potent tyrosinase inhibitor has been studied in detail by Zeng, Zheng, and Chen (2005). The results suggested that it might act as a reducing agent in the reaction of the tyrosinase catalytic substance L-DOPA. In addition, some literature reported that 4-hydroxybenzoic acid and gallic acid had potent tyrosinase inhibitory effects (Huang, Lin et al., 2006; Varda, Noach, & Varda, 1997; Xue et al., 2007).

Inspired by these results, we supposed that the condensations of vitamin C and 4-hydroxybenzoic acid or gallic acid had potent

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0308-8146/$ - see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2009.03.108

tyrosinase inhibitory activities with low side effects. In the present paper, therefore, compounds **1** and **2** (Fig. 1) were designed, synthesised and their inhibitory effects (inhibitory activity, inhibition mechanism, and inhibition kinetics) on mushroom tyrosinase were studied. Furthermore, the antioxidant activities of these compounds against hydroxyl radical scavenging, superoxide anion radical scavenging, and DPPH radical scavenging were also investigated. The aim of the present study was the discovery of feasible and efficient compounds as food additives or food preservatives, which can offer a clue to the design and synthesis of novel tyrosinase inhibitors with potent antioxidant activity.

2. Materials and methods

2.1. Chemicals and reagents

Melting points were determined on a WRS-1B digital instrument without correction. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury-Plus 300 NMR instrument (^{1}H 300 MHz; ^{13}C 75 MHz) in DMSO-d_6. Mass spectra were recorded on a Thermo Finnigan LCQ DECAXP ion trap mass spectrometer. IR spectra were recorded as potassium bromide pellets on a Bruker Equinox 55 FT/IR spectrometer. Elemental analyses (C, H, N) were carried out on an Elementary Vario EL series elemental analyser and the results were within ± 0.4%.

Tyrosinase and L-3,4-dihydroxyphenylalanine (L-DOPA), 1,1-diphenyl-2-picrylhydrazyl (DPPH) phenanthroline, and tertiary-butyl-hydroquinone (TBHQ) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Other chemicals were purchased from commercial suppliers and were dried and purified when necessary. The water used was re-distilled and ion-free. D-ascorbic acid-6-*p*-hydroxybenzoic acid ester (**1**) was available from our previous work (Wu et al., 2007).

2.2. Synthesis

A mixture of vitamin C (30 mmol), gallic acid (10 mmol) and H_2SO_4 (98%, 20 ml) was refluxed at 25 °C for 24 h. The residue was extracted with ethyl acetate several times and filtered. The filtrate was washed successively with saturated aqueous $NaHCO_3$ solution and ice-water, dried over $MgSO_4$, and evaporated. The obtained precipitate was recrystallised from water to afford compound **2** as a yellow solid. The structure of the synthesised compound **2** was established by IR, ^{1}H NMR, ^{13}C NMR, and ESI-MS, and the purity was confirmed by elemental analysis. The results were as follows: Melting point: 188.8–190.5 °C; IR (KBr, cm^{-1}): 3449, 3354, 1761, 1680, 1612, 1340, 1358, 1243, 1204, 1126, 1033, 1002; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 6.97 (s, 2H), 4.74 (s, 1H), 4.22 (m, 2H), 4.06 (m, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 172.5, 168.7, 154.4, 147.2 (2C), 139.3, 121.3, 119.1, 110.5 (2C), 76.3, 68.8, 66.2; ESI-MS: *m/z* 327 (M^{+-} 1, 100%); Anal Calcd for $C_{13}H_{12}O_{10}$: C, 47.57; H, 3.69; found: C, 47.33; H, 3.81.

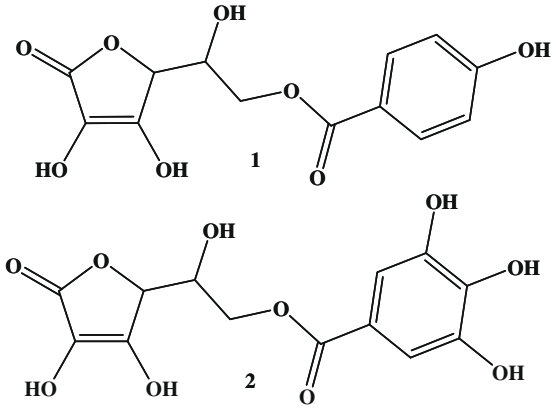

Fig. 1. Chemical structures of compounds **1** and **2**.

2.3. Assay of the diphenolase activity

The spectrophotometric assay for tyrosinase was performed according to the method reported by our groups (Liu et al., 2008; Yi et al., 2008) with some slight modifications. Both the synthesised compounds were screened for diphenolase inhibitory activity of tyrosinase, using L-DOPA as substrate. All the compounds were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the test solution was 2.0%. First, thirty units of mushroom tyrosinase (0.5 mg/ml) were pre-incubated with the sample in 50 mM phosphate buffer (pH 6.8) for 10 min at 25 °C. Then, the L-DOPA (0.5 mM) was added to the reaction mixture and the enzyme reaction was monitored for 1 min by measuring the change in absorbance at 475 nm, due to formation of the DOPAchrome. The measurement was performed in triplicate for each concentration and averaged before further calculation.

2.4. Assay of antioxidant activity of vitamin C esters 1 and 2

2.4.1. Assay of hydroxyl radical-scavenging activity

The hydroxyl radical-scavenging activity of samples was evaluated, according to a modified procedure from our previous report (Wu et al., 2007). First, 600 μl of 100 mM phosphate buffer (pH 5.5), 100 μl of aqueous solution of 7.5 mM $FeSO_4$, 100 μl of an ethanolic solution of 7.5 mM phenanthroline, and 100 μl of the sample solution (dissolved in ethanol) were put into a test tube. Then, 100 μl of H_2O_2 solution (dissolved in deionised water) were added to the tube and incubated at 37 °C in the dark for 1 h. The absorbance of the resulting solutions was measured at 536 nm. As a control, 100 μl of ethanol were added to the tube. Ascorbic acid and TBHQ were used as standard references. The extent of reduction by the addition of the sample was expressed as the percentage necessary for 50% reduction (EC_{50}).

2.4.2. Assay of superoxide anion radical-scavenging activity

The superoxide anion radical-scavenging activity of the samples was evaluated according to a modified procedure from our previous report (Wu et al., 2007). First, 100 μl of ethanolic solution of the sample was pre-incubated in 850 μl of 50 mM phosphate buffer (pH 8.2) at 25 °C for 10 min. Then, 50 μl of 3.0 mM pyrogallol solution (dissolved in 0.01 mM hydrochloric acid) were added to the reaction mixture and the oxidation reaction was monitored by measuring the rates at 320 nm for 100 s. As a control, 100 μl of ethanol were added to the tube. Ascorbic acid and TBHQ were used as standard references. The extent of reduction by the addition of the sample was expressed as the percentage necessary for 50% reduction (EC_{50}).

2.4.3. Assay of DPPH radical-scavenging activity

The DPPH radical-scavenging activity of the samples was evaluated according to a modified procedure from a previous report (Iwai, Kishimoto, Kakino, Mochida, & Fujita, 2004). First, 630 μl of 100 mM acetate buffer (pH 5.5) and 350 μl of an ethanolic solution of 0.3 mM DPPH were put into a test tube. Then, 20 μl of the sample solution (dissolved in DMSO) were added to the tube and incubated at 25 °C for 30 min. The absorbance of the

resulting solution was recorded at 517 nm. As a control, 20 μl of DMSO were added to the tube. Ascorbic acid and TBHQ were used as standard references. The extent of reduction by the addition of the sample was expressed as the percentage necessary for 50% reduction (EC_{50}).

3. Results

3.1. Effects of vitamin C esters 1 and 2 on the diphenolase activity of mushroom tyrosinase

Taking compound **1** and **2** (see Fig. 1 for structure) as the effectors, we proved their effect on the activity of mushroom tyrosinase for the oxidation of L-DOPA. The time courses of compound **1** were shown as curves **a** to **g** in Fig. 2A. For the first few minutes, absorptions increased slowly. After some time, the curve rose, mostly linearly, and gave a constant slope. The system reached a constant rate (the steady-state rate, V_{ss}) after the lag period, which was estimated by extrapolation of the curve to the abscissa (Xie, Chen, Huang, Liu, & Zhang, 2003). The kinetics course of the oxidation of the substrate in the presence of different concentrations of compounds **1** and **2** is shown in Fig. 2B and C, respectively. With increasing concentration of these compounds, the lag period increased exponentially. The result showed that compounds **1** and **2** could delay the lag time from 70 to 600 s at 0.75 mM and 10–410 s at 0.48 mM, respectively. On the other hand, the steady-state rate (V_{ss}) decreased with increasing concentration of compounds. The IC_{50} values of vitamin C, **1** and **2** were estimated to be 0.14, 0.58 and 0.16 mM, respectively. The results indicated that the inhibition strength followed the order: vitamin C ≈ compound **2** > compound **1**.

3.2. The inhibitory effects of compounds 1 (irreversible manner) and 2 (reversible manner) on the diphenolase activity of mushroom tyrosinase

The inhibition mechanism on mushroom tyrosinase by compounds **1** and **2** for the oxidation of L-DOPA was studied first. Fig. 3A shows the relationship between the enzyme activity and its concentration in the presence of compound **1**. The plots of the steady-state rate (V_{ss}) *versus* the concentrations of enzyme at different inhibitor concentrations gave a family of straight lines, which all ran parallel to each other. It indicated that compound **1** was an irreversible inhibitor of tyrosinase. However, for compound **2**, as shown in Fig. 3B, the plots gave a family of straight lines, which all passed through the origin. Increasing the inhibitor concentration resulted in a decrease in the slope of the line, indicating that compound **2** was a reversible inhibitor of tyrosinase.

3.3. Determination of the inhibitory types of the parent compound gallic acid and vitamin C esters 2 on mushroom tyrosinase

The inhibitory type of gallic acid on the diphenolase activity, during the oxidation of L-DOPA, was determined from Lineweaver–Burk plots. In the presence of gallic acid, the kinetics of the enzyme was shown in Fig. 4A. The plots of $1/V_{ss}$ *versus* $1/[S]$ gave a family of straight lines with different slopes but they intersected one another at the ordinate. The values of V_{max} remained the same and the values of K_m increased with increasing concentrations of the inhibitor, which indicated that gallic acid was a competitive inhibitor. The result showed that it could only bind with free enzyme. The inhibitory type of compound **2** on mushroom tyrosinase was determined by the same methods.

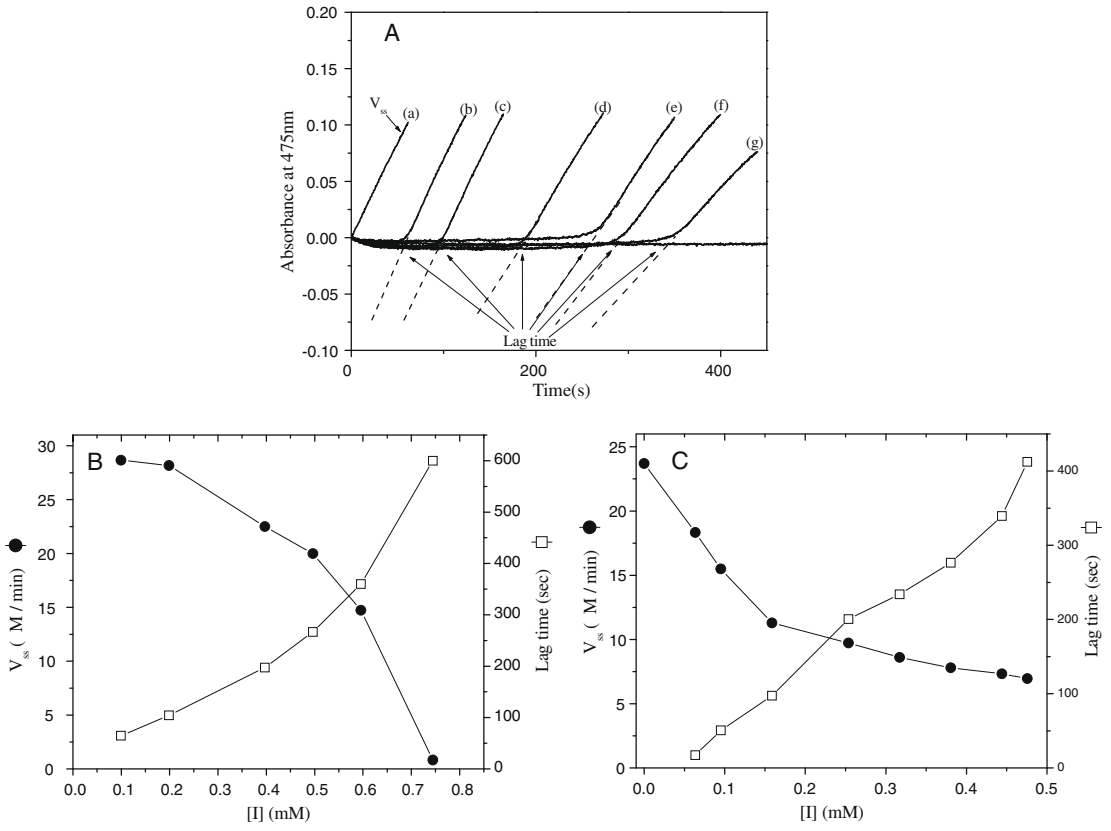

Fig. 2. Effects of vitamin C esters 1 and 2 on the diphenolase activity of mushroom tyrosinase. **A**: represents the progress curves for the inhibition of the diphenolase of mushroom tyrosinase by compound **1**. The concentrations of compound **1** for curves **a–h** were 0, 0.10, 0.20, 0.40, 0.50, 0.55, and 0.60 mM, respectively; **B** and **C** represent the effects of compounds **1** and **2** on the lag time and the steady-state-rate of the diphenolase of mushroom tyrosinase.

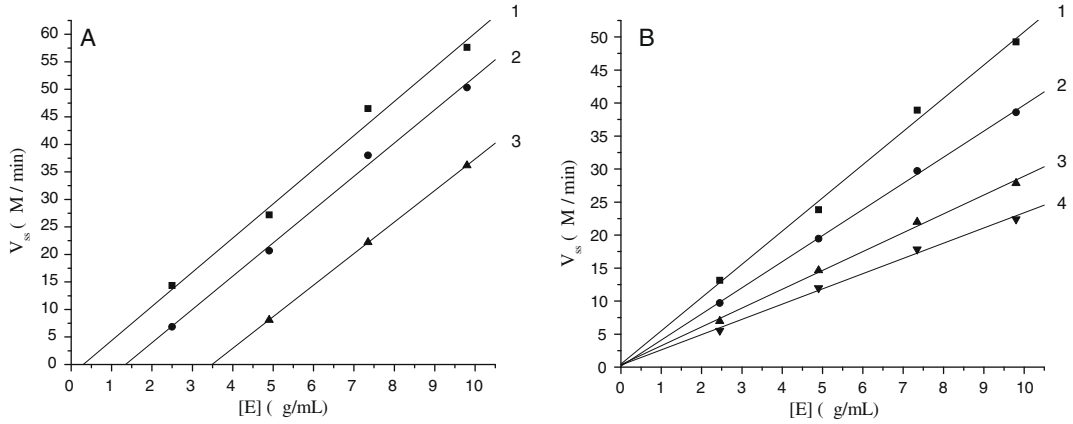

Fig. 3. Determination of the inhibitory effects of compound **1** and **2** on mushroom tyrosinase (**A** and **B**). The concentrations of compound **1** for curves 1–3 were 0.02, 0.13, and 0.34 mM, respectively. The concentrations of compound **2** for curves 1–4 were 0, 0.12, 0.24, and 0.36 mM, respectively.

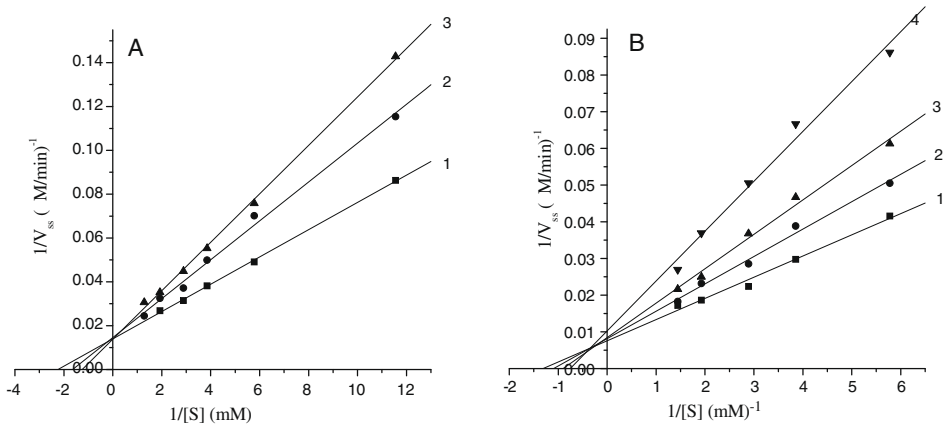

Fig. 4. Determination of the inhibitory types of gallic acid and compound **2** on mushroom tyrosinase (**A** and **B**). The concentrations of gallic acid for curves 1–3 were 0.03, 0.11, and 0.27 mM, respectively. The concentrations of compound **2** for curves 1–4 were 0, 0.03, 0.11, and 0.27 mM, respectively.

The results from Fig. 4B showed that compound **2** was a mixed-type inhibitor, since increasing its concentration resulted in a family of lines with different slopes, which intersected one another in the second quadrant. This result showed that compound **2** can bind, not only with free enzyme, but also with the enzyme–substrate complex (Huang, Lin et al., 2006; Huang, Chen et al., 2006).

3.4. Antioxidant activity of vitamin C esters 1 and 2

In the food industry, oxidation is one of the most important processes involved in food deterioration because it may affect food quality (colour, flavour and texture). Antioxidants may help preserve food quality by preventing the oxidative deterioration of lipids (Kinsella, Frankel, German, & Kanner, 1993). Furthermore, there is a considerable amount of evidence that points to an association between a diet rich in fresh fruits and vegetables and a decreased risk of cardiovascular disease and certain forms of cancer, and it is generally assumed that antioxidants (vitamins, carotenoids, sterols, polyphenols) are the active dietary constituents, which contribute to this protective effect.

Therefore, the antioxidant activities of compounds **1** and **2** against hydroxyl radical scavenging, superoxide anion radical scavenging and DPPH radical scavenging were also investigated. For the hydroxyl radical-scavenging activity, EC_{50} values of these compounds were determined and the results are shown in Fig. 5A. The scavenging strength follows the order: compound **2** > vitamin C > TBHQ > compound **1**. For the superoxide anion radical-scavenging activity, EC_{50} values of these compounds were determined and the results are shown in the Fig. 5B. The scavenging strength follows the order: compound **2** > compound **1** > vitamin C > TBHQ (EC_{50} = 5.1 mM, not shown). For the DPPH radical-scavenging activity, EC_{50} values of these compounds were determined and the results are shown in Fig. 5C. The scavenging strength follows the order: compound **2** > compound **1** > vitamin C ≈ TBHQ. These data showed that compounds **1** and **2** had potent antioxidant activities. In particular, compound **2** was found to be the most effective antioxidant, more potent than the well-known antioxidants vitamin C and TBHQ.

4. Discussion

Arbutin (4-hydroxybenzyl-*O*-β-D-glucoside) is an inhibitor of tyrosinase, which has been used widely in the cosmetic industry. Its IC_{50} value for diphenolase activity was measured as 7.30 mM (Yi et al., 2008). Our results in this paper showed that the IC_{50} values of compound **1** and its derivative **2** were 0.58 and 0.16 mM, respectively. This indicated that compound **1** and its derivative **2** could inhibit the diphenolase activity of tyrosinase at a lower concentration than that of arbutin, i.e., compound **1** and its derivative **2** were more effective inhibitors of diphenolase activity than arbutin. The results showed that these components might serve as new potent preservatives in the food industry or skin-whitening agents in cosmetics.

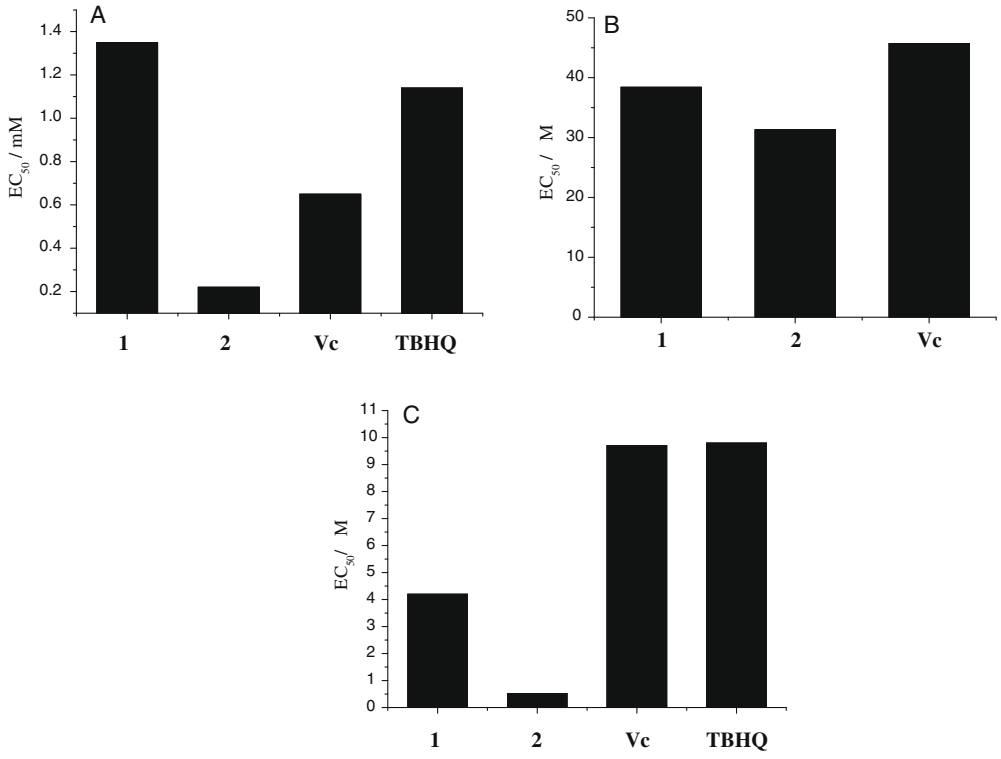

Fig. 5. EC_{50} values of compounds **1** and **2** on hydroxyl radical-scavenging activity (**A**), on superoxide anion radical-scavenging activity (**B**), and DPPH radical-scavenging activity (**C**). Here vitamin C (Vc) and TBHQ provide a comparison.

The dose–response curves demonstrated that compounds **1** and **2** not only lengthened the lag time, but also decreased the steady-state rate. Previous literature (Zeng et al., 2005) reported that the lag effect was caused by the reducing ability of vitamin C. On this basis, the action mechanism was concluded and summarised in Fig. 6. As shown in Fig. 6, the reason for the lag effects caused by vitamin C esters **1** and **2** might be that, the oxidation of L-DOPA to L-DOPAquinone occurred in the presence of tyrosinase and molecular oxygen. However, because of the existence of the vitamin C moiety, L-DOPAquinone was reduced to L-DOPA, and the redox reaction was repeated until the vitamin C moiety was completely transformed to the dehydrogenated vitamin C moiety. Undoubtedly, the greater the quantity of vitamin C existing in molecules, the longer the lag times as a result.

Recently, the crystallographic structure of tyrosinase has been reported, enabling a close look at its three-dimensional structure and a better understanding of its mechanism of action (Matoba, Kumagai, Yamamoto, Yoshitsu, & Sugiyama, 2006; Khatib et al., 2007) Within the structure, there are two copper ions in the active centre of tyrosinase and a lipophilic long-narrow gorge near to the active centre. Previous literature reported that vitamin C was defined as an irreversible inhibitor of tyrosinase, and suggested that

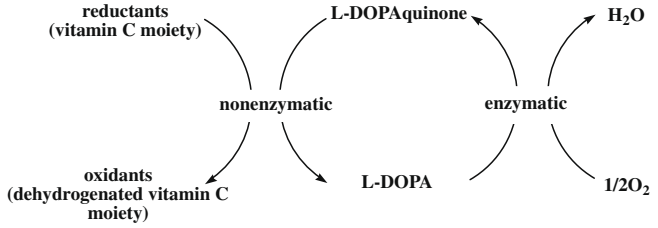

Fig. 6. The mechanism producing the lag effect in the inhibition of the diphenolase of mushroom tyrosinase by vitamin C esters.

it could exhibit strong affinity for copper ions in the active centre. Our report showed that the inhibitory effect of compound **1** was the same as vitamin C. However, its activity was four times lower than that of vitamin C. The result suggested that the introduction of 4-hydroxybenzoic acid into vitamin C might be detrimental to the tyrosinase inhibitory activity, which confirmed a previous report (Song et al., 2006) that the hydrophilic substituents, such as hydroxyl and carboxyl, might block the inhibitors that interact with the hydrophobic vicinity of the tyrosinase active centre. Interestingly, the inhibitory effect of compound **2** was obviously different from vitamin C and compound **1**. The kinetic analyses showed that the inhibition by compound **2** was reversible and its mechanism was mixed-type. From the result, it can be deduced that compound **2** might not combine with the binuclear copper ions of tyrosinase but with the hydrophobic pocket of the enzyme. Furthermore, it has been reported that hydrogen-bonding interactions could stabilise the *oxy*-form of *Streptomyces glaucescens* tyrosinase (Kubo & Kinst-Hori, 1999). Based on these observations, it could be supposed that an intermolecular hydrogen bond formed between the hydroxyl group of compound **2** and the sulfhydryl, amino, carboxyl or hydroxyl groups in the hydrophobic pocket of tyrosinase. Undoubtedly, the more hydrogen bonds formed between compound **2** and tyrosinase, the tighter and more stable the interaction between compound **2** and tyrosinase. In addition, the inhibition mechanism analysis showed that gallic acid was a competitive inhibitor of tyrosinase. By analogy with the chemical structures of gallic acid and compound **2**, the conclusion can be obtained that the introduction of a vitamin C moiety affected the binding mode of the substrate and the active site of tyrosinase.

Oxidation reactions, often radical initiated, are important processes in biological systems. A lot of pathophysiological conditions are considered to be initiated by radical reactions, and this oxidation can be protected by radical scavengers. Therefore, the present study also investigated the antioxidant activities of compounds **1** and **2**. The results showed that these compounds had potent

antioxidant activities. Comparison of compounds **1** and **2** indicated that the numbers of hydroxyl groups attached to the benzene ring might play a vital role in determining their antioxidant activities. The result further confirmed that the antioxidant activity depended on the number of donatable hydrogen atoms in the aromatic rings (Fagerlund, Sunnerheim, & Dimberg, 2009).

In summary, in the present paper, two novel vitamin C esters **1** and **2** were designed, synthesised and their inhibitory effects (inhibitory activity, inhibition mechanism, and inhibition kinetics) on mushroom tyrosinase were studied. Furthermore, their antioxidant activities against hydroxyl radical scavenging, superoxide anion radical scavenging, and DPPH radical scavenging were also investigated. All the data showed that compounds **1** and **2** might serve as new potent preservatives in the food industry or skin-whitening agents in cosmetics and suggested that further development of such compounds may be of interest.

Acknowledgments

The present investigation was supported by Grant 20032020 of the China Natural Science Foundation and Grant 2004B30101007 of the Natural Science Foundation of Guangdong Province.

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Food Chemistry 117 (2009) 387–392

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Macroporous resin purification of grass carp fish (*Ctenopharyngodon idella*) scale peptides with in vitro angiotensin-I converting enzyme (ACE) inhibitory ability

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ARTICLE INFO

Article history:
Received 15 November 2008
Received in revised form 5 April 2009
Accepted 7 April 2009

Keywords:
Adsorption
Carp fish scale
Macroporous resins (MARs)
Peptides
In vitro ACE (angiotensin-I converting enzyme) inhibitory ability

ABSTRACT

The adsorption dynamics and thermodynamics of grass carp fish scale peptides (FSPs) onto non-polar macroporous resins (MARs), DA201-C, have been investigated. The adsorption of FSPs was affected by time, pH and peptide concentration. The adsorption process followed the Langmuir adsorption isotherm, and was endothermic ($\Delta H < 43$ kJ/mol). The predominant force in adsorption of FSPs onto DA201-C was hydrophobic. Depending on this force, the dynamic adsorption and gradient desorption results showed that DA201-C resins were good at desalting and enriching peptides with higher contents of hydrophobic amino acids, and these peptides had higher ACE inhibitory capabilities in vitro. The lowest concentration at which the eluted fraction possessed half of its original ACE activity (IC_{50}) was 0.13 mg/ml. The results indicated that fish scale peptides produced showed good ACE-inhibitory effect in vitro and fish scales are a good source of peptides with in vitro ACE inhibitory activity.

1. Introduction

The grass carp fish (*Ctenopharyngodon idella*) is one of the largest members of the minnow family, which inhabits fresh warm water. The body is covered with large and heavy scales. The global production of cultured grass carp fish was only 10.5 tons in 1950 (Vannuccini, 2004). By 2002 it reached 3.6 million tons and accounted for 15% of global fresh water aquaculture production. During the processing of fish, large amounts of waste resulting from filleting are either discarded or utilised for low value products. Fish scales are dermally derived, specifically in the mesoderm, and are comprised of highly ordered type I collagen fibres and hydroxyapatite $Ca_{10}(OH)_2(PO_4)_6$, (Ikoma, Kobayashi, Tanaka, Walsh, & Mann, 2003). However, fish scales are stiff, and most of them are discarded during processing. Due to increasing costs, animal protein supply is limited, as is the potential for increasing the supply through increased animal production. Therefore, there is a need for efficient utilisation of inexpensive and available food resources.

One of the approaches for improving and upgrading the functional and nutritional properties of proteins is enzymatic hydrolysis. Proteins can be hydrolysed to produce biologically active peptides. Protein hydrolysates are diverse in their functional and bioactive properties. Inhibition of angiotensin-I converting enzyme (ACE), which results in decreased blood pressure, is the bioactive property of interest in this study. Many food protein-derived enzymatic hydrolysates and peptides with in vitro ACE inhibitory activities have been well demonstrated having in vivo inhibitory activities on ACE and antihypertensive effects after oral or intravenous administration in animal experiments using spontaneously hypertensive rats (SHR) and in clinical trails. For example, Nakamura, Yamamoto, Sakai, and Takano (1995) reported that oral administration of ACE inhibitory peptides Val-Pro-Pro and Ile-Pro-Pro derived from sour milk shows antihypertensive effects in SHR, and a placebo-controlled study in hypertensive human subjects by Hata et al. (1996) demonstrated a significant reduction in blood pressure after daily ingestion of 95 ml of Calpis sour milk containing these two kinds ACE inhibitory peptides. Kawasaki et al. (2002) reported that the vegetable drink with peptides such as Val-Tyr derived from sardine protein hydrolysates exhibited the antihypertensive effect in the subjects with mild hypertension. Fujita, Yamagami, and Ohshima (2002) showed that the thermolysin digest of dried bonito, which contains ACE inhibitory peptide Leu-Lys-Pro-Asn-Met, showed long lasting antihypertensive activity without any problematic side effects in a placebo-controlled, double-blind, randomised, cross-over study in 61 borderline and mildly hypertensive subjects. Fish scales can also be enzymatically hydrolysed to produce functional peptides.

There have been many studies of peptide structure and function with respect to ACE inhibitory activity. Large numbers of ACE inhibitory peptide sequences have been determined, and dipeptides and tripeptides are particularly useful. ACE is a well-characterised Zn^{4+} metallopeptidase, and it cleaves dipeptides from the C-terminus of the substrate peptides. The C-terminus is important

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doi:10.1016/j.foodchem.2009.04.015

for the binding of peptides to the ACE enzyme and for their inhibitory activity (Li, Le, Shi, & Shrestha, 2004; López-Fandiño, Otte, & Van Camp, 2006). The C-terminal catalytic site of the somatic form of ACE consists of three subsites, accommodating the three hydrophobic C-terminal residues of the substrate angiotensin I (Brew, 2003; Coates, 2003). Cheung, Wang, Ondetti, Sabo, and Cushman (1980) found that ACE has a higher affinity for substrates or competitive inhibitors that contain hydrophobic amino acid residues such as proline, phenylalanine and tyrosine at three positions from the C-terminus. Therefore, hydrophobic amino acids play an important role in ACE inhibitory peptides.

Generally, enzyme hydrolysates are purified first based on fractionation with respect to their molecular weight using ultrafiltration, size exclusion chromatography, or both (Kim, Whang, & Suh, 2004; López-Fandiño et al., 2006; Quirós et al., 2007; Vermeirssen, Van Camp, & Verstraete, 2005). Because of the lower capacity and film blocking problem, the purification process is limited. Although the hydrophobic amino acid content greatly affects the ACE inhibitory capabilities of these peptides, purification depends only on their molecular weight. Thus, these pre-purification methods result in reduced yields of ACE inhibitory peptides.

Recently, there has been a growing interest in employing macroporous resins (MARs) to separate bioactive components from crude extracts of herbal raw materials. MARs produce good recovery because of their unique adsorption properties and other advantages, including ideal pore structure and availability of various surface functional groups, low operational cost and easy regeneration (Tang, Zhou, & Duan, 2001; Zhang, Jiao, Liu, Wu, & Zhang, 2008). Generally, the main interactions between the absorbent and absorbate are known to be hydrophobic, electrostatic and hydrogen-bonding forces, but few reports have evaluated the adsorption mechanism between the peptides and MARs. In this paper, the adsorption behaviour and mechanism of water-soluble FSPs from aqueous solutions onto MARs have been studied. Specific MARs were chosen to desalt grass carp fish scale peptides (FSPs) and enrich the peptides with in vitro ACE inhibitory ability from the hydrolysates in order to provide purification information that may be useful for industry. We chose grass carp fish scales as the experimental material for production of peptides not only to ensure efficient exploitation of the by-products, but also to provide a potential value-added product to the market, thus solving the problem of waste disposal.

2. Materials and methods

2.1. Preparation of FSPs

Fresh grass carp fish scales were collected from market during the month of October, 2007 (Wuxi, Jiangsu province). The fish scales were washed twice in 1.5 mol/l NaCl solution to remove unnecessary proteins from the surface. Washes were performed by stirring the scales in the solution for 24 h. Demineralisation was achieved with 0.4 mol/l HCl solution (dry scales: solution = 1:15, w/v) for 90 min. The demineralised scales were washed thrice with distilled water and then dried. The dried fish scales were powdered using a crushing mill and a standard sample sieve with a particle diameter of about 0.42 mm, and then the powder was dispersed in water at a concentration of 50 g/l before hydrolysis. The neutral protease AS1398 (EC 3.4.24.28, Genencor, Wuxi, China) was used for hydrolysis of the protein at a ratio of 1:25 (w:w) enzyme:protein, respectively, at pH 7.0 and 50 °C (the optimal conditions for neutral protease AS1398).

The hydrolysis reaction was held at a constant pH value by continuous addition of 0.5 mol/l NaOH until a 16% degree of hydrolysis (DH) was achieved as determined using the pH stat method (Adler-Nissen, 1986). The hydrolysed mixtures were heated at 95 °C for 10 min after 16% DH had been reached. Mixtures were then filtered using Whatman 1 filter paper. The liquid was freeze-dried into FSP powder.

The nitrogen and ash contents of FSP powder were determined according to AOAC (1990) methods. The conversion factor for calculating the protein from nitrogen was 5.50. The content of carbohydrate was determined by phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.2. Preparation of adsorbent

Macroporous resin DA201-C (Jiangyin chemical industry factory, Wuxi, China) was used as the adsorbent. The macroporous resins were extensively washed with plenty of distilled water to remove salts and impurities. Prior to the adsorption experiments, adsorbents were washed with ethanol, followed with distilled water, and then dried in a vacuum at a temperature of 60 °C.

2.3. Static adsorption experiments

The static adsorption tests of FSPs were performed as follows: 1.0 g pretreated dry adsorbent was introduced into a 250 ml Erlenmeyer flask. First, the dried resins were swelled by anhydrous ethanol and washed by distilled water until there was no ethanol and the water was blotted up. Then, 50 ml of 10 mg/ml aqueous solution of FSPs was added to each flask. The flasks were shaken (160 rpm) at 25 °C in an incubator shaker (SHZ-88, Taicang Experiment Equipment Co., Jiangsu, China). The adsorption capacities of the resins were evaluated by the following equation:

$$\text{Adsorption ratio}\ (\%) = (C_0 - C_m)/C_0 \times 100\%$$

$$\text{Adsorption capacity}\ (\text{mg/g}) = (C_0 - C_m)V_a/W$$

where C_0 and C_m are the initial and equilibrium adsorption solution peptide concentrations (mg/ml), respectively; V_a is the adsorption solution volume (ml) and W is the dry weight of the resins (g).

The experiments to study the effect of the pH on adsorption were done as described above with the samples dissolved in buffers of different pH at 25 °C.

2.4. Isotherm adsorption experiments

The adsorption isotherms of FSPs on the selected DA201-C resins at different temperatures were also studied by allowing 100 ml of FSP sample solutions at different concentrations (from 100 to 1100 mg/l, the interval was 200 mg/l) to come into contact with the resins (0.1 g) in shakers at 15, 30 and 45 °C, respectively.

2.5. Dynamic adsorption and desorption experiments

Dynamic adsorption and desorption experiments were carried out at room temperature in glass columns (2.6 × 30 cm) wet-packed with DA201-C resin. The bed volume (BV) of the resin was 100 ml. Sample solution (20 mg/ml) flow rate through the glass column was 1/2 BV/h. During adsorptive equilibration, the adsorbate-laden column was washed with distilled water with a flow rate of 1 BV/h until the eluate had the same conductivity as distilled water. Then, the adsorbate was gradient-eluted with ethanol–water (20%, 40%, 60%, 80%, v/v) solution at a flow rate of 1 BV/h. The eluting solvent was changed when the absorbance of eluate at 220 nm showed little alteration. The eluate was received in an auto-partial sampler (15 min per test tube). SFP fractions eluted by different concentrations of ethanol were pooled, concentrated by rotary evaporation, and freeze-dried. The conductivity was determined by a conductivity metre (DDS-11C, Shanghai Leici

Apparatus Co., China). The desorption ratio was calculated according to the following equation:

$$\text{Desorption ratio}(\%) = C_d \cdot V_d/(C_0 \cdot V_a - C_{water} \cdot V_{water}) \times 100\%$$

where C_d is the peptide concentration in the desorbed solution (mg/ml), V_d is the desorption solution volume (ml), C_0 is the initial peptide concentration in the adsorption solution (mg/ml); C_{water} is the peptide concentration (mg/ml) in the distilled water elution solution. V_a is the adsorption solution volume (ml); V_{water} is the distilled water elution volume (ml).

2.6. Amino acid analysis

FSP powder was dissolved in 6 mol/l HCl solution at 110 °C for 22 h, and the hydrolysates were analysed by an amino acid analyser (HP1100, Agilent).

2.7. Molecular weight determination

Hydrolysates were analysed for molecular weight distribution using a Waters™ 600E Advanced Protein Purification System (Waters Corporation, Milford, MA, USA). A TSK gel, 2000SWXL, (7.8 × 300 mm) column was used with 10% acetonitrile + 0.1% TFA in HPLC grade water as the mobile phase.

The calibration curve was obtained with bovine carbonic anhydrase (29,000 Da), horse heart cytochrome C (12,400 Da), bovine insulin (5800 kDa), bacitracin (1450 Da), Gly-Gly-Tyr-Arg (451 kDa) and Gly-Gly-Gly (189 Da). The results were obtained and processed with the aid of Millennium^{32} Version 3.05 software (Waters Corporation, Milford, MA 01757, USA).

2.8. Assay of in vitro ACE inhibitory activity

In vitro ACE inhibitory activity was measured using RP-HPLC in a Waters™ 600E Advanced Protein Purification System (Waters Corporation, Milford, MA 01757, USA). A peptide sample solution was dissolved in distilled water and diluted to make five different concentrations: 0.1, 0.25, 0.5, 1.5 and 2.5 mg/ml (three parallel samples for each concentration). Then, from each dilution, 10 μl was mixed with 40 μl of 0.5 mol/l sodium borate buffer (pH 8.3) containing 6.5 mmol/l Hip-His-Leu and 0.3 mol/l NaCl and incubated for 6 min at 37 °C. The reaction was initiated by the addition of 20 μl of ACE dissolved in 0.5 mol/l sodium borate buffer (pH 8.3, containing 0.3 mol/l NaCl) (0.1 U/ml) and further incubated for 30 min at 37 °C. The reaction was stopped by adding 80 μl of 1 mol/l HCl. The hippuric acid liberated by ACE was determined directly at 228 nm. The concentration of ACE inhibitors needed to reduce activity by 50% was defined as the IC_{50} value. Plots of the peptide concentrations against the % inhibition were plotted and from its regress equation the IC_{50} value was estimated.

The inhibition of ACE was calculated as following:

The inhibition of ACE (%) = $(A_0 - A_C)/A_0 \times 100\%$
A_0: the hippuric acid peak area of the blank
A_C: the hippuric acid peak area of sample

3. Results and discussion

3.1. Chemical composition and molecular distribution of the grass fish scale hydrolysates

Grass carp fish scale hydrolysates mainly contained protein and ash, and little carbohydrate, with contents of 87.23%, 10.35% and 0.22%, respectively. In this experiment, in order to maintain the highest enzyme activities to hydrolyse fish scales and calculate the DH, 0.5 mol/l NaOH was used to maintain the pH value; thus the ash content was higher in the hydrolysates. The molecular weight and number of peptide residues remaining in the peptide chains after hydrolysis is essential for producing protein hydrolysates with the desired functional properties. Peptides with two to five amino acids can be absorbed in the intestine directly (Webb, 1990).When the DH reached 16%, FSPs with molecular weights (MW) ranging between 145 and 650 Da occupied 71.7%; these were assumed to be di- to pentapeptides, which is consistent with the manufacturer's claims. However, the salt content was too high for use as an orally-administered product and MARs could be used for desalting biological samples (Zhong, Liu, Ma, & Shoemaker Charles, 2007).

3.2. Effect of adsorption time on the adsorption of FSPs onto DA201-C

Based on previous experiment (results not shown), DA201-C resins with a larger surface area, smaller pore diameter and non-polarity exhibited stronger adsorption abilities for FSPs. Adsorption time directly affected the adsorption efficiency and adsorption capacity of DA201-C. The adsorption capacity increased with increased adsorption time before adsorption equilibrium. After 10 h, the adsorption of FSPs reached 290 mg/g. Increasing the adsorption time had almost no effect on the adsorption capacity, indicating that the adsorption tended towards equilibrium at 10 h.

3.3. Effect of adsorption solution pH on the adsorption of FSPs onto DA201-C

The pH value of the solution affected the electric charges of the adsorbents in the solution, which can change the electrostatic attraction and hydrogen bonding, thus influencing the adsorption capacity of the resins. These results can be seen in Fig. 1. The equilibrium absorption capacity first increased with an increase in solution pH from 4 to 6, but decreased when the pH value was higher than six. The amino acid composition of the FSPs is shown in table 1 (crude). The FSPs contained more acidic amino acids (Asp and Glu) than alkaline amino acids (Arg and Lys); the MAR DA201-C resin is non-polar, so in acidic solutions, the resin can have good adsorption. However, in lower pH solutions, proteins tended to aggregate and deposit, which can decrease the adsorption capacity. At pH 6, the DA201-C resins had the highest adsorption capacity, about 300 mg/g. This also suggests that the force between resins and FSPs was hydrophobic.

3.4. Adsorption isotherm of FSPs onto DA201-C

The equilibrium adsorption isotherms of FSPs onto DA201-C resin at three different temperatures 15, 30 and 45 °C are shown in Fig. 2. It was clear that the equilibrium adsorption of FSPs onto

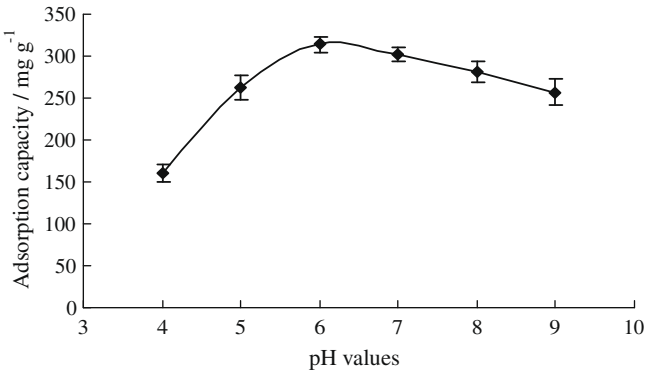

Fig. 1. The effect of pH on the adsorption capacity of FSPs onto DA201-C at 25 °C.

Table 1
Amino acid composition, desorption ratio and ash content of FSP fractions obtained by ethanol gradient elution.

Amino acid[a]	Crude	Ethanol concentration (%, v/v)			
		20	40	60	80
Asp	4.14	3.69	2.37	2.45	2.26
Glu	9.61	8.47	7.24	4.89	2.29
Ser	3.81	3.07	2.90	1.95	2.33
His	0.14	0.16	0.21	0.87	1.20
Gly	26.09	24.21	23.11	21.31	18.49
Thr	1.10	1.01	0.84	0.92	1.18
Arg	5.14	5.29	11.97	14.64	17.21
Ala	9.12	7.14	6.66	6.32	5.58
Tyr	1.35	1.37	1.46	1.65	2.53
Cys-s	6.58	6.20	5.98	4.97	5.01
Val	1.96	2.27	1.82	2.21	2.04
Met	1.35	1.73	0.96	0.94	1.23
Phe	1.83	2.42	2.68	3.39	5.19
Ile	2.04	2.36	2.53	2.86	3.02
Leu	3.37	3.66	3.76	4.58	4.78
Lys	2.09	2.17	3.48	5.05	5.54
Pro	20.23	21.73	21.98	20.96	20.09
Hydrophobic amino acids (%)	30.79	33.83	34.24	35.67	37.67
Average hydrophobic value[b] (kJ/mol)	3.89	4.25	4.38	4.62	4.92
IC_{50} value (mg/ml)	1.66	0.48	0.39	0.25	0.13
Desorption ratio (%)	–	43.84	22.03	15.26	10.06
Ash content (%)	10.35	1.20	0.84	0.71	0.68

[a] Trp was not detected (g/100 g protein).
[b] Average hydrophobic value was calculated as Q (kJ/mol) = $\sum[A_iF_i/M_i/\sum(A_i/M_i)]$, where A_i (g) is the weight of each amino acid in 100 g protein, M_i (g/mol) is the molecular weight of the amino acid and F_i (kJ/mol) is the hydrophobic value of the amino acid.

DA201-C increased with increasing temperature and initial concentration of the FSPs. When the adsorption reached saturation, there were no further FSPs adsorbed onto the resins with additional initial concentrations. However, increasing the initial concentration did shorten the adsorption equilibrium time. Application of the Langmuir equation, which has been widely used in model adsorption processes to explain the absorption phenomenon, indicates that each site of the adsorbent can adsorb only one particle (Du, Yuan, Zhao, & Li, 2007). Hence, the Langmuir equation describes the adsorption behaviour of a monomolecular layer. In the following Langmuir equation:

$$C_s/C_s^{\max} = K \cdot C_m/(1 + K \cdot C_m)$$

C_s represents the equilibrium adsorption capacity (mg/g) and C_m is the equilibrium concentration in liquid phases (mg/l), C_s^{max} is the maximum adsorption capacity (the maximum surface coverage, formation of the monolayer, mg/g). K is the Langmuir constant. The Langmuir equation was converted to the linear form $1/C_s = 1/(KC_s^{max}C_m) + 1/C_s^{max}$, with $1/C_s$ and $1/C_m$ as dependent and independent variables (Y and X). The experimental data were statistically analysed and R^2 values were obtained. Table 2 gives the correlative parameters of Langmuir adsorption isotherm equations for the FSPs at different temperatures. The correlation coefficients of Langmuir equations on DA201-C resin at three temperatures were rather high, implying that the adsorption behaviour fit the Langmuir adsorption isotherm equations. The values of K and C_s^{max}, which according to the Langmuir theory are relative indicators of adsorption capacity, increased with increasing temperature, suggesting that increased temperature is advantageous for the adsorption process.

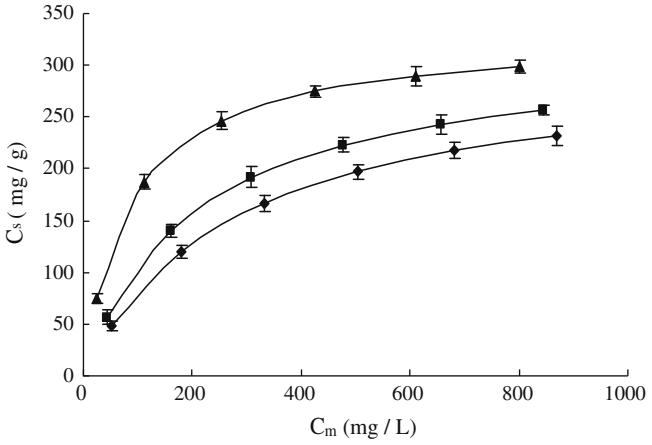

Fig. 2. Equilibrium adsorption isotherms for FSPs onto DA201-C at different temperatures. (♦) 15 °C; (■) 30 °C; (▲) 45 °C. C_s represents the equilibrium adsorption capacity (mg/g) and C_m is the equilibrium concentration in liquid phases (mg/l).

In general, adsorption involves the accumulation of molecules from a solution onto the exterior and interior surfaces of an adsorbent. These phenomena result from various interactions including those characterised by hydrophobic and electrostatic forces. Physical properties of resins (surface area, pore radius, pore distribution and porosity) can also play an important role in determining states and equilibria of adsorption (Koźlecki, Sokolowski, & Wilk, 1997). In this experiment, the resin with the larger surface area had a good adsorption capacity. Furthermore, the resin was non-polar and the FSPs were rich in hydrophobic amino acids. Therefore, the hydrophobic force was predominant in the adsorption of FSPs from the aqueous phase onto the non-polar adsorbent. Higher temperatures can strengthen the hydrophobic force (Pratt, 1985) and enhance the intraparticle diffusion rate of adsorbate into the pores; thus increasing the temperature can increase the amount of FSPs adsorbed onto the DA201-C resin.

Table 2
Langmuir adsorption parameters of FSPs on DA201-C resins at different temperatures.

Temperature (°C)	Langmuir equation	K	C_s^{max} (mg/g)	R^2
15	$Y = 0.9052X + 0.003272$	0.003615	305.6	0.9989
30	$Y = 0.6424X + 0.003138$	0.004884	318.7	0.9975
45	$Y = 0.2607X + 0.003028$	0.01162	330.3	0.9947

3.5. Isosteric enthalpies for FSPs adsorption

The isosteric adsorption enthalpy changes were calculated following a derivative of the Van't Hoff equation (Garcia-Delgado, Cotouelo-Minguez, & Rodriguez, 1992):

$$\mathrm{Ln}C_m = -\ln K_0 + \Delta H/R \cdot T$$

where C_m is the equilibrium concentration (mg/ml) at temperature T, ΔH is the isosteric enthalpy of adsorption, R is the gas constant (8.314 kJ/(mol K)), T is the absolute temperature (K) and K_0 is a constant. ΔH was calculated from the slope of the line plotted by ln C_m versus $1/T$, as shown in Table 3. The value of the enthalpy changes were positive, suggesting that the adsorption process was endothermic, and increasing temperature was favourable to adsorption. The value of the enthalpy change (<43 kJ/mol) also showed that the adsorption of FSPs on the DA201-C resins was physical (Huang & Cheng, 1997). The results were consistent with hydrophobic interaction. Therefore, the main force in the adsorption of FSPs on the surface of the DA201-C resins was hydrophobic.

3.6. Dynamic adsorption and desorption

The above static adsorption experiments showed that the force between adsorbates and resins was hydrophobic; thus peptides with different hydrophobic properties can be separated by gradient elution of the adsorbate resins with different concentrations of ethanol. In order to bring the separation to a larger scale and to shorten and control the desalting and gradient eluting process, dynamic adsorption of FSPs on DA201-C was used for the separation experiment. At a 1/2 BV loading rate, the loading volume at the penetration point (A_{220} = 0.05, at which peptides start to be overloaded and found in the eluate) was around 70 ml of FSP solution. The adsorbed resins were first eluted by distilled water. Due to the hydrophobic force between the resins and peptides, most of the salt was eluted with the distilled water, while only about 13% of the FSPs were eluted. When the conductivity ratio of the eluate was nearly equal to that of distilled water (about 8 BV), a gradient of ethanol solution was applied in order to elute the adsorbed resins, and about 90% of the adsorbates could be gradient-eluted. The yield and ash content of each eluted fraction are given in Table 1. The yield and ash content of fractions decreased with increased ethanol concentrations. The ash content in the crude dried FSPs was 10.35%, and it was lower than 1.5% after desalting by MAR. This suggests that using MAR adsorption is a good method to eliminate salt from peptides and to separate peptides with different hydrophobicities.

3.7. In vitro ACE inhibitory activity of different elution fractions

Fig. 3 shows the in vitro ACE inhibitory activities of different fractions. The crude hydrolysates without desalting had negative inhibitory activity at lower concentrations, indicating that the salt in the hydrolysates increases the activity of the ACE. ACE inhibition was found to increase with increased FSP concentrations, with IC_{50} values (in Table 1) of 1.66, 0.48, 0.39, 0.25 and 0.13 mg/ml. The IC_{50} values of different elution fractions became lower when fractions were eluted by higher concentrations of ethanol, suggesting that FSPs eluted by higher concentrations of ethanol had higher

Table 3
Thermodynamic values of FSP adsorption on DA201-C resins.

Adsorbent	C_s (mg/g)	ΔH (kJ/mol)
DA201-C	100	32.52
	150	33.34
	200	34.87

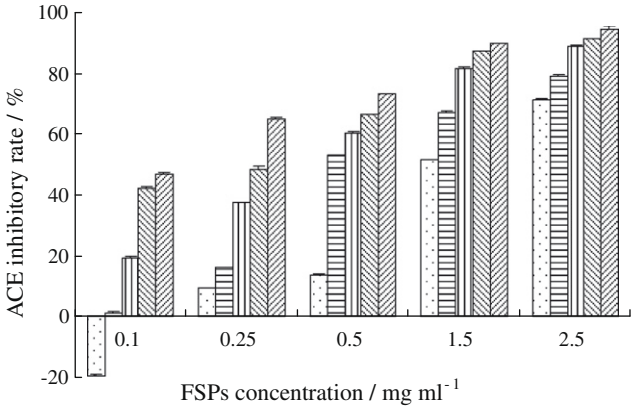

Fig. 3. In vitro ACE inhibitory abilities of different elution fractions. ⬚ Crude hydrolysates; ▭ fraction eluted by 20% ethanol solution; ▥ fraction eluted by 40% ethanol solution; ▧ fraction eluted by 60% ethanol solution; ▨ fraction eluted by 80% ethanol solution.

in vitro ACE inhibitory ability. The crude hydrolysates of grass carp fish scale protein had low ACE inhibitory activity (IC_{50} = 1.66 mg/ml) as compared to previously reported values for protein hydrolysates ranging from 0.021 to 1.73 mg/ml. However, when purified by MAR, hydrolysates had moderate ACE inhibitory activity (IC_{50} < 0.48 mg/ml). These results indicate that MAR DA201-C can separate peptides with different in vitro ACE inhibitory abilities by gradient ethanol elution in order to satisfy specific requirements of the consumer. Furthermore, the ethanol can also be recycled.

4. Amino composition of different fractions

In order to clarify the mechanism by which separation of peptides was achieved with gradient ethanol elution on MARs DA201-C, the amino acid composition of each peptide fraction was determined (Table 1). It was suggested that most naturally occurring ACE inhibitory peptides contain Pro, Lys or aromatic amino acid residues (Cheung et al., 1980; Kawakami & Kayahara, 1993; Suetsuna & Nakano, 2000), and that they are mostly hydrophobic. The present results showed that the content of hydrophobic amino acids (Ile, Tyr, Phe, Pro, Leu, Val and Lys) was greater in the fraction eluted with the higher ethanol concentration, and the contents of different fractions ranged from 30.79% to 37.67%, while the average hydrophobicity of each eluted fraction increased from 3.89 to 4.92 kJ/mol. These results suggest that the separation of FSPs with non-polar residues via ethanol gradient elution was based on hydrophobicity and that the in vitro ACE inhibitory ability of the FSPs was closely related to amino acid hydrophobic properties. In addition, the amino acids His, Thr and Arg were enriched. Suetsuna, Yamagami, and Kuwata (1988) studied the amino acid composition of ACE inhibitory peptides from 15 different types of fish and shellfish, and found that the ACE inhibitory peptides from fish were rich in Asp, Glu, Arg, Pro, Ile and Lys. As shown in Table 1 (crude), fish scale proteins were rich in these six amino acids, with a total percentage of 41.20%. This also indicates that grass carp fish scales are a good resource of ACE inhibitory peptides.

5. Molecular weight distribution of different fractions

Most food protein-derived peptides with ACE inhibitory abilities have relatively low molecular weight, generally ranging from dipeptides to pentapeptides with MW from 150 to 800 Da. However, ACE inhibitory peptides with six, seven or 11 amino acids have also been discovered (Haque & Chand, 2008; Otte, Shalaby Samah, Zakora, & Nielsen, 2007; Suetsuna & Nakano, 2000). Because

the FSPs separated by MAR DA201-C were distinguished based on hydrophobic force, the range in MW of the different fractions did not show great variability. However, peptides with MW from 750 to 330 Da were enriched from 25.68% to 45.12% as the ethanol concentration increased from 20% to 80%. Taking into account our previous findings that the fraction eluted by higher ethanol concentrations had the highest in vitro ACE inhibitory ability, the MW of most ACE inhibitory FSPs was deduced to be between 750 and 330 Da.

6. Conclusions

The non-polar MAR DA201-C with a larger surface area and smaller pore diameter proved to be efficient for separation of FSPs. The adsorption dynamics indicated that the DA201-C resins required about 10 h to reach equilibrium, and had a good adsorption capacity when the pH value of the adsorption solution was around 6. Higher temperatures favoured adsorption, indicating that the process was endothermic, and that hydrophobic forces were predominant in the adsorption of FSPs from the aqueous solution. Dynamic adsorption and desorption were used to study the process of desalting and gradient elution. The dynamic adsorption ratio of the FSPs reached 87%, and the dynamic desorption ratio of FSPs reached 90%. Most of the salt was removed from the FSPs. The in vitro ACE inhibitory ability was increased for fractions eluted using high ethanol concentration, and these fractions were mainly hydrophobic in nature. MAR DA201-C was thus a good material for desalting and enhancing peptides with in vitro ACE inhibitory properties. The results also indicate that fish scales are a good source for peptides with higher in vitro ACE inhibitory activity, which could be a potential value-added product in the market.

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Food Chemistry 117 (2009) 393–397

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Cooking effect on fatty acid profile of pork breakfast sausages enriched in conjugated linoleic acid by dietary supplementation or direct addition

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ARTICLE INFO

Article history:
Received 15 December 2008
Received in revised form 16 March 2009
Accepted 8 April 2009

Keywords:
Formulation
Diet
Pig
Meat product

ABSTRACT

The effectiveness of increasing CLA in pork products through animal dietary supplementation or direct addition in the product formulation has been studied, and the effect of grilling on dry matter and fat contents and fatty acid composition has been analysed. Sausages made with meat and back fat from pigs with CLA dietary supplementation had the highest saturated fatty acid content. Sausages from dietary supplementation and direct addition had CLA levels between 6% and 7% of total fatty acids. Moisture and fat contents decreased and increased respectively after cooking for the three sausage types (control, dietary supplementation, direct addition). Grilling had little effect on fatty acid levels, especially for sausages with direct addition in the product formulation. In general, saturated fatty acids increased and poly-unsaturated fatty acids decreased due to the increase of C16:0 and to the decrease of C18:2 $n-6c$ and C18:3 $n-3$ fatty acids. Added CLA, both from animal dietary supplementation or direct addition, remained at similar levels in cooked sausages to those found in raw sausages.

1. Introduction

The relationship between diet, health and lifestyle is now a key focal point for consumers, researchers and policy makers alike as we witness an increase in obesity and the rise of diet-related chronic diseases (Swinburn, 2009). A key objective in the European Technology Platform on Food for Live focuses on ensuring that the healthy choice is an easy choice for consumers. The meat industry is addressing this demand by adopting strategies to produce meat products which have more beneficial ingredient profiles in a value-added manner. One such strategy includes the incorporation of functional lipids into existing meat products to provide a healthier version of an existing product.

Conjugated linoleic acid (CLA) is a collective term for a group of octadecenoic acids that are geometric and positional isomers of linoleic acid (C18:2) (Pariza, Park, & Cook, 2001). CLA has been shown to have a variety of biological effects (Hur, Park, & Joo, 2007). Several health benefits, such as anticancer, anti-oxidation, anti-atherosclerosis and improving immuno-responses (Belury, Nickel, Bird, & Wu, 1996; Lee, Kritchevsky, & Pariza, 1994; Miller, Stanton, & Devery, 2001; Pariza & Hargraves, 1985; Park et al., 1999) have been reported for CLA. These substances have been found in the meat and milk of ruminants, where they are mainly formed by biohydrogenation of grass derived fatty acids. Pork contains only small amounts of CLA because pig is a mono-gastric animal (Chin, Liu, Storkson, Pariza, & Ha, 1992).

Interest in dietary supplementation with CLA for pigs increased during the last decade due to its potential to improve productive, carcass and meat quality traits and, at the same time, for obtaining meat and meat products enriched in CLA (Marco et al., 2009; Martín, Antequera, Muriel, Andrés, & Ruiz, 2008a; Schmid, Collomb, Sieber, & Bee, 2006). A second approach for increasing CLA in meat products is its direct addition as an ingredient during the manufacturing process (Hah et al., 2006; Martín, Ruiz, Kivikari, & Puolanne, 2008b). In addition to the healthy benefits of CLA, its addition into products provides a strategy for partial replacement of saturated fatty acids in the diet by unsaturated fatty acids (Martín et al., 2008a).

While strategies can be enacted to improve the ingredient profile of foodstuffs the cooking method can have an impact on the levels of the beneficial ingredient in the product which is ready to consume. Some studies focused on the influence of processing and cooking on CLA content in meat products that naturally contain CLA such as beef (Ma, Wierzbicki, Field, & Clandinin, 1999; Shantha, Crum, & Decker, 1994) or lamb meat (Badiani et al., 2004). However, little work has been presented which assesses the impact of cooking on the fatty acid profiles of CLA supplemented meat products.

The aim of the present study was to study the effects of grilling on the chemical and fatty acid composition of pork products enriched in CLA through animal dietary supplementation or through direct addition in the product formulation. In addition this study

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.04.023

aims to compare the useful of CLA dietary supplementation or direct addition into breakfast sausages.

2. Material and methods

2.1. Treatments

Sausages were manufactured according to three different protocols (control, dietary supplementation and direct addition in the formulation) using Boston Butt (*Musculus infraspinatus*, *M. supraspinatus*, *M. subscapularis* and *M. serratus ventralis*) and back fat removed from the pigs 24 h after slaughter. The CLA supplement used for both, diet and formulation supplementation, was Luta-CLA 60 (BASF, Germany), which consists of 56% (w/w) of the two main CLA isomers (*t*9, *c*11 and *c*10, *t*12), dissolved in a base of linoleic acid.

Dietary supplementation with CLA was carried as reported in Marco et al. (2009). Ten female pigs of approximately 40 kg live weight were selected and their diet was supplemented with 2.0% of CLA (3.57% of total oil added per tonne of feeding). Pigs were fed *ad libitum* from hopper feeders. The feeding trial lasted for 8 weeks, after which the animals were slaughtered having reached live weights of approximately 95 kg.

Pork breakfast sausages with CLA added in the formulation were prepared with meat and back fat from pigs with non-CLA enriched diets and live weights of approximately 95 kg, where 2% Luta-CLA (1.12% of CLA), substituting the same weight of back fat, was added to the mixture during the manufacturing process.

As a control, another treatment was prepared using meat and back fat from pigs fed with a non-CLA enriched diet and with no added CLA.

2.2. Sausage manufacture

Sausages were manufactured with the following formulation (w/w), 44.2% of lean meat, 18.7% of back fat, 2.5% seasoning, 7.0% rusk and 27.5% water. Prior to manufacture, back fat was chopped whilst frozen for 1 min at chopping speed 2 and bowl speed 2 (2, 2) using a bowl chopper (Fatosa C-35-2Z, Fatosa S.A., Sabadell, Spain) and then refrigerated. Diced lean meat, seasoning, overnight-hydrated rusk and ⅓ of the ice water were introduced into the bowl chopper and blended at speed (1, 1) for 20 s. The chopped fat was then added to the bowl along with the remaining ice water. All ingredients were then chopped for 2 min at speed (1, 1) and the mix was stuffed into collagen casings of 16 to the lb. The process was made by triplicate with meat and back fat from each animal (batch). For each batch, the sausages were then vacuum packed in bags containing six sausages, and stored at −20 °C.

Prior to cooking, raw samples were taken from all the treatments for subsequent analysis. Sausages were grilled for 30 min at 200 °C using a domestic oven grill (B-AH51-7 SIEMENS-Electrogerate, GmbH Germany), minced (R301 Ultra Robot Coupe, Robot Coupe UK Ltd., Middlesex, UK), vacuum packed and frozen for subsequent analysis. All results are expressed as the mean of six replicates of each treatment.

2.3. Analysis of fat and dry matter contents

Fat and dry matter contents were analysed with Smart Trac (CEM SMART Trac™ Fat and Moisture Analyzer, CEM Corporation, Matthews, USA), using a combination of microwave drying technology and Nuclear Magnetic Resonance (NMR). Minced samples (two replicates of 3 g) were dried in the microwave to calculate their dry matter content using the difference of weight before and after drying. Dried samples were placed in the NMR analyzer to calculate their fat content.

2.4. Fatty acid analysis

The total fatty acids were extracted, methylated and analysed with an adaptation of the method described by Aldai, Osoro, Barron, and Nájera (2006), which has been reported to be highly effective for poly-unsaturated analysis (Juárez et al., 2008). Separation and quantification of the fatty acid methyl esters was carried out using a gas chromatograph (GC, Varian Star 3400CX, Varian Associates Inc., California, USA) equipped with a flame ionisation detector automatic sample injector, and using a BPX-70 capillary column (120 m, 0.25 mm i.d., 0.2 μm film thickness, SGE, Australia). Tricosanoic acid methyl ester (C23:0 ME) at 10 mg/ml was used as an internal standard. Individual fatty acid methyl esters were identified by comparing their retention times with those of authenticated standards from Sigma (Sigma Chemical Co. Ltd., Poole, UK). Fatty acids were expressed as a percentage of total fatty acids identified and grouped as follows: saturated (SFA), monounsaturated (MUFA) and poly-unsaturated (PUFA) fatty acids. PUFA/SFA ratio and total CLA and Δ9-desaturase activities were calculated.

2.5. Statistical analysis

Statistical analyses were performed using Statistica 7.0 for Windows (StatSoft Inc., 2006). The effects of the different treatments (control, CLA dietary supplementation and CLA addition in the formulation) and cooking process as well as the interaction between them were studied using analysis of variance (multifactor ANOVA).

3. Results and discussion

In general, heat is applied to meat products in different ways to improve its hygienic quality by inactivation of pathogenic microorganisms to enhance its flavour and taste, and increase shelf life (Bognàr, 1998; Pokorny', 1999). During cooking, physicochemical reactions modify the food nutritional value. Cooking induces water loss in the food, increasing its lipid content, while only some fat is lost (García-Arias, Álvarez Pontes, García-Linares, García-Fernández, & Sánchez-Muniz, 2003; Yarmand & Homayouni, 2009).

In all the types of sausages, fat ($P < 0.01$) and dry matter ($P < 0.001$) contents increased after cooking with no interaction ($P > 0.05$) between cooking and treatment observed (Table 1). If expressed on dry matter basis, fat content of control, diet and formulation sausages decreased ($P < 0.001$) from 55.7%, 57.2% and 54.3% to 49.9%, 52.2% and 50.0% respectively, due to cooking losses. However, this was accompanied, by higher decreases in moisture content in all treatments following cooking, resulting in an apparent increase of fat content. This has been reported by other authors: for example Baggio and Bragagnolo (2006) in sausages, meat balls and hamburgers and by Dreeling, Allen, and Butler (2000) in beefburgers. Sheard, Wood, Nute, and Ball (1998) noted a similar effect in pork loin chops.

Fatty acid profiles of the sausages (Tables 2 and 3) were in line with those profiles reported elsewhere for pork products (Baggio & Bragagnolo, 2006; Lauridsen, Mu, & Henckel, 2005; Lo Fiego, Macchioni, Santoro, Pastorelli, & Corino, 2005; Pereira, Tarley, Matsushita, & Souza, 2000). The interaction between the studied factors (cooking and treatment) was significant ($P < 0.05$) for SFA and PUFA indices and for PUFA/SFA ratio (Table 2), as well as for several individual fatty acids (Table 3). Therefore cooking impacted in different ways depending on the type of sausage.

When the fatty acid profiles of the different types of sausages are compared, the levels of SFA of sausages from pigs with CLA dietary supplementation showed higher levels ($P < 0.001$) in comparison to those from the other two types. In this context, Dugan,

Table 1
Cooking effect on fat and dry matter contents (%) of control sausages and sausages with added CLA.

	Control		Diet		Formulation		SEM	Cooking		
	Raw	Cooked	Raw	Cooked	Raw	Cooked		Cooking	Treatment	×Treatment
Fat	19.10^{c}	19.90^{b}	19.55^{c}	21.16^{b}	21.01^{b}	21.92^{a}	0.328	**	***	ns
Dry matter	34.33^{d}	39.95^{bc}	34.18^{d}	40.49^{b}	38.70^{c}	43.89^{a}	0.267	***	***	ns

SEM: standard error mean. ns: $P > 0.05$; **: $P < 0.01$; ***: $P < 0.001$.
^{a,b,c,d}Indicate significant differences ($P < 0.05$) between samples.

Table 2
Cooking effect on fatty acid indices (% of total fatty acids) of control sausages and sausages with added CLA.

	Control		Diet		Formulation		SEM	Cooking		
	Raw	Cooked	Raw	Cooked	Raw	Cooked		Cooking	Treatment	× Treatment
SFA	37.16^{d}	37.76^{c}	43.03^{b}	44.79^{a}	32.78^{e}	33.34^{e}	0.190	*	***	*
MUFA	40.62^{a}	40.75^{a}	27.84^{c}	27.89^{c}	39.48^{b}	39.23^{b}	0.195	ns	***	ns
PUFA	22.22^{d}	21.50^{e}	29.16^{a}	28.40^{b}	27.74^{c}	27.42^{c}	0.255	*	***	*
Total CLA	0.10^{c}	0.14^{c}	6.37^{b}	6.17^{b}	6.93^{a}	6.79^{a}	0.123	ns	***	ns
PUFA/SFA	0.60^{c}	0.57^{c}	0.65^{b}	0.63^{bc}	0.85^{a}	0.82^{a}	0.015	ns	***	*
Δ9-C16	0.09^{a}	0.09^{a}	0.05^{b}	0.05^{b}	0.09^{a}	0.09^{a}	0.014	ns	***	ns
Δ9-C18	0.77^{a}	0.77^{a}	0.59^{b}	0.59^{b}	0.79^{a}	0.79^{a}	0.057	ns	***	ns

SEM: standard error of the mean. ns: $p > 0.05$; *: $p < 0.05$; ***: $p < 0.001$.
SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; CLA: conjugated linoleic acid.
Δ9-C16:C16:1/(C16:1 + C16:0).
Δ9-C18: (*cis*-C18:1n − 6 + *trans*-C18:1n − 11 + *cis*-C18:1n − 9*c*)/(C18:0 + *cis*-C18:1n − 6 + C18:1n − 11 + *cis*-C18:1n − 9).
^{a,b,c,d}Indicate significant differences ($P < 0.05$) between samples.

Table 3
Cooking effect on individual fatty acid contents (% of total fatty acids) of control sausages and sausages with added CLA.

	Control		Diet		Formulation		SEM	Cooking		
	Raw	Cooked	Raw	Cooked	Raw	Cooked		Cooking	Treatment	× Treatment
C14:0	1.62^{c}	1.68^{c}	1.97^{b}	2.13^{a}	1.31^{d}	1.36^{d}	0.030	*	***	*
C16:0	22.79^{c}	23.47^{b}	24.97^{a}	25.18^{a}	20.49^{d}	20.88^{d}	0.180	***	***	***
C16:1	2.29^{b}	2.43^{a}	1.21^{d}	1.24^{d}	2.10^{c}	2.16^{c}	0.030	*	***	*
C17:0	0.49^{b}	0.48^{b}	0.57^{a}	0.57^{a}	0.48^{b}	0.47^{b}	0.013	ns	***	ns
C17:1	0.39^{a}	0.37^{a}	0.14^{b}	0.14^{b}	0.39^{a}	0.37^{a}	0.010	ns	***	*
C18:0	11.71^{b}	11.62^{b}	16.86^{a}	17.14^{a}	10.13^{c}	10.21^{c}	0.165	ns	***	ns
C18:1 *n* − 6*c*	0.15^{d}	0.17^{d}	0.34^{b}	0.49^{a}	0.21^{c}	0.19^{cd}	0.016	***	***	***
C18:1 *n* − 11*t*	2.20^{a}	2.25^{a}	1.14^{c}	1.09^{c}	1.83^{b}	1.81^{b}	0.036	ns	***	ns
C18:1 *n* − 9*c*	37.01^{a}	37.05^{a}	23.21^{c}	23.15^{c}	35.98^{b}	35.73^{b}	0.163	ns	***	ns
C18:2 *n* − 6*t*	0.22^{a}	0.21^{a}	0.08^{b}	0.07^{b}	0.23^{a}	0.21^{a}	0.011	ns	***	*
C18:2 *n* − 6*c*	16.12^{b}	15.49^{c}	19.25^{a}	18.91^{a}	15.69^{c}	15.62^{c}	0.170	*	***	*
C18:3 *n* − 6	0.16^{a}	0.13^{a}	0.09^{b}	0.10^{b}	0.14^{a}	0.14^{a}	0.013	ns	***	ns
C18:3 *n* − 3	1.34^{a}	1.27^{b}	1.07^{c}	1.04^{c}	1.35^{a}	1.35^{a}	0.014	***	***	***
*c*9, *t*11-CLA	0.05^{c}	0.08^{c}	3.72^{a}	3.60^{a}	3.57^{b}	3.49^{b}	0.050	ns	***	ns
*t*10, *c*12-CLA	0.04^{c}	0.06^{c}	2.63^{b}	2.52^{b}	3.28^{a}	3.25^{a}	0.043	ns	***	ns
C20:1	0.25^{b}	0.23^{b}	0.29^{a}	0.31^{a}	0.28^{ab}	0.30^{a}	0.015	ns	*	ns
Cis-C20:4 *n* − 6	0.42^{ab}	0.46^{a}	0.43^{ab}	0.41^{ab}	0.41^{ab}	0.38^{b}	0.025	ns	*	ns

SEM: standard error of the mean. ns: $p > 0.05$; *: $p < 0.05$; ***: $p < 0.001$.
^{a,b,c,d}Indicate significant differences ($P < 0.05$) between samples.

Aalhus, and Kramer (2004) observed how CLA dietary addition increased SFA in pig meat and fat, increasing marbling and fat firmness. The increase of SFA in this study was accompanied by a decrease of MUFA ($P < 0.001$) in sausages with dietary supplementation. Schöne et al. (2003) reported that same effect, particularly in the back fat of pigs fed with CLA. According to Schöne et al. (2003), the shift from the MUFA to the SFA could result from the diminution of the Δ9-desaturase activities by the CLA, as observed (Table 2) for sausages with CLA dietary addition ($P < 0.001$). No cooking effect ($P > 0.05$) was observed for Δ9-desaturase activities from any treatment. However, as expected, PUFA levels of the sausages with dietary supplementation and direct addition were much higher ($P < 0.001$) than those of the control sausages, as a result of the added CLA.

The PUFA/SFA ratio for all the treatments was over 0.45, the minimum recommended by the British Department of Health (1994), showing cooking had no negative effect on this parameter. The highest ratio ($P < 0.001$) was that from CLA direct addition. CLA dietary supplementation led to an increase of both PUFA and SFA levels as compared to meat and fat from pigs with regular diet. Nevertheless the final PUFA/SFA ratio was greater than that from the control sausages.

Sausages enriched with CLA (diet and formulation) had CLA levels between 6% and 7%, whereas CLA levels of control sausages were very low (0.1% of total fatty acids). Cooking had no impact on the percent CLA present in all products. As mentioned earlier pork may content low amounts CLA, depending on the diet, as reported by Dhiman, Olson, MacQueen, and Pariza (1999) and Raes, De Smet, and Demeyer (2004) and reviewed by Chilliard, Ferlay, and Doreau (2001). From a nutritional point of view, the increase in PUFA and CLA levels would be positive. Therefore, the simplest way to increase CLA levels in breakfast sausages is the direct addi-

tion during the manufacturing process, as this method would be cheaper and does not result in increased SFA. It is important to note though that an increase in PUFA could lead to variations in the texture and flavour of sausages as seen in other similar emulsion type products; Turkish sausages (Yildiz-Turp & Serdaroğlu, 2008), and emulsion type sausages (Hah et al., 2006). Nevertheless, other authors have increased the $n-3/n-6$ ratio of sausages without significant variations in texture or sensory evaluation (Cáceres, García, & Selgas, 2008). Finally, CLA dietary supplementation has been shown to be able to get high CLA levels in sausages, while increasing PUFA and SFA and decreasing MUFA levels. Also PUFA/SFA level was greater than the minimum recommended.

Cooked control sausages had higher SFA ($P < 0.05$) and lower PUFA ($P < 0.05$) contents, as well as lower PUFA/SFA ratio ($P < 0.05$) compared to raw ones (Table 2). The increase of SFA was related to the increase ($P < 0.001$) of C16:0 (Table 3). The decrease of PUFA was due to the decrease of C18:2 $n-6c$ ($P < 0.05$) and C18:3 $n-3$ ($P < 0.001$). In the literature reduced PUFA on cooking has been related to triglycerides unsaturated fatty acid drip losses (Cobos, Veiga, & Díaz, 2008; Scheeder et al., 2001). While the overall MUFA levels were not affected by cooking ($P > 0.05$), the levels of C16:1 were higher ($P < 0.05$) and those of C17:1 lower ($P < 0.05$) in cooked than in raw control sausages.

One fatty acid from each type (SFA, MUFA and PUFA) was affected by cooking in sausages made from pigs with CLA dietary supplementation. An increase was observed for C14:0 ($P < 0.05$) and C18-1 $n-6c$ ($P < 0.001$), while C18:2 $n-6t$ levels decreased ($P < 0.01$) after cooking. Therefore, cooked sausages showed again higher levels of SFA and MUFA ($P < 0.05$) than raw ones. Increases in PUFAs after cooking have been observed in other studies. Maranesi et al. (2005) observed an increase in PUFA for lamb rib-loins after broiling and microwaving followed by final grilling. Some authors (Gerber, Scheeder, & Wenk, 2008; Igene, Pearson, & Gray, 1981; Rodríguez-Estrada, Penazzi, Caboni, Bertacco, & Lercker, 1997) have found an increase in PUFA levels of meat and meat products after cooking due to the lipid losses, containing mainly triacylglycerols of adipose tissues with relatively more SFA than PUFA, as suggested by Ramamurti (1986). However, in those studies, PUFA levels were much lower (1–6%) than those found in the sausages of the present study (21–29%). It is interesting to note that in a recent study, Sarriés, Murray, Moloney, Troy, and Beriain (2009) found no changes in the relative distribution of fatty acids upon cooking (140 °C for 30 min) in beef from animals with diets designed to enhance the concentration of CLA in tissue.

Unlike the other two types of sausages, no cooking effect ($P > 0.05$) was observed between the levels of any measure of fatty acid content when CLA was added during manufacturing process. This suggests that the oil added during manufacturing might help stabilise the presence of fatty acids during cooking.

No significant differences were found in CLA levels (total CLA and CLA isomers) between raw and cooked sausages within each treatment ($P > 0.05$). Maranesi et al. (2005), also reported no differences in total CLA levels before and after microwaving and broiling lean meat from rib loins. Therefore, CLA addition, either by dietary supplementation or direct addition in the formulation, resulted in similar levels to that added to the raw sausages after grilling.

4. Conclusions

The results from this study show that pork products can be modified to provide a significant increase in a functional lipid, which can have positive influences on health. Moreover, grilling had no clear effect on fatty acid levels in sausages with CLA added during manufacturing process. Grilling has a small but significant effect in sausages made with meat and back fat from pigs with a dietary CLA addition. Total CLA levels were not affected by grilling. Therefore, since CLA levels have been found to be stable during cooking, texture and sensory properties of both types of sausages should be evaluated in future studies to clarify the optimal process to increase CLA levels in pork breakfast sausages.

Acknowledgements

This research was carried out at Ashtown Food Research Centre (formerly known as the National Food Centre), Teagasc, which was designated as a Marie Curie Training Site in Biochemistry of Meat Quality under the Improving Human Potential and Socio-economic Knowledge Base Programme of the Fifth Framework. The authors acknowledge funding support for Aurora Marco and Manuel Juárez under this Marie Curie Fellowship scheme.

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Food Chemistry 117 (2009) 398–404

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Antioxidant activity and bioactive components of the aerial parts of *Hypericum perforatum* L. from Epirus, Greece

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ARTICLE INFO

Article history:
Received 2 February 2009
Received in revised form 3 March 2009
Accepted 7 April 2009

Keywords:
Hypericum perforatum L.
Aerial parts
Antioxidant activity
Bioactive components

ABSTRACT

The aerial parts of *Hypericum perforatum* L. were studied to assess their composition in basic categories of bioactive compounds and their antioxidant activity, employing commonly accepted assays. The results reveal that floral buds, during the development stage, bear the highest concentrations of hyperforin followed by the flowers of the plant and comparable concentrations of hypericin and pseudohypericin. Among the various plant fractions, shoots and branches show significant antioxidant activity, a fact which may be accounted for by the high phenolic content. The measurement of DPPH· antioxidant activity of extracts by an on-line HPLC-DPPH· antioxidant activity measurement set-up, delineated the pivotal contribution of hyperforin, adhyperforin and their analogues to this antioxidant property. The role of phenolic compounds on the total DPPH· scavenging activity was minor except chlorogenic acid and its derivatives. No encouraging results were obtained as regards the inhibition of the oxidative damage of proteins. All the extracts promote the damage of proteins exhibiting a functional pro-oxidant role.

1. Introduction

Oxidative damage of biological molecules in the human body is involved in degenerative or pathological processes such as aging, coronary heart disease, cancer, arteriosclerosis and rheumatism (Halliwell, 1999). In the past decade, lots of epidemiological studies have conformed that intake of exogenous antioxidants is effective in preventing or suppressing such diseases (Block, Patterson, & Subar, 1992; Singh & Downing, 1995). The use of naturally occurring antioxidants has attracted considerable attention and an increasing interest in natural antioxidants, mainly phenolic compounds, has appeared worldwide that might help prevent oxidative damage (Stalikas, 2007).

Plant species of the genus *Hypericum* are well known for their use in traditional medicine, due to the therapeutic efficacy of its many different species. One of the most important and recognised species of the genus is *Hypericum perforatum* L., St. John's wort, which has been used in herbal medicine, externally for the treatment of skin wounds, eczema and burns and, internally, for disorders of the central nervous system, the alimentary tract and other purposes (Barnes, Anderson, & Phillipson, 2001; Bombardelli & Morazzoni, 1995). Hypericum is the second most used antidepressant worldwide with more than two billion euros turnover annually in Europe. Several studies have revealed antidepressant, antiviral, wound healing, antioxidant and antimicrobial activity of various extracts of the flowers of *H. perforatum* (Barnes et al., 2001; Butterweck, Jürgenliemk, Nahrstedt, & Winterhoff, 2000; Sakar & Tamer, 1990). A survey of the recent literature showed that data on the antioxidant activity of all of the known varieties are rather scarce (Kirca & Arslan, 2008; Silva, Ferreres, Malva, & Dias, 2005; Silva, Malva, & Dias, 2008; Zou, Lu, & Wei, 2004).

Natural antioxidants are considered to be multifunctional and their activity depends on various parameters such as the multiplicity and heterogeneity of the matrix, the experimental conditions and mainly the reaction mechanism. In general, the effectiveness of an antioxidant relies on the interfacial phenomena and the distribution of the components between the lipophilic and hydrophilic phases. It is now known that the antioxidant properties of plant extracts cannot be evaluated by one single method due to the complex nature of phytochemicals. A thorough antioxidant assay of plant extracts should involve several activity studies.

Despite detailed studies on the flowers of *H. perforatum* L., information on the antioxidant activity and composition of different morphological parts of it has hitherto not been reported. Therefore, apart from the traditionally used flowers, it would be of interest to evaluate the antioxidant properties of extracts obtained from the different aerial parts of the plant, employing commonly accepted antioxidant activity assays. In this study, the aerial parts of *H. perforatum* were collected and subsequently extracted with organic and aqueous solvents. Their antioxidant activity was extensively evaluated using established protocols and their composition in

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doi:10.1016/j.foodchem.2009.04.016

terms of basic constituents was considered. More specifically, the plant was divided into five parts: shoots with leaves, non flower-bearing branches, flower-bearing branches, floral buds and flowers. The extracts were then analysed for their total phenolic and chlorophyll content and their concentration in hypericin, pseudohypericin and hyperforin which are well established active substances of the plant (Gioti, Skalkos, Fiamegos, & Stalikas, 2005). The potential correlation between the content of the extracts and their antioxidant activity was investigated.

2. Materials and methods

2.1. Chemicals

Hypericin, pseudohypericin and hyperforin (99%) were purchased from Alexis Corp. (Lausen, Switzerland). L-α-Phosphatidylcholine (PC), bovine serum albumin (BSA), nitro blue tetrazolium chloride (NBT), 5-methylphenazinium methosulphate (PMS), quercetin, β-nicotinamide adenine dinucleotide (β-NADH), chlorogenic acid, and *n*-octanol were obtained from Sigma (St. Louis, MO, USA). Linoleic acid, malondialdehyde (MDA), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·), 2,6-di-*tert*-butyl-4-methyl phenol (BHT), vitamin E, ferrozine and gallic acid were from Aldrich (Steinheim, Germany). Trichloroacetic acid, EDTA, ferric chloride, citric acid (99.5%), potassium chloride (p.a.), sodium carbonate, hydrochloric acid (37%, p.a.) and *n*-butanol were from Merck (Darmstadt, Germany). Guanidine hydrochloride (⩾98%), Folin-Denis reagent, Tween-20, sodium thiocyanate, 2-thiobarbituric acid (TBA) (⩾98%) and catechin (⩾96%) were obtained from Fluka Chemie (Buchs SG, Switzerland). Sodium dihydrogen phosphate, sodium monohydrogen phosphate (p.a.), ferrous chloride, ascorbic acid, sodium chloride, hydrogen peroxide, orthophosphoric acid (85% p.a.) and ethanol were from Riedel-de-Haen (Seelze, Germany). Finally, methanol and acetonitrile were of HPLC-grade and obtained from Labscan Ltd. (Dublin, Ireland).

2.2. Sample collection and extraction

Samples of *H. perforatum* were collected during the summer of 2003, from Epirus, Northwestern Greece. Voucher specimens of the collected plants are kept at the Agricultural University of Athens (code n. 1022). The collected samples were divided into five parts: (a) shoots with leaves (Sh), (b) non flower-bearing branches (NFBB), (c) flower-bearing branches (FBB), (d) floral buds (FB) and flowers (Fl). Floral buds were collected during their development, before opening stage and flowers during maturation. Each part was dried in the dark over a period of 30 days, ground to pass through a 0.4 mm sieve and extracted with: (I) MeOH at room temperature followed by EtOH–water (60:40) at 80 °C (organic extract) and (II) water at room temperature (aqueous extract) (Poutaraud, Lobstein, Girardin, & Weniger, 2001).

The collected extracts were filtered and the filtrates were evaporated into a flash evaporator and finally lyophilised. The dry residues were weighed and stored at −18 °C in dark-walled glass vials.

2.3. Solutions

Standard solutions of hypericin 0.02 mg/ml, pseudohypericin 0.1 mg/ml and hyperforin 0.05 mg/ml were prepared in MeOH and stored at −18 °C. Plant extracts of various concentrations (~0.02–1.00 mg/ml) were prepared by successive dilutions of a stock solution of 10 mg of dry extracts dissolved in 10 ml of methanol. A stock solution of MDA 10^{-2} M was prepared by hydrolysis of 1,1,3,3-tetraethoxypropane (Stalikas & Konidari, 2001).

2.4. Instrumentation

Spectrophotometric measurements were performed on a UV 2100 UV–Vis Shimadzu, Spectrophotometer (Shimadzu, Kyoto, Japan). Lyophilised extracts were obtained by an Alpha 1-2LD Lyophilisation apparatus (Christ, Germany).

Liquid chromatographic analysis of hypericin and pseudohypericin was performed on a Shimadzu HPLC system Shimadzu (Duisburg, Germany), as described elsewhere (Gioti et al., 2005). Hyperforin was chromatographed on the same HPLC system furnished with a Shimadzu spectrophotometric detector SPD 10AV at 276 nm, connected in series with a fluorescence detection, as per conditions for hypericins. Class LC10 software Version 1.6 (Shimadzu) was used for data analysis and processing.

Mass spectra were measured on an Agilent 1100 Series LC-MSD-Trap-SL spectrometer (Palo Alto, CA, USA) equipped with an electrospray interface using the positive ionisation mode, and an autosampler with autoinjector. Operating conditions were: accumulation time, 300 ms; dry temperature, 350 °C; capillary voltage, 3500 V; nebuliser, 40 psi; dry gas, helium at 12 l/min. Ion trap full-scan analysis was conducted from *m*/*z* 50 to 700 with an upper fill time of 200 ms. A 10 μl sample volume was injected. System control and data evaluations were performed using the HP ChemStation for LC/MS. The analytical column and chromatographic program was the same used for the isocratic analysis of hypericins and hyperforin.

Liquid chromatographic analysis of the extracts was performed by the above mentioned HPLC systems by gradient elution using MeOH as solvent A and aqueous formic acid 0.1% as solvent B at a flow rate of 0.5 ml/min. The gradient program started with 20% solvent A, raised to 95% after 70 min, and remained at this percentage for 30 min. The overall analysis time was 100 min and the column was kept at 30 °C throughout analysis.

The on-line post-column radical scavenging measurements were carried out by a hyphenated DPPH· method based on a system first proposed by van Beek et al., 2000 (Koleva, Niederlander, & Van Beek, 2000). The separated analytes reacted post-column with the DPPH· and any reduction in the concentration of radicals was detected as a negative peak by the absorbance detector at 515 nm. The DPPH· solution (3×10^{-5} M) was inserted in the HPLC effluents with the use of a second HPLC pump at a flow rate of 0.4 ml/min and the reaction took place in a 5.0 m Teflon reaction coil of 0.25 mm internal diameter. The bleaching of the DPPH· solution was recorded by a second UV–Vis detector (Exarchou, Fiamegos, van Beek, Nanos, & Vervoort, 2006).

2.5. Methods of analysis and antioxidant measurements

The determination of total phenolic content was based on the Folin-Denis method. Total chlorophylls and chlorophylls *a* and *b* were determined by measuring the absorbance at 663 and 640 nm.

The free radical scavenging activity was evaluated by the DPPH· method. The percentage of the superoxide anion scavenging activity of the hypericum extracts was determined by the nitro blue tetrazolium chloride method. The metal chelating activity of hypericum extracts was evaluated by the ferrozine–Fe^{2+}complex formation method. The reducing power of the extracts was evaluated by the ferricyanate–Fe^{3+}method. The inhibition of lipid oxidation was determined by measuring the antioxidant activity using PC as the lipid matrix. Prevention of oxidative damage to proteins was based on the Fenton/BSA reaction. Linoleic acid antioxidant activity was based on the inhibition of linoleic acid oxidation.

More details on the above methods are provided in Supplementary material.

3. Results and discussion

3.1. Composition of extracts from Hypericum perforatum aerial parts

Phloroglucinols, naphthodianthrones, phenolic acids and flavonoids are the most prominent constituents of *H. perforatum*. Its pharmaceutical activity, however, is attributed mainly to hypericins and hyperforin (Butterweck, Petereit, Winterhoff, & Nahrstedt, 1998; Skalkos et al., 2005). Table 1 summarises the results from the quantitative determination of the three major components of the extracts and their respective total phenolic contents. The organic extracts, as opposed to the aqueous ones, are rich in all components because of the demonstrated low solubility of hypericin, pseudohypericin and hyperforin in water. Moreover, among the three constituents, hyperforin is the most abundant in all extracts. Highest was the concentration of hyperforin in the FB extracts. This is due to the fact that hyperforin is photosensitive and its highest concentrations appear just before the blossom period (Ang et al., 2004; Orth & Schmidt, 2000). From Table 1, it is also clear that in all extracts under study, phenolics are in abundance (expressed as mg of gallic acid/g dry extract) with the Sh extracts to exhibit the highest and the FB the lowest concentrations. Phenolic compounds have a wide range of polarity and thus, both organic and aqueous extracts show significant phenolic concentrations.

Ward's hierarchical clustering and principal component analysis based on standardised data of Table 1 can distinguish between tested samples. Visual diagrams summarising all ten samples are given in Fig. 1. Both diagrams reveal that the extracts fall into two major clusters-groups consisting of small sub-groups. In this way, the organic extracts of flowers and floral buds prove to belong to a distinct group because of their highest concentrations in hypericin, pseudohypericin and hyperforin. Two significant subgroups are comprised of NFBB and FBB for both extraction media, which are characterised by comparable phenolic content and concentrations of active constituents.

As for chlorophylls, highest total quantities were observed in the organic extracts as a consequence of their strong hydrophobicity. FB and Fl presented lower concentrations of total chlorophylls (see Supplementary material) most of which were attributed to chlorophyll α which are better extracted to polar organic solvents than chlorophylls *b*.

Furthermore, the LC–MS analysis of the extracts made it possible to identify the most prominent constituents of the extracts, in combination with the injection of reference compounds, where possible. As expected, the organic extracts are richer in constituents being in higher quantities, as compared to the aqueous counterparts. The identified constituents of the extracts are the following: chlorogenic acid, rutin, taxifolin ramnoside, hyperoside, isoquercitrin, quercetin, biapigenin, pseudohypericin, hypericin, hyperforin, adhyperforin together with two unidentified hyperforin and adhyperforin analogues. Chlorogenic acid is the dominant compound in all extracts while taxifolin ramnoside, routin, hyperoside and isoquercitrin were found to be equally distributed between the organic and aqueous extracts. The FB and Fl-organic extracts comparatively bear the highest quantities of hyperforin, adhyperforin and their analogues. Chromatographic and spectral data of the identified peaks are provided in Supplementary material.

3.2. Antioxidant activity of H. perforatum aerial parts

The antioxidant activity of an extract, especially when dealing with food and biological samples depends on several parameters such as the matrix effect, the oxidation parameters and the antioxidant reaction conditions. In order to evaluate the antioxidant activity of a natural product, it is crucial to implement more than one antioxidant methods, taking into consideration the various oxidation aspects in the systems under scrutiny. In this context, the antioxidant properties of the aqueous and organic extracts of the five aerial parts of *H. perforatum* were studied and compared with the activity of the well known antioxidants vitamin E, quercetin, gallic acid and BHT. Moreover, the EC_{50} values, which refer to the smallest concentration of antioxidants necessary for 50% of reactivity were calculated. The lower the EC_{50} value the more reactive the compound under study.

Various concentrations of the extracts were investigated for *their DPPH· radicals scavenging activity* and they were compared with that of BHT. Increased scavenging activities were recorded in all cases for extract concentrations higher than 0.27 mg/ml. Optimum behaviour was presented by organic and aqueous Sh and Fl extracts (~100% activity) which was comparable to BHT. The activities of the rest of the extracts ranged from 84.6% to 93.9%. Weakest reactivity was rendered by the organic FB extract approaching 73.7% of scavenging activity in this particular assay. It is worth mentioning that for concentrations lower than 0.27 mg/ml the extracts behaved better than BHT.

Radicals present in living organisms are short-lived. Thus, the study of the antioxidant activity of a natural product should inevitably be correlated with the required reaction time. EC_{50} values when combined with the respective time $T_{EC_{50}}$ (time required to achieve 50% of antioxidant reactivity) afford the antioxidant efficiency factor (*AE*), given by the equation $AE = 1/EC_{50}T_{EC_{50}}$ (Villano, Fernandez-Pachon, Moya, Troncoso, & Garcia-Parrilla, 2007). The higher the *AE* value the stronger the antioxidant activity of the

Table 1
Phenolic content and concentrations of hypericin, pseudohypericin and hyperforin in the aerial parts of *Hypericum Perforatum* L. Average of three measurements ± SD.

Sample[a]	Constituent			
	Hypericin (mg/g of dry extract)	Pseudohypericin (mg/g of dry extract)	Hyperforin (mg/g of dry extract)	Phenolic content (mg of gallic acid/g of dry extract)
Organic extract				
Sh	0.49 ± 0.01	0.71 ± 0.01	20.1 ± 0.3	257 ± 4
NFBB	1.50 ± 0.01	2.25 ± 0.01	33.0 ± 0.2	231 ± 3
FBB	1.61 ± 0.01	2.08 ± 0.01	49.5 ± 0.3	233 ± 4
FB	3.04 ± 0.08	5.41 ± 0.07	195 ± 3	133 ± 6
Fl	4.75 ± 0.07	6.56 ± 0.08	74.2 ± 0.7	191 ± 5
Aqueous extract				
Sh	0.04 ± 0.01	0.11 ± 0.02	3.09 ± 0.06	228 ± 2
NFBB	0.13 ± 0.01	0.47 ± 0.02	2.73 ± 0.07	184 ± 2
FBB	0.09 ± 0.03	0.36 ± 0.01	2.36 ± 0.05	190 ± 2
FB	0.46 ± 0.04	1.71 ± 0.03	27.2 ± 0.7	125 ± 1
Fl	0.53 ± 0.08	1.95 ± 0.05	27.9 ± 0.7	162 ± 3

[a] Sh, shoots; NFBB, non-flower bearing branches; FBB, flower-bearing branches; FB, floral buds; Fl, flowers.

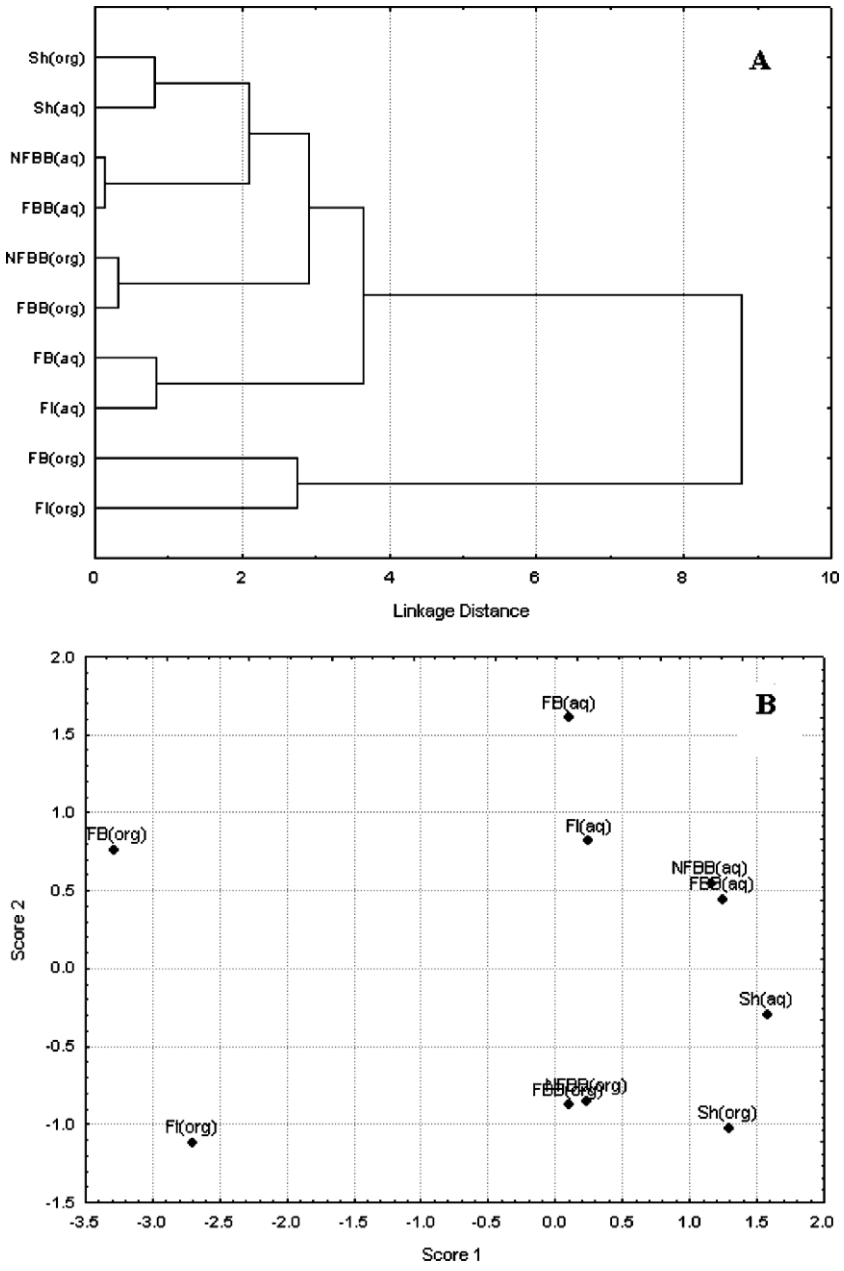

Figure 1. (A) Dendrogram indicating the grouping obtained by cluster analysis and (B) the scores plot obtained from principal component analysis of all plant extracts.

extract. If this approach is applied, the antioxidant potential of the different plant extracts is altered because of the different kinetic behaviour of the extracts. Resting on *AE* calculated values, the scavenging DPPH· activity decreases following the order: org-Sh > org-NFBB ∼ org-FBB > aq-Sh > aq-NFBB > aq-FBB > org-FB > org-Fl > aq-FB > aq-Fl.

On a subsequent step, a gradient elution program was applied to acquire on-line, post-column DPPH· scavenging measurements. Any reduction in the radical concentration was detected as a negative peak at 515 nm. Fig. 2 exhibits the chromatographic analysis of a methanol extract using the on-line DPPH· test. The negative peaks were observed with a time delay of 3.31 min which was attributed to the 5.0 m reaction coil. As expected, the negative peaks acquired exhibit low resolution and chromatographic peaks with close retention times are represented by a single broad peak. The contribution of each of the extracts components to the overall radical scavenging activity of the extract was correlated to the area of the respective negative peak. Since the reaction of 3.31 min is not adequate for the reactants to reach equilibrium, the overall procedure is a fixed-time method strongly depending on the kinetics of the reaction; thus, it is better described by the $T_{EC_{50}}$ value. Table 2 summarises the contribution of each of the main active ingredients, as a percentage of the overall activity of the extract.

Chlorogenic acid is the compound contributing the most to the DPPH· antioxidant activity; in the case of Sh, it accounts for ∼60% of the total activity. The antioxidant activity of chlorogenic acid, which appears in highest concentrations in both organic and aqueous extracts has been well established (Govindarajan et al., 2006). Worth mentioning is the high contribution of hyperforin, adhyperforin and their analogues which show up as main DPPH· antioxidants of the organic FE and Fl extracts. On the contrary, less significant is the contribution to the DPPH· scavenging activity of taxifolin ramnoside, rutin, hyperoside, isoquercitrin, quercetin and biapigenin.

The scavenging activity of the superoxide anion ($O_2^{\cdot-}$) of the extracts was evaluated against the activity of gallic acid. According to

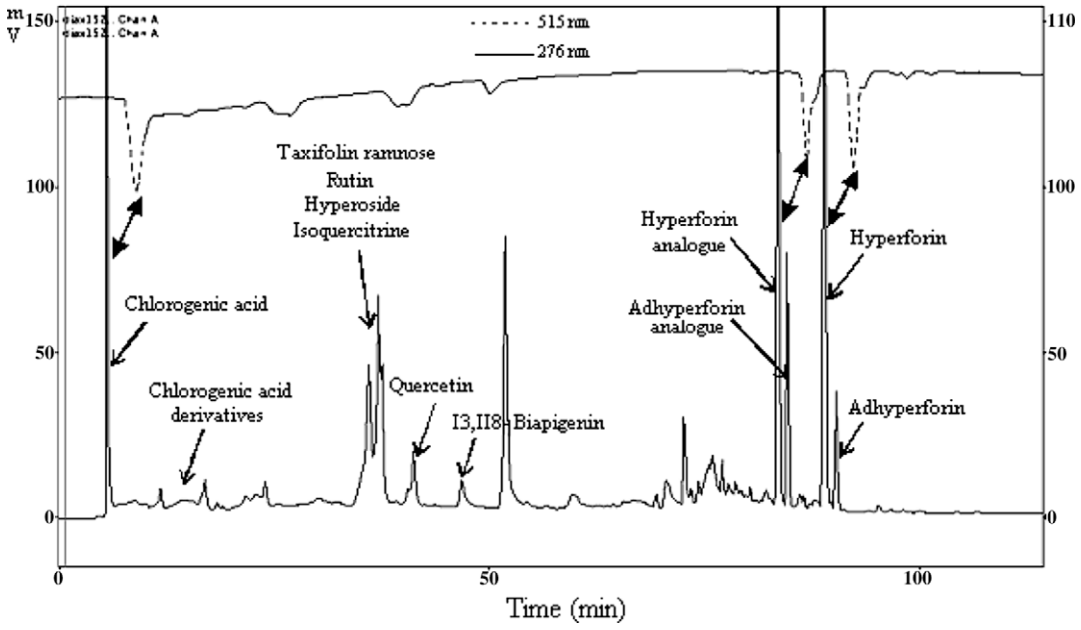

Figure 2. On-line, post-column radical scavenging chromatogram of the methanol extract of *Hypericum perforarum* L. flowers.

Table 2
Radical scavenging activity of the main constituents identified, presented as a percentage of the respective overall extract activity.

Sample	Extract constituents (%)						
	Chlorogenic acid	Chlorogenic acid derivatives	Rutin hyperoside	Quercetin	I3,II8-Biapigenin	Hyperforin and adhyperforin analogues	Hyperforin and adhyperforin
Organic extract							
Sh	60.6	22.6	8.9	–	–	0.7	6.0
NFBB	46.0	18.8	14.3	–	–	–	13.2
FBB	44.6	17.1	12.3	–	–	4.8	12.7
FB	19.2	5.6	3.7	5.1	4.0	20.3	30.0
Fl	25.7	12.4	10.7	0.4	4.3	21.2	22.0
Aqueous extract							
Sh	59.0	29.7	11.3	–	–	–	–
NFBB	51.9	21.5	19.1	1.0	–	–	–
FBB	53.5	21.0	18.6	0.7	–	0.1	0.6
FB	33.4	6.9	13.9	–	8.5	2.9	4.3
Fl	42.9	23.0	24.7	0.8	4.5	1.9	2.3

SDs of the values of the table are ranging between 0.3 and 0.8.

this study, the inhibition of the chromophor formation is directly correlated to the extract concentration, reaching 84% and 72% for the organic and aqueous extracts respectively. The activity of the extracts reaches the respective of gallic acid at concentrations above 1.0 mg/ml. This inhibitory effect for the aqueous extracts ranges from 68% to 72%, with the greatest activities corresponding to Sh and Fl. As regards the organic extracts, the respective percentages are 70–84% with the highest values corresponding to FBB and Fl. The EC_{50} values were between 0.18 and 0.23 mg/ml while the respective value for gallic acid was 0.020 mg/ml, indicating its superiority on superoxide anion scavenging activity. The scavenging activity of superoxide anion according to the calculated EC_{50} values follows the order: gallic acid > aq.-Sh > org.-FB > org.-Sh > aq.-FBB and aq.-Fl > org.-NFBB > aq.-NFBB and org.-Fl > aq.-FB > org.-FBB.

The occurrence of appropriate *chelating agents* into the extracts is substantial for the reduction of free transition metals which give rise to catalytic lipid peroxidation. These agents form σ-bonds with the metals stabilising their oxidised form (Elmastas, Gulcin, Beydemir, Kufrevioglu, & Aboul-Enein, 2006). It is known that Fe^{2+}generates lipid peroxidation through the Fenton reaction or by accelerating the dissociation of lipid hydroperoxides to the respective peroxy- and alkoxy-radicals. Thus, the ability of the extracts to bind with Fe^{2+}in the presence of ferrozine was compared with the respective of EDTA which is a strong chelating agent. If EDTA's chelating ability is taken as 100% then for a range of extract concentrations 0.17–1.00 mg/ml their chelating potential were determined. Organic and aqueous FE extracts exhibited best chelating behaviour, i.e. 34.1% and 45.5%, respectively. The corresponding EC_{50} values were between 0.242 and 1.411 mg/ml while for EDTA was 0.011 mg/ml. Although the chelating activity of the extracts was much lower than EDTA's, yet, values were not negligible for a natural product. The EC_{50} values of the chelating activity of the extracts is summarised as follows: EDTA > aq.-FB > org. FB > org.-NFBB > org.-Fl > aq.-Fl > aq.-FBB > org.-Sh and aq.-NFBB > aq.-Sh.

The *reducing power* of the extracts is directly associated to their antioxidant activity (Meir, Kanner, Akiri, & Philosoph-Hadas, 1995). A rise in the extract's concentration led to increase of the reducing power value. At extract concentrations between 0.01

and 0.12 mg/ml the highest reducing power values were acquired for Sh, NFBB and FBB extracts and lowest for the FB extracts, with the activity of BHT being by far the strongest.

Lipid phase antioxidant activity (TBARS method) was based on monitoring the behaviour of all the extracts under study after the induction of radical peroxidation in PC. Lipid peroxidation leads to the formation of MDA and other similar compounds which are characterised as thiobarbituric-reactive substances (TBARS). The inhibition of TBARS formation can directly be associated to the peroxidation inhibiting activity of extracts. BHT and quercetin, which is a well known natural antioxidant, were used as reference compounds. The activity of *H. perforatum* extracts was tested in the concentration range of 0.10–1.0 mg/ml. The synthetic BHT was the most active of the tested forms exhibiting lipid peroxidation protection of 89%. Quercetin presents behaviour closer to that of the extracts under study. Of the organic extracts, NFBB and Sh are more active, which accounts for 75.9% and 73.8% peroxidation inhibition, respectively. Less active are the aqueous extracts with aq.-Sh to demonstrate the inhibition of 65.4%. As for org.-FB, at concentrations higher than 0.50 mg/ml, its activity is reduced significantly and it acts as a pro-oxidant. The respective EC_{50} values shape the following decreasing order of activity: BHT (0.004) > quercetin (0.094) > org. extracts (0.101–0.203) > aq. extracts (0.192–0.330).

The inhibitory effect of quercetin against lipid peroxidation is attributed to its chelating properties. Quercetin forms complexes with Fe^{2+}which occurs in the PC oxidation solution and thus it is not possible for the hydroxyl radicals to be formed *via* the Fenton reaction. Moreover, quercetin promotes the oxidation of $Fe^{2+}$to Fe^{3+}rendering it less effective in the production of free radicals. Nonetheless, in metal-induced lipid peroxidation its antioxidant reaction dominates (Hajji, Nkhili, Tomao, & Dangles, 2006). On the other hand, BHT acts mainly as hydrogen donor, while its high lipophilicity facilitates its incorporation into the liposomes providing stronger protection against oxidation. This is probably the main activity mechanism of the extracts, as well. The antioxidant activity of all organic extracts against lipid phase oxidation may be attributed to a great number of compounds of different polarities and their protective orientation to the bilayer and polar surface of PC.

Oxidative damage of proteins is attributed to the presence of free radicals leading to the formation of carbonyl compounds. The ability of the extracts to *prevent protein oxidative damage* was evaluated based on the inhibition of carbonyls formation. This kind of oxidation is due to Fenton reaction, Fe^{2+}/H_2O_2 while H_2O_2 facilitates the production of free radicals. Here, BHT and quercetin were used as reference compounds at concentrations of 0.01–1.00 mg/ml. BHT is the only tested among compounds and extracts that showed an increase in the antioxidant activity with increasing concentrations. Quercetin, at concentrations of 0.01–0.20 mg/ml showed negative values of inhibition (−18.3%) manifesting itself as a pro-oxidant, in this concentration range. At concentrations greater than 0.20 mg/ml the pro-oxidative effect turns opposite with positive values of antioxidant activity (12.1% at 1.00 mg/ml). The plant extracts under study presented only increasing pro-oxidative activity. Thus, at concentrations of 1.00 mg/ml, pro-oxidative activities of 56.5 and 42.1% are produced by org. and aq.-Sh while org.- and aq.-FB extracts presented the lowest activity of 14.5 and 17.7%, respectively. This is not extraordinary since other researchers have noticed that several other plant extracts or pure antioxidants scarcely prevent metal-catalysed oxidation of proteins ((Makris & Rossiter, 2001; Moure et al., 2001). Unlike lipid-phase peroxidation, flavonoids in metal-catalysed protein oxidation may appear to have a pro-oxidative effect (Hajji et al., 2006). Besides, quercetin and other related phenolic substances are known to bind with proteins or amino acids, thus exhibiting reduced activity (Arts, Haenen, Voss, & Bast, 2001; Smith, Halliwell, & Aruoma, 1992). In addition to this phenomenon, the hydrophilicity of the proteinic system together with the occurrence of citric acid promotes the Fe^{2+}solubility which drives the elevated production of hydroxyl radicals. Although citric acid shows a tendency to form complexes with Fe^{2+}, this reaction is rather slow especially at neutral pH ambience. In addition, the pro-oxidation effect of the extracts may be attributed to the reduction of metal ions like $Fe^{3+}$to their considerably more active forms like Fe^{2+}(Moure et al., 2001). So, it is possible that the efficiency of the aqueous antioxidants may be negated by their pro-oxidant activity due to the occurrence of metal ions in their reduced form, which is more reactive.

Finally, the antioxidant activity of the extracts was tested with respect to the *prevention of oxidation of linoleic acid system*. The potential of the plant extracts under study to inhibit lipid peroxidation was measured in a time span of 1–72 h of incubation. In a previous work, we demonstrated that *H. perforatum* L. extracts inhibit the formation of pentanal and hexanal and work as antioxidants under induced oxidative conditions in oil-in-water systems (Gioti, Fiamegos, Skalkos, & Stalikas, 2007). The highest antioxidant activity was noticed at relatively low concentrations of extracts. From the experiments herein, it became evident that for 1 h incubation time the antioxidant activities of both types of extracts and the reference compounds were low ($\leqslant$37% for lipophilic BHT and vitamin E, $\leqslant$22% for org. extracts and $\leqslant$34% for aq. extracts). Increased incubation times led to an improvement in the activity with higher values to be observed in all cases in the concentration range 0.10–0.75 mg/ml and incubation time of 24 h (64–83% for org. extracts and 32–83% for aq. extracts). The extended antioxidant protection was achieved effectively at higher extract concentrations while aqueous extracts present lower antioxidant activity when lower extract concentrations were used in contrast to the respective organic extracts. The greater than 24 h the incubation period the higher the extract concentrations required in order to maintain enough antioxidant protection from lipid peroxidation. Vitamin E and BHT, as reference compounds, were superior for shorter incubation periods but their activities converged to the respective of the extracts for higher time periods (i.e. at concentrations higher than 0.50 for org. and 0.75 mg/ml for aq. extracts). For all the above antioxidant activity methods more data are provided in Supplementary material.

3.3. Correlation analysis of the measurements

It is widely accepted that the antioxidant activity of a plant extract is correlated to its phenolic content (Elmastas et al., 2006). However, the results of this study agree with researchers who question this possible correlation (Bocco, Cuvelier, Richard, & Berset, 1998; Oktay, Gulcin, & Kufrevioglu, 2003). In addition, experiments using pure compounds showed that the chemical structures of phenolic compounds have a decisive effect on their antioxidant properties. Thus, the antioxidant activity is stronger when a second hydroxyl or methyl group is present in *ortho* or *para* position (Tuberoso, Kowalczyk, Sarritzu, & Cabras, 2007). In order to elucidate the correlation between the phenol content of *H. perforatum* aerial parts and their antioxidant activity, linear regression analysis was performed, quoting the correlation coefficient (r) of each of the antioxidant tests performed. The significance test was based on the calculation of t_{exp} and its comparison with $t_{(0.05,8)}$ of the t-distribution, at 95% confidence (for $t_{exp} > t_{(0.05,8)}$ the correlation is statistically significant). It was obvious that the phenolic content in the extracts showed a strong correlation with $DPPH^{\cdot}$ scavenging activity ($r = 0.822$), reducing power ($r = 0.981$) and prevention of PC oxidation ($r = 0.946$). This implies that phenolics are undoubtedly responsible for such inhibition of *Hypericum perforatum* extracts. No significant correlation could be found between

phenolic content and superoxide anion scavenging activity and linoleic acid antioxidant activity.

The inhibition of protein oxidation is correlated negatively with phenolics, for reasons already mentioned. The same behaviour holds true for metal chelating activity. The pro-oxidant activity of metal chelating complexes is attributed to the capacity of the chelating agent to increase the solubility of the metal or to facilitating the participation of the metal on the redox cycle. In addition, the chelating ability highly depends on the pH of the solution. At pH values lower than the respective pK_a the active groups are protonated and their chelating power is substantially reduced. Moreover, the chelating ability is reduced in the presence of antagonistic chelating metals like Ca.

There are studies reporting that chlorophylls may present antioxidant activity while the pro-oxidant activity due to their light and heat instability is also supported (Tuberoso et al., 2007). In our case, a satisfactory correlation with DPPH· scavenging activity ($r = 0.660$) and reducing power ($r = 0.648$) exists, which entails stabilisation of the chlorophylls in the experimental conditions that allows their antioxidant activity to be expressed.

4. Conclusions

The *Hypericum perforarum* L. owes its widespread use in traditional and contemporary medicine to the high content of hypericins and hyperforins in its flowers. To reap the full benefits of the plant it was necessary to undertake a detailed antioxidant and compositional study of the entire aerial plant. Floral buds bear the highest concentrations of the foregoing constituents followed by the flowers; therefore, if extracts rich in bioactive substances is the case, floral buds should be extracted. Based on the antioxidant activity profile expressed through various assays, it can be concluded that, among the various plant fractions, shoots and branches show significant activity, which may be accounted for by the high phenolic content. Interestingly enough, the on-line HPLC-DPPH· antioxidant activity measurement of the extracts delineated the pivotal contribution of hyperforin, adhyperforin and their analogues to this antioxidant activity. The role of phenolic compounds on the total DPPH· scavenging activity was minor except chlorogenic acid and its derivatives. No encouraging results were obtained as regards the inhibition of the oxidative damage of proteins. All the extracts promote the damage of proteins exhibiting a functional pro-oxidant role. Finally, it is taken for granted that, the complex composition of plant extracts can lead to contradictory results if the antioxidant activity evaluation is carried out by a single method.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2009.04.016.

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Food Chemistry 117 (2009) 405–411

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Cytotoxicity of curcumin, resveratrol and plant extracts from basil, juniper, laurel and parsley in SH-SY5Y and CV1-P cells

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ARTICLE INFO

Article history:
Received 15 October 2008
Received in revised form 8 April 2009
Accepted 8 April 2009

Keywords:
Resveratrol
Curcumin
Basil
Laurel
Parsley
Juniper
Phytochemicals
Plant extracts
Cell viability

ABSTRACT

The health-promoting effects of natural phenolic compounds are attracting growing interest. In this study, we tested four plant extracts, namely basil, juniper, laurel and parsley, and two well-known pure phenolic compounds of natural origin, curcumin and resveratrol, for their possible cytotoxic effects *in vitro*. AraC was used as an apoptotic reference compound. The pure compounds were studied in two cell lines, SH-SY5Y neuroblastoma and CV1-P fibroblast cells, and the plant extracts in SH-SY5Y cells. Cytotoxicity was examined by MTT and LDH assays, and the molecular mechanisms by Western blot analysis of p53 protein in cells. Juniper extract decreased the cell viability and increased the amount of p53 in SH-SY5Y cells at lower concentrations than did other plant extracts, and its effects on the amount of p53 in cells were comparable to the treatment with 50 μM AraC. The actions of curcumin and resveratrol were dependent on the concentration and cell line. Curcumin decreased the cell viability and increased the amount of p53 in SH-SY5Y cells more effectively than in CV1-P cells. The comparison between the results of AraC and pure curcumin showed a similar effect on the amount of p53. Our results indicate a potential role of plant extracts and plant-derived compounds as health-promoting food constituents, as well as candidates for drug development.

1. Introduction

The role of cell death in health and disease has been intensively studied (Lockshin & Zakeri, 2007). Apoptosis is one form of programmed cell death and a closely regulated mechanism to control the removal of unwanted and/or harmful cells of an organism. Apoptosis is also needed for the maintenance and function of homoeostasis, differentiation, embryonic development and the immune system. Impairments in apoptosis can be associated with several diseases, e.g. cancer, autoimmune and neurodegenerative diseases and AIDS. An unregulated form of cell death is necrosis. It occurs due to various stress causes, e.g. toxins or physical damage, leading to uncontrolled death of a cell.

The p53 tumour suppressor protein is one of the key regulators of apoptosis and plays a pivotal role in mediating DNA damage-induced apoptosis. The role of p53 has also been widely studied in experimental models of apoptosis (Maňáková, Puttonen, Raasmaja, & Männistö, 2003; Roy, Baliga, Elmets, & Katiyar, 2005). Activation of p53 by a cellular stress, e.g. DNA damage and cytotoxic compounds, can lead the cells to apoptosis or cell cycle arrest by acting as a transcription factor and modulating the activity of other proteins related to apoptosis (Meulmeester & Jochemsen, 2008). The significance of p53 in cancer is, without dispute; thus an impaired p53 pathway is involved in most human cancers (Vogelstein, Lane, & Levine, 2000).

Natural phenolic compounds, abundant in fruits, berries and vegetables, have been shown to be capable of modulating cell signalling pathways related to cell death. Their role as chemopreventive agents is under intensive investigation and many promising compounds have been found (Pan, Ghai, & Ho, 2008). Indeed, the activities of two intensively studied plant phenolic compounds, resveratrol and curcumin, in the signalling mechanisms of a cell are indisputable. There is also strong evidence that natural phenolic compounds can alter cell signalling pathways related to tumour suppressor protein p53 in cancerous cell lines (Lin et al., 2008; Shukla & Gupta, 2008). Besides, there has been a growing interest in plant-derived extracts, instead of pure compounds. This is because the extracts have been used for therapy from past to present, and their health-promoting effects might involve several active compounds in the extracted material. A potential advantage of plant-derived compounds in healthcare is that their utilisation as food has a long history, and their use has been accepted as safe. The possible utilisation of plant-derived compounds and extracts as chemopreventive and health-promoting agents in the future has focussed increasing attention on the understanding of their molecular mechanisms and targets of action.

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.04.018

The aim of our study was to examine four plant extracts containing phenolic compounds (Hinneburg, Dorman, & Hiltunen, 2006), and two pure plant-originated phenolic compounds for their possible effects on cell viability and cell death. Therefore, we have tested the effects of water extracts from basil (*Ocimum basilicum* L.), juniper (*Juniperus communis* L.), laurel (*Laurus nobilis* L.) and parsley [*Petroselinum crispum* (Mill.) Nyman ex A.W. Hill] (Hinneburg et al., 2006) on the membrane integrity, metabolic activity and amount of protein p53 in SH-SY5Y neuroblastoma cells. Pure phenolic compounds, curcumin and resveratrol, were tested for their effects on the metabolic activity and amount of p53 in SH-SY5Y neuroblastoma and CV1-P fibroblast cells. In addition, the effects of plant extracts and pure compounds were compared with the effects of AraC, which was used as a positive control for apoptosis.

2. Materials and methods

2.1. Cell cultures and treatments

CV1-P fibroblast cells were cultured in Dulbecco's modified Eagle Medium (DMEM) containing L-glutamine, 1000 mg/l of D-glucose and sodium pyruvate (Gibco; Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated foetal bovine serum (HyClone, Thermo Fisher Scientific, Waltham, MA), and penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco). SH-SY5Y neuroblastoma cells were cultured in DMEM: nutrient mixture F-12 (1:1) containing 15 mM HEPES buffer and L-glutamine (Gibco) supplemented with 15% heat-inactivated foetal bovine serum (HyClone), penicillin (170 U/ml) and streptomycin (170 μg/ml) (Gibco) and 1% non-essential amino acids (Gibco). The cells were grown in 75 cm^2 cell culture flasks at 37 °C in a humidified atmosphere containing 5% CO_2 in air. CV1-P cells were seeded in 6-well plates and SH-SY5Y cells in 6-well plates or 60 mm culture dishes for Western blot analysis. Both cell lines were cultured in 96-well plates for cell viability assays. The cells were exposed to AraC, resveratrol, curcumin and, furthermore, SH-SY5Y cells to different plant extracts 18–24 h after plating. AraC was dissolved in milliQ H_2O, curcumin and resveratrol in DMSO: milliQ H_2O (1:1) and plant extracts in cell culture medium or milliQ H_2O. The final concentration of DMSO in all experiments was 0.1%. Control cells were exposed to the same amount of DMSO.

2.2. Preparation of plant extracts

Plant extracts from basil (*O. basilicum* L.), juniper berry (*J. communis* L.), laurel (*L. nobilis* L.) and parsley [*P. crispum* (Mill.) Nyman ex A.W. Hill] were prepared in our own laboratory (Hinneburg et al., 2006). Briefly, dried plant material was purchased from Paulig Oy, Finland. The extraction of plant material was performed by hot water under normal pressure and the volatile oils were removed by hydrodistillation. Plant extracts were freeze-dried, and stored at 4 °C.

2.3. MTT assay

An assay of MTT (methylthiazolyldiphenyl-tetrazolium bromide) reduction was used to examine the metabolic activity of the cells. The MTT assay is based on the reduction of the yellowish MTT to dark blue formazan by viable and metabolically active cells (Mosmann, 1983). The cells were incubated with different compounds for 12 or 24 h. Then, MTT (Sigma, Saint Louis, MO), dissolved in Hank's balanced salt solution, was added into each well to get the final concentration of MTT at 0.5 mg/ml. SH-SY5Y cells were incubated with MTT for 2.5 h and CV1-P cells for 3–4 h at 37 °C in 5% CO_2. The medium with MTT solution was removed and the formazan crystals were dissolved with DMSO. The absorbance was measured at 560 nm, subtracting the background at 655 nm, using a microplate reader (Bio Rad, model 550, Japan). The percentage metabolic activities were calculated using Eq. (1) from the values of 2–4 independent experiments.

$$\%\ \text{metabolic activity} = \left(\frac{\text{absorbance of treated cells}}{\text{absorbance of untreated cells}}\right) \times 100 \quad (1)$$

2.4. LDH assay

The membrane integrity of cells was measured using a commercial kit (CytoTox-ONE Homogeneous Membrane Integrity Assay; Promega, Madison, WI). The measurement is premised on the leakage of lactate dehydrogenase (LDH) from cells after different treatments, and the assay was performed according to the manufacturer's instructions. In brief, the cells were incubated with different extracts for 12 h and 50 μl of medium from treated cells were mixed with the 50 μl of CytoTox-ONE reagent. The plate was incubated for 30 min, shaking the first 10 min. The reaction was stopped adding 25 μl of CytoTox Stop Solution, and the fluorescence was determined with an excitation wavelength 560 nm and an emission wavelength 590 nm, using a fluorescence microplate reader (Varioskan, Thermo Scientific, Finland). The percentage membrane integrities were calculated using Eq. (2) from the values of 3–4 independent experiments.

$$\%\ \text{membrane integrity} = \left(\frac{\text{experimental-blank}}{\text{control-blank}}\right) \times 100 \quad (2)$$

2.5. Western blotting

Protein p53 is one of the key regulators of apoptosis. Therefore, we have measured the amount of p53 in cells after treatments using Western blotting. Both cell lines were incubated with different samples for 12 h and harvested into ice-cold PBS. The cells were spun down by centrifugation (SH-SY5Y: 5 min, 5000*g*, 4 °C and CV1-P: 5 min, 2000*g*, 4 °C) and the cell pellets were stored at −80 °C until the total protein was extracted. To extract the total protein from cells, the cell pellets were incubated with a lysis buffer (20 mM HEPES, 20% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 0.1% Triton X-100) supplemented with the cocktail of protease inhibitors (Complete Mini, Roche, Mannheim, Germany) for 30 min (SH-SY5Y) and 1 h (CV1-P) on ice. After incubation, the samples were sonicated 3 × 10 s and centrifuged (SH-SY5Y: 15 min, 16000*g*, 4 °C and CV1-P: 30 min, 13000*g*, 4 °C). The supernatants were collected and the amount of protein was determined by a colorimetric bicinchoninic (BCA) protein assay kit (Thermo Scientific, Rockford, IL). Protein samples were separated using 10–12% SDS–PAGE gel and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked with 5% non-fat milk powder in Tris-buffered saline (TBS) supplemented with 0.05% Tween 20 for 2 h at room temperature. Proteins p53 and β-actin were identified using a mouse monoclonal p53 antibody (DO-7, Novocastra, UK) and a mouse monoclonal β-actin antibody (A1973, Sigma, Saint Louis, MO). Primary antibodies were incubated with the nitrocellulose membranes overnight at 4 °C in TBS with 0.5% non-fat milk powder with samples from SH-SY5Y cells and with 5% non-fat milk powder with samples from CV1-P cells. Then, the membranes were washed with TBS with 0.5% Tween 20, and exposed to a secondary antibody (Anti-mouse IgG-HRP, HAF007, R&D Systems, Minneapolis, MN) for 1 h at room temperature. The membranes were washed and the protein bands were

detected by a chemiluminescent reagent (SuperSignal West Pico, Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. The protein bands were visualised by GeneSnap with GeneGnome (Syngene, Cambridge, UK). The results were analysed using the GeneTools (Syngene, Cambridge, UK) programme.

2.6. Statistical analysis

The results are shown as means ± SEM. Statistical significance was measured by one-way ANOVA, followed by Dunnett's multiple comparison test. $p < 0.05$ was defined significant.

3. Results

3.1. The effects of AraC, curcumin and resveratrol on the metabolic activity of SH-SY5Y and CV1-P cells

The effects of AraC, curcumin and resveratrol on the metabolic activity were measured by MTT reduction assay. Both cell lines were treated with various concentrations of compounds, and the metabolic activity of SH-SY5Y cells was determined after 12 h and that of CV1-P cells after 24 h. The reference compound, AraC, decreased the metabolic activity in CV1-P cells significantly at the concentrations of 200 and 400 μM, but it did not have any significant effect on SH-SY5Y cells (Fig. 1a). Curcumin significantly decreased the metabolic activity of SH-SY5Y cells at the concentrations of 25, 50 and 100 μM, but, in CV1-P cells, a significant decline was seen only upon treatment with 200 μM (Fig. 1b). Resveratrol significantly decreased the metabolic activity of CV1-P cells but had no significant effect on SH-SY5Y cells, although there was a slight decrease at 50 and 100 μM concentrations (Fig. 1c).

3.2. The effects of AraC, curcumin and resveratrol on the amounts of p53 in SH-SY5Y and CV1-P cells

The amount of p53 protein was determined from both cell lines by Western blotting after 12 h treatments with AraC, curcumin and resveratrol. AraC significantly increased the amount of p53 at all concentrations (5, 10, 25, 50 and 100 μM) in both cell lines (Fig. 2a). SH-SY5Y cells were more sensitive to AraC than were CV1-P cells, and the maximal increase of the amount of p53 was 250–300% in SH-SY5Y and 175–200% in CV1-P cells compared to the controls. Curcumin significantly increased the amount of p53 in SH-SY5Y but not in CV1-P cells (Fig. 2b). The maximal increase of the amount of p53 in SH-SY5Y cells exposed to 50 μM curcumin was 250% compared to the control cells. Resveratrol increased the amount of p53 in both cell lines, and CV1-P cells were slightly more sensitive to the effects of it than were SH-SY5Y cells. Resveratrol, at 100 μM, increased the amounts of p53, by 160% in SH-SY5Y and 200% in CV1-P cells. Other concentrations of resveratrol had no effects on the amount of p53 in cells.

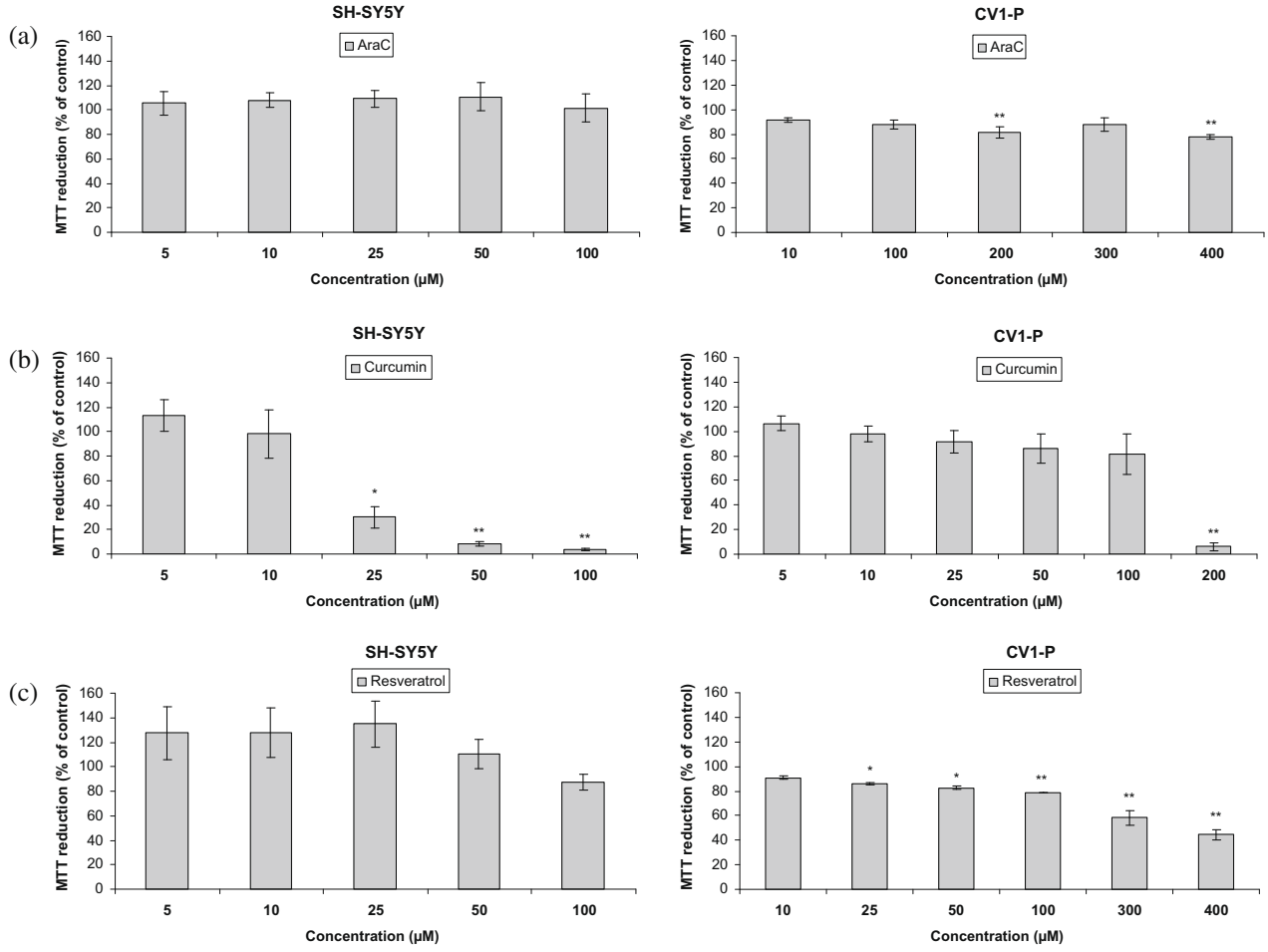

Fig. 1. Viability of SH-SY5Y and CV1-P cells after AraC (a), curcumin (b) and resveratrol (c) treatments, as measured by MTT reduction assay. The cells were exposed to the compounds for 12 h (SH-SY5Y) or 24 h (CV1-P) as indicated. The absorbance of MTT reduction was measured at the wavelength 550 nm and the background at 655 nm was subtracted. The values are expressed as means ± SEM from two independent experiments where the number of replications was 2x3 for SH-SY5Y and 2x6 for CV1-P cells. Statistical significance was determined by one-way ANOVA, followed by Dunnett's multiple comparison test; $^{*}p < 0.05$ and $^{**}p < 0.01$.

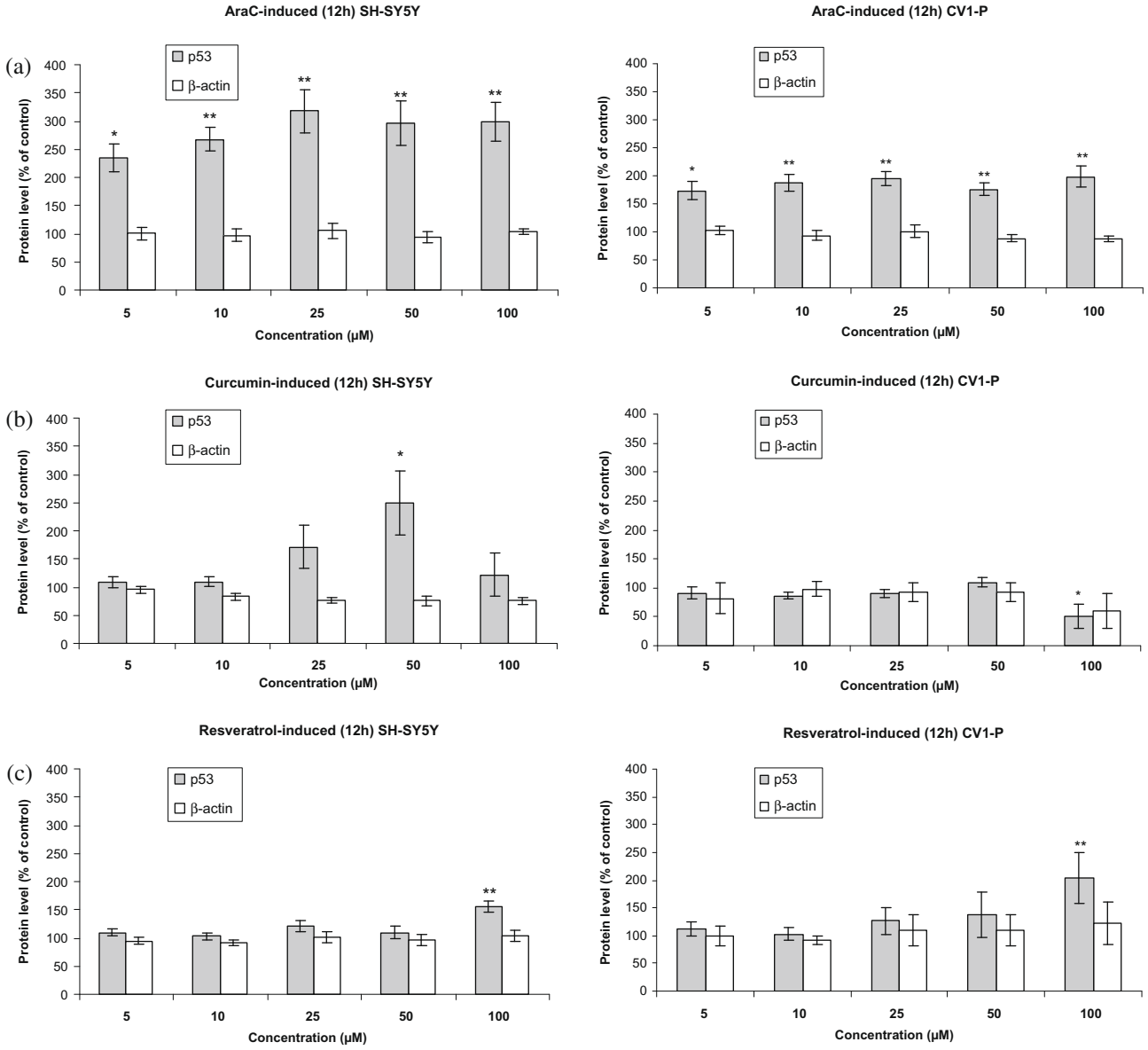

Fig. 2. Effects of AraC (a), curcumin (b) and resveratrol (c) on the amount of protein p53 in SH-SY5Y and CV1-P cells. The cells were treated for 12 h, and the levels of p53 and β-actin were examined by Western blot analysis. The values are means ± SEM from five independent experiments made in duplicate. Statistical significance was determined by one-way ANOVA, followed by Dunnett's multiple comparison test; $^{*}p < 0.05$ and $^{**}p < 0.01$.

3.3. The effects of plant extracts on the metabolic activity and membrane integrity in SH-SY5Y cells

Extracts from basil, juniper and laurel significantly decreased the metabolic activity of SH-SY5Y cells after 12 h of treatment (Fig. 3a). This effect was seen at all concentrations (0.01, 0.05, 0.1, 0.5 and 2.0 mg/ml) of juniper and laurel, but only at the highest concentration (2.0 mg/ml) of basil. The exposure of the cells to 2.0 mg/ml of basil, juniper and laurel decreased the metabolic activity by more than 50% compared to the control cells. A significant decrease in the integrity of cell membranes was observed after the exposure to 2.0 mg/ml of basil extract (Fig. 3b). However, evident increase of LDH leakage from cells is also observed after exposure to 2.0 mg/ml of juniper and laurel extracts, although the effects are not significant. Parsley extract had no effects on the metabolic activity or membrane integrity of cells. The exposure to 0.5 and 2.0 mg/ml of laurel extract produced a massive cell death which was not observed after treatments with the basil, juniper and parsley extracts.

3.4. The effects of plant extracts on the amounts of p53 in SH-SY5Y cells

The cells were treated with different concentrations of plant extracts for 12 h, as in the cell viability assays, and the amount of p53 was defined by Western blotting. Fig. 4 demonstrates that basil, juniper and laurel plant extracts increased the amount of p53 in SH-SY5Y cells. The exposure to 0.01 mg/ml juniper extract caused a significant rise of the amount of p53, although this effect was not observed at higher concentrations (Fig. 4b). The exposure of cells to the basil extract of 2.0 mg/ml significantly increased the amount of p53 in the cells (Fig. 4a). The laurel extract, at a concentration of 0.5 mg/ml, increased the amount of p53 up to 500% compared to the control cells in a single experiment, but there was also a considerable number of dead cells in this treatment (Fig. 4c). The laurel extract of 2.0 mg/ml caused remarkable cell death and therefore the amount of p53 was not possible to define. The parsley extract had no effect on the amount of p53 in SH-SY5Y cells (Fig. 4d).

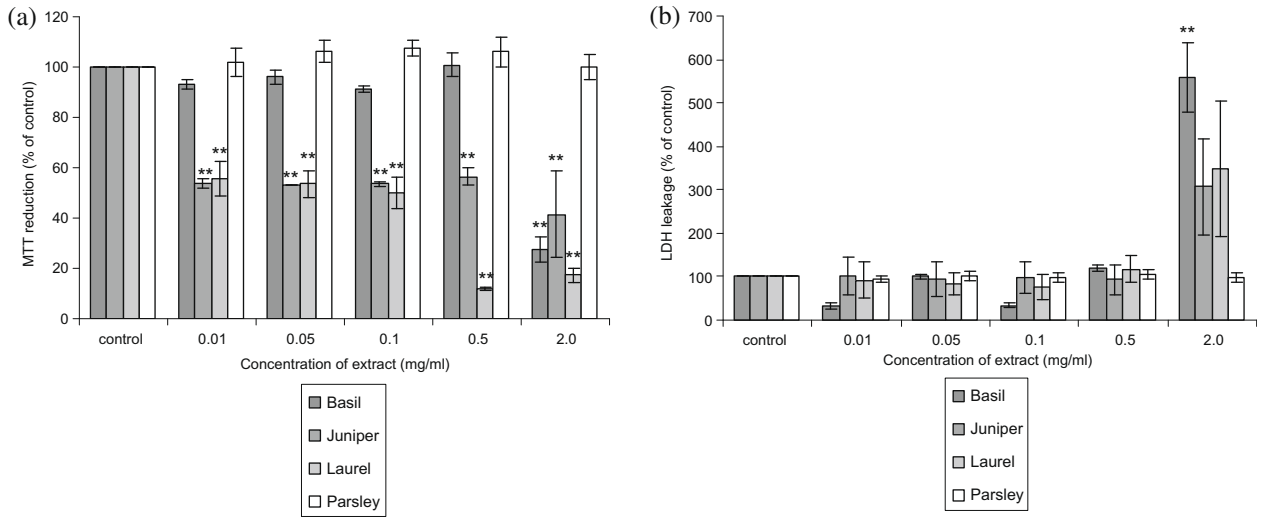

Fig. 3. The viability of SH-SY5Y cells after 12 h treatment with plant extracts from basil, juniper, laurel and parsley. The absorbance of MTT reduction was measured at 550 nm and the background at 655 nm was subtracted (a) and the leakage of LDH was determined using fluorescence with an excitation wavelength of 560 nm and an emission wavelength of 590 nm (b). The values of cell viability assays are means ± SEM of three or four independent experiments made with six replicates. Statistical significance was determined by one-way ANOVA, followed by Dunnett's multiple comparison test; $^{**}p < 0.01$.

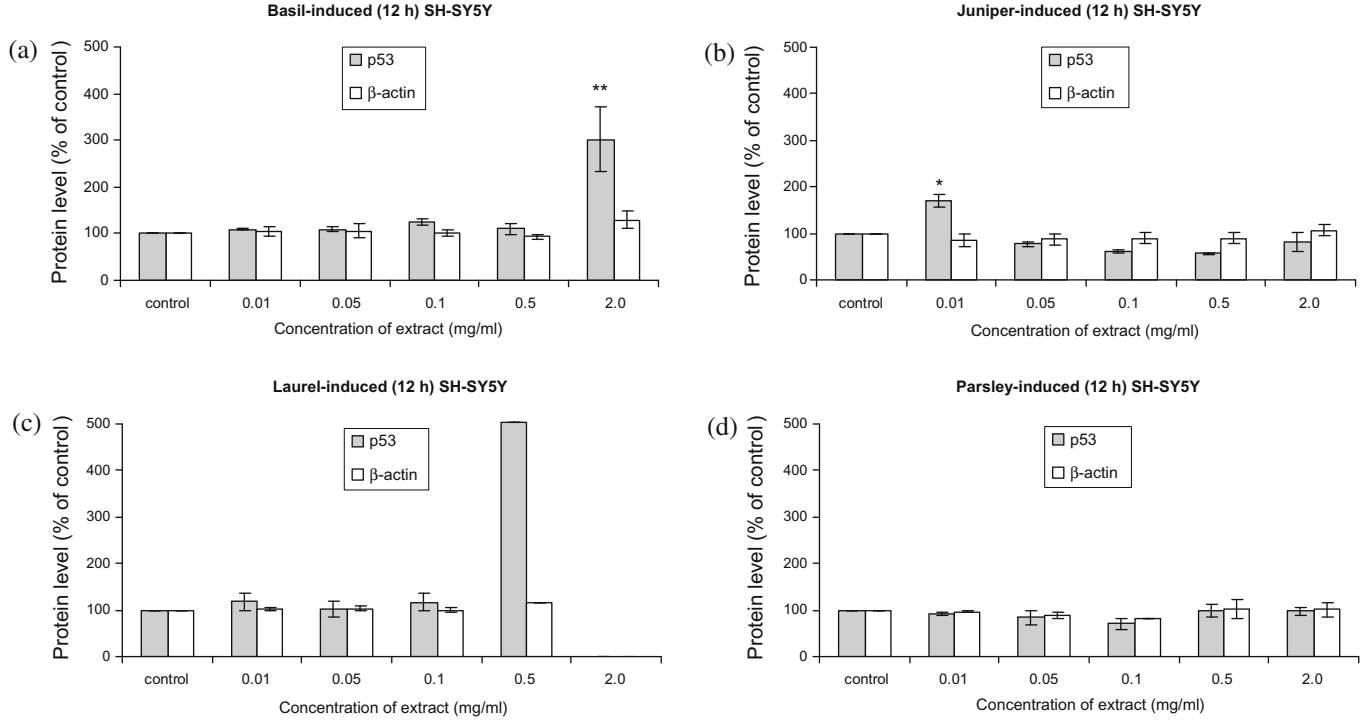

Fig. 4. The effects of basil (a), juniper (b), laurel (c) and parsley (d) extracts on the amounts of p53 protein in SH-SY5Y cells. The cells were treated for 12 h, and the amounts of proteins p53 and β-actin were examined by Western blot analysis. The values are means ± SEM from 2 to 3 independent experiments done in duplicate. Statistical significance was determined by one-way ANOVA, followed by Dunnett's multiple comparison test; $^{*}p < 0.05$ and $^{**}p < 0.01$.

3.5. The effects of juniper extract on the metabolic activity, membrane integrity and amount of p53 in SH-SY5Y cells

The effects of the juniper extract in SH-SY5Y cells were further defined with additional experiments. The cell viability decreased according to the MTT and LDH assays after the 12 h incubation, with concentrations between 1–30 μg/ml. The metabolic activity of cells decreased significantly at all concentrations (Fig. 5a), but the membrane integrity decreased significantly only after the exposure to the extract at concentrations between 20 and 30 μg/ml (Fig. 5b). Concentrations of 10, 12.5 and 15 μg/ml increased the amounts of p53 significantly (Fig. 5c), and the maximal increase of the amount of p53 (200% compared to the control cells) was observed after exposure to 12.5 μg/ml of juniper extract.

4. Discussion

There is growing evidence of the health-promoting effects of plant secondary metabolites, e.g. phenolic compounds. The beneficial connection between cancer and a diet rich in fruits and

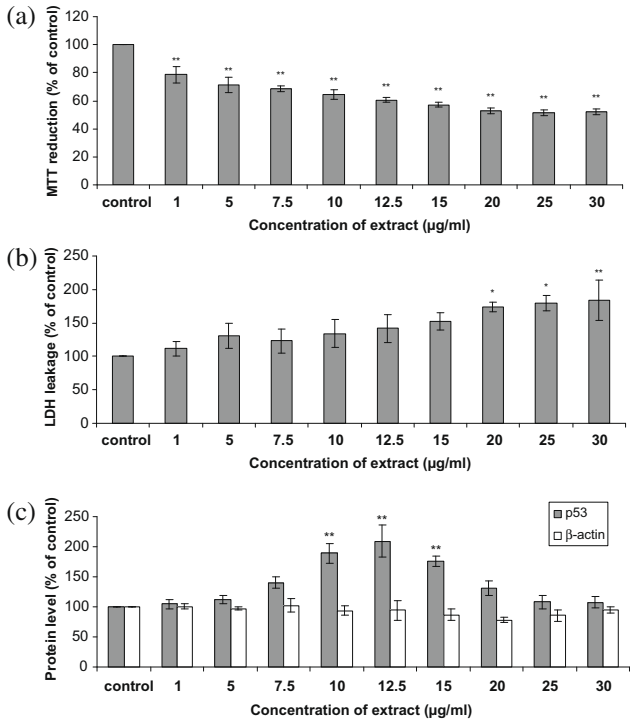

Fig. 5. The effects of juniper extract on the cell viability and the amount of p53 in SH-SY5Y cells. Cells were treated for 12 h, and the cell viability was determined by the MTT reduction in cells (a) and by the LDH leakage from cells (b). The amounts of proteins p53 and β-actin were examined by Western blot analysis (c). The values for the viability assays are means ± SEM from 4 to 5 independent experiments done with four replicates and for the Western blot from four independent experiments done in duplicate. Statistical significance was determined by one-way ANOVA, followed by Dunnett's multiple comparison test; $^{*}p < 0.05$ and $^{**}p < 0.01$.

vegetables is without dispute (Pan et al., 2008). In the present study, the roles of two phenolic compounds, namely curcumin and resveratrol, and four plant extracts from common spices, namely basil, juniper, laurel and parsley, were studied for their possible cytotoxic effects *in vitro*. Furthermore, their effects were also compared to those of AraC, a well known apoptotic compound. In earlier studies, AraC induced the apoptosis in SH-SY5Y cells and reduced the cell viability of SH-SY5Y and CV1-P cells (Maňáková et al., 2003; Puttonen, Lehtonen, Lampela, Männistö, & Raasmaja, 2008). The effects of AraC in this study are in agreement with those previous observations. Curcumin and resveratrol were chosen in our study as pure phenolic reference compounds for their proven effects on the apoptotic signalling and cell viability in several cell cultures and clinical trials (Francy-Guilford & Pezzuto, 2008). SH-SY5Y cells were used, in particular, because, in earlier studies, they have proved a good model to study cell death and have shown a high sensitivity to different toxins (Maňáková et al., 2003; Puttonen et al., 2008).

Curcumin and resveratrol decreased cell viability, measured by the metabolic activity of cells, more than did the reference compound AraC, and this decrease was markedly larger for curcumin than for resveratrol. Also, the toxic effect of curcumin was observed at lower concentrations, whilst that of resveratrol appeared only at higher ones. The sensitivity of cells to curcumin may also have some cell type-specificity since it was higher for SH-SY5Y than for CV1-P cells, whereas no clear difference was seen with resveratrol. Despite the less cytotoxic character of AraC, it was more potent at increasing the amount of p53 in both cell lines than were curcumin and resveratrol. Resveratrol caused a slight increase of the amount of p53 in both cell lines whilst the effect of curcumin occurred only in SH-SY5Y neuroblastoma cells and not in CV1-P fibroblast cells. In the literature, there are reports claiming the increased sensitivity of cancer cells compared to normal cells to the effects of natural phenolic compounds (Ahmad, Feyes, Nieminen, Agarwal, & Mukhtar, 1997; Widodo et al., 2007). Our results with curcumin support these reports. There is also evidence that the cancer cells prefer to take up curcumin more than do normal cells (Kunwar et al., 2008). That might explain the differences in toxicity to curcumin between used cell lines.

Plant extracts from basil, juniper, laurel and parsley have a long history in use as spices and herbal products, but the mechanisms of their possible actions on health are not fully understood. The extracts used in the present study were prepared using aqueous solvent and essential oils were removed by hydrodistillation (Hinneburg et al., 2006). The preparation process enables the elimination of organic solvents from cell experiments, and mimics the traditional and nutritional use of herbs mainly based on aqueous solvents. When the total phenols and antioxidant activity of extracts were defined by Hinneburg et al. (2006), the highest phenol contents were specified in the basil and laurel extracts. The juniper and parsley extracts contained from 12.5% to 32% of the amounts measured in extracts from basil and laurel. However, the juniper extract seemed to be the most efficient in increasing the amount of p53 amongst the tested plant extracts, and it also showed significant cytotoxic effects in SH-SY5Y cells. The concentrations of 10, 12.5 and 15 μg/ml significantly raised the amount of p53 in cells, and the cell viability decreased at all the concentrations. However, the reference compound AraC was more effective at increasing the amount of p53 compared to the control cells, raising it by 300% at the concentration of 50 μM whilst the juniper extract increased the amount of p53 by 200% at comparable concentrations (10, 12.5 and 15 μg/ml). The juniper extract has also shown cytotoxic effects in different cancer cell lines in a literature report (Bayazit, 2004).

The laurel extract decreased the cell viability at all concentrations whilst the basil extract had a significant effect on the cell viability and amount of p53 at the concentration of 2.0 mg/ml in SH-SY5Y cells. The amount of p53 was dramatically increased (500%) after the treatment with 0.5 mg/ml of laurel extract. Furthermore, massive cell death after treatments at concentrations of 0.5 and 2.0 mg/ml of the laurel extract, indicate that an unregulated form of cell death occurred. Also, in the study of Kaileh, Berghe, Boone, Essawi, and Haegeman (2007), a laurel extract had cytotoxic effects against fibrosarcoma cells but not against breast cancer cells.

In summary, the plant extracts from basil, juniper and laurel had an influence on the membrane integrity, metabolic activity and amount of p53 in SH-SY5Y cells. The juniper berry extract had effects at the lowest concentrations amongst other extracts studied found to be the most potent of our extracts. Our results do not clarify whether the effects are associated with apoptotic or necrotic cell death, and more profound experiments are needed to define the results. However, our results, and a long history of use of plants as herbal products, indicate that the plant extracts may be useful as health-promoting food constituents and for drug development. Since the juniper extract potently decreased the cell viability and increased the amount of p53, further studies are in progress to investigate the role of the juniper extract in the cell death.

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Food Chemistry 117 (2009) 412–416

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Effect of particle size upon the extent of extraction of antioxidant power from the plants *Agrimonia eupatoria*, *Salvia* sp. and *Satureja montana*

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ARTICLE INFO

Article history:
Received 15 September 2008
Received in revised form 2 March 2009
Accepted 8 April 2009

Keywords:
Agrimony
Sage
Savoury
ABTS·+
Sieve
Granulometry
Phenolic compounds

ABSTRACT

The dependence of the extent of aqueous extraction of antioxidant compounds on particle size and contact time was studied for three important medicinal plants, that are commonly used in infusions: agrimony, sage and savoury. The effect of extraction time was dependent on the plant considered; however, ca. 5 min can be taken as the minimum period required to assure an acceptable degree of extraction of those compounds. As expected, a smaller particle size led to a higher extraction extents; a typical value of 0.2 mm is accordingly recommended. Chlorogenic acid was the dominant phenolic compound extracted from agrimony, whereas caffeic acid dominated in the case of sage or savoury. A mathematical model based on Fick's law was developed from first principles, and its two parameters were suitably fitted to the experimental data generated – in attempts to predict the evolution of antioxidant capacity extracted during contact time, for each plant and each particle size.

1. Introduction

Agrimony, sage and savoury (common English names for *Agrimonia eupatoria*, *Salvia* sp. and *Satureja montana*, respectively) are plants often used in traditional medicine in Portugal, and which grow in the poor soils of the Mediterranean basin. The former has been claimed to control uric acid, favour the respiratory system, function as an analgesic or a diuretic aid, treat wounds and provide a rich source of antioxidants (Venskutonis, Škėmaitė, & Ragažinskienė, 2007). Besides application as condiment, sage has been used as an anti-diarrhoea vector, and to help in digestion, contribute to heal wounds, play an anti-inflammatory role, fight insomnia and decrease blood pressure; some of these biological activities have been associated with its contents of rosmarinic acid (Petersen & Simmonds, 2003) and L-salvianolic acid (Lu & Foo, 2001), which are two antioxidant compounds. Finally, savoury is also used as condiment, and has been prescribed to combat diarrhoea, help digestion and heal wounds, as well as a disinfectant (Gião et al., 2007). Agrimony exhibits the highest antioxidant capacity and total phenolic content within the aforementioned three plants (Gião et al., 2007, 2008) – probably because of its rich contents of coumarins, flavonoids, tannins and terpenoids (Copland et al., 2003); savoury and sage come second (in this order) in those features.

Upstream of food (or beverage) production or formulation effective, harvesting antioxidants as nutraceuticals from the aforementioned plants depends on maximisation of their extraction. However, it is widely known that the efficiency of solid/liquid extraction processes is affected by critical processing parameters, such as temperature, nature of solvent, structure of solid matrix (mainly particle size) and extraction time (Franco, Pinelo, Sineiro, & Núñez, 2007). This means that each plant matrix/extraction solvent pair behaves in a unique way, so it should be studied as such. On the other hand, both the particle size of the plant matrix and the temperature of the extraction process are easily manipulated physical conditions. In general, a smaller size and a higher temperature facilitate mass transfer (Cacace & Mazza, 2003; Waterman & Sutton, 2003), but quantification of such heuristic rules for each plant source is required before optimisation efforts can be rationally developed.

The major goal of this research study was to model the influences of particle size and time of exposure on the efficiency of aqueous extraction of antioxidant power from solid dried material from given plants. A second goal was to characterise the chemical profiles of extracts in terms of phenolic compounds (which have been often associated with comparatively high antioxidant power).

2. Materials and methods

2.1. Sample preparation

Three plants were considered: agrimony, sage and savoury, all of which were a kind gift from ERVITAL (Castro Daire, Portugal). These plants had been cultivated as organic products, and were supplied in their commercial form of dried leaves: ca. 4 g was then

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doi:10.1016/j.foodchem.2009.04.020

Nomenclature

a	outer specific area	k	mass transfer coefficient based on the liquid side
A_S	area of solid phase	R	ratio of volumes of solid and liquid phases
C_{AL}	antioxidant power of liquid phase	V_L	volume of liquid phase
$C_{AL,0}$	initial antioxidant power of liquid phase	V_S	volume of solid phase
C^*_{AS}	antioxidant power of solid phase, expressed as equilibrium value in the liquid phase		

crushed (using a coffee mill) for 1 min, so as to obtain the corresponding powder. This powder was consecutively passed through bolters of 0.2, 0.25 and 0.3 mm mesh sizes (Haver and Boecker, Oeld, Germany), and the four different fractions thus collected were duly weighed.

Each fraction (ca. 1 g) was finally contacted, under uniform stirring, with 110 ml of boiling distilled water – so as to mimick ready-to-drink infusion preparations; samples were collected every minute up to 10 min, and an extra two samples by 12 and 15 min. All those samples were kept in ice, so as to quench the extraction process prior to analysis. Samples were assayed for their total antioxidant capacity, as described below. All experiments were run in duplicate.

For chromatographic analyses, samples were taken of aqueous infusions after 5 min, and were filtered through a 0.45 μm filter before injection.

2.2. Antioxidant capacity assessment

Determination of the antioxidant capacity was as detailed previously by Gião et al. (2007). The $ABTS^{\bullet+}$ stock solution was prepared via addition, at 1:1 (v/v), of 7 mM ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic) acid) diammonium salt (Sigma–Aldrich, St. Louis, MO, USA) to a solution of 2.45 mM potassium persulphate (Merck, Damstadt, Germany); the developing reaction took place in the dark, for 16 h. In order to obtain an absorbance of 0.700 ± 0.020 at 734 nm, measured with an UV 1203 spectrophotometer (Shimadzu, Tokyo, Japan), the aforementioned stock solution was diluted in as much ultra-pure water as necessary. A 10 μl-aliquot of the sample was assayed for inhibition percentage (between 20% and 80%, so as to guarantee a linear response of the analytical method), after 6 min of reaction with 1 ml of diluted $ABTS^{\bullet+}$ solution; triplicates of each sample were averaged to generate each datum point (which implies a total of six replicates per plant). The final result was expressed as equivalent concentration of ascorbic acid (in $g\,l^{-1}$), using a calibration curve previously prepared with such a reference compound.

2.3. Phenolic compound profiling

The chromatographic system consisted of a Prostar 210 LC pump (Varian, Walnut Creek, CA, USA), coupled with a 1200 triple quadrupole mass spectrometer (Varian) with electrospray ionisation (ESI) in both positive and negative modes. A 5 μm C_{18} column (4.6 mm × 100 mm, Merck) was used for separation, at a flow rate of eluant of 0.4 ml min^{-1}. The LC–MS/MS method was based on that followed by Politi, Rodrigues, Gião, Pintado, and Castro (2008). Chromatographic separation was performed within 33 min, using the following gradient pattern of eluant A (water with 0.1% (v/v) formic acid) and eluant B (methanol with 0.1% (v/v) formic acid): from 0 min with 90% A, to 12.05 min with 78% A, to 22.05 min with 50% A, to 27.05 min with 95% A, and to 30 min with 95% A. ESI–MS/MS detection used a capillary voltage of 55 V; for MS/MS fragmentation, Ar was used (under 1.20 mtorr, with a collision energy of 15 V). An in-house LC–MS/MS library was created in advance, by injecting 33 chromatographic standards of phenolic compounds, under similar analytical conditions. Identification of the phenolic compounds in the samples was by direct injection and comparison with the spectra of the in-house library; for simplicity, the results of quantification were presented as percent abundance.

2.4. Data treatment

Starting from mechanistic modelling based on Fick's law (Çengel, 2007), the balance of the antioxidant concentration throughout contact time is expected to be given by

$$V_L \frac{dC_{AL}}{dt} = kA_S(C^*_{AS} - C_{AL}) \qquad (1)$$
$$t = 0, \quad C_{AL} = C_{AL,0}$$

where V_L is the volume of the liquid phase, C_{AL} is the concentration of antioxidants in the liquid phase, t is the contact time, k is the mass transfer coefficient based on the liquid phase, A_S is the area of the solid phase, C^*_{AS} is the solubility of antioxidants in the liquid phase, and $C_{AL,0}$ is the initial value of C_{AL}. Defining a as the specific area of the powder, calculated as

$$a = \frac{A_S}{V_S} = \frac{6}{d} \qquad (2)$$

where d is the average particle diameter, and defining R as the volume ratio of the solid to the liquid phases, namely

$$R = \frac{V_S}{V_L} \qquad (3)$$

then Eq. (1) can be rewritten as

$$\frac{dC_{AL}}{dt} = kaR(C^*_{AS} - C_{AL}) \qquad (4)$$

Assuming that there is an excess of solute in the solid phase, then C^*_{AS} can be taken as essentially constant; consequently, integration of Eq. (4) from the initial condition set forth in Eq. (1) leads finally to

$$C_{AL}(t) = C^*_{AS}(1 - e^{-kaRt}) \qquad (5)$$

where (kaR) and C^*_{AS} accordingly become the only two adjustable parameters.

2.5. Statistical analyses

Non-parametric tests were applied to each set of experimental data, owing to their intrinsic heteroschedasticity. Friedman and Wilcoxon tests were thus chosen to check whether time influenced the observed results. Kruskal–Wallis tests were applied to check whether, at each time, plant source and particle size influenced the results produced. Mann–Whitney tests were, in turn, applied to unfold possible differences between plant source and particle size pairs. Principal component analysis for categorical data (PCA) was used to ascertain differences between fractional mass distributions. Finally, Tukey's tests were considered to quantify dif-

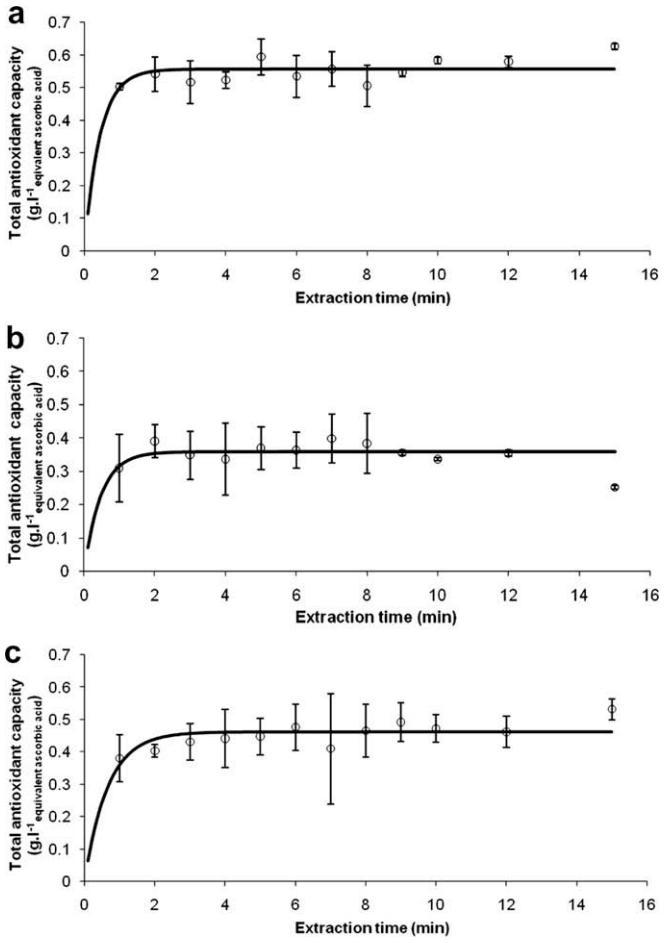

Fig. 1. Total antioxidant capacity, experimentally obtained (○, mean ± standard error) and theoretically predicted (—), of (a) agrimony, (b) sage and (c) savoury, as a function of extraction time, for particle sizes between 0.2 and 0.25 mm.

ferences between plants. All tests were used at a significance level of 0.05. The predictive model denoted as Eq. (5) was fitted by non-linear regression analysis to experimental data points, using the Levenberg–Marquardt estimation method. All statistical tests were performed using SPSS, v. 16.0.0 (Chicago, IL, USA).

3. Results and discussion

The total antioxidant capacities of the infusions obtained from the three plants, for various contact times and at particle sizes between 0.2 and 0.25 mm (for the sake of illustration), are depicted in Fig. 1. As expected (Waterman & Sutton, 2003), the antioxidant power increased with increasing extraction time and decreasing particle size; this means that an increase in the time and in the surface area available for molecular transport contribute to a more extensive mass transfer of solutes between phases – according to the general principles underlying Fick's law.

Non-linear regression analysis was sequentially applied to fit Eq. (5) to the data pertaining to each plant and to each particle size range; typical curves are depicted also in Fig. 1, whereas estimates of the associated model parameters (coupled with relevant statistical information) are comprehensively tabulated in Table 1.

The correlation coefficient between parameters C^*_{AS} and kaR was low, so the parameter estimates were relatively independent of each other – and thus no further uncoupling was required for efficient estimation. Inspection of Table 1, reveals that the range of equilibrium antioxidant power in the liquid phase is 0.3–0.7 g l^{-1} of equivalent ascorbic acid min^{-1}, whereas the characteristic time scale for mass transfer ranges from 0.14 to 0.33 min; extraction was accordingly efficient and fast. The maximum rate of extraction, observed when $t = 0$, ranged in turn from 0.3 to 1.6 g l^{-1} of equivalent ascorbic acid min^{-1}, and increased when the particle size decreased; it was also higher for agrimony and savoury than for sage. On the other hand, the likelihood associated with the model initially postulated and later fitted is high – as concluded from visual inspection of the goodness of fit illustrated in Fig. 1.

Following statistical analysis of our data, several conclusions can be drawn. When considering all three plants together, Friedman's test indicated that extraction time influenced total antioxidant capacity. On the other hand, Wilcoxon's test allowed definition of six time groups, the first group including only 1 min, the second only 2 min, the third, 3 and 4 min, the fourth, 5 until 9 min, the fifth, 10 and 12 min, and the sixth only 15 min. The initial minutes are thus particularly important regarding extraction – as extraction rate slows down considerably between 5 and 10 min (Fig. 1). These results are consistent with the choice of 5 min for extraction time, as proposed elsewhere (Gião et al., 2007, 2008).

The total antioxidant capacity extracted in the first 2 min, for each of the three plants studied, can be seen in Fig. 2 for the various particle size ranges. For particle sizes below 0.2 mm, agrimony showed significantly higher total antioxidant capacity; this difference decreased in magnitude with increasing particle size, and essentially vanished for particles larger than 0.3 mm. A strong decrease (ca. 0.1 g l^{-1} of equivalent ascorbic acid) of the total antioxidant capacity can also be observed as the particle size increases (see Fig. 2a–d). Upon calculation of average values among the different particle size ranges, a value similar to that obtained in previous studies (Gião et al., 2007) could be obtained.

In terms of particle size, Kruskal–Wallis' test unfolded differences at all extraction times. In an attempt to discriminate which sizes led to better performance at each extraction time, Mann–Whitney's test was applied; no influence of particle size was ob-

Table 1
Parameter values (estimate ± standard deviation) entertained by the mathematical model labelled as Eq. (5), for each plant and granulometry.

Plant	Granulometry (diameter: x)	C^*_{AS} (g l^{-1} equivalent ascorbic acid)	kaR (min^{-1})	Initial rate, $C^*_{AS} \cdot kaR$ (g l^{-1} equivalent ascorbic acid min^{-1})
Agrimony	$x < 0.2$ mm	0.694 ± 0.007	2.27 ± 0.31	1.58
	0.2 < x < 0.25 mm	0.557 ± 0.007	2.27 ± 0.38	1.26
	0.25 < x < 0.3 mm	0.582 ± 0.009	1.30 ± 0.17	0.76
	$x > 0.3$ mm	0.340 ± 0.010	0.84 ± 0.15	0.28
Sage	$x < 0.2$ mm	0.404 ± 0.009	2.56 ± 0.95	1.03
	0.2 < x < 0.25 mm	0.360 ± 0.010	2.13 ± 0.73	0.77
	0.25 < x < 0.3 mm	0.372 ± 0.013	1.51 ± 0.48	0.56
	$x > 0.3$ mm	0.388 ± 0.009	0.68 ± 0.09	0.26
Savoury	$x < 0.2$ mm	0.515 ± 0.008	2.97 ± 0.97	1.53
	0.2 < x < 0.25 mm	0.461 ± 0.010	1.51 ± 0.30	0.70
	0.25 < x < 0.3 mm	0.342 ± 0.008	2.37 ± 0.83	0.81
	$x > 0.3$ mm	0.301 ± 0.014	0.99 ± 0.30	0.30

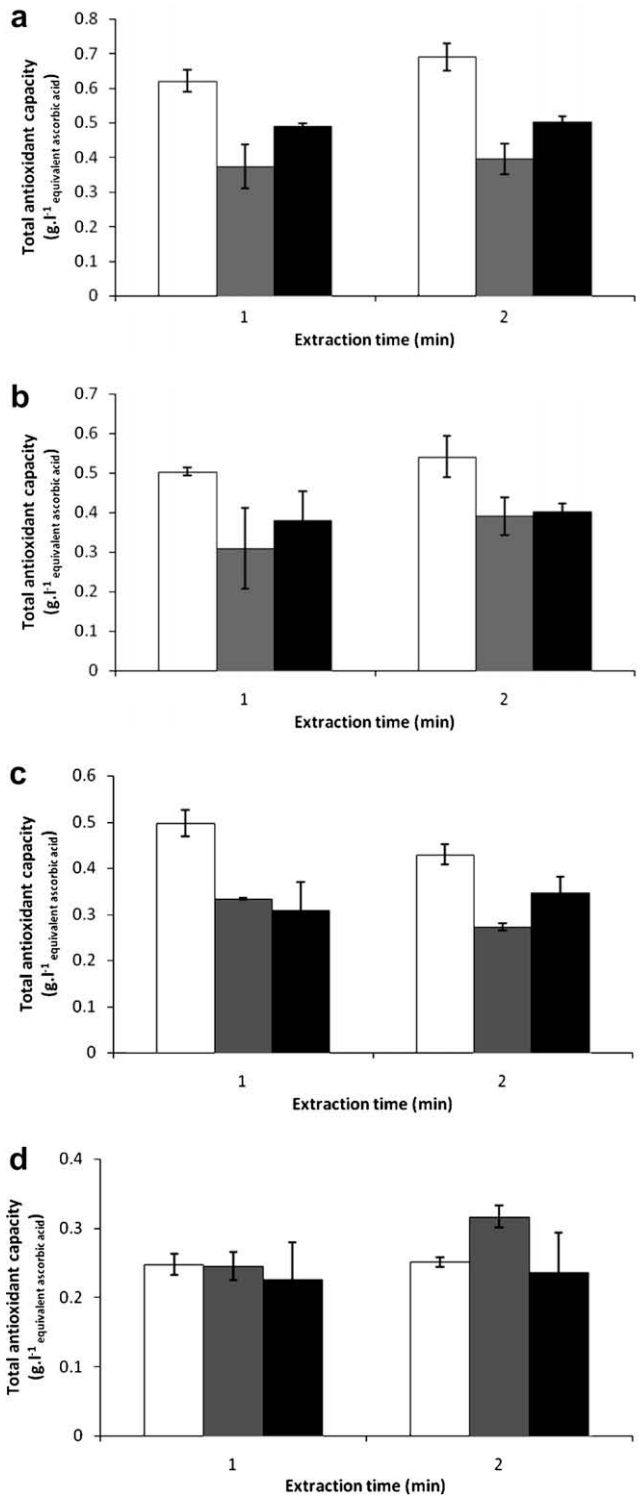

Fig. 2. Total antioxidant capacity, experimentally obtained by 1 and 2 min of extraction, of (□) agrimony, (▩) sage and (■) savoury, for particle sizes (a) below 0.2 mm, (b) between 0.2 and 0.25 mm, (c) between 0.25 and 0.3 mm, and (d) above 0.3 mm.

served only at 2 min (and this justifies the choice below of 2 min-extracted samples, for phenolic compound profiling). For 1 and 4 min, three different groups could in addition be pinpointed; one related to sizes below 0.2 mm, a second was associated with intermediate sizes (between 0.2 and 0.3 mm), and a third encompassed sizes above 0.3 mm; for 3 min, and from 5 to 15 min, only sizes above 0.3 mm appeared to be statistically different from the others.

Table 2
Fractional concentration (%) of phenolic compounds, resolved by HPLC and identified by MS/MS, for each plant.

Phenolic compound	M-1	Plants		
		Agrimony	Sage	Savoury
Protocatechuic acid	153–109	1.59	3.95	0.84
Coumaric acid	163–119	1.80	19.62	1.74
Gallic acid	169–125	3.98	0.17	–
Caffeic acid	179–135	–	64.96	72.94
Ferulic acid	193–134	–	–	–
Naringenin	271–151	–	–	1.86
Quercetin	301–151	–	–	–
Isorhamnetin	315–300	–	0.19	–
Chlorogenic acid	353–191	91.30	10.78	2.26
Prunin	433–271	–	0.13	–
Isoorientin	447–327	–	–	–
Quercitrin	447–301	1.32	–	–
Rutin	609–301	–	0.19	20.36
Total		100	100	100

Note: –, not detected.

Setting a given extraction time, a clear trend of particle size as a function of fractional mass distribution is apparent. PCA was accordingly applied, and indicated that a total variance of 79.9% was associated with the three major groups: one accounted for particles below 0.2 mm in diameter, a second was characterised by particles between 0.2 and 0.3 mm, and another included particles above 0.3 mm. These three groups could be pinpointed for all three plants – and a similar performance was associated with each plant, concerning the mass distribution.

When plants were considered separately, Kruskal–Wallis' test indicated differences arising throughout extraction time: agrimony appeared as different from either sage or savoury at all times, whereas the latter two were similar throughout extraction time. Meanwhile, Friedman's test unfolded significant differences between extraction times; this is why Wilcoxon's test was applied, so as to understand which times accounted for that difference for each plant separately. In the case of agrimony, the effect of extraction time allowed identification of three major groups: a first one associated with 1 and 2 min, a second with 3 to 9 min, and a third with 10 to 15 min. For sage, only 1 min stood out, and both 1 and 15 min appeared different in the case of savoury.

The phenolic compositions of the extracts, by 2 min of extraction and irrespective of particle size range, as obtained by LC–MS/MS, are depicted in Table 2. The identification of such compounds was achieved by comparison with chromatographic grade standards, injected previously using the same separation and detection conditions. Our in-house library of phenols contains 33 compounds – 13 of which could be found and identified in the extracts (Table 2); chlorogenic and caffeic acids were the most abundant, in agrimony and sage/savoury, respectively. Note that the majority of phenolic compounds are not highly soluble in water at room temperature, yet they were found in our extracts – because high temperature aqueous extraction was employed. The aforementioned compounds were already found in aqueous extracts of plants such as green tea (Bastos et al., 2007), yerba maté (Bastos et al., 2007) and *Mentha cervina* (Politi et al., 2008).

4. Conclusions

In general, statistically significant differences exist, in terms of total antioxidant capacity, among the three plants at each specific particle size range. Regarding extraction of antioxidants from the powdered plant material via boiling water, an exposure period of

5 min appears sufficient to assure that most antioxidant power is recovered. On the other hand, an indicative particle size of 0.2 mm, which is the smallest obtained via conventional coffee mill grinding, is sufficient to assure acceptable rates of extraction. The extraction phenomenon follows the typical asymptotic exponential behaviour predicted by Fick's law, with initial rates of extraction in the range of 0.3–1.6 g l^{-1} of equivalent ascorbic acid min^{-1}.

The data generated and the model proposed are innovative, and relevant to rational attempts to predict the antioxidant response of plant extracts, and how the degree of division of the plant material affects it.

Acknowledgements

Partial funding for this research work was via a PhD fellowship (Ref. SFRH/BD/19601/2004), granted to author M.S.G. and administered by Fundação para a Ciência e a Tecnologia (Portugal) and a Project Grant (EXTRAVIDA, Ref. 13-05-04-FDR-00020), scientifically coordinated by author F.X.M. and administered by Agência de Inovação (Portugal).

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Food Chemistry 117 (2009) 417–425

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Rheological properties of aqueous blends of high purity barley β-glucan with high purity commercial food gums

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ARTICLE INFO

Article history:
Received 28 February 2008
Received in revised form 12 March 2009
Accepted 13 April 2009

Keywords:
β-Glucan
Barley
Synergism
Flow behaviour
Viscosity
Thixotropy
Viscoelastic

ABSTRACT

Rheological properties such as flow behaviour, viscosity, viscoelasticity, and thixotropy of solutions of β-glucan purified from barley fibre concentrate and twelve commonly used food gums, alone and in combinations, were characterised using an oscillatory rheometer. Pure gums and gum combinations were evaluated at 0.5% and 0.75% (w/w) total gum concentration in aqueous medium, whereas the β-glucan/gum ratios were kept at 90/10 or 80/20 (w/w). Viscosity synergism was observed for β-glucan solutions in combination with xanthan, *iota*-carageenan, and carboxymethyl cellulose. However, barley β-glucan blends with *lambda*-carageenan, Konjac, high- and low-methoxyl pectin, microcrystalline cellulose, alginate, and gum arabic showed marked lowering of the viscosity compared to β-glucan alone. In addition, β-glucan/xanthan gum blends demonstrated improved shear tolerance compared to xanthan dispersions alone, and soft gel transformation. Non-thixotropic behaviour was observed for 0.5 and 0.75% (w/w) β-glucan dispersions and its gum combinations. None of the gum combinations studied demonstrated thixotropy.

1. Introduction

β-Glucan, a soluble dietary fibre component present in the cell walls of barley and oat endosperm, possesses physiological functionalities, which confer a number of health benefits, including the reduction of heart disease risk as indicated in the health claims approved by the Food and Drug Administration of USA for oats (FDA, 1997) and barley (FDA, 2005). In spite of this, barley remains an underutilised crop. Only 5% of barley produced in Canada is currently being utilised for human consumption. There is potential to incorporate β-glucan into beverages and other food products thereby classifying β-glucan as an important functional food ingredient.

Due to functionality and cost considerations, blends of food gums are often used in food formulations (Le Gloahec, 1951; Nnanna & Dawkins, 1996; Schorsch, Garnier, & Doublier, 1997; Tako, Qi, Yoza, & Toyama, 1998; Casas, Mohedano, & Garcia-Ochoa, 2000; Hernandez, Dolz, Dolz, Delegido, & Pellicer, 2001). An important parameter that dictates the acceptability of gum blends in food and beverages is the stability of the blends throughout the product shelf life.

Studies directed towards understanding how barley β-glucan interacts with other food gums and its applications in common foods and beverages are limited. Factors such as the concentration and the type of gum (positively or negatively charged) may have a profound effect on the physicochemical properties of β-glucan in aqueous media (Bansema, 2000). Interactions between gums modify the rheological properties of gum mixtures and are important for new product development as well as improving the quality of the existing food products. For instance, the addition of *kappa*-carageenan to locust bean gum produces highly stable thermoreversible gels with important synergistic effects (Tako, Qi, Yoza, & Toyama, 1998). A mixture of gum arabic and carrageenan as an ice cream stabiliser has been patented (Le Gloahec, 1951) since it functions to retard both ice crystal formation and growth. The synergistic effect on viscosity due to blending two polysaccharides is the most studied interaction. It has much significance to the industry and it is quite an inexpensive method of manipulating rheology and texture of food products. Hence, establishing the fundamental rheological properties of gum blends and understanding the interactions of barley β-glucan with other food gums are of great importance.

This study was designed to provide insight into physical and functional properties of β-glucan in aqueous systems in combination with other food gums. The main objective of the present study was to investigate the rheological properties of aqueous dispersions of barley β-glucan (BBG) and binary gum blends consisting of BBG and commonly used food gums, namely sodium alginate

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doi:10.1016/j.foodchem.2009.04.027

(ALG), carageenan (CAR) (*kappa*, *lambda*, and *iota*), carboxymethyl cellulose (CMC), gum arabic (GAR), guar gum (GUA), high-methoxyl pectin (HMP), Konjac gum (KOG), locust bean gum (LBG), low-methoxyl pectin (LMP), microcrystalline cellulose (MCC), and xanthan (XAN). Various rheological evaluations were performed, with a focus on flow behaviour, viscosity, viscoelasticity, and thixotropy of pure gums as well as blended gum systems.

2. Materials and methods

2.1. Materials

A barley β-glucan concentrate (60–65%, w/w, β-glucan) was obtained from Cevena Bioproducts Inc. (Edmonton, AB). XAN was provided by ADM Inc. (Decatur, IL), whereas CMC, GAR, GUA, HMP, LBG, and LMP were from TIC Gums Inc. (Belcamp, MD). ALG, CAR, KOG, and MCC were procured from FMC BioPolymer (Princeton NJ). Sodium carbonate, citric acid, and hydrochloric acid were procured from BDH Inc. (Toronto, ON) and Fisher Scientific Co. (Nepean, ON), respectively. Ethanol and Termamyl 120 LN, a thermostable α-amylase (E.C. 3.2.1.1) of *Bacillus licheniformis*, were procured from Commercial Alcohols Inc. (Brampton, ON) and Novozyme North America Inc. (Toronto, ON), respectively.

2.2. Extraction and purification of BBG from barley β-glucan concentrate

β-Glucan concentrate (50 g) was dissolved in 3.75 L deionized water at pH 10 by mixing the slurry continuously for 30 min at 50 °C. After adjusting the slurry pH to 6.5, thermostable α-amylase was stirred in at 90 °C for 1 h. The thermostable α-amylase was inactivated by adjusting the slurry pH to 4 and holding for 10 min. The slurry was then held for 10 min at pH 5.5 at room temperature to precipitate proteins followed by centrifugation at 13,000×*g* for 10 min. The supernatant was neutralised to pH 7 and then β-glucan was recovered by alcohol precipitation (i.e. adding absolute ethanol to the supernatant in order to bring the final ethanol concentration to 50% v/v and storing overnight at 4 °C) and centrifugation. The recovered purified β-glucan was washed once with 250 mL of absolute ethanol and then dried overnight at 40 °C.

2.3. Chemical analyses

Contents of moisture, BBG, starch, protein ($N \times 6.25$), and lipid of dried samples were determined in duplicate according to the methods of McCleary and Glennie-Holmes (1985), Megazyme assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland), Holm, Bjorck, Drews, and Asp (1986), FP-428 Nitrogen Determinator (Leco Corp., St. Joseph, MI), and AACC (1982), respectively.

2.4. Determination of viscosity and thixotropy

Dispersions of BBG alone and its blends with common food gums (e.g. XAN, KOG, MCC, CAR, ALG, HMP, LMP, GUA, LBG, CMC, and GAR) were prepared at a total gum concentration of 0.5% and 0.75% (w/w) in ratios of 80/20 and 90/10 (w/w). For all binary blends, BBG was the major gum ingredient used. All gum dispersions were prepared separately, heated at 90 °C for 1 h and were allowed to cool down to room temperature. The gum blend dispersions were prepared by weighing and mixing at 80/20 and 90/10 (w/w) ratios of gum dispersions that were prepared individually. The samples were then mixed for 20 min at room temperature to ensure uniformity.

Viscosity tests were performed for BBG and binary blend dispersions. Viscosity was determined at consecutive fixed shear rates of 1.29–129 s^{-1} using a Paar Physica UDS 200 rheometer (Glenn, VA). The viscometer was equipped with a Peltier heating system that controlled the sample temperature. All viscosity tests were performed at 20 °C using DG 27 cup and bob geometry with a 7 ± 0.005 g sample. Shear rate was reported in s^{-1} after multiplying rpm by a conversion factor of 1.29 s^{-1} as specified by the manufacturer.

Thixotropy tests were also performed on both BBG and binary blend dispersions using DG 27 cup and bob geometry with a 7 ± 0.005 g sample at 20 °C. These tests were performed at a series of fixed shear rates that were consecutively increased from 1.29 to 3870 s^{-1} and then immediately decreased to the original shear rate of 1.29 s^{-1}. All analyses on gum blends were performed at least in duplicate.

2.5. Determination of viscous synergism index (I_v)

When blended gum dispersion exhibited greater viscosity than the sum of the viscosities of the individual gum dispersions determined separately, the situation was considered as synergism. These interactions were quantified using a "viscous synergism index", I_v, that was defined as:

$$I_v = \frac{\eta_{i+j}}{\eta_i + \eta_j} \quad (1)$$

where i and j represent the two gums forming the mixed system, $i + j$. The aqueous dispersions of the systems i, j, and $i + j$ were prepared at the same total gum concentration, i.e., $c_i = c_j = c_{i+j}$ (Hernandez et al., 2001). According to Eq. (1), I_v always has a positive value. If $0 < I_v < 0.5$, the viscosity of the mixed system will be less than the sum of the viscosities of its two component gums and also less than both of them individually, the situation is described as antagonistic interaction. However, if $I_v = 0.5$ and both gums are of equal viscosity (when considered separately at identical concentrations), so that $\eta_{i+j} = \eta_i = \eta_j$ then the situation is described as no interaction. On the other hand, if $0.5 < I_v < 1$, synergism occurs, provided η_{i+j} is more than η_i and η_j individually. If $I_v > 1$, indicating that the viscosity of the mixed system is greater than the sum of the viscosities of the two individual systems i.e., $\eta_{i+j} > \eta_i + \eta_j$, then synergism has also occurred (Hernandez et al., 2001; Pellicer et al., 2000). For economical and practical reasons, blending of two pure gums together to increase the viscosity is not necessary when the viscosity of one of the pure gums, η_i or η_j, is greater than η_{i+j} at identical gum concentrations (Hernandez et al., 2001).

2.6. Determination of viscoelastic properties of gum blends

All dispersions of individual and blended gums were prepared using a similar procedure to the one described under sample preparation for viscosity and thixotropy determinations. Since the viscoelastic properties are strongly dependent on time and temperature, all systems were allowed to equilibrate for 15 min at ambient temperature. Storage modulus (G') and loss modulus (G'') were obtained at 20 °C using a 7 ± 0.005 g sample placed in a DG 27 cup and bob geometry of a Paar Physica UDS 200 rheometer. The rheometer was set in amplitude sweep controlled shear displacement (CSD) mode with a constant frequency of 1 Hz and controlled strain of 0.25–20% and 0.75–120% for 0.5% and 0.75% total gum concentration, respectively.

3. Statistical analysis

Purification experiments were performed in duplicate. All chemical analyses were performed in duplicate for each β-glucan sample. Rheological measurements were performed for each of

the purified samples in duplicate. Viscosity tests for the gums or gum blends were also performed in duplicate for each of the purified samples. Means were reported for all parameters measured. Regression analysis used to determine the Power Law model parameters.

4. Results and discussion

4.1. Recovery and composition of purified BBG

Recovery is defined as the ratio between the amount of BBG in the purified sample and the amount of BBG present in the barley β-glucan concentrate. The recovery and purity of laboratory purified BBG were 82% and 94.7% (w/w, dry weight), respectively. Moisture, starch, and protein contents were 3.8%, 0.9%, and 1.7% (w/w), respectively. Lipid content was 0% (w/w) in the barley β-glucan concentrate used and hence it was assumed that the purified barley β-glucan contains no lipids.

4.2. Viscosity of gum blends

The viscosity of 0.5% and 0.75% (w/w) pure gums at 20 °C determined over a shear rate range of 1.29–129 s^{-1} is presented in Table 1. GAR, HMP, LMP, and MCC showed low viscosity at both concentrations of 0.5% and 0.75% (w/w). As expected, the viscosity of all gum dispersions increased when the concentration was increased from 0.5 to 0.75% (w/w). The flow curves of individual gums and blends showed shear thinning behaviour, while yield stress was observed only in dispersions containing XAN, CAR, and ALG. The concentration and shear rate effects on rheological properties were dependent upon the type of food gum used. The effect of concentration (0.5% and 0.75%, w/w) on viscosity enhancement was more pronounced in BBG, *iota*-CAR, and *kappa*-CAR dispersions as shown in Table 1. For XAN dispersions, however, the viscosity increased from 368 to 481 mPa s at shear rate of 12.9 s^{-1} upon increasing the gum concentration from 0.5% to 0.75% (w/w). This may be attributed to the near saturation of XAN dispersions at the concentrations tested.

In fluid flow behaviour studies, the Power Law model describes the pseudoplastic behaviour of gums (Marcotte, Hoshahili, & Ramaswamy, 2001). The following equation represents the Power Law model:

$$\tau = c\gamma^n \quad (2)$$

where τ is the shear stress (N/m^2), γ is the shear rate (s^{-1}), c is the consistency coefficient and n is the flow behaviour index or Power Law index. The flow behaviour index and consistency coefficient of 0.5% and 0.75% (w/w) pure gum dispersions are shown in Table 2. At 0.5% (w/w) concentration, ALG, *iota*-CAR, GAR, HMP, and LMP were almost Newtonian. Moreover, at 0.75% (w/w) gum concentration, HMP and LMP continued to behave almost like Newtonian with $n \sim 0.99$, over a shear rate range of 1.29–129 s^{-1}. BBG was highly pseudoplastic with a flow behaviour index of 0.74 and 0.59 at 0.5% and 0.75% (w/w) concentrations, respectively. In comparison to other gums at 0.5% (w/w) concentration, XAN demonstrated high pseudoplasticity with $n = 0.2$, followed by GUA with $n = 0.38$. In terms of the flow behaviour index, BBG at 0.5% (w/w) was comparable to CMC, KOG, and LBG.

Table 1
Viscosity (mPa s) of 0.5% and 0.75% (w/w) pure gum dispersions at shear rates of 1.29–129 s^{-1} and a temperature of 20 °C.

Pure gums systems	Shear rate (1/s)					
	1.29	6.46	12.9	25.8	64.6	129
0.5% (w/w) Gum concentration						
ALG	24	20	18	17	16	78
BBG	287	237	203	166	118	87
CMC	378	283	235	189	135	101
GAR	1	1	1	1	1	1
GUA	1193	667	466	310	172	108
HMP	6	4.2	3.9	3.8	3.7	3.7
i-CAR	31	30	30	29	28	26
KOG	550	455	389	316	221	159
k-CAR	71	59	51	43	32	25
LMP	4	4	4	4	3	3
LBG	394	360	327	279	200	144
l-CAR	196	166	146	123	92	70
MCC	12	7	6	6	5	4
XAN	2317	652	368	209	101	60
0.75% (w/w) Gum concentration						
ALG	91	71	65	59	50	44
BBG	1890	1190	891	640	389	256
CMC	733	522	421	329	225	164
GAR	4	3	2	2	2	2
GUA	3407	1693	1130	721	382	231
HMP	10	10	9	9	9	9
i-CAR	378	300	255	208	148	110
KOG	1720	1270	1020	768	489	326
k-CAR	4043	1340	743	438	207	109
LMP	5.5	5.2	5.1	5.1	5.0	5.1
LBG	1447	1191	994	764	480	315
l-CAR	3317	1030	570	322	158	97
MCC	10	8	7	7	6	5
XAN	2908	834	481	277	132	78

Values are means of duplicate determinations; ALG = alginate; BBG = barley beta-glucan; CMC = carboxymethyl cellulose; GAR = gum Arabic; GUA = Guar gum; HMP = high-methoxyl pectin; *i*-CAR = *iota*-carageenan; KOG = Konjac; *k*-CAR = *kappa*-carageenan; LMP = low-methoxyl pectin; LBG = locust bean gum; *l*-CAR = *lambda*-carageenan; MCC = microcrystalline cellulose; XAN = xanthan.

Table 2
Flow behaviour index (n) and consistency coefficient (c) at 0.5% and 0.75% (w/w) concentration of pure food gum dispersions over a shear rate range of 1.29–129 s^{-1} and a temperature of 20 °C.

Pure gum systems	Flow behaviour index (n)	Consistency coefficient (c)	R^2
0.50% (w/w) Gum concentration			
ALG	0.890	0.024	1.000
BBG	0.740	0.353	0.992
CMC	0.710	0.453	0.995
GAR	1.004	0.001	1.000
GUA	0.380	2.170	0.994
HMP	0.897	0.006	0.996
i-CAR	0.965	0.032	0.999
KOG	0.730	0.690	0.990
k-CAR	0.776	0.083	0.997
LMP	0.991	0.003	1.000
LBG	0.690	0.696	0.992
l-CAR	0.770	0.234	0.994
MCC	0.795	0.011	0.997
XAN	0.200	2.838	0.998
0.75% (w/w) Gum concentration			
ALG	0.840	0.096	0.999
BBG	0.590	2.296	0.995
CMC	0.670	0.893	0.994
GAR	0.825	0.004	0.995
GUA	0.440	4.334	0.989
HMP	0.960	0.010	1.000
i-CAR	0.220	4.150	0.991
KOG	0.680	2.075	0.989
k-CAR	0.230	5.150	0.990
LMP	0.987	0.004	1.000
LBG	0.660	1.772	0.989
l-CAR	0.730	0.460	0.993
MCC	0.840	0.011	1.000
XAN	0.210	3.580	0.999

Values are means of duplicate viscosity determinations; ALG = alginate; BBG = barley beta-glucan; CMC = carboxymethyl cellulose; GAR = gum Arabic; GUA = Guar gum; HMP = high-methoxyl pectin; *i*-CAR = *iota*-carageenan; KOG = Konjac; *k*-CAR = *kappa*-carageenan; LMP = low-methoxyl pectin; LBG = locust bean gum; *l*-CAR = *lambda*-carageenan; MCC = microcrystalline cellulose; XAN = xanthan.

Blending of gums resulted in changes in certain rheological properties such as viscosity, compared to the corresponding values for single components. The viscosities of gum blends having total gum concentrations of 0.5% and 0.75% (w/w), determined at shear rates of 1.29–129 s^{-1} at 20 °C, are presented in Table 3.

At total gum concentrations of 0.5% and 0.75% (w/w), dispersions of BBG blended with CMC and XAN showed marked enhancement in viscosity. Additionally, dispersions of BBG blended with *lambda*-CAR at 0.5% total concentration and BBG blended with *iota*-CAR at 0.75% total concentration exhibited viscosity enhancement. At total gum concentrations of both 0.5% and 0.75%, BBG blended with ALG, GAR, HMP, KOG, LMP, and MCC showed marked lowering of viscosity compared to BBG alone. At the 0.75% concentration, BBG blended with *lambda*-CAR also showed marked lowering of the viscosity.

As shown in Table 1, at a shear rate of 64.6 s^{-1}, 0.5% (w/w) BBG and XAN individually exhibited viscosities of 118 and 101 mPa s, respectively, whereas in Table 3 and 0.5% (w/w) BBG/XAN blended in 80/20 and 90/10 (w/w) ratios demonstrated viscosities of 158 and 174 mPa s, respectively. Thus, the BBG/XAN blend was more

Table 3
Viscosity (mPa s) of 0.5% and 0.75% (w/w) BBG/other gum blend dispersions at shear rates of 1.29–129 s^{-1} and a temperature of 20 °C.

Gum blend		1.29	6.46	12.9	25.8	64.6	129	*n*	c
Shear rate (1/s)									
0.5% (w/w) Gum concentration									
BBG/ALG	80/20	232	192	166	139	102	84	0.74	0.29
	90/10	289	235	201	164	118	87	0.71	0.38
BBG/CMC	80/20	763	493	381	284	182	126	0.58	1.02
	90/10	681	443	345	258	167	116	0.59	0.90
BBG/GAR	80/20	104	95	86	75	59	46	0.81	0.12
	90/10	176	152	134	113	84	64	0.76	0.22
BBG/GUA	80/20	408	308	252	196	131	93	0.65	0.56
	90/10	375	292	242	192	132	95	0.67	0.51
BBG/HMP	80/20	151	134	120	103	79	62	0.87	0.21
	90/10	210	180	158	132	97	73	0.85	0.23
BBG/*i*-CAR	80/20	289	240	206	169	120	88	0.71	0.41
	90/10	314	256	217	175	123	90	0.69	0.43
BBG/KOG	80/20	276	232	200	165	119	88	0.70	0.45
	90/10	272	226	194	159	114	85	0.71	0.44
BBG/*k*-CAR	80/20	219	183	158	130	94	87	0.69	0.38
	90/10	254	203	173	141	100	88	0.72	0.37
BBG/LMP	80/20	144	127	114	98	75	58	0.78	0.18
	90/10	155	136	121	103	79	61	0.77	0.19
BBG/LBG	80/20	304	256	222	184	133	98	0.73	0.40
	90/10	324	264	226	184	130	96	0.71	0.43
BBG/*l*-CAR	80/20	583	407	321	242	156	107	0.67	0.77
	90/10	506	358	285	216	141	99	0.67	0.80
BBG/MCC	80/20	153	120	103	85	63	49	0.81	0.27
	90/10	200	163	140	116	84	64	0.73	0.35
BBG/XAN	80/20	1277	540	378	261	158	108	0.46	1.47
	90/10	1090	531	390	278	174	121	0.51	1.29
Gum blend		1.29	6.46	12.9	25.8	64.6	129	*n*	c
0.75% (w/w) Gum concentration									
BBG/ALG	80/20	1193	788	610	454	290	200	0.58	1.62
	90/10	1413	920	706	519	326	220	0.57	1.95
BBG/CMC	80/20	2607	1480	1074	751	444	290	0.49	3.61
	90/10	2580	1480	1076	752	444	290	0.50	3.58
BBG/GAR	80/20	625	465	377	290	192	135	0.64	0.84
	90/10	1033	709	554	413	262	178	0.60	1.40
BBG/GUA	80/20	1870	1150	857	608	362	234	0.52	2.66
	90/10	1720	1100	830	598	363	239	0.54	2.45
BBG/HMP	80/20	841	603	482	368	242	169	0.62	1.14
	90/10	1243	840	653	486	308	210	0.58	1.73
BBG/*i*-CAR	80/20	2323	1370	1000	697	402	255	0.49	3.36
	90/10	2217	1320	970	681	400	257	0.50	3.16
BBG/KOG	80/20	1733	1140	874	638	394	261	0.56	2.46
	90/10	1840	1180	895	648	397	262	0.54	2.61
BBG/*k*-CAR	80/20	1720	1030	768	550	334	221	0.53	2.37
	90/10	1827	1124	841	601	364	239	0.53	2.57
BBG/LMP	80/20	692	503	404	310	204	143	0.63	0.92
	90/10	1073	736	574	426	270	183	0.58	1.51
BBG/LBG	80/20	1740	1160	891	651	399	262	0.55	2.59
	90/10	1797	1170	890	645	394	259	0.55	2.57
BBG/*l*-CAR	80/20	1327	868	669	492	308	207	0.57	1.84
	90/10	1593	1020	779	566	349	231	0.55	2.24
BBG/MCC	80/20	1017	627	476	348	218	149	0.56	1.36
	90/10	1380	858	647	469	290	195	0.55	1.89
BBG/XAN	80/20	3868	1634	1100	726	408	260	0.40	4.90
	90/10	4643	2049	1386	913	511	324	0.41	5.98

Values are means of duplicate determinations.
n = Flow behaviour index; *c* = consistency coefficient; ALG = alginate; BBG = barley beta-glucan; CMC = carboxymethyl cellulose; GAR = gum Arabic; GUA = Guar gum; HMP = high-methoxyl pectin; *i*-CAR = *iota*-carageenan; KOG = Konjac; *k*-CAR = *kappa*-carageenan; LMP = low methoxyl pectin; LBG = locust bean gum; *l*-CAR = *lambda*-carageenan; MCC = microcrystalline cellulose; XAN = xanthan.

shear tolerant as evident by the higher flow behaviour index value (Table 3) than XAN alone.

The total gum concentration and ratio of gums in a blend affected the rate and the type of interaction (synergistic or antagonistic) as demonstrated by the viscosity measurements. These interactions were quantified using "viscous synergism index", I_v, as defined previously in Eq. (1).

Tables 4 and 5 show the I_v calculated for 0.5% and 0.75% (w/w) BBG/other gum blends, respectively, using the viscosity data determined at a shear rate of 6.46 s^{-1} (to mimic the approximate shear that exists in human mouth) at 20 °C. Synergistic interactions were observed in blends of BBG with CMC or *iota*-CAR under all studied conditions. Synergistic interactions were also observed between BBG and *lambda*-CAR at the 0.5% concentration level, and between BBG and XAN at the 0.75% concentration level, for both 80/20 or 90/10 blend ratios. All other gum blends and showed no interaction or antagonistic interactions.

Synergistic interactions between gums in a blend are governed by hydrogen bonding (Bresolin, Milas, Rinaudo, & Ganter, 1998). Increased intermolecular interactions between hydrocolloid polymers enhances the stability of the molecular entanglements, suppressing phase separation and improving shear tolerance, and the viscosity of the solution.

4.3. Thixotropy of gum blends

Thixotropy can be defined as a decrease in viscosity due to destruction of structure under a constant shear rate or a consecutively increasing shear rate that is fixed for a period of time at selected shear rates followed by the structural network redevelopment when shear is withdrawn (Muller, 1973; Schramn, 1994). The viscosity of non-thixotropic systems does not decrease with time when exposed to constant shear rates. Under consecutively increasing shear rates the viscosity decreases, but regains over time when shear is withdrawn.

In the present study, the thixotropy was examined, using consecutively increasing shear rates in the range of 1.29–3870 s^{-1} for fixed intervals of time and then decreasing it immediately to the original shear rate of 1.29 s^{-1}. Non-thixotropic behaviour was observed for BBG dispersions at 0.5% and 0.75% (w/w) gum concentration. Autio, Myllymaki, and Malkki (1987) also reported a similar behaviour for β-glucan dispersions. Table 6 summarises the thixotropy of pure BBG and gum blend dispersions at 20 °C at the total gum concentrations of 0.5% and 0.75% (w/w). None of the gum blends used in the study demonstrated thixotropy except BBG/MCC. Both 0.5% and 0.75% (w/w) BBG/MCC blends showed inability of viscosity redevelopment at 1.29 s^{-1} after being exposed to high shear (3870 s^{-1}), hence, showing thixotropy. BBG/XAN blended at a ratio of 80/20 (w/w) at 0.5% and 0.75% (w/w) total gum concentrations recovered its original viscosity in 10–15 s. For pure BBG dispersions, the time required for the viscosity to redevelop at 1.29 s^{-1} exceeded 4–6 min. Interestingly, during the thixotropy testing, 80/20 and 90/10 (w/w) BBG/XAN blends demonstrated an unusual increase in viscosity upon immediately decreasing the shear rate from 3870 to 1.29 s^{-1} compared to the original viscosity at the starting shear rate of 1.29 s^{-1}. This shear-induced thickening of the blend dispersion suggested typical "rheopectic" behaviour. Such rheopectic behaviour is possibly

Table 4
Viscous synergism index, I_v, of 0.5% (w/w) BBG/other gum blend dispersions at a shear rate of 6.46 s^{-1} and a temperature of 20 °C.

Gum blend	Viscosity at 6.46 s^{-1}					Interaction
	$\eta(i)$	$\eta(j)$	$\eta(i)+\eta(j)$	$\eta(i+j)$	I_v	
Blend ratio 80/20 (w/w)						
BG/ALG	237	20	257	192	0.75	Antagonism
BBG/CMC	237	283	520	493	0.95	Synergism
BBG/GAR	237	1.1	238.1	95	0.40	Antagonism
BBG/GUA	237	667	904	308	0.34	Antagonism
BBG/HMP	237	4.2	241.2	134	0.56	Antagonism
BBG/*i*-CAR	237	30	267	240	0.90	Synergism
BBG/KOG	237	455	692	232	0.34	Antagonism
BBG/*k*-CAR	237	59	296	183	0.62	Antagonism
BBG/LMP	237	3.5	240.5	127	0.53	Antagonism
BBG/LBG	237	360	597	256	0.43	Antagonism
BBG/*l*-CAR	237	166	403	407	1.01	Synergism
BBG/MCC	237	7	244	120	0.49	Antagonism
BBG/XAN	237	652	889	540	0.61	Antagonism
Blend ratio 90/10 (w/w)						
BBG/ALG	237	20	257	235	0.91	Antagonism
BBG/CMC	237	283	520	443	0.85	Synergism
BBG/GAR	237	1.1	238.1	152	0.64	Antagonism
BBG/GUA	237	667	904	292	0.32	Antagonism
BBG/HMP	237	4.2	241.2	180	0.75	Antagonism
BBG/*i*-CAR	237	30	267	256	0.96	Synergism
BBG/KOG	237	455	692	226	0.33	Antagonism
BBG/*k*-CAR	237	59	296	203	0.69	Antagonism
BBG/LMP	237	3.5	240.5	136	0.57	Antagonism
BBG/LBG	237	360	597	264	0.44	Antagonism
BBG/*l*-CAR	237	166	403	358	0.89	Synergism
BBG/MCC	237	7	244	163	0.67	Antagonism
BBG/XAN	237	652	889	531	0.60	Antagonism

Values are means of duplicate determinations. All viscosity measurements [$\eta(i)$, ($\eta(j)$ and $\eta(i+j)$] were performed at identical total gum concentration (0.5%, w/w). ALG = alginate; BBG = barley beta-glucan; CMC = carboxymethyl cellulose; GAR = gum Arabic; GUA = Guar gum; HMP = high-methoxyl pectin; *i*-CAR = *iota*-carageenan; KOG = Konjac; *k*-CAR = *kappa*-carageenan; LMP = low-methoxyl pectin; LBG = locust bean gum; *l*-CAR = *lambda*-carageenan; MCC = microcrystalline cellulose; XAN = xanthan.

Table 5
Viscous synergism index, I_v, of 0.75% (w/w) BBG/other gum blend dispersions at a shear rate of 6.46 s^{-1} and a temperature of 20 °C.

Gum blend	Viscosity at 6.46 s^{-1}					Interaction
	$\eta(i)$	$\eta(j)$	$\eta(i)+\eta(j)$	$\eta(i+j)$	I_v	
Blend ratio 80/20 (w/w)						
BBG/ALG	1190	71	1261	788	0.62	Antagonism
BBG/CMC	1190	522	1712	1480	0.86	Synergism
BBG/GAR	1190	3	1192	465	0.39	Antagonism
BBG/GUA	1190	1693	2883	1150	0.40	Antagonism
BBG/HMP	1190	10	1199	603	0.50	Antagonism
BBG/*i*-CAR	1190	300	1490	1370	0.92	Synergism
BBG/KOG	1190	1270	2460	1140	0.46	Antagonism
BBG/*k*-CAR	1190	1340	2530	1030	0.41	Antagonism
BBG/LMP	1190	5	1195	503	0.42	Antagonism
BBG/LBG	1190	1191	2381	1160	0.49	No interaction[a]
BBG/*l*-CAR	1190	1030	2220	868	0.39	Antagonism
BBG/MCC	1190	8.1	1198	627	0.52	Antagonism
BBG/XAN	1190	834	2024	1634	0.81	Synergism
Blend ratio 90/10 (w/w)						
BBG/ALG	1190	71	1261	920	0.72	Antagonism
BBG/CMC	1190	522	1712	1480	0.86	Synergism
BBG/GAR	1190	3	1192	709	0.59	Antagonism
BBG/GUA	1190	1693	2883	1100	0.38	Antagonism
BBG/HMP	1190	10	1199	840	0.70	Antagonism
BBG/*i*-CAR	1190	300	1490	1320	0.88	Synergism
BBG/KOG	1190	1270	2460	1180	0.48	Antagonism
BBG/*k*-CAR	1190	1340	2530	1124	0.44	Antagonism
BBG/LMP	1190	5	1195	736	0.61	Antagonism
BBG/LBG	1190	1191	2381	1170	0.49	No interaction[a]
BBG/*l*-CAR	1190	1030	2220	1020	0.46	Antagonism
BBG/MCC	1190	8	1198	858	0.71	Antagonism
BBG/XAN	1190	834	2024	2049	1.01	Synergism

[a] Marginally antagonistic; Values are means of duplicate determinations. All viscosity measurements [$\eta(i)$, $\eta(j)$ and $\eta(i+j)$] were performed at identical total gum concentration (0.75%, w/w); ALG = alginate; BBG = barley beta-glucan; CMC = carboxymethyl cellulose; GAR = gum Arabic; GUA = Guar gum; HMP = high-methoxyl pectin; *i*-CAR = *iota*-carageenan; KOG = Konjac; *k*-CAR = *kappa*-carageenan; LMP = low-methoxyl pectin; LBG = locust bean gum; *l*-CAR = *lambda*-carageenan; MCC = microcrystalline cellulose; XAN = xanthan.

Table 6
Thixotropy of 0.5% and 0.75% (w/w) BBG/other gum blend dispersions during a shear cycle[a] at a temperature of 20 °C.

Gum blend[c]		Onset shear 1.29 (1/s)	Peak shear 3870 (1/s)	Post shear 1.29 (1/s)	Flow behaviour
Viscosity (mPa s)[b]					
0.5% (w/w) Gum concentration					
BBG	–	287	18	284	NT[d]
BBG/ALG	80/20	232	21	224	NT
	90/10	289	20	286	NT
BBG/CMC	80/20	763	22	743	NT
	90/10	681	22	666	NT
BBG/GAR	80/20	104	17	102	NT
	90/10	176	18	169	NT
BBG/GUA	80/20	408	19	402	NT
	90/10	375	20	370	NT
BBG/HMP	80/20	151	19	148	NT
	90/10	210	19	209	NT
BBG/*i*-CAR	80/20	289	20	284	NT
	90/10	314	19	309	NT
BBG/KOG	80/20	276	20	274	NT
	90/10	272	19	266	NT
BBG/*k*-CAR	80/20	219	19	218	NT
	90/10	254	20	253	NT
BBG/LMP	80/20	144	18	145	NT
	90/10	155	19	157	NT
BBG/LBG	80/20	304	20	299	NT
	90/10	324	20	320	NT
BBG/*l*-CAR	80/20	583	20	581	NT
	90/10	506	19	505	NT
BBG/MCC	80/20	153	16	118	T[e]
	90/10	200	18	182	T
BBG/XAN	80/20	1277	22	1525	R[f]
	90/10	1090	24	1395	R
0.75% (w/w) Gum concentration					
BBG	–	1890	27	1872	NT
BBG/ALG	80/20	1193	30	1159	NT
	90/10	1413	30	1386	NT
BBG/CMC	80/20	2607	34	2609	NT
	90/10	2580	33	2567	NT
BBG/GAR	80/20	625	23	612	NT
	90/10	1033	25	1004	NT
BBG/GUA	80/20	1870	28	1866	NT
	90/10	1720	30	1694	NT
BBG/HMP	80/20	841	26	820	NT
	90/10	1243	28	1206	NT
BBG/*i*-CAR	80/20	2323	28	2283	NT
	90/10	2217	28	2166	NT
BBG/KOG	80/20	1733	30	1702	NT
	90/10	1840	30	1799	NT
BBG/*k*-CAR	80/20	1720	27	1699	NT
	90/10	1827	28	1807	NT
BBG/LMP	80/20	692	24	682	NT
	90/10	1073	26	1046	NT
BBG/LBG	80/20	1740	30	1704	NT
	90/10	1797	29	1756	NT
BBG/*l*-CAR	80/20	1327	27	1306	NT
	90/10	1593	28	1565	NT
BBG/MCC	80/20	1017	23	900	T
	90/10	1380	26	1285	T
BBG/XAN	80/20	3868	33	4727	R
	90/10	4643	36	4758	R

[a] The shear cycle started at 1.29 s^{-1} (onset shear), increased stepwise to 3870 s^{-1} (peak shear) and then instantly decreased to 1.29 s^{-1} (post shear).
[b] Values are means of duplicate determinations.
[c] ALG = alginate; BBG = barley beta-glucan; CMC = carboxymethyl cellulose; GAR = gum Arabic; GUA = Guar gum; HMP = high-methoxyl pectin; *i*-CAR = *iota*-carageenan; KOG = Konjac; *k*-CAR = *kappa*-carageenan; LMP = low-methoxyl pectin; LBG = locust bean gum; *l*-CAR = *lambda*-carageenan; MCC = microcrystalline cellulose; XAN = xanthan.
[d] NT = Non-thixotropic.
[e] T = Thixotropic.
[f] R = Rheopectic.

due to a change in polymer conformation. Changes in XAN conformations in aqueous media have been reported elsewhere, but the changes occurred due to heating (Bresolin et al., 1998; Kovacs & Kang, 1977). In the present study, the shear rate of 3870 s^{-1} employed during thixotropy testing might have resulted in unwinding of the ordered helical conformation of XAN into disordered random coil conformation, a cellulose-like conformation, thus increasing the hydrodynamic volume and hence the increased viscosity.

4.4. Elastic modulus of gum blends

Storage modulus (G') and loss modulus (G'') define the viscoelastic properties of gum solutions (Mandala & Palogou, 2003; Skendi, Biliaderis, Lazaridou, & Izydorczyk, 2003). G' and G'' at controlled strain and constant frequency (1 Hz) were recorded in order to locate the linear viscoelastic region (Dickinson & Merino, 2002; Mandala & Palogou, 2003). A typical curve of G' and G'' values versus strain defining a linear viscoleastic region has been discussed elsewhere (Mandala & Palogou, 2003). Deviations from linearity occur when the gel is strained to a point at which certain weak physical bonds of the aggregated network structure are destroyed. Formation of new bonds will also influence the linear viscoelastic region. In general, gels have much shorter linear regions than cross-linked polymer gels (Dickinson & Merino, 2002).

In the present study, an amplitude sweep was applied where stress and strain were increased linearly at a constant frequency of 1 Hz. Dependence of G' and G'' on frequency was not performed because it was beyond the scope of the present study. Frequency sweep is important to determine the time required for polymer entanglements to form or break within the variable periods of oscillations (Lazaridou, Biliaderis, & Izydorczyk, 2003). A constant frequency of 1 Hz was selected to allow sufficient time for network (polymer entanglements) to form and break because at higher frequencies, the molecular chains cannot disentangle during the short periods of oscillation (Lazaridou et al., 2003).

A gel-like material shows distinct behaviour that is different from liquid or concentrated solution when subjected to amplitude sweep in a rheometer at constant frequency. Freshly prepared BBG dispersions have been reported to behave like a viscoelastic liquid ($G'' > G'$) where the G' and G'' are highly dependent on frequency (Skendi et al., 2003). Formation of an elastic gel-like network ($G' > G''$) depends on the gum concentration as well as the induction time of gelation. Once the gel-like viscoleastic properties are gained, G' and G'' become less dependent on frequency (Lazaridou et al., 2003).

Comparison of G' and G'' for 0.5 and 0.75% (w/w) BBG dispersions was performed at linearly increasing strain of 0.25–20% and 0.75–120%, respectively, at a constant frequency of 1 Hz. For 0.5% (w/w) gum dispersions, the ramp of strain was carefully selected to ensure that the stress used would not exceed 1 Pa. A strain range of 0.25–20% was selected based on observations for preliminary experiments with 0.5% (w/w) gum dispersions and blends at different levels of strain sweep in order to prevent the destruction of physical bonds that contribute to the elastic properties. However, for 0.75% (w/w) gum and blend dispersions, strain sweep of 0.075–120% was selected to ensure the stress used would not exceed 10 Pa. The main reason for selecting a maximum stress of 1 Pa for 0.5% (w/w) and 10 Pa for 0.75% (w/w) gum and gum blend dispersions was to enable the comparison of linear viscoelastic regions of different BBG/other gum blends to that of pure BBG dispersions.

Figs. 1A and 2A show comparison of G' and G'' for 0.5% and 0.75% (w/w) BBG dispersions, respectively, determined at 20 °C. Both 0.5% and 0.75% (w/w) BBG dispersions demonstrated viscoelastic behaviour since $G'' > G'$. This finding is in agreement with other viscoelastic studies of oat and barley β-glucan dispersions at different concentrations (Lazaridou et al., 2003). Fig. 1B–G presents a comparison of G' and G'' for 0.5% BBG/other gum blends. Both gum ratios of 80/20 and 90/10 (w/w) of 0.5% (w/w) BBG/CAR, BBG/CMC,

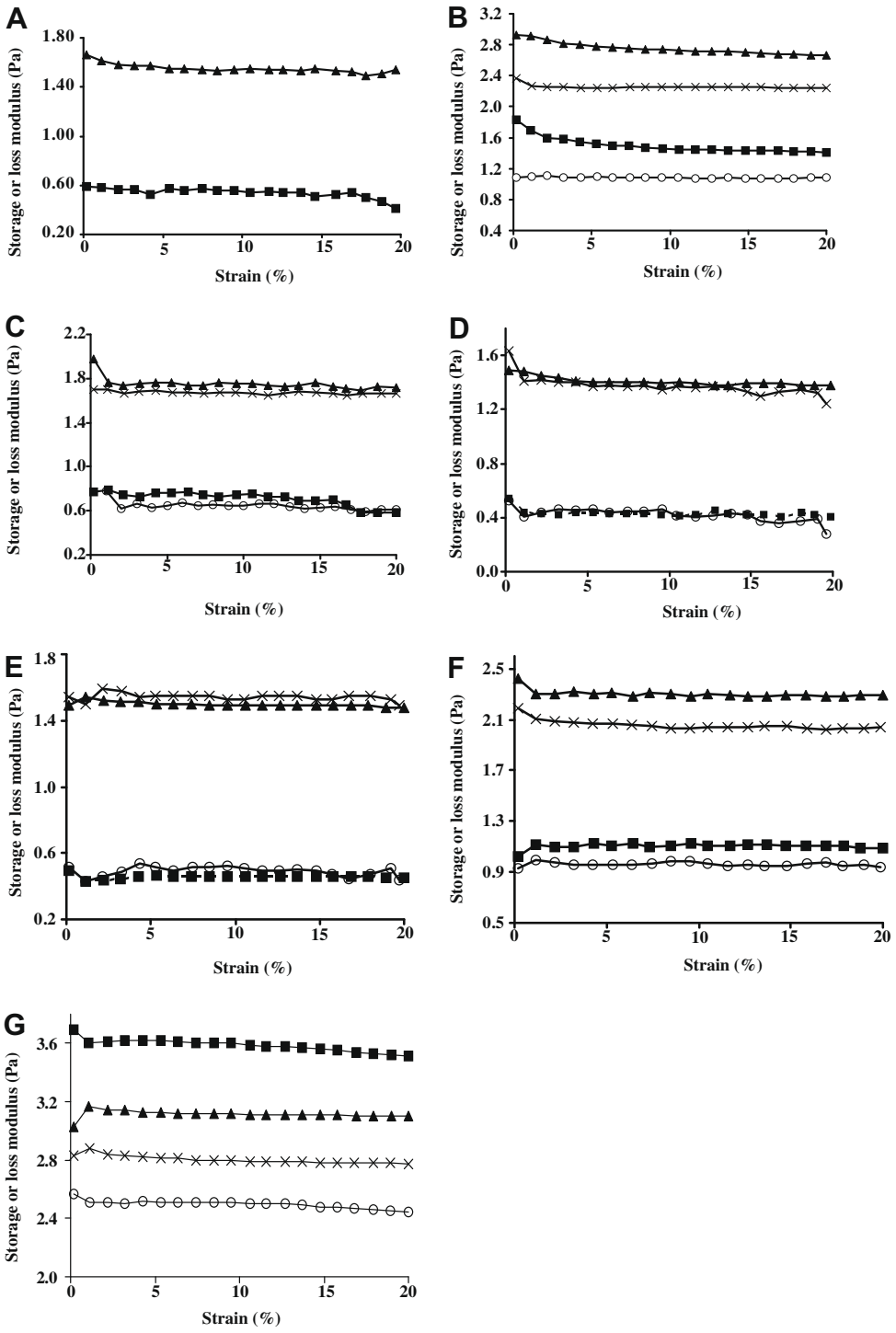

Fig. 1. Storage modulus (G') and loss modulus (G'') of 0.5% BBG alone and its blends (■) G' of 80/20, w/w, (▲) G'' of 80/20, w/w, (○) G' of 90/10, w/w, (×) G'' of 90/10, w/w. (A) 0.5%, w/w BBG alone, (B) BBG/CMC, (C) BBG/GUA, (D) BBG/KOG, (E) BBG/LBG, (F) BBG/*lambda*-CAR, (G) BBG/XAN.

BBG/GUA, BBG/KOG, and BBG/LBG blends exhibited viscoelastic behaviour with $G'' > G'$. However, 0.5% (w/w) BBG/XAN blend mixed at a ratio of 80/20 (w/w) became typical of an elastic gel network with $G' > G''$. Such an elastic gel-like behaviour was not exhibited by 90/10 (w/w) BBG/XAN blends at 0.5% (w/w) total gum concentration. Hence, BBG/XAN in a ratio of 80/20 (w/w) and mixed at 0.5% (w/w) total gum concentration is critical for the development of a gel-like network (Fig. 1G). Elastic network formation may be the reason for faster recovery time observed soon after the network destruction at 3870 s^{-1} during thixotropy testing. G' and G'' values decreased as the proportion of XAN increased from 10% to 20% (w/w) in 0.5% (w/w) BBG/XAN blend. Blends containing BBG and ALG, *iota*-CAR, GAR, HMP, LMP, and MCC having a total gum concentration of 0.5% (w/w), could not be measured using viscoelastic tests as the stress applied (1 Pa) during the amplitude sweep exceeded the strength of the network.

Fig. 2B–I shows the viscoelastic behaviour of 0.75% (w/w) BBG/other gum blends determined at 20 °C. For both gum ratios of 80/20 and 90/10 (w/w) of 0.75% (w/w) BBG/XAN blend, crossover of G' and G'' was observed. The crossover of G' and G'' defines a change from a viscoelastic fluid to a viscoelastic solid (Lazaridou et al., 2003). This indicated a soft gel formation when total gum blend

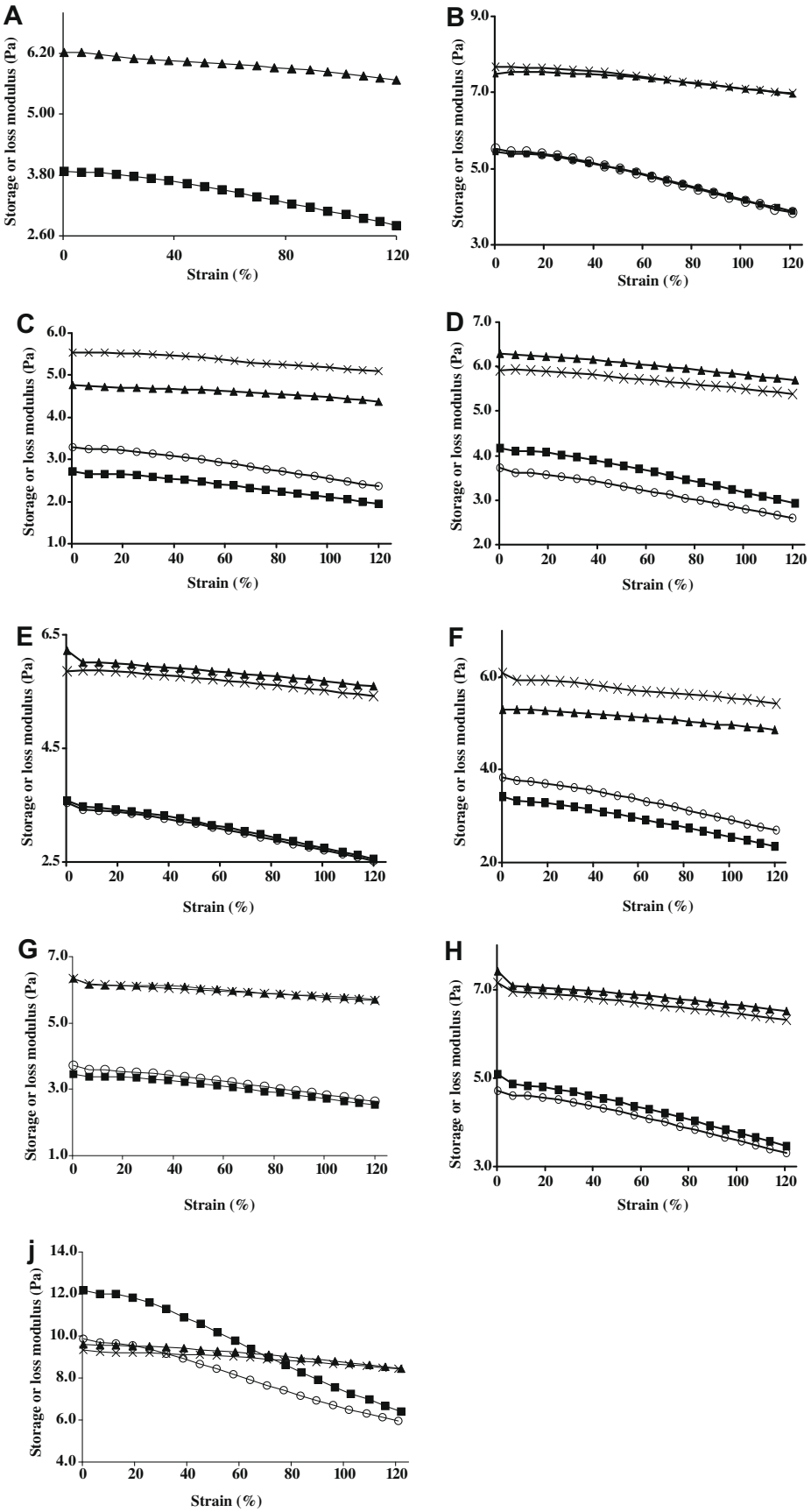

Fig. 2. Storage modulus (G') and loss modulus (G'') of 0.75% (w/w) BBG alone and its blends. (■) G' of 80/20, w/w, (▲) G'' of 80/20, w/w, (○) G' of 90/10, w/w, (×) G'' of 90/10, w/w. (A) 0.75%, w/w BBG alone, (B) BBG/CMC, (C) BBG/GUA, (D) BBG/*iota*-CAR, (E) BBG/KOG (F) BBG/*kappa*-CAR, (G) BBG/LBG, (H) BBG/*lamda*-CAR, (I) BBG/XAN.

concentration was increased from 0.5% to 0.75%, w/w. In addition to the gum concentration, the gel setting or gelation time has been reported to be affected by time and temperature of storage (Lazaridou et al., 2003). In the present study, critical time of G' and G'' crossover for the gum blends was not measured. Gum blends containing BBG and ALG, GAR, HMP, LMP, and MCC at a total gum concentration of 0.75% (w/w) were not subjected to viscoelastic tests as the stress applied (10 Pa) during the amplitude sweep exceeded the strength of the network.

5. Conclusions

BBG in binary systems exhibited synergistic interactions with XAN at 0.75%, *iota*-CAR, and CMC at both 0.5% and 0.75%, and *lambda*-CAR at 0.5% total gum concentration. Thus, the nature of the interactions depended on the blending ratios and the total gum concentrations. The improved shear tolerance (observed from flow behaviour index) of BBG/XAN blends may be beneficial in food applications where enhanced shear tolerance is required. The soft gel transformation (a change from viscoelastic fluid to viscoelastic solid) when BBG was blended with XAN may provide a unique consistency needed to achieve a "solids suspension property" much desired in products such as salad dressings or beverages with suspended particles. The addition of XAN or CMC to aqueous dispersions of BBG improved the solution viscosities at high shear rates as compared to BBG alone, meaning that at particular shear rates (e.g. intestinal shear rates), blends of BBG with XAN or CMC will maintain higher viscosities than BBG alone. The use of such blends could relate to an improved satiety effect of BBG within the human body and may be particularly useful in the creation of diet products intended to maintain a feeling of fullness. The evidence gathered from the present study also indicates potential applications for BBG in the functional beverage industry.

Acknowledgments

The authors thank Dr. Mahinda Wettasinghe who helped in the research as well as in the technical discussions. Authors wish to thank Alberta Agricultural Research Institute (AARI), Alberta Crop Industry Development Fund (ACDIF), Alberta Barley Commission (ABC) and Natural Sciences and Engineering Research Council of Canada (NSERC) for funding this research.

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Food Chemistry 117 (2009) 426–431

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Changes of flavonoid content and antioxidant capacity in blueberries after illumination with UV-C

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ARTICLE INFO

Article history:
Received 26 September 2008
Received in revised form 1 April 2009
Accepted 14 April 2009

Keywords:
Anthocyanins
Phenolics
Phytochemicals
Postharvest
Vaccinium corymbosum

ABSTRACT

The levels of flavonoids in blueberries (*Vaccinium corymbosum* L.) were found to increase after illumination with UV-C. Phytochemicals affected included resveratrol, myricetin-3-arabinoside, quercetin-3-galactoside, quercetin-3-glucoside, kaempferol-3-glucuronide, delphinidin-3-galactoside, cyanidin-3-galactoside, delphinidin-3-arabinoside, petunidin-3-galactoside, petunidin-3-glucoside, petunidin-3-arabinoside, malvidin-3-galactoside, malvidin-3-arabinoside, and chlorogenic acid as analyzed by HPLC. Significantly higher antioxidant capacity was detected in fruit treated with 2.15, 4.30, or 6.45 kJ m^{-2} compared to the control fruit. UV-C dosage of 0.43 kJ m^{-2} also increased phenolics and anthocyanins, but to a lesser extent. The optimum doses of UV-C for enhancing phytochemical content in blueberries were 2.15 and 4.30 kJ m^{-2}. These data suggest that proper use of UV-C illumination is capable of modifying the phytochemical content of blueberries. Time course measurements of the effects of UV-C revealed that the strongest responses of fruit to UV-C treatment occurred instantly after the illumination and the effects diminished with time. Therefore, even though residual effects were evident following UV-C exposure, the best results were obtained immediately after the treatment.

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1. Introduction

In recent years, increasing attention has been paid by consumers to the health and nutritional aspects of horticultural products (Scalzo, Politi, Pellegrini, Mezzetti, & Battino, 2005). Fruits and vegetables contain high levels of biologically active components that impart health benefits beyond basic nutritional value (Larson, 1988). Blueberries are one of the richest sources of natural antioxidants among fruits and have high antioxidant capacity against peroxyl radicals, superoxide radicals, hydrogen peroxide, and singlet oxygen (Heinonen, Meyer, & Frankel, 1998; Wang & Jiao, 2000). These antioxidants are capable of acting as free radical scavengers, peroxide decomposers, singlet oxygen quenchers, enzyme inhibitors and synergists (Larson, 1988).

UV-C illumination as a postharvest treatment has proven to be beneficial in delaying postharvest fruit senescence and reducing decay in various fruits and vegetables (Allende & Artes, 2003; Erkan, Wang, & Wang, 2008; Gonzalez-Aguilar, Wang, Buta, & Krizek, 2001). A previous study (Perkins-Veazie, Collins, & Howard, 2007) has indicated that postharvest application of UV-C might be effective in stimulating the antioxidant content of blueberries. However, no information is available on the effect of UV-C illumination on specific flavonols and flavonoids in blueberries. In addition, the residual effect of UV-C exposure has not been studied and little is known about what happens after UV-C treatment. The purpose of this study was to evaluate not only the changes in antioxidant capacity but also the response of individual flavonoid compounds in blueberries exposed to different UV-C illumination dosages, and the time course change of the responses of blueberries to the UV-C treatment.

2. Materials and methods

2.1. Chemicals

2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA Inc. (Richmond, VA, USA). Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 2,2-di-(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH), and disodium fluorescein were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Acetonitrile, methanol, acetone, and water were of HPLC grade and were purchased from Baxter (Muskegon, MI, USA). All anthocyanins and aglycons were obtained from Indofine Chemical Co. Inc. (Somerville, NJ, USA). Other authentic standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA).

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0308-8146/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2009.04.037

2.2. Fruit sample handling and illumination with UV-C

Blueberries (*Vaccinium corymbosum* L., cv. Duke) were hand-picked from Butler's Orchard in Maryland at a commercially mature stage. Approximately 2000 fruits were harvested and sorted to eliminate damaged, shriveled, and unripe fruit, and selected for uniform size and colour. Nine hundred and ninety fruits were selected, randomized, and used for this study. The UV-C illumination device consisted of two banks (upper and lower sides) of a 15 UV-C lamp (Model UVLMS-38: 3UV EL Series UV Lamp, 8W, LW/MR/SW, Upland, CA, USA) equipped with a filter (98-0016-02, Upland, CA, USA) to have only one wavelength at 254 nm. The intensity of the UV-C lamp was determined with a Blak-Ray J-225 photometer (Ultra-Violet Products Inc., San Gabriel, CA, USA). The UV-C lamps, reflectors, and treatment area were enclosed in a wooden box covered with aluminium foil and supported by a metal frame to provide protection for the operators. Fruit were placed on a polystyrene net and illuminated with UV-C from both the upper and lower sides. Four UV-C illumination durations and dosages were applied to blueberry fruit. These durations were 1, 5, 10, and 15 min which were equal to the dosages of 0.43, 2.15, 4.30, and 6.45 kJ m^{-2}, respectively, on each side of the berries. Non-illuminated blueberries were considered as controls. Experiments were carried out in triplicates with 30 fruits for each treatment. After UV-C illumination, control and illuminated fruit were frozen in liquid nitrogen and the samples were stored at −80 °C until analysis.

2.3. Measurement of residual effect after UV-C treatment

A separate batch of blueberries were illuminated with 4.30 kJ m^{-2} UV-C and then placed at 20 °C and samples were taken at 0, 60, 180, 300, 540, and 1440 min after UV-C illumination. Likewise, experiments were carried out in triplicates with 30 fruits for each timing in each replicate. The samples were then frozen in liquid nitrogen and stored at −80 °C until analysis.

2.4. Total anthocyanin and total phenolic content

Three 5-g composite samples from 30 berries were extracted twice with 25 mL 80% acetone containing 0.2% formic acid using a Polytron (Brinkmann Instruments Inc., Westbury, NY, USA). The homogenised samples from the acetone extracts were then centrifuged at 14,000 × *g* for 20 min at 4 °C. The supernatants were transferred to vials, then stored at −80 °C, and later used for total anthocyanin and total phenol analysis. Total anthocyanin content in fruit extracts was determined using the pH differential method (Cheng & Breen, 1991). Absorbance was measured using a Shimadzu Spectrophotometer UV-160 (Shimadzu Corp., Columbia, MD, USA) at 510 and 700 nm in buffers at pH 1.0 and 4.5, using $A = [(A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}]$ with a molar extinction coefficient of cyanidin-3-glucoside (29,600). Results were expressed as milligrams of cyanidin-3-glucoside equivalent per gram of fresh weight (mg/g fw). Total soluble phenolics in the berry fruit extracts were determined with the Folin–Ciocalteu reagent by the method of Slinkard and Singleton (1977) using gallic acid as the standard. Results were expressed as mg gallic acid (GA) equivalents per gram fresh weight (mg GA/g fw).

2.5. Oxygen radical absorbance capacity (ORAC) assay

Three 5-g composite samples from 30 berries were extracted twice with 25 mL 80% acetone containing 0.2% formic acid. The ORAC assay was carried out according to Huang, Ou, Hampsch-Woodill, Flanagan, and Prior (2002) using a high-throughput instrument platform consisting of a robotic eight-channel liquid handling system. A FL800 microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, VT, USA) was used with fluorescence filters for an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm. The plate reader was controlled by software KC4 3.0 (revision 29) (Bio-Tek Instruments Inc., Winooski, VT, USA). Sample dilution was accomplished by a Precision 2000 automatic pipetting system managed by precision power software (version 1.0) (Bio-Tek Instruments Inc., Winooski, VT, USA). The ORAC values were determined by calculating the net area under the curve (AUC) of the standards and samples (Huang et al., 2002). The standard curve was obtained by plotting Trolox concentrations against the average net AUC of the two measurements for each concentration. Final ORAC values were calculated using the regression equation between Trolox concentration (6.25–50 μM) and the net AUC and were expressed as micromole Trolox equivalents per gram of fresh weight (Huang et al., 2002).

2.6. Hydroxyl radical scavenging capacity (·OH; HOSC) assay

Three 5-g composite samples from 30 berries were extracted twice with 25 mL of 50% acetone. The ·OH in aqueous media is generated through the Fenton reaction. The HOSC assay was conducted with acetone solutions according to a previously published protocol (*21*) with some modifications. The assay was carried out using a high-throughput instrument platform consisting of a robotic eight-channel liquid handling system and a 96-well microplate with a FL800 microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Fluorescence was measured every minute for 3 h with an excitation wavelength of 485 nm and emission wavelength of 535 nm. The plate reader was controlled by software KC4 3.0 (revision 29). Sample dilution was accomplished by a Precision 2000 automatic pipetting system managed by precision power software (version 1.0) (Bio-Tek Instruments Inc., Winooski, VT, USA). Reaction mixtures consisted of 115 μL of 3.352×10^{-6} M FL prepared in 75 mM sodium phosphate buffer, 20 μL of standard or sample or blank, 25 μL of 0.1990 M H_2O_2, and 41 μL of 3.43 mM $FeCl_3$. Trolox prepared in 50% acetone at concentrations of 12.5, 25, 50, and 100 μM was used to prepare the standard curve for HOSC quantification. The HOSC values were determined by calculating the net area under the curve (AUC) of the standards and samples. The standard curve was obtained by plotting Trolox concentrations against the average net AUC of the two measurements for each concentration. Final HOSC values were calculated using the regression equation between Trolox concentration and the net AUC and were expressed as micromole Trolox equivalents (TE) per gram of fresh weight.

2.7. 2,2-Di-(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) assay

To determine the antioxidant activity of different extracts, 2,2-di-(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) radicals were used. In the radical form, this molecule has an absorbance at 515 nm that disappears with acceptance of an electron from an antioxidant compound to become a stable diamagnetic molecule. The method described by Cheng, Moore, and Yu (2006) was used with some modifications. A high-throughput instrument platform consisting of a robotic eight-channel liquid handling system and a microplate with a FL800 microplate UV–visible spectrometer reader (Bio-Tek Instruments Inc., Winooski, VT, USA) was utilised for this assay. The automated sample preparation was performed using a Precision 2000 instrument with an automatic pipetting system managed by precision power software (version 1.0) (Bio-Tek Instruments Inc., Winooski, VT, USA). The plate reader was controlled by software KC4 3.0 (revision 29). Three 5-g composite samples from 30 berries were extracted twice with 25 mL of 50% acetone, and 50 μL of this extract was diluted with 150 μL of 50%

acetone. Then, 40 μL of this diluted extract was used for assay. An aliquot (160 μL) of the DPPH solution (3.3 mg/50 mL 100% ethanol) was added to each well. The mixtures were shaken gently and allowed to stand for 40 min in the dark. The decrease in absorbance was measured at 515 nm against a blank (50% acetone) without extract using a FL800 microplate UV–visible spectrometer reader (Bio-Tek Instruments Inc., Winooski, VT, USA). The DPPH values were determined by calculating the endpoint of the standards (gallic acid) and samples. Final DPPH values were calculated using the regression equation between standard gallic acid concentrations (6.25–50 μM) and were expressed as micromole gallic acid equivalents per gram of fresh weight (μmol GA/g fw).

2.8. HPLC analysis of blueberry fruit flavonoids

High performance liquid chromatography (HPLC) was used to separate and determine individual anthocyanins and phenolic compounds in blueberry fruit tissue. Three 5-g composite samples from 30 berries were extracted twice with 20 mL 80% acetone containing 0.2% formic acid using a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY, USA) for 1 min. Extracts (40 mL) were combined and concentrated to 1 mL using a Buchler Evapomix (Fort Lee, NJ, USA) in a water bath at 35 °C. The concentrated sample was dissolved in 10 mL of acidified water (3% formic acid) and then passed through a C_{18} Sep-Pak cartridge (Waters Associated, Millipore, Milford, MA, USA), which was previously activated with methanol followed by water and then 3% aqueous formic acid. Anthocyanins and other phenolics were adsorbed onto the column while sugars, acids, and other water-soluble compounds were eluted. The anthocyanins and other phenolics were then recovered with 2 mL of acidified methanol containing 3% formic acid. The methanol extract was passed through a 0.45-μm membrane filter (Millipore, MSI, Westboro, MA, USA) and 20 μL was analyzed by HPLC. The samples were analyzed using a Waters (Waters Associated, Millipore, Milford, MA, USA) HPLC system equipped with two pumps (600 E system Controller) coupled with a photodiode array detector (Waters 990 Series). Samples were injected at ambient temperature (20 °C) onto a reverse phase NOVA-PAK C_{18} column (150 × 3.9 mm, particle size 4 μm) (Waters Associated, Millipore, Milford, MA, USA) with a guard column (NOVA-PAK C_{18}, 20 × 3.9 mm, particle size 4 μm) (Sentry guard holder universal). The mobile phase was acidified water containing 2.5% formic acid (A) and acetonitrile (B). The flow rate was 1 mL/min, with a gradient profile consisting of A with the following proportions (v/v) of B: 0 min, 3%, 1–10 min, 3–6% B; 10–15 min, 6% B; 15–35 min, 6–18% B; 35–40 min, 18–20% B; 40–45 min, 20–100% B; 45–50 min, 100% B. The phenolic compounds in fruit extracts were identified by their UV spectra, recorded with a diode-array detector and by chromatographic comparison with authentic markers. Individual flavonols and anthocyanins were quantified by comparison with an external standard of chlorogenic acid, resveratrol, myricetin, quercetin, kaempferol, or cyanidin-3-galactoside. Scanning between 250 and 550 nm was performed and data were collected by the Waters 990 3-D chromatography data system.

2.9. Statistical analysis

Data were subjected to analysis of variance using NCSS (NCSS 2007, Kaysville, UT, USA). ORAC and DPPH values, total phenolics, and total anthocyanin were evaluated by the Tukey–Kramer Multiple-Comparison test. Differences at $P \leqslant 0.05$ were considered significant. Correlation coefficients were calculated using the software, Microsoft Excel (Microsoft, 2003, Roselle, IL, USA).

3. Results

3.1. Instant effect of various doses of UV-C illumination

3.1.1. Effect on total phenols, anthocyanins, and antioxidant capacities

Different doses of UV-C illumination induced different degrees of response in blueberry fruit. Samples taken immediately after UV-C illumination showed that all UV-C doses used in this study including 0.43, 2.15, 4.30, and 6.45 kJ m^{-2} increased the levels of total phenols and anthocyanins compared to control (Table 1). The increases appeared to be dose-dependent for 0.43 and 2.15 kJ m^{-2}. However, UV-C doses higher than 4.3 kJ m^{-2} did not further enhance the levels of total phenols and anthocyanins. Instead, a slight decline in these levels were observed after blueberries were exposed to 6.45 kJ m^{-2}.

Antioxidant capacities measured by ORAC were also increased by the UV-C treatment. UV-C doses at 2.15 and 4.30 kJ m^{-2} were the most effective in promoting antioxidant capacities (Table 1). The DPPH and hydroxyl radical scavenging capacities had a similar response as the oxygen radical absorbance capacities to the UV-C exposure.

3.1.2. Effect on major phenolics and flavonoid content

Major phenolics and flavonoid components in blueberries include chlorogenic acid, resveratrol, myricetin-3-arabinoside, quercetin-3-galactoside, quercetin-3-glucoside, kaempferol-3-glucoside, and kaempferol-3-glucuronide with quercetin-3-galactoside as the predominant constituent and kaempferol-3-glucoside as a minor compound (Table 2). Resveratrol and myricetin-3-arabinoside were present at moderate amounts. Treatment with a UV-C dose at 0.43 kJ m^{-2} significantly increased the amounts of myricetin-3-arabinoside, quercetin-3-galactoside, and kaempferol-3-glucoside. Exposure to 2.15 or 4.30 kJ m^{-2} increased the levels of all the major phenolics and flavonoids detected including chlorogenic acid, resveratrol, and quercetin derivatives, in addition to myricetin-3-arabinoside, quercetin-3-galactoside, and kaempferol-3-glucoside. It appears that all individual phenolic compounds were affected similarly by the UV-C treatment. UV-C doses at 2.15 or

Table 1
Effect of different doses of UV-C illumination on total phenolics, anthocyanins, and antioxidant capacities [oxygen radical absorbance capacity (ROO·; ORAC), 2,2-di-(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) and hydroxyl radical scavenging capacity (·OH; HOSC)] in blueberries (Duke).[a,b]

Treatment (kJ m^{-2})	Total phenolics (mg/g fwt)	Anthocyanins (mg/g fwt)	ORAC (μmol TE/g fwt)	HOSC (μmol TE/g fwt)	DPPH (μmol GAE/g fwt)
0	3.12 ± 0.06	2.02 ± 0.03	40.4 ± 3.2	48.4 ± 0.6	30.5 ± 0.6
0.43	4.05 ± 0.07	2.38 ± 0.06	54.4 ± 7.6	59.7 ± 1.4	38.3 ± 0.8
2.15	4.97 ± 0.09	2.87 ± 0.04	63.2 ± 3.0	66.8 ± 2.1	40.8 ± 0.7
4.30	4.96 ± 0.11	3.11 ± 0.09	59.6 ± 2.0	79.3 ± 0.8	43.8 ± 0.9
6.45	4.72 ± 0.03	2.42 ± 0.12	54.1 ± 5.1	66.4 ± 0.7	34.6 ± 6.8
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Significance[c] Treatment	*	*	*	*	*

[a] Samples were taken immediately after UV-C illumination.
[b] Data expressed as mean ± SE ($n = 3$).
[c,*] Significant at $P \leqslant 0.05$.

Table 2
Effects of different doses of UV-C illumination on major phenolics and flavonoid content in blueberries (Duke).[a,b]

Treatment (kJ m^{-2})	Chlorogenic acid[c]	Resveratrol[d]	Myricetin-3-arabinoside[e]	Quercetin-3-galactoside[f]	Quercetin-3-glucoside[f]	Kaempferol-3-glucoside[g]	Kaempferol-3-glucuronide[g]
0	40.6 ± 4.8	13.0 ± 0.7	12.8 ± 0.2	98.7 ± 7.5	54.5 ± 1.3	2.1 ± 0.2	24.9 ± 1.2
0.43	38.4 ± 0.2	14.6 ± 2.0	15.3 ± 3.2	146.6 ± 9.7	62.9 ± 2.4	4.5 ± 0.1	29.9 ± 0.5
2.15	55.3 ± 6.8	16.2 ± 0.9	17.2 ± 0.8	177.3 ± 3.0	75.5 ± 4.2	3.3 ± 0.4	32.7 ± 2.6
4.30	45.1 ± 6.1	17.4 ± 0.2	17.2 ± 0.4	181.9 ± 2.4	83.4 ± 4.8	3.9 ± 0.9	32.3 ± 0.4
6.45	46.0 ± 5.3	17.0 ± 0.3	14.9 ± 0.9	176.2 ± 5.1	70.8 ± 1.7	3.2 ± 0.3	29.0 ± 3.3
P value	0.12053	0.03626	0.00294	0.01105	0.00171	0.03076	0.09677
Significance[h]							
Treatment	ns	*	*	*	*	*	ns

[a] Samples were taken immediately after UV-C illumination.
[b] Data expressed as mean ± SE (n = 3).
[c] Data expressed as μg of chlorogenic acid equivalents per g of fresh weight.
[d] Data expressed as μg of *trans*-resveratrol equivalents per g of fresh weight.
[e] Data expressed as μg of myricetin equivalents per g of fresh weight.
[f] Data expressed as μg of quercetin equivalents per g of fresh weight.
[g] Data expressed as μg of kaempferol equivalents per g of fresh weight.
[h,*,ns] Significant or non-significant, respectively, at $P \leqslant 0.05$.

4.30 kJ m^{-2} induced comparable quantitative increases, but no qualitative changes.

3.1.3. Effect on major anthocyanin content

Malvidin-3-galactoside was found to be the main anthocyanin in blueberries followed by malvidin-3-arabinoside, petunidin-3-galactoside, petunidin-3-glucoside, petunidin-3-arabinoside, delphinidin-3-galactoside, cyanidin-3-galactoside, and delphinidin-3-arabinoside (Table 3). The levels of malvidin-3-galactoside, delphinidin-3-galactoside, delphindin-3-arabinoside, and petunidin-3-arabinoside were increased by the UV-C exposure at 0.43 kJ m^{-2}. The higher the UV-C doses up to 2.15 kJ m^{-2}, the higher the stimulation of the individual anthocyanins detected in this study. However, a decline in the levels of most of the individual anthocyanins was found when the UV-C dose increased to 4.3 or 6.45 kJ m^{-2}. UV-C illumination increased the concentrations of all individual anthocyanins, but did not influence the ratios of the flavonols and anthocyanins.

3.2. Residual effect of UV-C illumination

3.2.1. Changes of total phenols, anthocyanins, antioxidant capacities, and hydroxyl radical scavenging capacities in blueberries after UV-C illumination

Little information is available on the residual effect of UV-C illumination. In our study, we measured the changes of total phenols, anthocyanins, antioxidant capacities, and hydroxyl radical scavenging capacities in blueberry fruit at various time intervals after

Table 3
Effects of different doses of UV-C illumination on major anthocyanin content in blueberries (Duke).[a,b,c]

Treatment (kJ m^{-2})	Delphinidin-3-galactoside	Cyanidin-3-galactoside	Delphinidin-3-arabinoside	Petunidin-3-galactoside	Petunidin-3-glucoside	Petunidin-3-arabinoside	Malvidin-3-galactoside	Malvidin-3-arabinoside
0	90.2 ± 12.7	62.4 ± 8.1	32.4 ± 0.4	164.4 ± 12.2	157.4 ± 8.5	121.2 ± 4.1	788.6 ± 14.3	370.0 ± 6.2
0.43	136.0 ± 13.6	66.7 ± 7.8	51.3 ± 0.9	156.4 ± 7.0	147.9 ± 4.5	174.1 ± 9.1	1087.7 ± 20.9	530.3 ± 7.5
2.15	199.1 ± 4.2	72.8 ± 1.1	81.6 ± 1.7	307.5 ± 0.7	218.5 ± 13.2	192.4 ± 12.1	1251.9 ± 12.1	664.8 ± 9.4
4.30	189.6 ± 3.6	76.1 ± 10.1	70.2 ± 0.4	218.8 ± 8.7	237.7 ± 6.4	186.0 ± 10.0	1237.7 ± 13.9	612.0 ± 6.8
6.45	130.3 ± 11.9	62.1 ± 1.4	34.3 ± 0.2	145.9 ± 9.9	136.5 ± 4.8	155.0 ± 9.1	1113.3 ± 16.7	544.5 ± 3.8
P value	0.0316	0.60716	<0.0001	0.0003	0.0137	0.0029	0.0013	0.00215
Significance[d]								
Treatment	*	ns	*	*	*	*	*	*

[a] Samples were taken immediately after UV-C illumination.
[b] Data expressed as mean ± SE (n = 3).
[c] Data expressed as μg of cyaniding-3-galactoside equivalents per g of fresh weight.
[d,*,ns] Significant or non-significant, respectively, at $P \leqslant 0.05$.

Table 4
Changes of total phenolics, anthocyanins, and antioxidant capacities [oxygen radical absorbance capacity (ROO$^{\cdot}$; ORAC), 2,2-di-(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) and hydroxyl radical scavenging capacity ($^{\cdot}$OH; HOSC)] in blueberries (Duke) after being illuminated with 4.30 kJ m^{-2} UV-C and held for various lengths of time at 20 °C.[a]

Time after UV-C illumination	Total phenolic (mg/g fwt)	Anthocyanin (mg/g fwt)	ORAC (μmol TE/g fwt)	HOSC (μmol TE/g fwt)	DPPH (μmol GAE/g fwt)
0 min	4.96 ± 0.11	3.12 ± 0.09	59.6 ± 2.0	79.3 ± 0.9	43.8 ± 1.3
60 min	4.29 ± 0.14	2.65 ± 0.06	58.5 ± 2.6	75.7 ± 0.8	37.9 ± 0.8
180 min	3.76 ± 0.12	2.57 ± 0.08	47.3 ± 1.9	68.9 ± 1.4	34.7 ± 0.6
300 min	3.54 ± 0.08	2.48 ± 0.04	47.6 ± 2.8	62.4 ± 0.7	34.5 ± 0.2
540 min	3.39 ± 0.06	2.29 ± 0.05	45.4 ± 2.0	58.6 ± 1.2	33.0 ± 0.6
1440 min	3.31 ± 0.05	2.13 ± 0.04	44.1 ± 3.1	52.1 ± 0.9	32.6 ± 0.3
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Significance[b]					
Treatment	*	*	*	*	*

[a] Data expressed as mean ± SE (n = 3).
[b,*] Significant, at $P \leqslant 0.05$.

the fruit had been exposed to 4.3 kJ m^{-2} UV-C. We found that the levels of these components and capacities were stimulated immediately after UV-C illumination as described in the previous sections. However, these levels gradually declined with time (Table 4). Samples taken at 1440 min after UV-C treatment contained much lower amounts of total phenols and anthocyanins than those taken immediately after the illumination. The decreases were not linear over time. The greatest decline occurred within the first 180 min following UV-C exposure and then gradually tapered off. After 1440 min, the concentrations of phenolic compounds were back to levels comparable to those of the initial samples (Table 1).

3.2.2. Changes of major phenolics and flavonoid content in blueberries after UV-C illumination

The main phenolics, chlorogenic acid, increased markedly immediately following UV-C illumination (Table 5). However, it declined steadily with time. After 1440 min following UV-C treatment, the amount of chlorogenic acid in treated samples decreased to a level comparable to those of the non-illuminated samples (Table 2). The major flavonoids such as quercetin-3-galactoside, myricetin-3-arabinoside, and resveratrol reacted to the UV-C treatment in a similar way. Residual effects of UV-C were evident from the change of levels of these flavonoids after the illumination, but the effects weakened with time.

3.2.3. Changes of major anthocyanins content in blueberries after UV-C illumination

Similar to phenolics and flavonoids, major anthocyanins increased substantially right after UV-C exposure (Table 6). Although there were obvious residual effects after UV-C treatment, these effects diminished with time. This phenomenon was especially noticeable in malvidin-3-galactoside, malvidin-3-arabinoside, petunidin-3-glucoside, petunidin-3-galactoside, delphindin-3-galactoside, and cyanidin-3-galactoside.

4. Discussion

Anthocyanins and phenolic compounds are largely responsible for the antioxidant capacity in plant tissues (Larson, 1988). Natural anthocyanins are glycosides which release aglycone forms (anthocyanidins) by hydrolysis. Anthocyanins have been reported to help reduce damage caused by free-radical activity such as low-density lipoprotein oxidation, platelet aggregation, and endothelium-dependent vasodilation of arteries (Heinonen et al., 1998). The increases of anthocyanins, phenolics, and antioxidant capacities in UV-C irradiated blueberries may allow for additional quenching of active oxygen species. Enhancement of anthocyanin levels by UV-C treatment also has been reported in strawberries and sweet cherries (Baka, Mercier, Corcuff, Castaigne, & Arul, 1999; Kataoka, Beppu, Sugiyama, & Taira, 1996). Fruit undergoing UV-C (9.2 kJ m^{-2}) and heat treatment (45 °C) retained better fruit quality and antioxidant activity than control fruit in boysenberries (Vicente et al., 2004). In grapes, accumulation of anthocyanin occurred in the skins when berry sections were exposed to UV irradiation (Kataoka, Sugiyama, & Beppu, 2003).

Stimulation of the biosynthesis of phenolic compounds in epicarp and mesocarp cells and biochemical reinforcement of the cell wall through lignification and suberization induced by UV-C treatment have been reported in tomatoes (Charles, Goulet, & Arul, 2008). These modifications have been implicated in the increased

Table 5
Changes of major phenolics and flavonoid content in blueberries (Duke) after being illuminated with 4.3 kJ m^{-2} UV-C and held for various lengths of time at 20 °C.[a]

Time after UV-C illumination	Chlorogenic acid[b]	Resveratrol[c]	Myricetin-3-arabinoside[d]	Quercetin-3-galactoside[e]	Quercetin-3-glucoside[e]	Kaempferol-3-glucoside[f]	Kaempferol-3-glucuronide[f]
0 min	45.1 ± 6.1	17.4 ± 0.2	16.7 ± 1.6	181.9 ± 2.4	83.4 ± 4.8	3.9 ± 0.9	32.3 ± 0.3
60 min	44.6 ± 2.7	16.4 ± 1.1	14.6 ± 0.3	171.1 ± 9.5	78.9 ± 8.9	2.6 ± 0.6	28.6 ± 2.4
180 min	42.1 ± 2.4	16.9 ± 2.1	12.2 ± 1.4	152.7 ± 6.1	71.5 ± 8.6	2.3 ± 0.8	26.8 ± 2.2
300 min	42.8 ± 3.2	15.2 ± 0.3	10.8 ± 0.8	134.6 ± 1.8	63.6 ± 0.3	2.2 ± 0.3	25.1 ± 0.8
540 min	41.7 ± 2.1	13.8 ± 0.8	9.2 ± 0.6	117.9 ± 6.3	60.2 ± 5.8	2.1 ± 0.6	24.9 ± 3.1
1440 min	40.8 ± 0.4	13.6 ± 0.2	8.7 ± 1.3	109.4 ± 1.2	57.1 ± 1.7	2.3 ± 0.7	25.7 ± 2.8
P value	0.0035	0.0429	0.0058	0.0009	0.0163	0.0398	0.0221
Significance[g]							
Treatment	*	ns	*	*	*	ns	*

[a] Data expressed as mean ± SE ($n = 3$).
[b] Data expressed as μg of chlorogenic acid equivalents per g of fresh weight.
[c] Data expressed as μg of *trans*-resveratrol equivalents per g of fresh weight.
[d] Data expressed as μg of myricetin equivalents per g of fresh weight.
[e] Data expressed as μg of quercetin equivalents per g of fresh weight.
[f] Data expressed as μg of kaempferol equivalents per g of fresh weight.
[g,*,ns] Significant or non-significant, respectively, at $P \leqslant 0.05$.

Table 6
Changes of major anthocyanins in blueberries (Duke) after being illuminated with 4.30 kJ m^{-2} UV-C and held for various lengths of time at 20 °C.[a,b]

Time after UV-C illumination	Delphinidin-3-galactoside	Cyanidin-3-galactoside	Delphinidin-3-arabinoside	Petunidin-3-galactoside	Petunidin-3-glucoside	Petunidin-3-arabinoside	Malvidin-3-galactoside	Malvidin-3-arabinoside
0 min	189.6 ± 3.6	76.1 ± 10.1	70.2 ± 0.4	218.8 ± 13.6	237.7 ± 6.4	186.0 ± 9.6	1237.7 ± 19.9	612.0 ± 2.8
60 min	181.4 ± 9.3	68.9 ± 7.2	67.4 ± 3.2	197.8 ± 17.1	198.5 ± 4.3	187.4 ± 11.6	1204 ± 14.2	561.3 ± 0.3
180 min	170.8 ± 7.6	65.3 ± 3.1	65.6 ± 0.8	185.6 ± 12.6	189.3 ± 12.2	183.8 ± 6.2	1054.1 ± 12.2	463.4 ± 7.0
300 min	162.7 ± 3.2	66.3 ± 8.6	54.3 ± 1.6	180.8 ± 8.2	172.1 ± 8.4	171.2 ± 7.8	986.4 ± 10.2	459.1 ± 7.7
540 min	156.9 ± 4.5	64.8 ± 7.8	51.7 ± 2.8	169.8 ± 5.6	163.9 ± 4.1	165.6 ± 3.6	871.2 ± 15.3	447.4 ± 8.9
1440 min	147.2 ± 5.8	63.2 ± 6.9	46.5 ± 1.1	167.2 ± 7.8	166.9 ± 9.6	152.4 ± 2.7	861.1 ± 6.2	385.7 ± 4.5
P value	<0.0001	0.0762	<0.0001	0.0050	0.0014	<0.0001	<0.0001	<0.0001
Significance[c]								
Treatment	*	ns	*	*	*	*	*	*

[a] Data expressed as mean ± SE ($n = 3$).
[b] Data expressed as μg of cyaniding-3-galactoside equivalents per g of fresh weight.
[c,*] Significant, at $P \leqslant 0.05$.

disease resistance in UV-C treated tomato fruit. The beneficial effect of UV-C has also been linked to the activation of phenylalanine ammonia lyase (Nigro, Ippolito, Iattanzio, Di-Venere, & Salerno, 2000; Pan, Vicente, Martinez, Chaves, & Civello, 2004) and the accumulation of phytoalexins (Mercier, Arul, & Julien, 1993; Rodov, Ben-Yehoshua, Kim, Shapiro, & Ittah, 1992). In our study, we have found that UV-C illumination also enhanced levels of flavonoids and other antioxidant compounds.

UV-C exposure may act by inducing stress in plants. Increases in flavonoids and antioxidant capacities as described in this study might be part of the defense mechanism produced by blueberries in reacting to stress induced by UV-C illumination. The enrichment of stilbenes, such as resveratrol, in grapes in response to stress induced by postharvest UV-C irradiation has been described (Cantos, Espín, & Tomás-Barberán, 2002). The biological activity of resveratrol as an anticarcinogenic and an antioxidant has been reported (Jang et al., 1997). The increases of total phenols and anthocyanins in blueberries by UV-C illumination in our study appeared to be dose-dependent at lower doses (0.43–2.15 kJ m^{-2}). However, higher doses (4.30–6.45 kJ m^{-2}) tended to suppress these increases. This phenomenon has also been reported in strawberries where high doses of UV-C exposure was thought to cause too much stress and possibly resulted in injury (Baka et al., 1999).

Previous studies have showed that UV-C exposure retarded fungal growth in various commodities, and consequently reduced decay development in several fruits and vegetables after harvest (Stevens et al., 1996; Wilson et al., 1997). UV-C light has been considered as a germicidal agent and the use of this treatment has been demonstrated to directly inhibit microbial growth (Allende & Artes, 2003; Stevens et al., 1996). UV-C may also retard the mold development by enhancing the resistance of plant tissues against pathogenic attack (Droby et al., 1993). Thus, adequate UV-C treatment can be beneficial and have practical impact for commercial practices in terms of reducing the spoilage of fresh produce. Any other benefits obtained from UV-C treatment would be additive. Even though our study showed that the effect of UV-C on antioxidant capacities, phenolics, and anthocyanins might be short-term in blueberries, the responses of other commodities or the effect using different dosages could be different. Further investigations may also be warranted to explore if combining UV-C illumination with other postharvest techniques would have a long lasting effect in increasing the levels of antioxidant activity and potential health-promoting nutraceutical compounds.

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Food Chemistry 117 (2009) 432–437

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Fatty acid accumulation in the different fractions of the developing corn kernel

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ARTICLE INFO

Article history:
Received 6 January 2009
Received in revised form 14 April 2009
Accepted 15 April 2009

Keywords:
Fatty acids,
Maturation
Corn kernel
Endosperm
Germ
Pericarp

ABSTRACT

In the food industry, the use of the oil is determined by the composition of fatty acids, and this is highly dependent on its natural origin. The fatty acid composition of whole corn kernel was determined in three varieties of corn (*Astro*, *GH*2547, and *Local*). Linoleic acid was the predominant fatty acid in the oil of *Astro* and *Local* at all collection dates, whereas it was the major fatty acid in the oil of *GH*2547 only between 40 and 60 days after pollination (DAP). The fatty acid accumulation in the endosperm, pericarp and germ fractions of the corn kernel during maturation was determined. The accumulation pattern of oil content was different in these three kernel fractions. The highest levels of oil content in the endosperm (2.2%), germ (34.3%) and pericarp (10.8%) fractions were detected at 20, 40 and 30 (DAP), respectively. The fatty acid accumulation patterns were different amongst the analysed kernel parts, indicating a numerous differences between the three corn kernel parts. Throughout the sampling periods, the endosperm fraction was distinguished by the highest and the lowest levels of oleic and linoleic acids, respectively. At all stages of kernel development, the pericarp fraction had the highest levels of total polyunsaturated fatty acids, which has numerous healthy applications. These results may be useful in the understanding of the potential source of the beneficial unsaturated fatty acids amongst the different fractions of the corn kernel during maturation.

1. Introduction

Plant oils and animal fats constitute one of the major classes of food product, for the provision of energy and essential fatty acids. Animal sources, such as butter and lard, are characterised by high concentrations of saturated fatty acids. It is well known that a diet rich in saturated fatty acids forms a risk factor for hypercholesterolemia, atherosclerosis and others diseases in humans. In order to reduce the saturated fat content of processed foods, the food industry in developed countries moves progressively from animal fat to vegetable fat sources (Ruiz-Jiménez, Priego-Capote, & Luque de Castro, 2004). The change in consumer preferences is mostly due to consumers demanding food products that combine a pleasant flavour with nutritional benefits. Vegetable oils have a high content of unsaturated fatty acids, which are heart-healthy. The nutritional value of *n*-3 polyunsaturated fatty acids (PUFA) in the human diet is well recognised, and increased consumption of these fatty acids has been recommended (Department of Health, 1994). The precursor of the *n*-3 series of long-chain PUFA is the essential fatty acid linolenic acid (C18:3), from which man can synthesise the longer chain PUFA such as eicosapentaenoic acid and docosahexaenoic acid (Elmore et al., 2005). Recently, interest in the PUFA, as health-promoting nutrients, has expanded dramatically.

The intensive research period up to the 1990s, focused on the modification of the fatty acid composition of oilseeds by biotechnology. Information on the fatty acid distribution in seed parts provides a possible way to produce vegetable oils with high additional value. Since, based on the understanding of composition, biosynthetic and metabolic pathways of fatty acids in seed parts it is practically applicable to genetically alter the lipid composition of cells through the alteration of the encoding genes of enzymes that are responsible for lipid synthesis or metabolism. Commercially exploited seeds such as soya and rape have been the subject of many years of breeding programmes to obtain oils with particular fatty acid patterns (Stenberg, Svensson, & Johansson, 2005). Successful advances in plant breeding, such as the elimination of erucic acid from rapeseed, have drastically improved the oil quality. The established limits of fatty acid contents could be used for the detection of fraud of the olive oil with corn oil, at levels of adulteration up to 5% (Christopoulou, Lazaraki, Komaitis, & Kaselimis, 2004). In industry, the use of the oil is determined by the composition of fatty acids, and this is highly dependent on its natural origin. The fatty acid composition of oils from vegetable sources varies depending on plant origin, genetic factors, ripening grade of fruits and specific climatic conditions (Davis & Poneleit, 1974; Velasco, Rojas-Barros, & Fernandez-Martinez, 2005). In exploring the role of lipids in cells and tissues, it is useful to know which lipids are present and in what proportions. Furthermore, one of the main objectives of industry is to identify plant matrices rich in

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doi:10.1016/j.foodchem.2009.04.038

some compounds, which have large industrial applications. Thus, Bruni et al. (2002) analysed Ecuadorian *Theobroma subincanum* seed parts to determine quali-quantitative tocopherol, fatty acids and sterols distributions. A number of studies have been reported concerning the developmental changes in the fatty acid composition of the whole corn kernel (Poneleit & Davis, 1972; Weber, 1969). However, there is limited information on the fatty acid distribution within corn kernel during maturation. The objective of the present research was to monitor the accumulation pattern of fatty acids in the endosperm, germ and pericarp fractions of the corn kernel during maturation. This study seems to be very useful in understanding the source of nutritionally and industrially fatty acids in the corn kernel fractions and for many aspect of vegetable oil production.

2. Experimental

2.1. Reagents and standard

Methanol, chloroform acetone and pentane, solvents of HPLC grade, were purchased from Panreac Quimica SA. (Barcelona, Spain). Triflorure Bore (BF_3) was from Fisher Scientific SA (Loughborough, Spain). Fatty acid standards were acquired from Sigma Aldrich (Madrid, Spain).

2.2. Samples

Sweet corn (variety *GH*2547*)* was obtained from USA. The sweet corn (*Zea mays saccharata*) differs from dent corn (*Zea mays indentata*) by a single recessive gene which prevents some of the sugar to being converted to starch (Bland, 1971). *GH*2547 variety was grown in restricted zones (15 × 3 m) on the Agronomy farm of the INRAT (Institut National Recherche Agronomie Tunis, North of Tunisia) from the middle of April until the end of August 2005. Ear samples were collected at intervals after the date of hand-pollination. Corn kernels were steeped for 3 h in 0.1% sodium metabisulfite solution at 50 °C before dissection.

2.3. Lipid extraction

The total lipids were extracted by the method of Folch, Lees, and Sloane Stanley (1957) modified by Bligh and Dyer (1959). Seeds (2.5 g) were washed with boiling water for 5 min to denature the phospholipases and then crushed in a mortar with a mixture of $CHCl_3$–MeOH (2:1, V/V). The water of fixation was added and the homogenate was centrifuged at 3000*g* for 15 min. The lower chloroformic phase containing the total lipids was kept and dried in a rotary evaporator at 40 °C.

2.4. Fatty acid analyses

The fatty acid composition of the seed oil was determined by gas chromatography of fatty acid methyl esters. Fatty acid methyl esters were prepared by simultaneous extraction and methylation following the procedure described by Mectalfe, Schmitz, and Pellka (1966) modified by Lechvallier (1966). Fatty acid methyl esters were analysed by gas chromatography (GC) using a HP 4890 chromatograph equipped with a flame ionisation detector (FID) on a capillary column coated with supelcowaxTM 10 (30 m length, 0.25 mm id, 0.2 μm film thickness). Column temperature was programmed from 150 to 200 °C (4 °C min^{-1}. Temperatures of detector and injector were 250 and 230 °C, respectively. The fatty acids were identified by comparison of their retention times with those of standards. The quantitative evaluation was carried out on the base of gas chromatography peak areas of the different methyl esters. The response factor for fatty acids, calculated by using heptadecanoic methyl ester, was 0.95.

2.5. Statistical analysis

Statistical analysis was performed by using the Proc ANOVA in SAS (Software version 8). Duncan's Multiple Range Test was used. For each oil sample, three determinations have been done.

3. Results and discussion

3.1. Oil content

Numerous studies reported the developmental changes in the oil content of the maturing whole corn kernel (Harrabi et al., 2007; Weber, 1969). The distribution of the total lipid in the different fractions of mature corn kernel has been examined (Harrabi et al., 2008). In this study, the maturing corn kernels were divided into three parts: germ, endosperm and pericarp. At 20 days after pollination (DAP), the corn kernel was made up mainly of the pericarp (37.1%) and endosperm (52.7%) fractions; however the proportion of the germ part was 10.2%. From 20 to 30 DAP, the percentages of the endosperm and germ parts rapidly increased, whilst those of the pericarp fraction decreased (Fig. 1). This result suggested that the biosynthesis of organic matter such as lipids, carbohydrates and protein was more important in the endosperm and germ fractions than that in the pericarp part. The endosperm development was divided into phases: the first was characterised by a rapid accumulation of soluble constituents, and the second

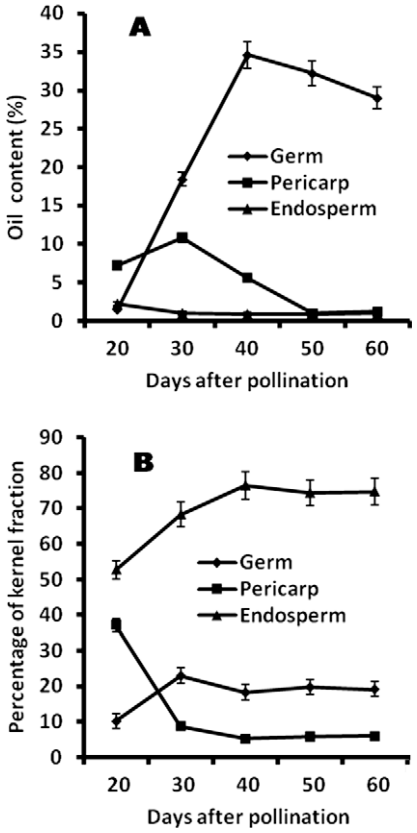

Fig. 1. Changes in the oil content (percentage dry weight (A)) and proportions of corn kernel fractions (B) during maturation of *GH*2547 variety. Pericarp fraction is presented by (■), germ part is presented by (♦) and endosperm part is presented by (▲).

by the utilisation of those soluble constituents to cause an increase in the protein content (Davis & Poneleit, 1974).

During the corn kernel development, the total lipid content decreased gradually from 2.2% at 20 DAP to about 0.9% at 40 DAP in the endosperm fraction (Fig. 1). Conversely, the oil content in the pericarp fraction increased to a maximum (10.8%) at 30 DAP, and then decreased gradually to reach a constant value of 1.2% at 40 DAP. The oil which was accumulated early in the pericarp fraction was apparently metabolised later. The greatest increase of oil content was observed in the germ part, resulting in much higher final oil content (29.0%) in this kernel fraction. The final oil content in the three corn kernel parts was influenced by the duration and rate of oil synthesis. This result suggested numerous differences between the corn kernel parts, such as the amounts and activities of enzymes catalysing the various lipid synthetic processes. The oil is presumably synthesised and stored by the germ fraction for eventual use as energy during germination and other processes. During the corn kernel development, the endosperm and germ fractions stores primarily starch and lipids, respectively.

3.2. Fatty acid profiles of the whole corn kernel and its parts at maturity

The fatty acid profiles of the mature whole corn kernel were compared amongst the three varieties (Table 1). For all the three corn varieties, linoleic acid (49.7–62.7%) was the predominant fatty acid components, followed by oleic (23.5–34.9%) and palmitic (9.5–11.5%) acids. This contrasts with the composition of olive oil where oleic acid was the most abundant fatty acid (78.1%), followed by palmitic (10.2%) and linoleic (7.1%) acids (Rezanka & Rezankova, 1999). *Astro* was distinguished by the highest level of linoleic acid (62.7%) and the lowest for oleic acid (23.5%), whilst *GH*2547 showed the highest value for oleic acid (34.9%) and the lowest for linoleic acid (49.7%). The percentage of linolenic acid was nearly the same for the three corn varieties (1.7–2.1%). Considering the fact that the three varieties were grown in the same experimental conditions, we suggested that the variability in fatty acid composition was mainly due to genetic effects.

The fatty acid profile of the principal parts of the mature corn kernel was also investigated. A comparison between the three corn kernel parts demonstrated that there was a significant difference ($p < 0.05$) in their fatty acid percentages (Table 1). In the endosperm fraction, the percentage of oleic acid was higher than that of linoleic acid, whilst the reverse is true in the germ and pericarp fractions. *GH*2547 endosperm had significantly ($p < 0.05$) higher oleic acid percentage (66.3%) than had *Astro* endosperm (52.7%) and *Local* endosperm (48.5%). Oleic acid was converted to linoleic acid by Δ12 desaturase, which is a membrane bound enzyme; thus we suggested that this desaturation step was less important in the endosperm part. In addition, this result could be linked to the activity of phosphatidyl choline oleoyl desaturase, enzyme which catalyses the desaturation of oleic acid to linoleic acid (Velasco et al., 2005).

Amongst the three corn kernel parts, germ part had the most levels of palmitic acid (11.4–15.6%), whilst pericarp part had the lowest levels (5.3–7.6%) of this fatty acid. Tan and Morrison (1979) reported that the levels of this fatty acid in the pericarp, germ and endosperm fractions were: 19.4–24.7%, 10.6–12.0% and 24.7–27.5%, respectively. However, Moreau, Singh, Nuñez, and Hicks (2000) reported that the germ, fibre and aleurone fractions had similar proportions of palmitic acid. Abundance of this fatty acid might be linked to the abundances and activities of 3-ketoacyl-ACP (Acyl Carrier Protein) synthase II and thioesterase specific for saturated acyl-ACP. In fact, the palmotyl-ACP synthesised in plastid could be hydrolysed by thioesterases or converted to stearoyl-ACP by 3-ketoacyl-ACP synthase II (Ohlrogge & Jaworski, 1997).

The oil extracted from the pericarp fraction had significantly ($p < 0.05$) higher amount of linolenic acid than had the endosperm and germ fractions. This result suggested that Δ15-desaturase, enzyme which converted linoleic acid to linolenic acid, was more abundant and active in the germ fraction than that in the endosperm and pericarp fractions. The percentage of stearic acid (0.5–2.2%) was very lower in all of the corn kernel fractions, as it was in the whole corn kernels of the three varieties. According to Bruni et al. (2002), the seed parts of sterculiaceae (*T. subincanum*) had high amounts of stearic acid (24.3–40.7%). That result underlines the differentiation between the seed parts of sterculiaceae and those of corn. The abundance of various fatty acids in the different seed parts might be considered as characteristic of the vegetal species.

3.3. Fatty acid accumulation in the whole corn kernel

Differences in the percentages of the various fatty acids over the course of maturation of the three corn varieties are evident in Fig. 2. As the corn kernel developed, the percentage of palmitic acid decreased rapidly between 20 and 30 DAP and then remained nearly stable until maturity, in agreement with the results of Poneleit and Davis (1972) and Weber (1969). Oleic acid and linoleic acid exhibited contrasting accumulation patterns in the maturing whole corn kernel; the percentage of linoleic acid increased whilst the oleic acid percentage decreased. The patterns of fatty acid accumulation were different amongst the vegetable seeds. In fact, the

Table 1
Fatty acid composition (percentage of the total fatty acids) of dissected corn kernels, at maturity.

Sample		C16:0	C18:0	C18:1 *n*-9	C18:2 *n*-6	C18:3 *n*-3	C20:0
Astro	G	11.4 ± 0.9a	2.1 ± 0.3a	19.1 ± 0.4a	65.4 ± 1.3a	1.8 ± 0.1a	0.2 ± 0.1a
	P	6.2 ± 0.6b	1.3 ± 0.1a	19.4 ± 0.2a	68.6 ± 1.7b	4.2 ± 0.3b	0.3 ± 0.1a
	E	8.7 ± 0.4c	0.5 ± 0.2b	52.7 ± 1.1b	36.3 ± 1.3c	0.9 ± 0.3c	0.8 ± 0.2b
	W k	9.5 ± 1.0c	1.9 ± 0.2a	23.5 ± 0.7c	62.7 ± 1.0a	2.1 ± 0.4a	0.3 ± 0.1a
Local	G	13.2 ± 0.5a	0.9 ± 0.3a	17.6 ± 0.9a	67.4 ± 1.7a	0.8 ± 0.3a	0.1 ± 0.0a
	P	5.3 ± 0.7b	1.1 ± 0.2a	27.5 ± 0.8b	60.4 ± 0.9b	5.2 ± 0.4b	0.2 ± 0.1a
	E	9.2 ± 1.0c	2.2 ± 0.1b	48.5 ± 1.1c	38.1 ± 1.3c	0.9 ± 0.2a	1.1 ± 0.3b
	W k	10.4 ± 0.5c	1.7 ± 0.4b	26.3 ± 0.6b	59.3 ± 0.8b	1.8 ± 0.1c	0.4 ± 0.2a
*GH*2547	G	15.6 ± 0.7a	1.5 ± 0.1a	32.3 ± 0.4a	49.6 ± 1.1a	0.7 ± 0.2a	0.2 ± 0.1a
	P	7.6 ± 0.6b	1.6 ± 0.2a	40.5 ± 1.1b	44.3 ± 0.8b	5.8 ± 0.3b	0.2 ± 0.0a
	E	10.2 ± 0.5c	0.9 ± 0.2b	66.3 ± 1.5c	20.5 ± 0.6c	1.2 ± 0.1c	0.4 ± 0.2a
	W k	11.5 ± 0.5c	1.7 ± 0.1a	34.9 ± 1.3a	49.7 ± 1.2a	1.7 ± 0.1c	0.3 ± 0.1a

Mean values with different letters within a column for the different parts of the same variety are significantly different, at $p \leqslant 0.05$.
C16:0, palmitic acid; C18:0, stearic acid; C18:1 *n*-9, oleic acid; C18:2 *n*-6, linoleic acid; C18:3 *n*-3, linolenic acid; C20:0, arachidic acid; G, germ; P, pericarp; E, endosperm; Wk, whole kernel.
Each value is a mean ± standard deviation (SD) of a triplicate analysis performed on different samples.

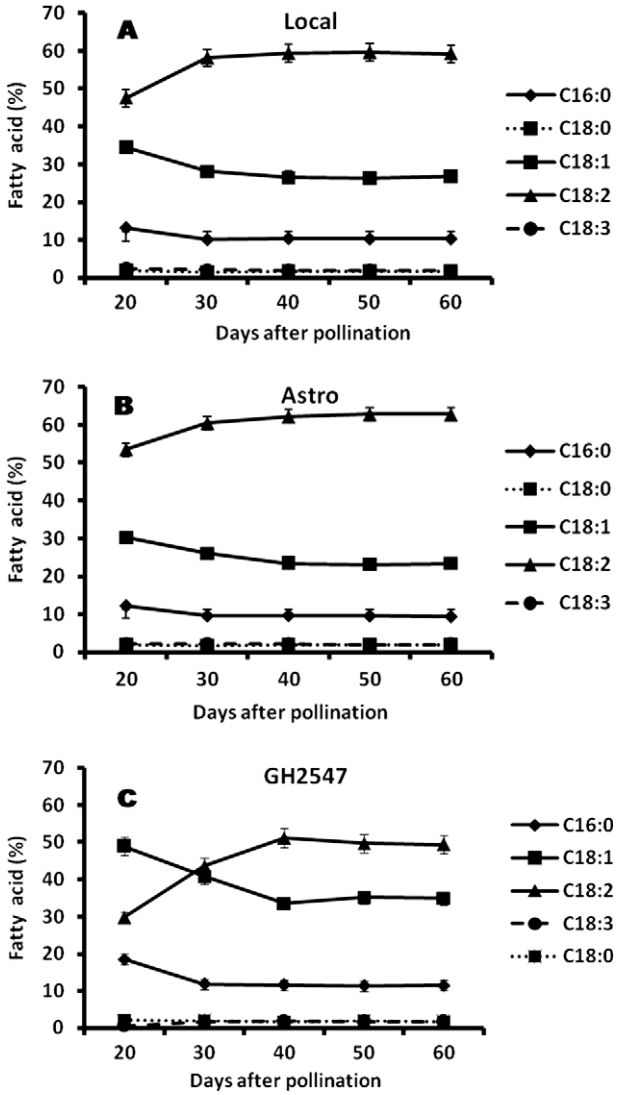

Fig. 2. Fatty acid composition (percentage of the total fatty acids) of the whole kernel during maturation of three corn varieties: (A) *Local*, (B) *Astro*, (C) *GH*2547. Palmitic acid (♦), linoleic acid (▲), oleic acid (■), stearic acid (...■...), linolenic acid (●).

percentage of linoleic acid decreased in the developing seeds of castor (Velasco et al., 2005). Linoleic acid was the predominant fatty acid in the oil of *Astro* and *Local* at all collection dates, whereas it was the major fatty acid in the oil of *GH*2547 only between 40 and 60 DAP. Although *GH*2547 accumulated linoleic acid at faster rate than did *Astro* and *Local*, *GH*2547 had the lowest levels of this unsaturated fatty acid at all collection dates. This result suggested that the percentage of linoleic acid should be influenced by the highly efficient of Δ12 desaturase in the early development period before 20 DAP. The increase in the amount of linoleic acid was not accompanied by a concomitant raise of linolenic acid percentage, which suggested that the very low level of linolenic acid could be the result of a lower efficiency of the desaturation of linoleic acid in the maturing corn kernel.

3.4. Fatty acid accumulation in the endosperm, pericarp and germ fractions

The patterns of fatty acid accumulation were different amongst the three corn kernel parts (Fig. 3). In the germ and pericarp fractions, oleic and linoleic acids exhibited contrasting accumulation patterns, as it was in the whole corn kernel. However, these two fatty acids followed a similar accumulation pattern in the endosperm fraction, where their percentages increased slightly from 20 to 30 DAP and then remained unchanged. As the corn kernel developed, the highest levels of oleic acid (62.4–67.5%) and the lowest for linoleic acid (14.2–21.3%) were detected in the endosperm fraction. This result suggested a lower efficiency of the desaturation of oleic to linoleic acid and indicated that this desaturation step was not favoured by the accumulation of the substrate. At all collection dates, the percentages of stearic acid was very small (0.9–4.3%) in the three corn kernel parts. Stearic acid was converted to oleic acid by Δ9 desaturase, a reaction that occurs in plastids. Therefore, it can be thought that this desaturation step occurred mainly before 20 DAP. The interplay between the fatty acid synthase, Δ9 desaturase, and thioesterases determines the levels of palmitic and stearic acids. Considering the facts that cell membranes are constituted mainly by phospholipids and that the early period of seed development are characterised by an intense cell divisions (Cresti, Blackmore, & Went, 1992), we suggested that the highest rate of the biosynthesis of C18:1 and C18:2 was required by cells for the synthesis of phospholipids to regulate membrane permeability and fluidity. Ohlrogge and Jaworski (1997) reported that the rate of fatty

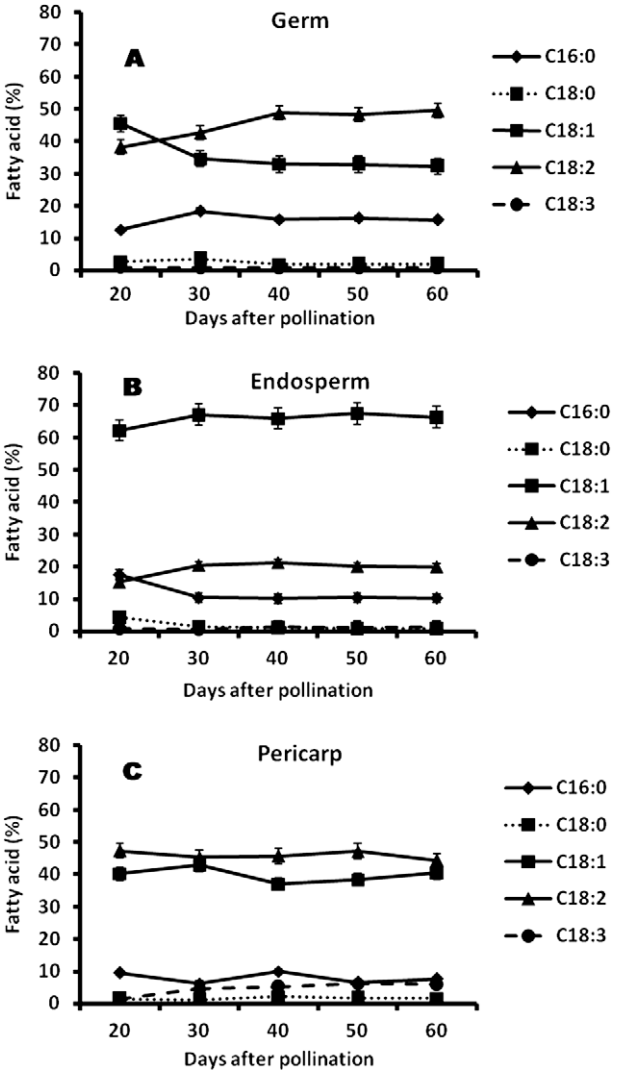

Fig. 3. Fatty acid composition (% of the total fatty acids) of the germ (A), endosperm (B), and pericarp (C) fractions, during maturation of *GH*2547 variety. Palmitic acid (♦), linoleic acid (▲), oleic acid (■), stearic acid (...■...), linolenic acid (●).

acid synthesis can apparently be regulated by the demand for fatty acids, which were needed for membranes synthesis. This could explained why the increase in the amounts of oleic and linoleic acids occurred during the most rapid rate of endosperm mitosis, in fact it has been reported that cell divisions in the endosperm part of corn kernel (variety Wf9) was essentially completed within 28 DAP (Ingle, Beitz, & Hageman, 1965).

The pattern of palmitic acid accumulation varied amongst the three corn kernel parts. In the endosperm fraction, palmitic acid exhibited the same trend observed for the whole corn kernel. Whereas in the germ fraction, the percentage of palmitic acid increased from 12.6% at 20 to 18.3% at 30 DAP, and then decreased to reach a constant value about 15.5% at 40 DAP. This result is in contrast with the result of Jellum (1970), which indicated that palmitic acid proportion decreased in the germ fraction. Differences in variety were likely the reason for the observed result. In fact, Goffman and Böhme (2001) reported that variation for fatty acid composition of corn oils was principally due to genetic differences amongst the hybrids. In the pericarp fraction, the amount of palmitic acid fluctuated and no definite trend was evident; although the highest level (9.9%) was observed at 40 DAP. Arachidic acid amount in the germ and pericarp fractions (0.1–0.2%) was relatively constant during corn kernel development; however it increased slightly in the endosperm fraction from 0.1% at 40 to 0.4% 60 DAP. As the corn kernel developed, the amount of linolenic acid in the pericarp oil rapidly increased from 1.3% at 20 to 4.5% at 30 DAP, whereas it was nearly the same in the endosperm and germ fractions. Fatty acid synthesis increases in response to the high demand for acyl chain for the synthesis of glycerolipids in the endoplasmic reticulum. At all stages of kernel development, the pericarp fraction was richer in linolenic acid than the endosperm and germ fractions. Considering the fat that Δ15 desaturase catalyses the conversion of linoleic to linolenic, we suggested that this enzyme was more abundant and active in the pericarp part than in the endosperm and germ parts. The highest amounts of total polyunsaturated fatty acids were detected in the pericarp fraction, at all stage of corn kernel development (Fig. 4). This result suggested the high healthy nutritional value of the pericarp oil. In fact, polyunsaturated fatty acids have been suggested to be beneficial in reducing the risk of certain chronic diseases, such as cardiovascular, inflammatory and atherosclerosis diseases (Finley & Shahidi, 2001; Riemersma, 2001).

Ricinoleic acid, which synthesised by the hydroxylation of oleic acid, was not detected in the three corn kernel fractions. This result suggested the absence of Δ12 hydroxylase, a microsomal enzyme which convert oleic acid to ricinoleic acid (Bafor, Smith, Jonsson, Stobart, & Stymne, 1991) in the different parts of corn kernel. In contrast, this fatty acid was detected in the seeds of castor (Velasco et al., 2005).

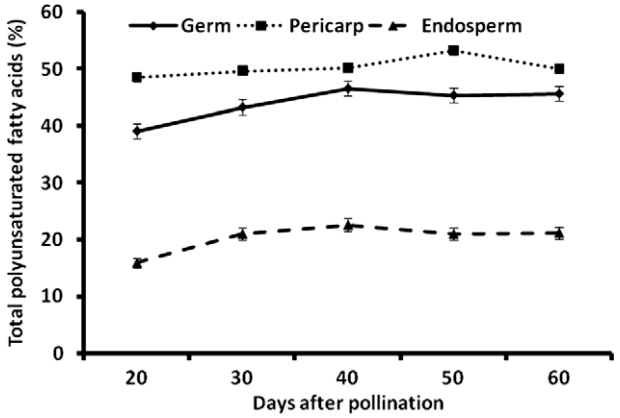

Fig. 4. Pattern of variation in polyunsaturated fatty acid composition (percentage of the total fatty acids) for the three fractions of the corn kernel during maturation (variety *GH*2547). Pericarp fraction is presented by (■), germ part is presented by (♦) and endosperm part is presented by (▲).

4. Conclusion

The fatty acid composition showed a different pattern in endosperm, germ and pericarp fractions of the maturing corn kernel. In the endosperm fraction, the early accumulation of oleic acid did not promote its desaturation to linoleic acid, which remained unchanged during corn kernel development. The pericarp fraction accumulated linolenic acid for a longer period and at faster rate than did the germ and endosperm fractions. As a result, at all collection dates the highest levels of linolenic acid were detected in the pericarp fraction. The highest amounts of polyunsaturated fatty acids detected in the pericarp fraction, at all stage of corn kernel development, suggested its high healthy nutritional value.

Acknowledgements

The authors gratefully acknowledge Dr. Hamadi Ben Saleh at INRAT for technical assistance in conducting the culture of corn. They thank Dr. Clem W. Kazakoff and Annick St-Amand for their technical advice.

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Food Chemistry 117 (2009) 438–443

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Development of antioxidant rich peptides from milk protein by microbial proteases and analysis of their effects on lipid peroxidation in cooked beef

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ARTICLE INFO

Article history:
Received 28 December 2008
Received in revised form 13 April 2009
Accepted 14 April 2009

Keywords:
Milk peptides
Antioxidant
Lipid peroxidation
Ground beef
ORAC
DPPH
TBARS

ABSTRACT

Three microbial proteases, validase (Val) from *Aspergillus oryze*, alkaline protease (AP) from *Bacillus licheniformis*, and neutral protease (NP) from *Bacillus subtilis*, were investigated for producing antioxidant hydrolysates/peptides from milk protein isolate. The hydrolysates were fractionated by sequential ultrafiltration and their antioxidant properties examined. The oxygen radical absorbance capacity (ORAC) of the twelve hydrolysate fractions varied significantly ranging from 62.9 to 246.6 μmol Trolox equivalents (TE) per gram with Val-F3 fraction possessing the highest value. The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH·) scavenging activities of the hydrolysate fractions also varied significantly ranging from 3.1 to 35.4 μmol TE/g. The results revealed that most hydrolysate fractions exerted weak chelating activities of ranging from 0.1 to 0.9 mg ethylenediaminetetraacetic acid (EDTA) equivalents/g. However, the NP-F2 fraction exerted an exceptionally higher activity of 4.7 mg EDTA equivalents/g. Val-F3, Val-F2, and NP-F2 were incorporated into ground beef to determine their impact on lipid peroxidation. Only the Val-F3 fraction (200 μg/g in the meat) significantly reduced meat lipid peroxidation. Increasing the dosage of VAL-F3 (800 μg/g) did not enhance the inhibition on meat lipid oxidation.

1. Introduction

Lipid oxidation is a key process that occurs in food products which correlates to the deterioration of food quality. Incorporating antioxidant compounds into food products have proved to be an effective measure for inhibiting lipid oxidation which extends shelf-life and improves food quality by preserving flavour and nutritional value (Laguerre, Lecomte, & Villeneuve, 2007; Rojas & Brewer, 2007). The identification and development of food-derived peptides with antioxidant properties has attracted increased attention due to heightened safety concerns over the use of synthetic antioxidants and the consumer's preference for naturally derived products (Sakai, Miyata, & Takahashi, 1997; Shahidi & Zhong, 2008). Once proven safe and effective naturally derived antioxidant peptides could have beneficial utility/application in the food industry as of result of their incorporation into fat-rich meat products replacing synthetic antioxidants such as BHA to inhibit lipid oxidation and improve shelf life. Interest in the development of peptides as an alternative antioxidant stems from studies in which a variety of food proteins, such as milk, soy, zein, canola, capelin, and porcine collagen, were found to inhibit lipid oxidation in commonly consumed foods and in *in vitro* models (Amarowicz & Shahidi, 1997; Cervato, Cazzola, & Cestaro, 1999; Chen, Muramoto, Yamauchi, & Nokihara, 1996; Cumby, Zhong, Naczk, & Shahidi, 2008; Li, Chen, Wang, Ji, & Wu, 2007). These antioxidant properties are believed to be attributed to unique sequences inherent in the protein macromolecular structure (Giovanna, Roberta, & Benvenuto, 1999; Wang, Fujimoto, Miyazawa, & Endo, 1991). For example, clear evidence of the antioxidant activities associated with specific peptides were demonstrated in oil-in-water emulsions, beef homogenates, liposomal suspensions, and β-carotene/linoleate model system (Diaz, Dunn, McClements, & Decker, 2003; Kong & Xiong, 2006; Sakanaka, Tachibana, Ishihara, & Juneja, 2005; Shahidi & Amarowicz, 1996). Certain hydroxyl- or sulphate-bearing amino acids such as Tyr, Met, His, Lys, and Trp have been shown to possess some degree of antioxidant properties (Chen et al., 1996; Saito et al., 2003). Furthermore, specific peptides have been reported to have enhanced antioxidant activities when compared to constituent amino acid mixtures (Guo, Kouzuma, & Yonekura, 2009). Consequently, it has been suggested that the synergistic effect of specific amino acid sequences in peptides, molecular structures, and functional groups are all major factor that contribute to the antioxidant potential of peptides.

A variety of methods have been employed with the purpose of generating potentially functional peptides. For example, food-derived peptides with biological activities are primarily produced through enzymatic hydrolysis, fermentation, and chemical or enzymatic synthesis (Gill, Lopez-Fandino, Jorba, & Vulfson, 1996;

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doi:10.1016/j.foodchem.2009.04.040

Hernandez-Ledesma, Davalos, Bartolome, & Amigo, 2005; Robert, Razaname, Mutter, & Juillerat, 2004; Shahidi & Zhong, 2008). Amongst these processes, enzymatic hydrolysis is often the method of choice in which common digestive enzymes are exploited, such as trypsin and pepsin, to generate peptides with potential antioxidant properties (Jiang, Chen, Ren, Luo, & Zeng, 2007; Kanda, Hisayasu, Abe, Katsura, & Mashimo, 2007). During hydrolysis the protease specificity is essential because it dictates the amino acid sequence of the resultant peptides and their biofunctional properties (Murray & FitzGerald, 2007). Use of commercial proteases of microbial or plant-origin has gained increased popularity due to their unique functions and cost-effectiveness. For instance, a commercially developed and marketed *Bacillus* protease enzyme, Protamex® by Novozyme, was used to generate catfish protein hydrolysates with notable antioxidant activities (Theodore, Raghavan, & Kristinsson, 2008). Furthermore, the plant derived papain enzyme has been attributed to utility in the production of a variety of protein hydrolysates with different functions in food science (Hicks, Onuorah, & Surjawan, 2000; Kong & Xiong, 2006). Additional enzyme hydrolysis research is merited due to the fact that there is a general recognition that the type of enzyme used in the hydrolysis process will impact the potency of bioactive properties such as antioxidant capacity.

In this study, three commercial microbial proteases, validase from *Aspergillus oryze*, alkaline protease from *Bacillus licheniformis*, and neutral protease from *Bacillus subtilis*, were utilised for the generation of functional peptides. The three selected proteases are commonly used for digesting food proteins but with considerably different substrate specification and reacting characteristics. The purpose of this study was to determine whether these commercial microbial proteases can, through hydrolysis of milk protein, generate functional antioxidant peptides as a strategy to improve food quality and extend shelf-life by inhibiting lipid oxidation. The milk protein hydrolysates were fractionated by sequential ultra-filtration. The acquired fractions were assessed for their antioxidant properties by various methods such as oxygen radical absorption capacity (ORAC), 1,1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\cdot}$) scavenging ability, and metal ion chelating activities. To test for a potential inhibitory effect on the lipid peroxidation process associated with the development of rancidity, off flavours, and shorter shelf life, fractions identified to have potent antioxidant activities were selected for further antioxidant assessment against lipid peroxidation in cooked ground beef by the thiobarbituric acid reactive substances (TBARS) method.

2. Material and methods

2.1. Materials

The milk protein isolate (MPI 5003) was obtained from Protient Inc. (St. Paul, MN, USA) with 90% purity on a dry basis. Protease Validase® FP concentrate (Val) and alkaline protease concentrate (AP) was kindly provided by Valley Research Corporate (South Bend, IN). Neutral protease (NP) was provided by Bio-CAT Inc (Troy, VA). Val is a fungal peptidase complex produced by submerged fermentation of a selected strain of *A. oryzae* and contains both endoprotease and exopeptidase activities. The AP is a serine alkaline protease prepared from *B. licheniformis* which contains endopeptidase activity. The NP is an extracellular endopeptidases produced from *B. subtilis*, these enzymes are active in the neutral pH range. These proteases were selected because they have been commonly used in industrial processes with high-quality thermostability. Fluorescein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). 2,2′-azobis(2-amino-propane) dihydrochloride (AAPH) was purchased from Wako Chemicals (Richmond, VA). All other chemicals and solvents were of analytical or HPLC-grade. The ultra-filtration system (Model No. 8050) and cellulose membranes for the preparation of protein hydrolysates were purchased from Millipore Co. (Billerica, MA).

2.2. Preparation of milk protein hydrolysates

For digestion of the milk protein isolate, three microbial proteases, Val, AP, and NP, were used according to the peptide guidelines from the manufacturer with slight modification. An amount of 50 g of the milk protein was homogenised in 500 mL water and hydrolysed by the individual proteases at a concentration of 2% (m/m, enzyme/substrate). The enzymatic hydrolysis was conducted for 6 h in a water bath at 55 °C under shaking. For Val, the reaction mixture was adjusted to pH 7.0 using 0.5 M NaOH or 0.6 M HCl during hydrolysis. For AP, the solution was adjusted to the optimum pH 10. The enzymatic reactions were terminated by boiling the mixtures for 5 min. Each mixture was centrifuged at 15,000*g* and the soluble fraction was then filtered using filtration paper (Whatman 4) for further fractionation.

2.3. Determination of the degree of hydrolysis (DH)

The DH of milk protein isolate by the three different proteases (AP, NP, and Val) was calculated by determining free amino groups after reacting with the trinitrobenzenesulphonic acid (TNBS) according to a previously reported protocol with modification (Adler-Nissen, 1979). The sample solution was mixed with 0.2 M sodium phosphate buffer (pH 8.2) and 0.1% TNBS, followed by incubation in the dark for 60 min at 50 °C. The reaction was quenched by adding 4.0 mL of 0.1 M HCl, and the absorbance was read at 340 nm. A 1.5 mM L-leucine solution was used as the standard. The DH was calculated by comparing the results to the standard curve for each particular protein substrate.

2.4. Fractionation of protein hydrolysates by ultrafiltration

The collected protein hydrolysates were ultra-filtered sequentially using a Millipore 8050 ultra-filtration unit through cellulose membranes with different molecular weight (MW) limits. Briefly, the hydrolysates were diluted with water and then ultra-filtered through a membrane with 10 kDa nominal molecular weight limit (NMWL) under 40 psi nitrogen gas. This process yielded two fractions: retentate (fraction 1, F1, represented hydrolysates > 10 kDa) and permeate (MW < 10 kDa). The permeate was further ultra-filtered through a 3 kDa NMWL membrane to obtain the second retentate (fraction 2, F2, represented hydrolysates between 3 and 10 kDa) and permeate. The second permeate was ultra-filtered through a 1 kDa NMWL membrane to yield the third retentate (fraction 3, F3, represented hydrolysates between 1 and 3 kDa) and permeate (fraction 4, F4, represented hydrolysates < 1 kDa). All retentates and permeates were lyophilised and stored at −20 °C until further analysis.

2.5. Measurement of oxygen radical absorbance capacity (ORAC)

The ORAC assay was conducted to kinetically measure the peroxyl radical scavenging activity of peptides with Trolox™ as the antioxidant standard according to the method reported previously (Moore et al., 2005). The Fluorescein was used as the fluorescent probe and the peroxyl radicals were generated from AAPH in 75 mM phosphate buffer (pH 7.4). The Trolox™ standards and samples were plated in duplicate to the inside wells of a black 96-well flat bottom plate. The fluorescence of the reaction mixture was

monitored and recorded every minute (λ_{ex} = 485 nm and λ_{em} = 535 nm) maintained at 37 °C until the reading had declined to less than 5% of the initial reading by a Victor^3 multilabel plate reader (Perkin–Elmer, Turku, Finland). Results for ORAC were determined by using a regression equation relating Trolox™ concentrations and the net area under the kinetic fluorescein decay curve. The ORAC value was expressed in micromoles of Trolox equivalents per gram dry weight milk protein (μmol TE/g).

2.6. Measurement of DPPH· scavenging activity

To test the DPPH· scavenging ability of milk samples a reaction mixture containing 100 μL of each sample and 100 μL of 0.208 mM DPPH solution were prepared. The initial absorption at 515 nm was determined immediately when the reaction was initiated by gentle shaking using a Victor^3 multilabel plate reader (Perkin–Elmer, Turku, Finland). For this kinetic assay each standard and sample were measured once every minute for 1.5 h in duplicate on a 96-well plate. The relative DPPH· scavenging ability for each sample was expressed as μmol of Trolox equivalents per gram of dry weight milk protein (μmol TE/g).

2.7. Measurement of metal chelating activity

The 2,2′-bipyridyl competition assay was conducted to measure the Fe^{2+} chelating activity of each fraction (Zhou, Yin, & Yu, 2005). The reaction mixture contained 0.1 mL of 1 mM $FeSO_4$ solution, 50 μL of fraction sample, 0.3 mL of 10% hydroxylamine-HCl, 0.4 mL of 2,2′-bipyridyl solution (0.1% in 0.2 M HCl), and 0.8 mL of Tris–HCl buffer (pH 7.4). The absorbance at 522 nm was measured and used to determine Fe^{2+} chelating activity using EDTA as the reference standard. The Fe^{2+} chelating activity was expressed as mg of EDTA equivalents per gram of dry weight milk protein (mg EDTA/g) in duplicate.

2.8. Preparation of ground beef with protein hydrolysates and cooked meat samples

The milk protein hydrolysate fractions with strong ORAC or DPPH· scavenging activities were further assessed for their antioxidant activity against lipid oxidation in ground beef. To accomplish this part of the study, beef trims were obtained from the Virginia Tech Meat Laboratory, ground through a 3.175 mm plate twice and mixed for 2 min at 4 °C. The ground beef sample was vacuum-packed and stored at −20 °C until further analysis.

To determine the impact of each milk hydrolysate fraction on lipid oxidation, 250 g of the prepared ground beef was homogenised with 25 mL of diluted solution for 2 min by a macro-ultrafine generator (Virtishear 225318, The Virtis Company Inc., Gardiner, NY). The homogenised samples were placed into 50 mL centrifuge tubes, weighed, and centrifuged at 100g for 10 min to remove trapped air. Next, the beef mixtures were cooked to an internal temperature of 71 °C in a water bath (Model 10l, Fisher Scientific, Pittsburgh, PA). The internal temperature was monitored by a needle-size thermocouple inserted into the thermal centre of the tubes during cooking. The cooked meat samples incorporated with protein hydrolysate fractions were re-weighed and the weight loss was calculated. After cooling to room temperature, the cooked meat samples homogenised with select hydrolysate fractions were placed in trays, covered with PVC film, and stored at 4 °C. The extent of lipid peroxidation in each meat sample was determined by the TBARS assay on days 1, 8, and 15 post cooking.

2.9. Lipid peroxidation determination by TBARS

The thiobarbituric acid reactive substances (TBARS) assay was used for screening and monitoring lipid peroxidation of the beef samples using a previously reported protocol with slight modification (O'Keefe & Wang, 2006; Shahidi & Hong, 1991). In brief, the 6-tetramethoxypropane (TMP) standard solutions were prepared at 0.0 (blank), 2.5, 5.0, 7.5, and 10.0 μM. An amount of 5 g of each meat sample, in triplicate, were homogenised with 50 mL of distilled water, 0.1 mL of 10% sodium dodecyl sulphate (SDS), and 10 mL of solution III (0.01% propyl gallate and 0.02% ethylenediaminetetraacetic acid, EDTA). Each homogenate was thoroughly stirred and 1.0 mL of homogenate was immediately transferred into a 15 mL centrifuge tube. The homogenate or the standard solution was further mixed with 4.0 mL of a solution containing 0.4% TBA, 0.5% SDS, and 9.3% acetic acid. The mixtures were heated in 95 °C in a water bath for 60 min under shaking. After cooling, 5.0 mL of pyridine/*n*-butanol (1:15) solution was added and mixed. Following centrifugation at 10 °C, the upper organic layer was collected and measured at 532 nm on a spectrophotometer (Model 21D Spectronic, Milton Roy Co., Rochester, NY). The final unit of TBARS value was calculated with the standard curve and expressed as mg of malonaldehyde (MDA) equivalents per kilogram of sample (mg MDA equivalent/kg).

2.10. Statistical analysis

The mean values within each test were compared by a two-sample *t*-test. Data is presented as mean ± SD (standard deviation). Difference was considered statistically significant when the *P* value was < 0.05 (SPSS for Windows, version 10.0.5., 1999). A two-tailed Pearson correlation test was conducted to determine the correlations amongst means.

3. Results and discussion

3.1. Generation and fractionation of milk protein hydrolysates and degree of hydrolysis

Protease digestion of milk protein isolate was conducted under the same conditions except using different optimum pH for each enzyme which included validase (Val) from *A. oryze*, neutral protease (NP) from *B. subtilis*, and alkaline protease (AP) from *B. licheniformis*. The degrees of hydrolysis (DH) value of the milk protein isolate were 27.9%, 35.5%, and 30.7% for NP, Val, and AP, respectively (Table 1). The result suggested that all three selected micro-

Table 1
The degree of hydrolysis (DH) by three microbial proteases and the resulting protein hydrolysate fractions after sequential ultrafiltration.

Proteases	DH (%)	F1 (g) (NMWL > 10 K)	F2 (g) (NMWL: 3–10 K)	F3 (g) (NMWL: 1–3 K)	F4 (g) (NMWL < 1 K)	Total (g)
NP	27.9	3.392	4.399	2.692	1.588	12.071
Val	35.5	4.59	2.326	4.827	5.206	16.949
AP	30.7	14.346	3.602	1.913	3.002	22.863

The digestive proteases included: validase from *Aspergillus oryze* (Hernandez-Ledesma et al., 2005), neutral protease from *Bacillus subtilis* (NP), and alkaline protease from *Bacillus licheniformis* (AP). NMWL refers to the nominal molecular weight limit.

bial proteases have notable affinity and digesting efficiency for milk protein with Val resulting in the highest activity. The DH values are remarkably higher when compared to biologically relevant digestive enzymes such pepsin and trypsin (data not shown). These findings indicate that commercial microbial proteases may provide a practical option for producing potentially functional peptides from milk protein. To accomplish the experiments, the soluble protein hydrolysates from each protease were fractionated into four fractions by sequential ultra-filtration with 10, 3, and 1 kDa NMWL membranes. This ultra-filtration technique has been used for separations of food protein hydrolysates because it is both cost-effective and can be readily scaled-up; however, it is acknowledged that the membrane separation based on molecular weight is not as precise as that of gel-filtration chromatography. For NP, a total of 12.1 g of water-soluble protein hydrolysates were recovered from 50 g milk protein isolate, of which, 4.3 g penetrated the 3 kDa NMWL membrane which was designated as small peptides (F3 and F4) with F4 containing free amino acids. These small peptide fractions accounted for 35.5% of total protein hydrolysates. The Val enzyme generated approximately 16.9 g soluble protein hydrolysates in which 59.2% of the hydrolysates were small peptides. Amongst the three microbial proteases investigated, the AP generated the largest amount of the protein hydrolysates (22.9 g). The majority of AP-derived hydrolysates were the retentates after filtration with 10 kDa NMWL membrane. In particular, amongst the three tested proteases, Val exhibited the highest degree of hydrolysis on milk protein and also produced the largest amount of small peptides under similar digestive conditions. However, this does not conclusively indicate that Val is superior compared to the other proteases for hydrolysing milk protein because the digesting conditions for each individual proteases were not optimised but rather conducted under the same conditions except in regards to the optimal pH of each enzyme. It is recognised that the hydrolysing conditions, including reaction temperature, incubation time, and substrate concentration, can significantly affect the efficiency of an individual protease for specific protein substrates.

3.2. ORAC activity of milk protein hydrolysates

The ORAC assay was used to assess the capacity of the twelve obtained protein hydrolysate fractions to quench the generated peroxyl radicals; the fractions with strong activity were then identified as potential antioxidant candidates for ensuing beef-model testing of lipid peroxidation. The ORAC values of each of the protein hydrolysate fractions varied significantly ranging from 62.9 to 246.6 μmol Trolox equivalents (TE) per gram dried weight, with the majority of hydrolysate fractions ranging from 95 to 145 μmol TE/g (Fig. 1). On average, the ORAC values of the protein hydrolysates with molecular weights between 1 and 10 kDa (i.e. F2 and F3 with 159.0 and 164.6 μmol TE/g, respectively) were higher than the hydrolysates greater than 10 k Dalton (F1) with 93.6 μmol TE/g and less than 1 kDa (F4) with 98.5 μmol TE/g. The F3 fraction prepared from Val provided the highest ORAC value of 246.6 μmol TE/g which was distinctly higher than the other protein hydrolysates. Interestingly, the F4 fraction prepared from the same protease Val had the lowest ORAC value (62.9 μmol TE/g) which was 74.5% lower than that of the Val-F3 fraction. These findings are similar with previous research which demonstrated that a variety of peptides, albeit primarily produced from pepsin and pancreatin, exhibited antioxidant potential in regards to their ORAC activities (Theodore et al., 2008). Overall, the ORAC results suggest a strong potential to generate specific peptides with potent antioxidant activity by using commercial microbial proteases.

3.3. DPPH· scavenging activity

DPPH· scavenging experiments were used as a second measure for assessing the antioxidant ability of the different milk hydrolysate fractions. Similar to the ORAC results, the DPPH· scavenging activities of the hydrolysate fractions varied significantly ranging between 3.1 and 35.4 μmol TE/g (Fig. 2). The Val-F2 fraction had the strongest activity against DPPH· followed by Val-F3 and NP-F3 with 32.6 and 28.6 μmol TE/g, respectively. The AP-F1 fraction had the lowest activity (3.1 μmol TE/g). Consistent with the ORAC results, the F2 and F3 fractions, on average, had the highest antiox-

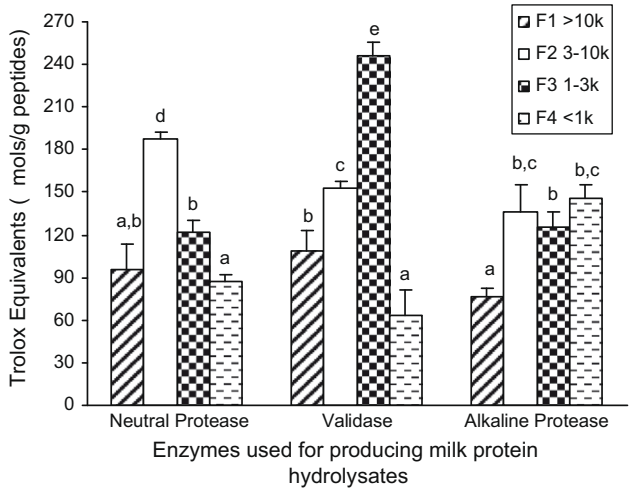

Fig. 1. Oxygen radical absorbance capacity (ORAC) values for milk hydrolysate fractions hydrolysed with three different microbial proteases. The digestion enzymes included: neutral protease (NP), validase (Val), and alkaline protease (AP). Each milk fraction of different molecular weights (F1, F2, F3, and F4 correspond to samples > 10, 3–10, 1–3, or <1 kDa, respectively) was tested in duplicate with ORAC values expressed in Trolox equivalents per gram of dry weight of milk hydrolysates (μmol TE/g). For each measurement, the data marked by different letters are significantly different ($P < 0.05$).

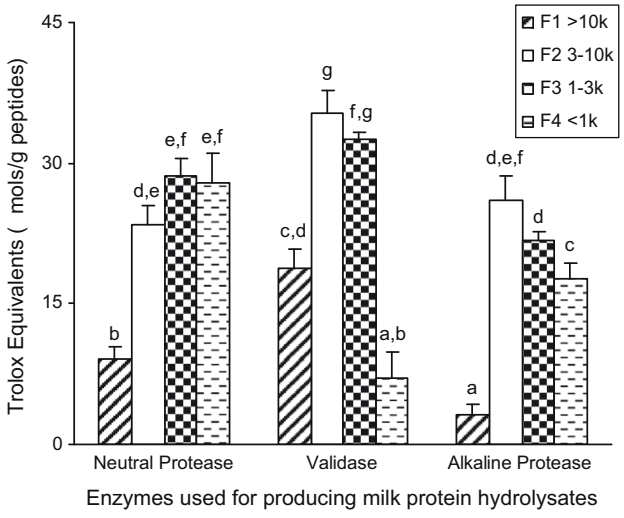

Fig. 2. DPPH· scavenging capacity for milk protein hydrolysate fractions hydrolysed with three different microbial proteases. The digestion enzymes included: neutral protease (NP), validase (Val), and alkaline protease (AP). Each milk fraction of different molecular weights (F1, F2, F3, and F4 correspond to samples > 10, 3–10, 1–3, or < 1 kDa, respectively) was tested in triplicate with the DPPH· scavenging ability expressed in Trolox equivalents per gram of dry weight of milk protein (μmol TE/g). For each measurement, the data marked by different letters are significantly different ($P < 0.05$).

idant activity as compared to the F1 and F4 milk hydrolysate fractions. However, there was no significant correlation detected between the ORAC and the DPPH· scavenging activities. This may be due to the possibility that the peptides were involved in separate redox scavenging mechanisms such as electron transfer (ET) whilst interacting with DPPH· radicals, whilst the ORAC assay was conducted based on hydrogen atom transfer (HAT) reactions. As such, a report of higher ORAC activity does not necessarily suggest a stronger DPPH· scavenging ability. The type of enzyme employed in protein hydrolysis has been reported to influence antioxidant potential. For example, Cumby and colleagues prepared canola protein hydrolysates using commercial enzymes (i.e. Alcalase and Flavourzyme) and reported that the hydrolysates exhibited notable antioxidant activity as determined by the DPPH radical scavenging capacity and reducing power assays (Cumby et al., 2008). The hydrolysate prepared using Flavourzyme exhibited higher antioxidant activity than those prepared using a combination of the two enzymes, or Alcalase alone. The authors suggested that the enzyme used for protein hydrolysis will impact antioxidant potential due in part to the mechanism of action of enzyme and subsequent presence or absence of functional groups such as hydroxyl groups on phenolic compounds (Cumby et al., 2008). Taken as a whole, our results and others indicate that the type of enzyme employed in the hydrolysis process will impact the antioxidant capacity of the hydrolysates.

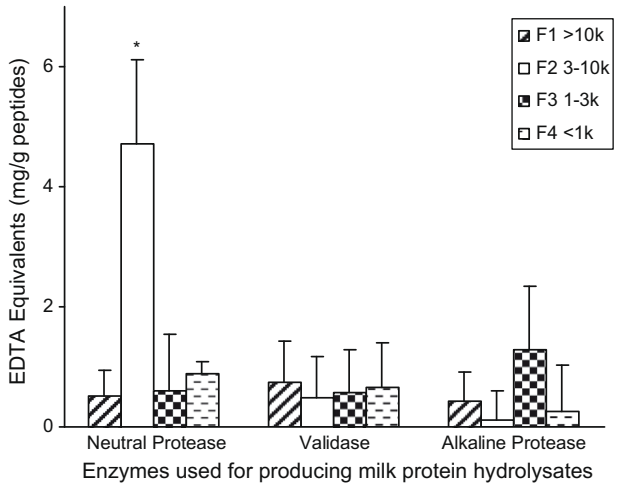

Fig. 3. Metal chelating activity for milk protein hydrolysate fractions hydrolysed with three different microbial proteases. neutral protease (NP), validase (Val), and alkaline protease (AP). Each milk fraction of different molecular weights (F1, F2, F3, and F4 correspond to samples > 10, 3–10, 1–3, or < 1 kDa, respectively) was tested in triplicate were the Fe^{2+} metal chelating activity was expressed as mg EDTA equivalents per gram of dry weight (mg EDTA/g). For each measurement, the data marked by different letters are significantly different ($P < 0.05$).

3.4. Metal chelating activity

Transitional metals are well-known promoters of lipid oxidation and their chelation facilitates in the delay of peroxidation and subsequently prevent food rancidity. Thus the assessment of metal chelating activity is an important measure of antioxidant properties. The ability of the protein hydrolysates to chelate transition metal ions was tested by assessing Fe^{2+} ion chelation with ethylenediaminetetraacetic acid (EDTA) as a positive control. The results showed that most hydrolysate fractions exerted weak chelating activities of 0.1–0.9 mg EDTA equivalents/g and there was no significant difference detected amongst these fractions except for the NP-F2 sample (Fig. 3). Under our experimental conditions, the NP-F2 was the only milk protein hydrolysate fraction that exerted a remarkably higher chelating activity (4.7 mg EDTA equivalents/g). Similar to these findings, previous studies have demonstrated that certain food-derived peptides such as caseinophospho peptides (CPP) and protein hydrolysates possess weak to moderate chelating activities (Diaz et al., 2003; Theodore et al., 2008). For example, the sunflower protein hydrolysates, after Alcalase and Flavourzyme digestion, were shown to inhibit beta-carotene oxidation induced by copper (Megias et al., 2007). Canola protein hydrolysates prepared using Alcalase and Flavourzyme also possessed significant DPPH· scavenging activities (Cumby et al., 2008). These results suggest that the NP-F2 sample warrants further investigation into the characterisation of its potentially active constituents involved in Fe^{2+} chelating activities. Once identified and analysed these particular peptides may have functional utility in emulsions and other lipid food applications in the food industry.

3.5. Lipid peroxidation in cooked beef

Lipid-rich products such as meat have been widely used as food models to evaluate the potential of functional agents in preventing or reducing lipid oxidation. For our study, cooked ground beef was prepared in order to determine whether peptides with notable antioxidant properties (as determined by ORAC, DPPH·, and Fe^{2+} chelating activities) can effectively inhibit lipid peroxidation in lipid-rich meat samples. After analysing the antioxidant and metal chelating data, three protein hydrolysate fractions, Val-F2, Val-F3, and NP-2, were selected for additional evaluation of their inhibitory activity on lipid oxidation in ground beef. The effects of the selected peptides on the lipid oxidation of the ground beef at different storage times are presented in Table 2. As reflected by the TBARS results, there was clear indication of lipid peroxidation in the beef samples which progressively increased as a function of storage time. For instance, at day 1, all the meat samples had experienced some degree of lipid peroxidation with the TBARS concentration varying from 7.6 to 11.4 mg MDA equivalents/kg of beef. Amongst the treatments with the selected hydrolysate mix-

Table 2
Lipid peroxidation determined by TBARS assay in cooked beef after treatments with the selected protein hydrolysate fractions at three different storage times post cooking.

Selected Fractions	Concentration in the meat (μg/g)	TBARS (mg/kg) at different storage time		
		Day 1	Day 8	Day 15
Control	0	11.35 ± 1.14	32.66 ± 1.74	44.49 ± 2.16
Val-F3	200	7.64* ± 1.24	21.71* ± 1.04	28.69* ± 1.03
Val-F3	800	9.57* ± 1.03	33.87 ± 1.67	41.82 ± 1.92
Val-F2	200	10.60 ± 0.47	31.29 ± 3.41	41.47 ± 1.35
Val-F2	800	10.79 ± 1.24	35.04 ± 0.48	40.54 ± 0.75
NP-F2	200	10.46 ± 0.86	32.55 ± 1.47	43.12 ± 1.83
NP-F2	800	11.01 ± 0.94	33.41 ± 1.24	41.13 ± 1.51

The final unit of TBARS value was calculated with the standard curve and expressed as mg of malonaldehyde equivalent per kilogram of sample (mg MDA equivalent/kg). The data marked by an asterisk is significantly different as compared to the control meat sample ($P < 0.05$).

tures, the treatment doses of 200 and 800 μg/g with the Val-F3 significantly ($P < 0.05$) reduced the meat lipid peroxidation at day 1. For the same measurements at day 1, the Val-F2 and NP-2 samples revealed a slight tendency to reduce lipid peroxidation in the meat but the effects did not achieve statistical significance. Interestingly, Val-F3 at a concentration of 200 μg/g appeared more effective in inhibiting oxidation than at a higher concentration of 800 μg/g. At days 8 and 15, the treatment doses of 200 μg/g with the Val-F3 sample continued to have a pronounced inhibitory effect on lipid oxidation in the meat in which the TBARS concentrations were significantly reduced by 33.5% and 35.6% compared to the control, respectively. However, at the storage day 8 and 15 under the 800 μg/g treatment condition, the Val-F3 sample did not enhance but rather reduced the inhibition on lipid oxidation of the meat. This result was reflected by a significantly higher TBARS values associated with the 800 μg/g treatment compared to 200 μg/g treatment condition. These observations indicate that Val-F3 was a more effective antioxidant at lower concentration. It is known that some antioxidants at high concentration may promote or may accelerate the rate of autoxidation (Pratt, 1996). For instance, previous studies have shown that vitamin E in the presence of transitional metals (Maiorino, Zamburlini, Roveri, & Ursini, 1993) and some phenolic compounds at higher concentration exerted pro-oxidant effects (Laughton, Halliwell, Evans, & Hoult, 1989; Sakihama, Cohen, Grace, & Yamasaki, 2002). Therefore, further experiments are needed in order to clarify and determine the appropriate dose of Val-F3 needed for improving the quality of meat-based products in regards to antioxidant activity without initiating an pro-oxidant scenario. In summary, the results demonstrate the usefulness of specific microbial proteases such as validase from *A. oryze* for generating potentially useful functional peptides with antioxidant application. Continued enzyme hydrolysis research efforts are warranted in order to development natural antioxidant peptides for utility in improving the quality and shelf-life of food products.

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Food Chemistry 117 (2009) 444–450

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Evaluation of the anti-inflammatory efficacy of *Lotus corniculatus*

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ARTICLE INFO

Article history:
Received 8 December 2008
Received in revised form 20 March 2009
Accepted 13 April 2009

Keywords:
Lotus corniculatus
Anti-inflammatory activity
Pleurisy
Mediators of inflammation
Mice

ABSTRACT

The anti-inflammatory effects of the crude extract (CE) of *Lotus corniculatus* var. São Gabriel and its derived hexane (HEX), ethyl acetate (AcOEt), *n*-butanol (BuOH) and aqueous (Aq) fractions and isolated compounds kaempferitrin, oleanolic acid and β-sitosterol, in a mouse model of pleurisy induced by carrageenan were investigated. Swiss mice were used in the *in vivo* experiments. The crude extract of *L. corniculatus* and its derived fractions, and also its isolated compounds, inhibited leukocytes, exudation, and myeloperoxidase (MPO) and adenosine-deaminase (ADA) activities, as well as nitrite/nitrate concentration and interleukin-1 beta (IL-1β) level ($p < 0.05$). *L. corniculatus* showed important anti-inflammatory activity by inhibition not only of leukocytes and/or exudation, but also of pro-inflammatory enzymes and mediators such as MPO, ADA, and IL-1β. The constituent's kaempferitrin, oleanolic acid and β-sitosterol may well account for it.

1. Introduction

Plants are a good source of useful anti-inflammatory agents. The continuing search for novel anti-inflammatory substances especially from plants with historically documented or pharmacological properties holds considerable nutraceutical and/or pharmaceutical promise (Kaplan et al., 2007). *Lotus corniculatus* var. São Gabriel (Fabaceae), also known as "Cornichão" in Brazil, is a plant used as a ruminant feed since it promotes an important increase in essential amino acid absorption (Waghorn, Ulyatt, John, & Fisher, 1987), ovulation rate, and production of milk protein and lactose (Wang, Douglas, Waghorn, Barry, & Foote, 1996). This herb is also used as an important anti-helmintic substance in these animals (Aerts, Barry, & McNabb, 1999).

Studies had demonstrated that these effects are due to the presence of secondary metabolites named proanthocyanidins, or condensed tannins (Xie & Dixon, 2005). The proanthocynidins also have important antioxidant (Kandil et al., 2002), immunomodulatory (Lin, Kuo, & Chou, 2002), analgesic and anti-inflammatory effects (Subarnas & Wagner, 2000).

Previous investigations into the chemical composition of this herb have identified the following compounds: flavonoids (kaempferol and quercetin) (Reynaud & Lussignol, 2005), oleanolic acid and saponins (Walter, 1961). Studies have also reported that kaempferol and quercetin modulate the inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), reactive C-protein (CRP), and nuclear factor kappa B (NF-κB) (García-Mediavilla et al., 2007). Other studies have shown that oleanolic acid and saponins possess anti-inflammatory, (Giner-Larza et al., 2001; Wang, Gao, Kou, Zhu, & Yu, 2008) and antitumoral properties (Chen et al., 2008; Wang, Zhang et al., 2008).

Motivated by rare studies of the anti-inflammatory properties of *L. corniculatus*, the crude extract and derived fractions of this herb was studied by analyzing its effects upon leukocyte migration, exudation concentration and myeloperoxidase/adenosine-deaminase activities, as well as nitrite/nitrate concentration and interleukin-1 beta levels, in the inflammation induced by carrageenan in the mouse model of pleurisy. We also isolated and identified the components from *L. corniculatus* that were responsible for the anti-inflammatory activity.

2. Material and methods

2.1. Plant material

L. corniculatus L. var. São Gabriel was collected in November 2006 in Lages, Santa Catarina State, Brazil. The material was identified by the botanist Prof. Dr. Daniel de Barcelos Falkenberg at the Department of Botany of the Federal University of Santa Catarina, Florianópolis, SC, Brazil. A voucher specimen was deposited in the Herbarium at the same university (FLOR 18.770).

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doi:10.1016/j.foodchem.2009.04.044

2.2. Preparation of plant extracts

The aerial parts of *L. corniculatus* var. São Gabriel were air-dried and protected from light at room temperature (25 °C) for one week. Subsequently the dried aerial parts (620 g) were grounded into particles (1.5 mm) using a knife mill (Mill TE-651, Tecnal, Piracicaba, SP, Brazil). The ground material was extracted with 5 L of ethanol 96% (plant 1:8, w/v) at room temperature. After two days, the extract obtained was filtered (Watmann, no. 1) and the ethanol was removed by rotavapor (Fisatom-802, São Paulo, SP, Brazil) at 55 °C under reduced pressure (460 mm Hg) (Vacuum Q-355A2, Quimis, Diadema, SP, Brazil). This procedure was repeated fifteen times in a period of one month to obtain the maximal yield of the crude extract (78 g).

The CE was fractionated by liquid–liquid extraction using solvents in growing order of polarity, resulting in hexane (HEX: 7.82 g), ethyl acetate (AcOEt: 11.4 g), *n*-butanol (BuOH: 5.24 g) and aqueous (Aq: 30.8 g) fractions.

2.3. Preliminary phytochemical analysis

A preliminary phytochemical screening of the crude extract of *L.* corniculatus was carried out according to Harborne (1998) to detect the presence of phenols, tannins, anthocyanins, anthocyanidins, flavonoids, xanthones, steroids, triterpenes and saponins. In these protocols we used the following reactions: the cyanidin test or Shinoda's test for flavonoids, anthocyanins and anthocyanidins; the Lieberman-Burchard reactions for sterols and triterpene; Stanishy's reaction to characterize the presence of condensed tannins and hydrolyzed tannins; the foam formation test to detect the presence of saponnins and finally for xanthones using chloridic acid and metallic magnesium, in which the appearance of red colour indicates the presence of xanthones (Harborne, 1998).

2.4. Isolation and identification of the compounds

The hexane fraction was subjected to silica gel column chromatography and eluted with a gradient of HEX/EtOAc, resulting in the isolation of two terpenoids: β-sitosterol as colourless crystals, m.p 137–139 °C, 76 mg, from fraction eluted with HEX/EtOAc (90:10, v/v), and oleanolic acid, as a white powder, m.p. 279–282 °C, 25 mg, from HEX/EtOAc (70:30, v/v). The chromatographic fractionation on silica gel of the EtOAc fraction afforded a crude flavonoid from EtOAc/EtOH (50:50, v/v) eluate that was further purified by flash chromatography using ethyl acetate/water/formic acid/acetic acid (70:20:3:2, v/v/v/v) as eluent, yielding 45 mg of kaempferitrin as a yellow needle crystals, m.p. 198.5–201.3 °C. The structures of the known compounds were identified by their spectroscopic data (^{1}H NMR, ^{13}C NMR (Varian AS-400 – Palo-Alto, CA, USA), and IR – Perkin Elmer FTIR 16 PC, Beaconsfield, England) measurement, comparison with spectral data obtained from the literature (Hung & Yen, 2001; Uurgoankar & Shaw, 2007), and co-TLC with authentic samples.

2.5. Structure elucidation of the compounds

The chemical structure of each isolated compound was determined on the basis of its physical characteristics, spectral data produced by infrared analysis (Perkin Elmer FTIR 16 PC, Beaconsfield, England) and nuclear magnetic resonance (^{1}H and ^{13}C NMR) recorded on a Varian AS-400 (Palo-Alto, CA, USA) spectrometer operating at 400 and 100 MHz, respectively. Thin Layer Chromatography (TLC) was carried out on silica gel 60 F_{254} plates (Macherey-Nagel, Düren, Germany). Finally, the structures of the compounds isolated were confirmed by comparing with reference data previously reported from available reliable sources.

2.6. Animals

Swiss mice, weighing 18–25 g, were housed under standardized conditions (room at constant temperature (22 ± 2 °C) with alternating 12 h periods of light and darkness), humidity 50–60%, and they were fed on a standard mouse diet with water *ad libitum* before use. This study was approved by the Committee for Ethics in Animal Research of the Federal University of Santa Catarina (Protocol number – PP00180), and experiments were performed in accordance with the norms of the Brazilian College of Animal Experimentation.

2.7. Experimental protocol

Initially, for analysis of the dose–response curve, different groups of animals were treated with different doses of crude extract (CE: 100–400 mg/kg) of *L. corniculatus* var. São Gabriel or its derived fractions or isolated compounds: hexane fraction (HEX: 50–200 mg/kg), ethyl acetate fraction (AcOEt: 100–400 mg/kg), *n*-butanol fraction (BuOH: 50–200 mg/kg), aqueous fraction (Aq: 25–200 mg/kg), kaempferitrin (50 and 100 mg/kg), oleanolic acid (10–100 mg/kg) or β-sitosterol (10–100 mg/kg) administered by intraperitoneal route (i.p.) 0.5 h before pleurisy induction by carrageenan (Cg 1%) that was administered by intrapleural route (i.pl.). In parallel, some animals received an injection of either sterile saline (NaCl, 0.9%) (negative – control group) or carrageenan (positive – control group) administered by intrapleural (i.pl.) route. After 4 h the animals were killed with an overdose of ether, the thorax was opened, and the pleural cavity was washed with 1.0 mL of sterile phosphate buffered saline (PBS) (pH 7.6), composition: NaCl (130 mmol), Na_2HPO_4 (5 mmol), KH_2PO_4 (1 mmol) and distillated water (1000 ml) containing heparin (20 IU/mL). Leukocytes and exudation were then evaluated.

In another set of experiments employed to establish the time course profile, different groups of animals were pre-treated with a single dose of CE (200 mg/kg), HEX (100 mg/kg), AcOEt (200 mg/kg), BuOH (100 mg/kg), Aq (100 mg/kg), kaempferitrim (100 mg/kg), oleanolic acid (50 mg/kg) or β-sitosterol (50 mg/kg) administered at different time points (0.5–4 h) and the same inflammatory parameters were evaluated 4 h after carrageenan administration.

After choosing the best dose and period of pre-treatment required for the crude extract of *L. corniculatus* and its derived fractions, as well as its isolated compounds, that inhibit leukocytes and/or exudation, different groups of animals were treated with CE (200 or 400 mg/kg), HEX (100 or 200 mg/kg), AcOEt (200 or 800 mg/kg), BuOH (100 mg/kg), Aq (50 or 400 mg/kg), kaempferitrin (100 mg/kg), oleanolic acid (50 mg/kg) or β-sitosterol (50 mg/kg) administered 0.5 h prior to pleurisy induction to analyze their effects upon myeloperoxidase (MPO) and adenosine-deaminase (ADA) activities, as well as nitrite/nitrate concentration (NO^x) and interleukin-1 beta (IL-1β) levels.

Dexamethasone (potent inhibitor of phospholipase A_2, of the expression of both induced NOS and of COX-2, among others, 0.5 mg/kg) and indomethacin (cyclooxygenase inhibitors, 5 mg/kg, i.p.) administered by intraperitoneal route (i.p.) 0.5 h before pleurisy induction, were used as anti-inflammatory drugs.

2.8. Quantification of leukocyte migration and exudation

After killing the animals, samples of the fluid leakage of the pleural cavity were collected to determine the total and differential leukocyte contents, and exudation. Total leukocyte counts were determined in a Newbauer chamber, and cytospin preparations of fluid leakage were stained with May-Grünwald-Giemsa for the differential count (Saleh, Calixto, & Medeiros, 1996). The degree

of exudation was determined by measuring the amount of Evans blue dye extravasation. Thus, in each experimental group, animals were challenged 0.5 h before the inflammation induction with a solution of Evans blue dye (25 mg/kg) administered by intravenous route (i.v.) in order to evaluate the exudation in the pleural cavity. On the day of the experiment, a batch of stored samples was thawed at room temperature and the amount of dye was estimated by colorimetric using an ELISA plate reader (Organon Teknika, Roseland, NJ, USA) at 620 nm, by interpolation from a standard curve of Evans blue dye in the range of 0.01–50 μg/mL.

2.9. Quantification of nitrite/nitrate concentration

Nitric oxide and its breakdown products nitrite (NO_2^-) and nitrate (NO_3^-) were measured using the Griess method (Green et al., 1982). Samples of exudates from the pleural cavity were collected, separated and stored at −20 °C. Nitrite/nitrate concentration was determined and the concentrations were estimated by means of colorimetric measurement at 450 nm on an ELISA plate reader (Organon, Teknika, Roseland, NY, USA) by interpolation from a standard curve (0–150 μM). The results were expressed as μM.

2.10. Quantification of myeloperoxidase activity

Standard samples with different concentrations of myeloperoxidase (from human neutrophils, Sigma: M6908, St. Louis, MO, USA) were prepared in order to obtain a standard curve in the range of 0.07–140 mU/mL. Pleural cavity fluid samples (40 μL) and standards were transferred to cuvettes and the reaction was initiated with the addition of 360 μL of assay buffer (0.167 mg/mL of *o*-dianisidine (3,3′-dimethoxybenzidine; fast blue B) dihydrochloride and 0.0005% H_2O_2). The reaction was stopped with sodium azide 1%. Afterwards, the samples were centrifuged at 50*g* for 5 min, the supernatants were separated, and the rates of changes in absorbance at 520 nm were determined. The mieloperoxidase activity was estimated by interpolation from the standard curve by means of colorimetric measurements on an ELISA plate reader (Organon Teknika, Roseland, NJ, USA) (Rao, Curie, Shaffer, & Isakson, 1993). The results were expressed as mU/mL.

2.11. Quantification of adenosine deaminase activity

Initially, standard samples (final volume of 500 μL) with different volume concentrations of $NaH_2PO_4{\cdot}H_20$ (35 mM), $Na_2HPO_4{\cdot}H_2O$ (15 mM) and NH_3SO_4 (15 mM) were prepared in order to obtain a standard curve in the range of 10–50 U/L. Pleural cavity fluid samples (20 μL) were transferred to cuvettes and the reaction was initiated by the addition of adenosine phosphate buffered solution (pH 6.5, 500 μL, composition: $NaH_2PO_4{\cdot}H_2O$ (35 mM), $Na_2HPO_4{\cdot}12H_2O$ (15 mM) and adenosine (0.5 mM)). After incubation for 1 h at 37 °C, the reaction was halted with the addition of a solution (1000 μL) of phenol (1 mM) and nitroprussiate (0.17 mM), plus alkaline buffer (1000 μL: NaCl: 11 mM). This solution (final volume 2000 μL) was also added to the cuvettes with the different standard samples. Afterwards, the rate of change in absorbance at 620 nm was determined. ADA activity was estimated by interpolation from the standard curve by means of colorimetric measurements on an ELISA plate reader (Organon Teknika, Roseland, NJ, USA) (Giusti & Galanti, 1984). The results were expressed as U/L.

2.12. Quantification of IL-1β levels

For analysis of IL-1β levels, samples of exudates were collected and immediately prepared for the analysis of cytokine levels. In this protocol, commercially available kits were used with monoclonal-specific antibodies for each cytokine. The cytokine level was measured by enzyme-linked immunosorbent assay (ELISA), using the kits according to the manufacturers' instructions. The range of values detected by this assay was: IL-1β (100–6400 pg/mL). The intra- and inter-assay coefficients of variation (CV) for IL-1β were: intra CV: IL-1β = 6.2 ± 0.4%; inter CV: IL-1β = 5.1 ± 0.6%, with a sensitivity value of IL-1β = 1.7 pg/mL. The cytokine concentration was estimated by means of colorimetric measurements at 450 nm on an ELISA plate reader (Organon Teknika, Roseland, NJ, USA) by interpolation from a standard curve.

2.13. Drugs

The following drugs and reagents were used: carrageenan (degree IV), human neutrophil myeloperoxidase, indomethacin, (Sigma Chemical Co., St. Louis, MO, USA), dexamethasone, (Ache pharmaceutical laboratories S.A., São Paulo, SP, Brazil), and Enzyme-linked immunosorbent assay (ELISA) for quantitative determination of rat IL-1β. Organic solvents: acetone, chloroform, *n*-hexane, ethyl acetate, *n*-butanol, methanol, and ethanol, all analytical grade, were purchased from Synth (Diadema, SP, Brazil). Other reagents used were also of analytical grade and were obtained from different commercial sources.

2.14. Statistical analysis

The data is reported as the mean ± SEM. Significant differences between groups were determined by two-way analysis of variance (ANOVA) followed by Student's-Newman-Keuls post-hoc tests, and the significant difference was set at $p < 0.05$.

3. Results

3.1. Phytochemical analysis

In this study, preliminary phytochemical analysis showed that the crude extract of *L. corniculatus* var. São Gabriel contained a significant amount of flavonoids, steroids and terpenoids. Representing the steroids and terpenoids classes, we isolated the β-sitosterol (compound **1**) (Fig. 1) and the oleanolic acid (compound **2**) (Fig. 2) from hexane fraction. In the ethyl acetate fraction, the flavonoid *O*-heteroside kaempferitrin (compound **3**) (Fig. 3) as representative of flavonoids was isolated. These compounds isolated from the specie *L. corniculatus* not reported previously to this variety. β-Sitosterol represented 2.08% and the oleanolic acid 0.67% of the hexane fraction. Further, the kaempferitrin represented 0.51% of the ethyl acetate fraction.

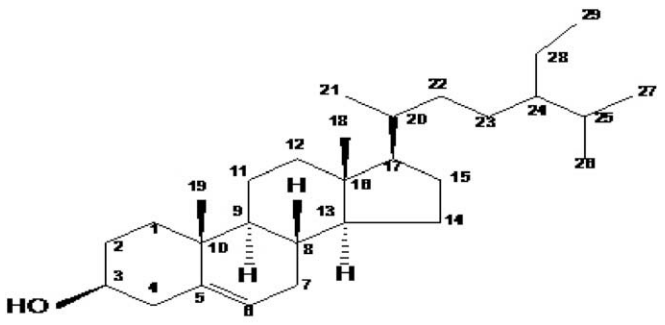

Fig. 1. The chemical structure of compound **1** (β-sitosterol) isolated from *n*-hexane fraction of *Lotus corniculatus* var. São Gabriel (Fabaceae) aerial parts.

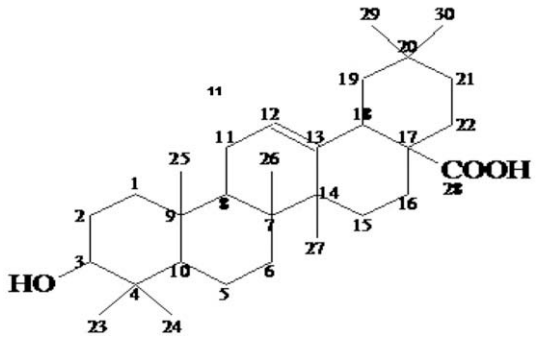

Fig. 2. The chemical structure of compound **2** (oleanolic acid) isolated from *n*-hexane fraction of *Lotus corniculatus* var. São Gabriel (Fabaceae) aerial parts.

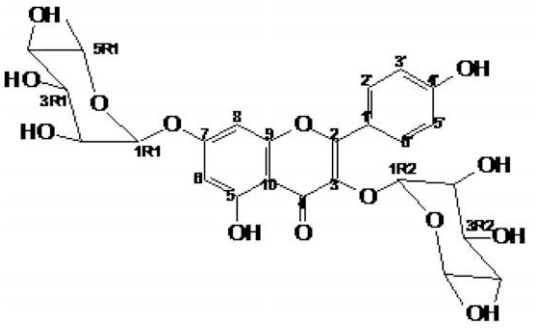

Fig. 3. The chemical structures of compound **3** (kaempferitrin) isolated from ethyl acetate fraction of *Lotus corniculatus* var. São Gabriel (Fabaceae) aerial parts.

3.2. Effects of crude the extract of L. corniculatus var. São Gabriel, its derived fractions and isolated compounds upon leukocyte migration and exudation

The crude extract of *L. corniculatus* (CE: 200 and 400 mg/kg) significantly decreased leukocyte migration from 38.7 ± 5.8% to 48.0 ± 7.7% ($p < 0.01$), neutrophils from 37.7 ± 5.2% to 46.4 ± 8.4% ($p < 0.01$), mononuclears from 44.2 ± 9.4% to 57.3 ± 11.6% ($p < 0.05$), and exudation from 25.4 ± 5.3% to 50.5 ± 2.2% ($p < 0.05$). CE (100 mg/kg) did not modify the studied inflammatory parameters ($p > 0.05$) (Table 1).

The hexane fraction (HEX: 100 and 200 mg/kg) significantly suppressed leukocytes from 26.1 ± 5.0% to 53.2 ± 12.5% ($p < 0.01$) and neutrophils from 21.8 ± 6.7% to 68.3 ± 17.3% ($p < 0.05$). HEX (100 mg/kg) also inhibited mononuclears by 45.5 ± 12.2% ($p < 0.05$) and at the dose of 200 mg/kg, HEX also inhibited exudation by 51.7 ± 12.0% ($p < 0.05$). The HEX fraction (50 mg/kg) did not alter any of the studied inflammatory parameters ($p > 0.05$) (Table 1).

The ethyl acetate fraction (AcOEt: 200 and 400 mg/kg) produced a significant inhibition of leukocyte migration from 21.0 ± 6.5% to 41.9 ± 8.7% ($p < 0.05$), neutrophils from 20.9 ± 7.4% to 56.1 ± 5.8% ($p < 0.05$) and exudation from 33.6 ± 6.6% to 34.8 ± 10.9% ($p < 0.05$). Nevertheless, the AcOEt fraction (100 mg/kg) did not inhibit these inflammatory parameters ($p > 0.05$). Mononuclears also were not inhibited by the AcOEt fraction ($p > 0.05$) (Table 1).

The *n*-butanol fraction (BuOH: 100 and 200 mg/kg) produced a significant decrease of leukocytes from 34.9 ± 9.1% to 39.2 ± 4.9% ($p < 0.01$) and neutrophils from 36.0 ± 5.8% to 36.3 ± 8.8% ($p < 0.01$). The BuOH fraction (200 mg/kg) also decreased mononuclears by 54.8 ± 6.2% ($p < 0.05$), and the dose of 50 mg/kg did not vary the leukocyte content ($p > 0.05$), but caused a significant enhancement of exudation by 50.7 ± 16.0% ($p < 0.05$) (Table 1).

The aqueous fraction (Aq: 50–200 mg/kg) caused a significant inhibition of leukocytes from 31.6 ± 8.1% to 43.0 ± 5.8% ($p < 0.05$), and neutrophils from 30.7 ± 8.5% to 44.2 ± 6.7% ($p < 0.01$), but failed to reduce mononuclears ($p > 0.05$). The Aq fraction (200 mg/kg) was also effective in inhibiting exudation by 44.7 ± 3.8% ($p < 0.05$). The doses of 25 and 50 mg/kg of this fraction increased exudation from 31.4 ± 12.3% to 38.2 ± 5.9% ($p < 0.05$). The Aq fraction (25 mg/kg) also did not inhibit total and differential leukocytes ($p > 0.05$) (Table 1).

The study of the effect of the isolated compounds showed that kaempferitrin, at the dose of 100 mg/kg, was also effective in sig-

Table 1
Effects of the crude extract of *Lotus corniculatus* and its derived fractions upon leukocyte migration and exudation in the inflammation induced by carrageenan in the mouse model of pleurisy.

Groups/doses (mg/kg)	Leukocytes ($\times 10^6$)	Neutrophils ($\times 10^6$)	Mononuclear cells ($\times 10^6$)	Exudation (μg/mL)
C[a]	6.20 ± 0.30	5.08 ± 0.30	1.12 ± 0.20	11.50 ± 1.00
CE 100[b]	5.77 ± 0.60	4.85 ± 0.50	0.91 ± 0.10	9.03 ± 0.50
CE 200[b]	3.80 ± 0.40**	3.17 ± 0.20**	0.63 ± 0.10*	8.58 ± 0.60*
CE 400[b]	3.21 ± 0.50**	2.73 ± 0.40**	0.48 ± 0.10**	5.70 ± 0.20**
HEX 50[b]	5.23 ± 1.20	4.60 ± 1.00	0.63 ± 0.20	8.91 ± 2.10
HEX 100[b]	4.58 ± 0.30**	3.97 ± 0.30*	0.61 ± 0.10*	8.85 ± 1.50
HEX 200[b]	2.90 ± 0.70**	1.62 ± 0.80**	1.28 ± 0.40	5.56 ± 1.40*
AcOEt 100[b]	6.97 ± 0.70	5.56 ± 0.90	1.49 ± 0.10	10.6 ± 1.80
AcOEt 200[b]	4.91 ± 0.40*	4.02 ± 0.30*	0.89 ± 0.10	7.64 ± 0.70*
AcOEt 400[b]	3.38 ± 0.70**	2.26 ± 0.40**	1.12 ± 0.40	7.11 ± 1.40*
BuOH 50[b]	7.04 ± 0.40	6.14 ± 0.40	0.90 ± 0.10	17.30 ± 1.80*
BuOH 100[b]	4.07 ± 0.50**	3.36 ± 0.50**	0.71 ± 0.09	9.59 ± 1.10
BuOH 200[b]	3.77 ± 0.30**	3.26 ± 0.30**	0.51 ± 0.07*	9.48 ± 0.60
Aq 25[b]	5.70 ± 0.30	5.01 ± 0.20	0.68 ± 0.10	15.90 ± 0.70*
Aq 50[b]	4.24 ± 0.50*	3.53 ± 0.40**	0.71 ± 0.10	15.10 ± 1.40*
Aq 100[b]	3.73 ± 0.80**	2.84 ± 0.60**	0.90 ± 0.20	10.40 ± 1.20
Aq 200[b]	3.52 ± 0.30**	2.83 ± 0.30**	0.68 ± 0.20	6.37 ± 0.40**
Dex 0.5[b]	1.75 ± 0.30**	1.33 ± 0.20**	0.44 ± 0.10**	6.51 ± 0.50**
Indo 5[b]	2.26 ± 0.30**	1.78 ± 0.20**	0.48 ± 0.20*	7.93 ± 0.50*

The crude extract (CE: 100–400 mg/kg) of *Lotus corniculatus* and its derived fractions, hexane fraction (HEX: 50–200 mg/kg), ethyl acetate fraction (AcOEt: 100–400 mg/kg), *n*-butanol fraction (BuOH: 50–200 mg/kg) or aqueous fraction (Aq: 25–200 mg/kg) administered 0.5 h before the pleurisy induction by carrageenan (1%). C = response in animals treated only with carrageenan. Dex = response in animals pre-treated with dexamethasone (0.5 mg/kg). Indo = response in animals pre-treated with indomethacin (5.0 mg/kg). *$p < 0.05$ and **$p < 0.01$. The data is reported as the mean ± SEM. a = administered by intrapleural route, b = administered by intraperitoneal route. $n = 5$ animals.

Table 2
Effects of the isolated compounds of *Lotus corniculatus* upon leukocytes migration and exudation in the inflammation induced by carrageenan in the mouse model of pleurisy.

Groups/doses (mg/kg)	Leukocytes ($\times 10^6$)	Neutrophils ($\times 10^6$)	Mononuclear cells ($\times 10^6$)	Exudation (μg/mL)
C[a]	6.20 ± 0.30	5.08 ± 0.30	1.12 ± 0.20	11.50 ± 1.00
Kaempferitrin 5[b]	7.26 ± 0.80	6.87 ± 0.70	0.39 ± 0.20*	7.68 ± 1.10*
Kaempferitrin 100[b]	4.00 ± 0.50*	3.37 ± 0.40*	0.63 ± 0.10*	7.25 ± 0.40*
Oleanolic acid 10[b]	6.15 ± 0.60	4.83 ± 0.40	1.32 ± 0.20	9.20 ± 1.10
Oleanolic acid 25[b]	4.88 ± 0.30*	3.66 ± 0.40*	1.23 ± 0.30	7.44 ± 0.20*
Oleanolic acid 50[b]	3.08 ± 0.60**	2.42 ± 0.60**	0.66 ± 0.10	6.61 ± 0.40*
Oleanolic acid 100[b]	2.95 ± 0.70**	1.45 ± 0.10**	1.50 ± 0.60	10.80 ± 1.20
β-Sitosterol 10[b]	5.63 ± 0.10	4.09 ± 0.60	1.55 ± 0.50	8.42 ± 0.90
β-Sitosterol 25[b]	5.27 ± 0.10*	4.11 ± 0.10*	1.16 ± 0.10	7.69 ± 0.20*
β-Sitosterol 50[b]	4.42 ± 0.70**	3.66 ± 0.70**	0.76 ± 0.10	8.26 ± 1.10*
β-Sitosterol 100[b]	4.10 ± 0.60**	3.22 ± 0.60**	0.88 ± 0.30	9.38 ± 1.70
Dex 0.5[b]	1.75 ± 0.30**	1.33 ± 0.20**	0.44 ± 0.10**	6.51 ± 0.50**
Indo 5[b]	2.26 ± 0.30**	1.78 ± 0.20**	0.48 ± 0.20*	7.93 ± 0.50*

Kaempferitrin (50 and 100 mg/kg), oleanolic acid (10–100 mg/kg) and β-sitosterol (10–100 mg/kg) isolated from *Lotus corniculatus* administered 0.5 h before the pleurisy induction by carrageenan (1%). C = response in animals treated only with carrageenan. Dex = response in animals pre-treated with dexamethasone (0.5 mg/kg). Indo = r-esponse in animals pre-treated with indomethacin (5.0 mg/kg). $^{*}p < 0.05$ and $^{**}p < 0.01$. The data is reported as the mean ± SEM. a = administered by intrapleural route, b = administered by intraperitoneal route. $n = 5$ animals.

nificantly inhibiting leukocytes by 35.5 ± 8.0% and neutrophils by 33.7 ± 8.8% ($p < 0.05$) (Table 2).This compound (50 and 100 mg/kg) also inhibited mononuclears by 65.2 ± 20.9% and 43.5 ± 5.7%, and exudation by 33.3 ± 9.8% and 37.0 ± 3.3% ($p < 0.05$) (Table 2).

The oleanolic acid (25–100 mg/kg) significantly suppressed leukocytes from 21.2 ± 5.8% to 52.4 ± 11.2% ($p < 0.05$) and neutrophils from 27.9 ± 7.5% to 71.6 ± 2.8% ($p < 0.05$). This compound failed to change mononuclears ($p > 0.05$). The dose of 10 mg/kg of this compound did not inhibit the inflammation caused by carrageenan ($p > 0.05$). Under the same conditions, oleanolic acid (25 and 50 mg/kg) reduced exudation levels from 35.3 ± 1.9% to 42.6 ± 3.9% ($p < 0.05$) (Table 2).

The β-sitosterol (25–100 mg/kg) significantly decreased leukocytes from 15.1 ± 1.9% to 33.9 ± 9.4% ($p < 0.05$) and neutrophils from 19.1 ± 2.9% to 36.7 ± 12.6% ($p < 0.05$). This compound did not modify mononuclears ($p > 0.05$), but at doses of 25 and 50 mg/kg it significantly inhibited the exudation from 28.2 ± 10.0% to 33.1 ± 2.3% ($p < 0.05$). The dose of 10 mg/kg did not vary the inflammatory parameters ($p > 0.05$) (Table 2).

The time course profile for the crude extract of *L. corniculatus* and its derived fractions, as well as its isolated compounds, showed that they were effective in inhibiting the studied inflammatory parameters when they were administered 0.5 h before carrageenan. It is important to note that the BuOH fraction (100 mg/kg), as well as the AcOEt fraction (200 mg/kg), had a long-lasting anti-inflammatory effect, since they were able to decrease the inflammation caused by carrageenan for up to 2 h of pre-treatment (results not shown).

As expected, dexamethasone (0.5 mg/kg, i.p.) and indomethacin (5.0 mg/kg, i.p.) significantly inhibited leukocytes by 71.7 ± 5.0% and 63.5 ± 5.0% ($p < 0.01$), neutrophils by 73.8 ± 4.2% and 64.9 ± 5.0% ($p < 0.01$), mononuclears by 60.7 ± 2.0% and 57.1 ± 1.0% ($p < 0.05$), and exudation by 43.3 ± 7.8% and 31.0 ± 5.0% ($p < 0.05$), in the inflammation response induced by carrageenan, respectively (Tables 1 and 2).

3.3. Effects of the crude extract of L. corniculatus var. São Gabriel, its derived fractions and isolated compounds upon myeloperoxidase and adenosine-deaminase activities

The pre-treatment (0.5 h) of animals with crude extract of *L. corniculatus* and its derived fractions, and also its isolated compounds, caused a significant decrease in myeloperoxidase (% of inhibition: CE (200 mg/kg): 63.7 ± 6.0, HEX (100 mg/kg):

Table 3
Effects of crude extract of *Lotus corniculatus*, its derived fractions and isolated compounds upon myeloperoxidase and adenosine-deaminase activities, and IL-1β levels in the inflammation induced by carrageenan in the mouse model of pleurisy.

Groups/doses (mg/kg)	MPO (mU/mL)	ADA (U/L)	IL-1β (pg/mL)
C[a]	334.00 ± 36.7	9.80 ± 0.30	1160.00 ± 119.00
CE 200[b]	121.00 ± 19.90**	3.20 ± 0.60**	871.00 ± 80.00*
HEX 100[b]	176.00 ± 38.80*	4.49 ± 1.40**	659.00 ± 100.00*
AcOEt 200[b]	141.00 ± 14.80**	0.63 ± 0.10**	732.00 ± 279.00*
BuOH 100[b]	303.00 ± 45.70	3.24 ± 0.80**	719.00 ± 200.00*
Aq 50[b]	281.00 ± 35.70	3.50 ± 0.80**	–
Aq 100[b]	220.00 ± 42.10*	3.21 ± 0.90**	744.00 ± 30.00*
Kampferitrin 100[b]	31.50 ± 11.30**	2.25 ± 0.70**	451.00 ± 160.00*
Oleanolic acid 50[b]	182.00 ± 72.20**	8.23 ± 3.70	1870.00 ± 229.00*
β-Sitosterol 50[b]	85.80 ± 56.30**	3.06 ± 0.60**	1040.00 ± 213.00
Dex 0.5[b]	135.80 ± 14.60**	2.80 ± 0.70**	516.00 ± 47.30*
Indo 5[b]	120.10 ± 13.70**	3.48 ± 0.10**	586.00 ± 20.00*

The crude extract (CE: 200 mg/kg) of *Lotus corniculatus* and its derived fractions, hexane fraction (HEX: 100 mg/kg), ethyl acetate fraction (AcOEt: 200 mg/kg), *n*-butanol fraction (BuOH: 100 mg/kg) or aqueous fraction (Aq: 50 or 100 mg/kg), and isolated compounds, kaempferitrin (100 mg/kg), oleanolic acid (50 mg/kg) and β-sitosterol (50 mg/kg) administered 0.5 h before the inflammation induction by carrageenan (1%). C = response in animals treated only with carrageenan. Dex = response in animals pre-treated with dexamethasone (0.5 mg/kg). Indo = response in animals pre-treated with indomethacin (5.0 mg/kg). $^{*}p < 0.05$ and $^{**}p < 0.01$. The data is reported as the mean ± SEM. a = administered by intrapleural route, b = administered by intraperitoneal route. $n = 5$ animals.

47.4 ± 11.6, AcOEt (200 mg/kg): 57.9 ± 4.4, Aq (100 mg/kg): 34.3 ± 12.6, kaempferitrin (100 mg/kg): 90.6 ± 3.4, oleanolic acid (50 mg/kg): 45.5 ± 21.6 and β-sitosterol (50 mg/kg): 73.0 ± 13.1) ($p < 0.05$), and adenosine-deaminase activities (% of inhibition: CE (200 mg/kg): 67.5 ± 6.8, HEX (100 mg/kg): 54.4 ± 15.0, AcOEt (200 mg/kg): 94.0 ± 1.4, BuOH (100 mg/kg): 67.1 ± 9.0, Aq (50 mg/kg): 64.5 ± 8.8, kaempferitrin (100 mg/kg): 77.1 ± 7.9, β-sitosterol (50 mg/kg): 68.7 ± 6.0) ($p < 0.01$), except for the BuOH fraction that did not inhibit MPO, and oleanolic acid that did not decrease ADA activities ($p > 0.05$) (Table 3).

Dexamethasone and indomethacin were effective in inhibiting myeloperoxidase by 59.4 ± 7.0% and 64.4 ± 7.0% ($p < 0.01$), and adenosine-deaminase activities by 71.4 ± 6.0% and 64.4 ± 6.0%, respectively ($p < 0.05$) (Table 3).

3.4. Effects of the crude extract of L. corniculatus var. São Gabriel, its derived fractions and isolated compounds upon IL-1 levels

The crude extract of *L. corniculatus* and its derived fractions, as well as its isolated compounds, caused a significant decrease of IL-1β (% of inhibition: CE (200 mg/kg): 24.9 ± 6.9, HEX (100 mg/kg): 43.2 ± 8.6, AcOEt (200 mg/kg): 36.9 ± 24.0, BuOH (100 mg/kg): 38.1 ± 17.2, Aq (100 mg/kg): 35.9 ± 2.6, and kaempferitrim (100 mg/kg): 61.1 ± 13.8) ($p < 0.05$). The oleanolic acid (50 mg/kg) caused a significant increase of the IL-1β levels by 61.4 ± 19.7% ($p < 0.05$), and β-sitosterol (50 mg/kg) did not alter the IL-1β level ($p > 0.05$) (Table 3).

Dexamethasone and indomethacin also significantly inhibited the IL-1β level by 55.5 ± 6.0% and 49.8 ± 6.0%, respectively ($p < 0.05$) (Table 3).

3.5. Effects of the crude extract of L. corniculatus var. São Gabriel, and its derived fractions and isolated compounds upon nitrite/nitrate concentration

Although the best dose of the crude extract of *L. corniculatus* and its derived fractions was determined, only higher doses caused a significant decrease of nitrite/nitrate concentration (% of inhibition: CE (400 mg/kg): 30.4 ± 5.6, HEX (200 mg/kg): 34.3 ± 9.9, AcOEt (800 mg/kg): 38.4 ± 5.4 and Aq (400 mg/kg): 44.1 ± 4.2) ($p < 0.05$). The isolated compounds also inhibited this inflammatory parameter (% of inhibition: kaempferitrin (100 mg/kg): 32.9 ± 4.2, oleanolic acid (50 mg/kg): 35.9 ± 5.6 and β-sitosterol (50 mg/kg): 35.2 ± 3.2) ($p < 0.05$). Dexamethasone and indomethacin pre-treatment of animals presented an inhibitory effect on nitrite/nitrate concentration by 75.6 ± 2.6% and 50.0 ± 1.3%, respectively ($p < 0.01$) (Table 4).

Table 4
Effects of the crude extract of *Lotus corniculatus*, its derived fractions and isolated compounds upon nitrite/nitrate concentration in the inflammation induced by carrageenan in the mouse model of pleurisy.

Groups/doses (mg/kg)	NO_x (μM)
C[a]	16.40 ± 1.50
CE 400[b]	11.40 ± 0.90*
HEX 200[b]	10.80 ± 1.60*
AcOEt 800[b]	10.10 ± 0.90**
BuOH 100[b]	14.90 ± 0.60
Aq 400[b]	9.16 ± 0.70**
Kaempferitrin 100[b]	11.0 ± 0.70*
Oleanolic acid 50[b]	10.5 ± 0.90*
β-Sitosterol 50[b]	10.6 ± 0.50*
Dex 0.5[b]	4.00 ± 0.76**
Indo 5[b]	8.21 ± 1.48**

The crude extract (CE: 400 mg/kg) of *Lotus corniculatus* and its derived fractions, hexane fraction (HEX: 200 mg/kg), ethyl acetate fraction (AcOEt: 800 mg/kg), *n*-butanol fraction (BuOH: 100 mg/kg), aqueous fraction (Aq: 400 mg/kg), and isolated compounds, kaempferitrin (100 mg/kg), oleanolic acid (50 mg/kg) and β-sitosterol (50 mg/kg) administered 0.5 h before the pleurisy induction by carrageenan (1%). C = response in animals treated only with carrageenan. Dex = response in animals pre-treated with dexamethasone (0.5 mg/kg). Indo = response in animals pre-treated with indomethacin (5.0 mg/k). * $p < 0.05$ and ** $p < 0.01$. The data is reported as the mean ± SEM. a = administered by intrapleural route, b = administered by intraperitoneal route. $n = 5$ animals.

4. Discussion

Data from this study indicates that the crude extract of *L. corniculatus* and its derived fractions had an important anti-inflammatory effect in a murine model of pleurisy. Although, different doses of *L. corniculatus* and its derived fractions were necessary to inhibits this inflammatory reaction. The results show that this herb exhibits a distinct inhibitory profile when compared to conventional drugs, such as indomethacin and dexamethasone.

The anti-inflammatory effect of *L. corniculatus* was more pronounced in relation to the inhibition of leukocytes and exudation. To understand the modulation of leukocytes by this herb, we also studied its effect upon MPO and ADA activities, which are known to be markers of activated neutrophils and mononuclears, respectively (Fröde & Medeiros, 2001). Our results demonstrated that the crude extract of *L. corniculatus* and its derived fractions significantly attenuated both MPO and ADA activities. These results reveled that *L. corniculatus* not only inhibited the leukocyte influx to the site of the inflammatory response, but also the activated leukocytes (Fröde & Medeiros, 2001).

The role of IL-1β in the inflammatory response is well known. The effect of *L. corniculatus* upon IL-1-β levels was also analyzed. In this experiment we observed a significant inhibition of this important mediator by *L. corniculatus* and its derived fractions.

Nitric oxide (NO) is another important pro-inflammatory substance that is released in the acute and chronic inflammatory response and is related to the exudation and cellular chemotaxis (Tripathi, Tripathi, Kashyap, & Singh, 2007). Once again, *L. corniculatus* showed an anti-inflammatory response, since the crude extract of this herb and its derived fractions, except for BuOH, caused a significant decrease of nitrite/nitrate concentration.

All the studied fractions isolated from *L. corniculatus* presented an important anti-inflammatory effect. The distinct biological effect may be linked to differences among chemical structures. This fact is observed mainly in the HEX and AcOEt fractions, which revealed a more pronounced anti-inflammatory response than the other fractions, since they were able to inhibit all the studied inflammatory parameters. On the contrary, BuOH did not inhibit either MPO activity or nitrite/nitrate concentration.

In an attempt to evaluate the anti-inflammatory potential of the isolated compounds from these fractions, first we isolated kaempferitrin from the AcOEt fraction, and β-sitosterol and oleanolic acid from the HEX fraction.

Likewise, we tested the effects of these compounds upon the same studied pro-inflammatory parameters. Our results revealed that all compounds inhibited leukocytes, neutrophils, exsudation, and MPO activity, as well as NO levels.

Although, β-sitosterol was a main compound isolated from *L. corniculatus*, kaempferitrin demonstrated a better anti-inflammatory effect since it was 3.8-fold, 1.5-fold and 1.3-fold more effective in inhibiting MPO, ADA and IL-1β levels, respectively, than indomethacin, and 4.3-fold and 1.2-fold more effective in inhibiting MPO and ADA activities than dexamethasone.

Similar results have also been observed with kaempferitrin that inhibits nitric oxide levels, as well as pro-inflammatory cytokines such as TNF-α and IL-12, in murine macrophages stimulated by LPS/IFN-γ (Fang, Rao, & Tzeng, 2005).

In relation to oleanolic acid, studies from literature have demonstrated the anti-inflammatory effect of this substance by reducing the paw edema induced by dextran, and also inhibiting the nitric oxide release via down-regulation of NF-κB in murine macrophage cells induced by LPS (Singh, Singh, Bani, Gupta, & Banerjee, 1992; Suh et al., 2007).

For β-sitosterol, studies have demonstrated important anti-inflammatory activity not only by inhibiting IL-12 from human Jukart T cells stimulated by concanavalin (ConA) or by phorbol-12-myristate-13-acetate plus ionomycin (PMA + IoM) (Aherne & O'Brien, 2008), but also by decreasing the eosinophil influx, mucus secretion, and IL-4/ IL-5 expression in a murine model of asthma induced by ovalbumin (Yuk et al., 2007), and the ear oedema induced by acetone in mice (Mavar-Manga et al., 2008).

In conclusion, *L. corniculatus* showed an important anti-inflammatory property, and its constituent's kaempferitrin, oleanolic acid and β-sitosterol may well account for it. These compounds have potential as novel lead compounds for the future development of therapeutic intervention in the treatment of patients with inflammatory disorders.

Acknowledgements

The authors thank MSc researcher MSc Ulisses de Arruda Córdova of EPAGRI (Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina S.A, Pesquisa, Estação Experimental de Lages) for the kind donation of *Lotus corniculatus* var. São Gabriel. We would also like to thank the Coordenação de Aperfeiçoamento Docente CAPES, Brazil, for financial support.

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Food Chemistry 117 (2009) 451–455

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Characterisation of alkylphenols in pistachio (*Pistacia vera* L.) kernels

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ARTICLE INFO

Article history:
Received 18 November 2008
Received in revised form 31 March 2009
Accepted 14 April 2009

Keywords:
Pistachio
Gas chromatography
Mass spectrometry
3-Alkylphenols

ABSTRACT

Pistachio kernels were extracted with *n*-hexane and the oil obtained was saponified with methanolic KOH. The extracted unsaponifiable matter was fractionated on Thin Layer Chromatography (TLC) plates. The band with $R_f = 0.47$, analysed by Gas Chromatography–Mass Spectrometry (GC–MS), showed the presence of long-chain alkylphenols: this is the first time that alkylphenols are reported in pistachio kernels. To elucidate the structures, Nuclear Magnetic Resonance (NMR) and GC–MS analyses (on the trimethylsilyl derivatives) were used. Sixteen different 3-alkylphenols (cardanols) with a saturated, monounsaturated and diunsaturated chain were detected. A reaction with dimethyl disulphide was successfully used to determine the double bond position in the monounsaturated compounds. Three phenols were new natural compounds. The most abundant cardanols were 3-(8-pentadecenyl)-phenol, 3-(10-pentadecenyl)-phenol, 3-pentadecyl-phenol and 3-(10-eptadecenyl)-phenol. Total amount of cardanols in the oils (mean of five samples) was roughly evaluated as 440 ± 95 mg kg^{-1}.

1. Introduction

Pistachio (*Pistacia vera* L.) is a plant from the Anacardiaceae family. The pistachio nut is mainly cultivated in Iran, Turkey, USA, Greece and Syria; in Italy it is almost exclusively cultivated in Sicily, near the volcano Etna, in the area of Bronte (FAO, 2005). Pistachio seeds are usually used as ingredients in the food industry or roasted and consumed as snack food.

Chemical studies on pistachio kernels mainly regarded the fatty acids and sterols content (Arena, Campisi, Fallico, & Maccarone, 2007; Chahed et al., 2006; Chahed et al., 2008; Daneshared & Aynehchi, 1980; Okay, 2002; Phillips, Ruggio, & Ashraf-Khorassani, 2005; Ryan, Galvin, O'Connor, Maguire, & O'Brien, 2006; Satil, Azcan, & Baser, 2003; Venkatachalam & Sathe, 2006; Yildiz, Turcan Gurcan, & Ozdemir, 1998); other studies concern the kernel pigments characterisation (Bellomo & Fallico, 2007; Giuffrida, Saitta, La Torre, Bombaci, & Dugo, 2006; Wu & Prior, 2005), the resveratrol recovery in seeds (Gentile et al., 2007; Grippi et al., 2008; Tokusoglu, Unal, & Yemis, 2005) and the presence of anacardic acids in the outer green shell (Yalpani & Tyman, 1983).

Several reports, conducted on other Anarcadiaceae, especially on the cashew (*Anacardium occidentale* L.) demonstrated that phenolic compounds such as anacardic acids (6-alkylsalicylic acids), cardols (5-alkylresorcinols) and cardanols (3-alkylphenols) with 15 carbon atoms in the side chain, saturated and unsaturated, were found on the external part of the hull (Paramashivappa, Phani Kumar, Vithayathil, & Srinavasa Rao, 2001; Phani Kumar, Paramashivappa, Vithayathil, Subba Rao, & Srinivasa Rao, 2002; Strocchi & Lercker, 1979; Trevisan et al., 2006; Tyman, Tychopoulos, & Colenutt 1981). The cardanols were also found in other vegetable species (Alen et al., 2000; Du, 1990; Franke, Masaoud, & Schmidt, 2001; Pramono, Gleye, Moulis, Debray, & Stanislas, 1985; Tan, Shen, Wang, & Yu, 2001).

Members of the Anacardiaceae characteristically produce a series of compounds based on unsaturated fatty acid precursors. Biosynthetically, usually the CoA ester of palmitoleic acid serves as a precursor of this group of compounds by chain elongation with three acetate groups; the side chain structure of active compounds can be mono-, di-, or triunsaturated, suggesting that a number of unsaturated acyl-CoA starter units can also be employed (Dewick, 1997). Some pure compounds are very active human allergens (Dewick, 1997).

Natural phenols are compounds with high antioxidant activity (Moure et al., 2001); this activity was studied on the phenolic compounds of pistachio hull extracts (Goli, Barzegar, & Sahari, 2005) and on the alkyl phenols of the cashew (Trevisan et al., 2006). Other studies about the Anacardiaceae phenols regarded the cytotoxic activity of anacardic acids and cardols against both BT-20 breast and HeLa epithelioid cervix carcinoma cells (Kubo, Ochi, Vieira, & Komatsu, 1993) and the antinematodal properties of some cardanols respect to the pine wood nematodes *Bursaphelenchus xylophilus* (Alen et al., 2000).

To the best of our knowledge, in the literature, studies about the identification of alkylphenols in pistachio kernels are not present; during our research on the composition of the unsaponifiable matter of pistachio oils, we found the presence of long-chain alkylphenols: in this paper we report the recovery of 3-alkylphenols (cardanols) and their characterisation.

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.04.043

2. Materials and methods

2.1. Pistachio seeds

Five fresh commercial samples of shelled pistachio (50–100 g each) were used. Two samples were from Bronte (Italy), while other labels did not specify the provenience.

2.2. Reference compounds

Solvents and reagents were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). Bis(trimethylsilyl)trifluoroacetamide and trimetylchlorosilane (BSTFA:TMCS 99:1) were purchased from Supelco (Bellefonte, Pennsylvania, USA).

2.3. Extraction of pistachio oils

Pistachio seeds were accurately ground with a homogenizer and then 30 g were weighed and extracted with 150 mL of *n*-hexane in a screw-cap flask, in the dark with magnetic stirring, at room temperature for 2 h. Hexane was separated by settling and the residue was extracted again with 150 mL of fresh *n*-hexane. A total of five extractions were carried out; to the combined solvent 5 g of anhydrous Na_2SO_4 were added and then *n*-hexane was filtered. The solvent was removed under vacuum at room temperature; the oil obtained was flushed with a stream of dry nitrogen to volatilise the solvent residues and then was weighed. All the oil samples were stored at −5 °C before the analysis. The oils yields obtained were in the range 46.2–52% (the mean of the five samples was 48.5 ± 2.3%).

2.4. Extraction of 3-alkylphenols

Five grams of pistachio oil were saponified with 50 mL of 2 N KOH in methanol, refluxing for 60 min. After cooling, 100 mL of distilled water were then added and the unsaponifiable matter was extracted three times in a separatory funnel with diethyl ether (75 + 50 + 50 mL). The combined organic layers were washed with distilled water (50 mL) and the obtained ethereal extract was filtered on anhydrous Na_2SO_4. Then the solvent was removed under vacuum at room temperature. The obtained unsaponifiable matter was fractionated by using Thin Layer Chromatography (TLC). The plates (20 × 20 cm) were layered with a slurry of 12 g of silica gel G in 25 mL of 2 N NaOH and then activated by heating at 110 °C for 90 min. Diethyl ether solutions of the unsaponifiable were put on the plates using a 100 μl syringe. Elution was performed with 100 mL of *n*-hexane:diethyl ether (60:40) in a glass developing chamber (27.0 x 26.5 x 7.0 cm), using two plates at a time. Elution time was about 45 min. Plates were then sprayed with a 0.2% (w/v) ethanolic solution of 2,7-dichlorofluorescein to highlight the bands under an UV source (366 nm). The band with R_f = 0.47 was separated and extracted from the silica gel with hot chloroform (3 + 2 + 2 mL). After removing the chloroform under vacuum, the residue was dissolved in 0.2 mL of *n*-hexane and then analysed by GC–MS.

2.5. Derivatization of 3-alkylphenols

The residues were also analyzed after derivatization by using 0.2 mL of bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (BSTFA–TMCS 99:1) at room temperature, for 30 min. Trimethylsilyl derivatives were analyzed by GC–MS.

For double bond location, dimethyldisulphide derivatives (Christie, 1997) were prepared as follows: to 2 mg of cardanols, 0.5 mL of dimethyldisulphide and 0.1 mL of iodine solution (60 mg/mL in diethyl ether) were added; the reaction mixture was stirred at room temperature for 24 h and then 5 mL of *n*-hexane were added and two washings with 5 mL of $Na_2S_2O_3$ 0.01 N were done. The organic phase was separated, filtered on anhydrous Na_2SO_4 and the solvent was removed under vacuum at room temperature. Hydroxyl groups were then silylated with BSTFA–TMCS as previously reported and the dimethyldisulphide–trimethylsilyl derivatives were analyzed by GC–MS.

2.6. GC analysis

Analysis was carried out using a Finnigan MAT GCQ GC–MS system equipped with a splitless-split injector and a Restek RTX-5MS column (30 m, 0.25 mm i. d., 0.25 μm film thickness).

Underivatized samples were analyzed in the following oven conditions: initial temperature 60 °C (2 min isothermal), from 60 to 275 °C at 21.5 °C/min and then isothermal for 33 min.

For the trimethylsilyl derivatives analysis, the oven temperature was programmed as follows: initial temperature 60 °C, from 60 to 150 °C at 15 °C/min, from 150 to 275 at 5 °C/min and then isothermal for 24 min.

Injector was set at 250 °C, transfer line at 275 °C and ion source at 200 °C. Injections were performed with a 60 s splitless with He as carrier gas, at 40 cm/s constant rate. The mass spectrometer was used in full scan EI mode (70 eV), from 40 to 800 amu, 1 spec/s.

2.7. NMR analysis

^{1}H NMR spectra were taken on a Varian Gemini 300 (300 MHz) in $CDCl_3$ using tetramethylsilane (TMS) as an internal standard. All the signal were referenced to TMS to within ± 0.01 ppm.

3. Results and discussion

The analyses carried out on the hexanic solution of the band with R_f = 0.47 showed chromatograms with a tailing peak (main compound) and some very little ones (Fig. 1). The mass spectrum of the main compound had base peak at *m/z* 108 and hypotethic molecular ion at *m/z* 302 (Fig. 2). The ions at *m/z* 77, 91 and 108 suggested the presence of a benzene ring with a phenolic hydroxyl and the ions at *m/z* 133, 147, 161, 175 and 206, 220, 234 suggested the presence of an alkyl chain. The molecular weight of 302 could be compatible with the structure of a phenol with a monounsaturated C-15 chain.

To evaluate this hypothesis, 25 mg of the band were collected and analyzed by ^{1}H NMR spectroscopy. The regiochemistry for

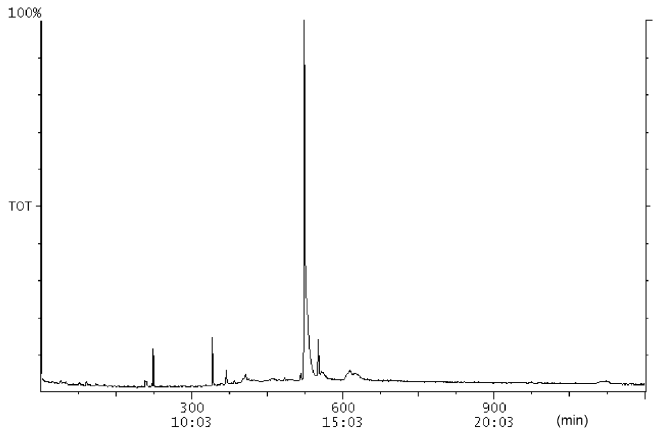

Fig. 1. Chromatogram of the extract of the band with R_f = 0.47.

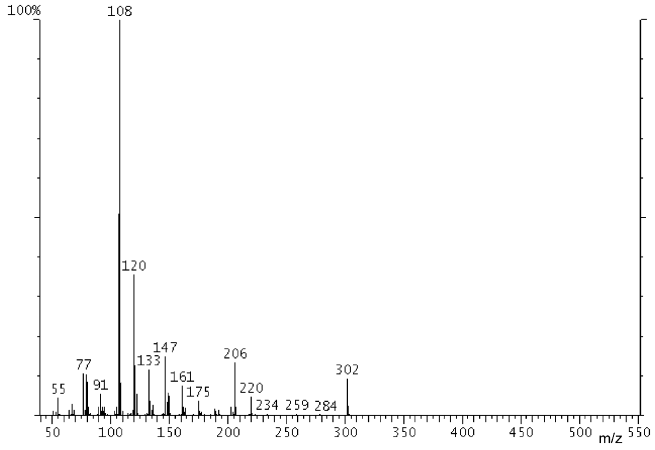

Fig. 2. Mass spectrum of the main compound.

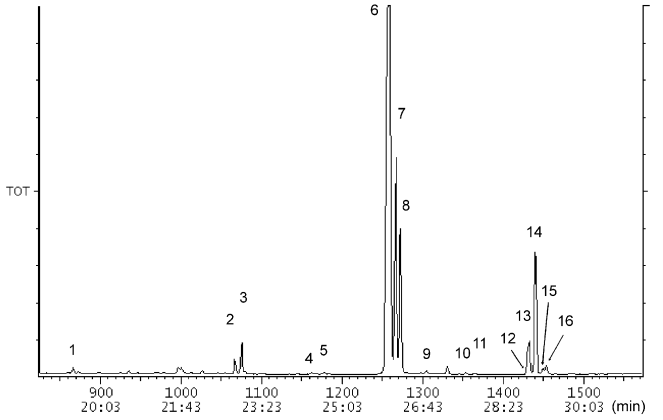

Fig. 3. Chromatogram of the alkylphenols (trimethylsilyl derivatives) of a pistachio sample. Peaks identification as in Table 3.

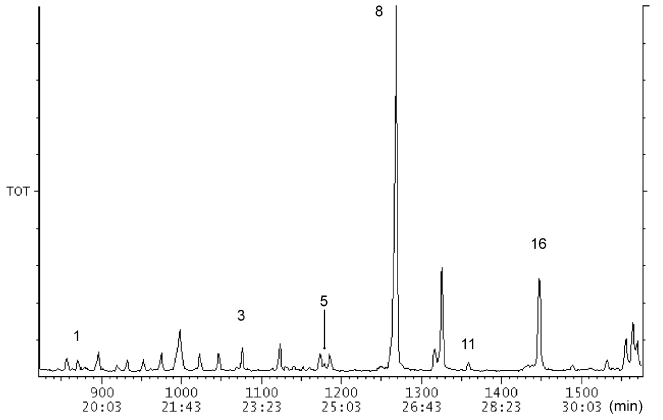

Fig. 4. Chromatogram of the alkylphenols (dimethyldisulphide–trimethylsilyl derivatives) of a pistachio sample (Part 1). Peaks identification as in Table 3.

the main compound was evident from its ^{1}H NMR spectrum which contained an aromatic pattern (4H, s, t and two d, all of equal intensity between 7.26 and 7.83 δ) distinctive of 1,3 substitution. The configuration of the double bond along the aliphatic chain was deduced from the coupling of the double bond protons; in fact, the characteristic resonance for these protons (2H, *m*, δ 5.96–5.92, J_{HH} = 10.4 Hz) points out a Z configuration of the double bond along the aliphatic chain. All the other ^{1}H NMR peaks [δ 3.18 (2H, *t*, –CH_2Ar), 2.47–2.68 (6H, *m*, –CH_2–), 2.12–2.26 (6H, *m*, –CH_2–), 1.80–2.01 (10H, *m*, –CH_2–), 1.44 (3H, *t*, –CH_3)], are consistent with the presence of a long aliphatic chain. Additionally, a characteristic feature of ^{1}H NMR spectrum was the appearance of the signal indicating the presence of a hydroxyl group [δ 5.29 (1H, br, OH)], just evidenced through the mass spectroscopy.

Literature data agree that long-chain alkylphenols recovered in plant materials are 3-alkylphenols and that the configuration of the double bond of the unsaturated compounds is Z (Alen et al., 2000; Paramashivappa et al., 2001; Phani Kumar et al., 2002; Tan et al., 2001).

Then, a GC–MS analysis on the trimethylsilyl derivatives was carried out to improve the separation; thus, the tailing peak of Fig. 1 was splitted in three narrow peaks (Fig. 3, peaks 6, 7 and 8) and the chromatograms obtained showed the presence of sixteen different alkylphenols. In Table 1 is reported a summary of the mass spectrometric characteristics of these compounds.

Molecular weights suggested the presence of alkylic chains from C11 to C17, mainly odd and monounsaturated; some compounds had the same molecular weight (peaks 6–7 and peaks 13–14–15), but the spectral characteristics did not allow a characterisation because there were not evident differences (Table 1). Fragmentation did not permit to determine the double bond positions, so the analysis of the dimethyldisulphide–trimethylsilyl derivatives was attempted. The dimethyldisulphide derivatives were chosen because they are easy to prepare, the increase in the molecular weights is smaller than the classic hydroxylation–trimethylsilylation, the reaction involves simple addition of dimethyl disulphide across the double bond and several applications have been described for the structural elucidation of fatty acids by

Table 1
GC–MS data of the trimethylsilyl derivatives of the cardanols in pistachio oil.

Peak	Retention time (min)	$[M]^{+\cdot}$, *m/z* (%)	Major fragment ions, *m/z* (%)
(1)	19:30	320 (18)	222 (7), 180 (100)
(2)	22:51	346 (11)	264 (4), 250 (9), 180 (100)
(3)	23:01	348 (22)	222 (8), 180 (100)
(4)	24:25	360 (14)	278 (6), 264 (9), 180 (100)
(5)	24:39	362 (21)	180 (100)
(6)	25:56	374 (23)	292 (6), 278 (13), 222 (5), 180 (100)
(7)	26:07	374 (17)	292 (4), 278 (10), 222 (5), 180 (100)
(8)	26:15	376 (32)	222 (8), 180 (100)
(9)	26:46	372 (30)	273 (7), 260 (5), 246 (13), 219 (21), 205 (15), 180 (100)
(10)	27:37	388 (15)	292 (9), 180 (100)
(11)	27:46	390 (20)	180 (100)
(12)	28:52	400 (25)	287 (4), 273 (10), 260 (5), 246 (13), 232 (8), 219 (16), 205 (13), 180 (100)
(13)	28:54	402 (20)	320 (4), 306 (11), 222 (5), 180 (100)
(14)	29:03	402 (21)	320 (4), 306 (11), 222 (5), 180 (100)
(15)	29:11	402 (18)	320 (5), 306 (10), 222 (5), 180 (100)
(16)	29:16	404 (38)	222 (9), 180 (100)

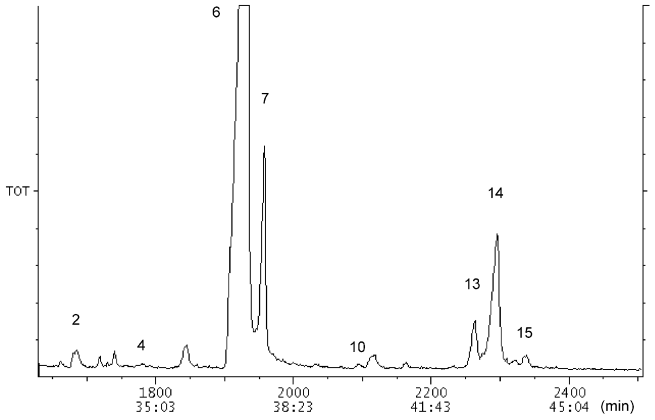

Fig. 5. Chromatogram of the alkylphenols (dimethyldisulphide–trimethylsilyl derivatives) of a pistachio sample (Part 2). Peaks identification as in Table 3.

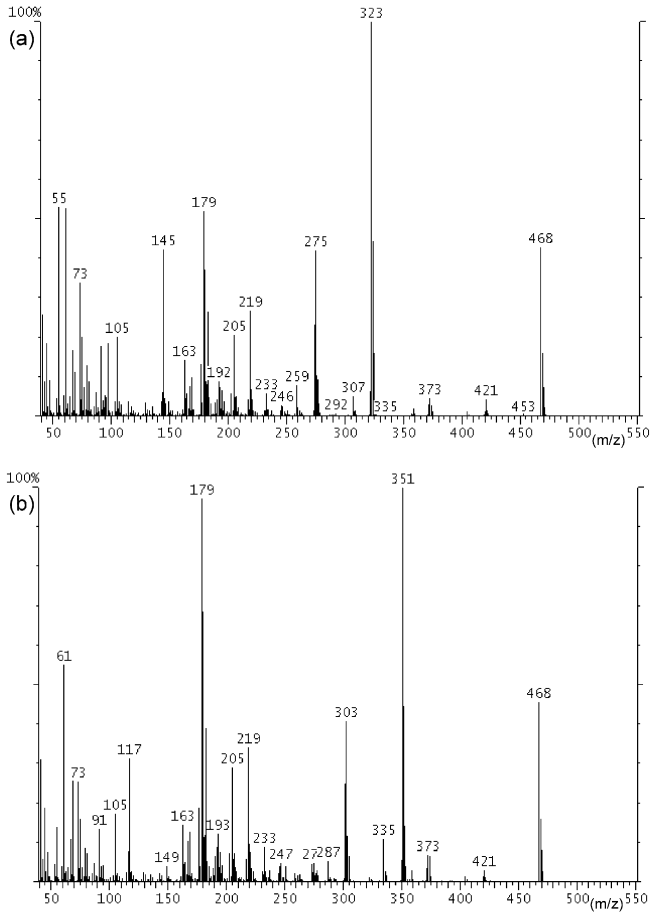

Fig. 6. (a) Mass spectrum of peak 6 and (b) mass spectrum of peak 7 (dimethyldisulphide–trimethylsilyl derivatives).

Table 3
Percentage composition of the cardanols in pistachio oil (five samples).

Peak	Compound	Range (%)	Mean ± SD (%)
(1)	3-Undecyl phenol	0.19–0.32	0.26 ± 0.02
(2)	3-(8-Tridecenyl) phenol	0.52–0.74	0.62 ± 0.10
(3)	3-Tridecyl phenol	1.20–1.53	1.35 ± 0.13
(4)	3-(8-Tetradecenyl) phenol	0.06–0.12	0.09 ± 0.02
(5)	3-Tetradecyl phenol	0.05–0.08	0.06 ± 0.01
(6)	3-(8-Pentadecenyl) phenol	66.37–68.91	67.38 ± 1.02
(7)	3-(10-Pentadecenyl) phenol	9.18–11.56	10.59 ± 1.20
(8)	3-Pentadecyl phenol	7.58–10.22	8.65 ± 1.04
(9)	3-(*x*,*y*-Pentadecadienyl) phenol	0.14–0.21	0.17 ± 0.03
(10)	3-(10-Hexadecenyl) phenol	0.03–0.06	0.04 ± 0.01
(11)	3-Hexadecyl phenol	0.02–0.03	0.02 ± 0.00
(12)	3-(*x*,*y*-Heptadecadienyl) phenol	0.75–1.18	0.99 ± 0.18
(13)	3-(8-Heptadecenyl) phenol	1.60–1,99	1.82 ± 0.17
(14)	3-(10-Heptadecenyl) phenol	6.62–7.28	6.95 ± 0.25
(15)	3-(12-Heptadecenyl) phenol	0.33–0.50	0.40 ± 0.07
(16)	3-Heptadecyl phenol	0.58–0.66	0.61 ± 0.01

mass spectrometry, in particular for monoenoic ones (Christie, 1997).

Fig. 4 reports the first part of a chromatogram of the dimethyldisulphide–trimethylsilyl derivatives. In this figure, the peaks 1, 3, 5, 8, 11 and 16 were saturated compounds, and no differences in retention times and spectra (as reported in Table 1) were observed, respect to the trimethylsilyl derivatives. In Fig. 5, the second part of the chromatogram showed the dimethyldisulphide–trimethylsilyl derivatives of the monounsaturated compounds (peaks 2, 4, 6, 7, 10, 13, 14 and 15); the addition of the dimethyldisulphide group on the double bond introduces characteristic features on the mass spectra: fragmentations occur where the former unsaturation existed (Christie, 1997). Thus, differences can now be observed in the spectra of peaks 6 and 7 (Fig. 6): the first had base peak at *m/z* 323 and an intense ion at *m/z* 145, corresponding to the structures $[(CH_3)_3SiO–C_6H_4–(CH_2)_7–CHSCH_3]^+$ and $[CH_3–(CH_2)_5–CHSCH_3]^+$, respectively; the second had base peak at *m/z* 351 and an intense ion at *m/z* 117, corresponding to the structures $[(CH_3)_3SiO–C_6H_4–(CH_2)_9–CHSCH_3]^+$ and $[CH_3–(CH_2)_3–CHSCH_3]^+$, respectively. Compound **6** was then 3-(8-pentadecenyl)-phenol and compound **7** was 3-(10-pentadecenyl)-phenol. The same considerations could be done for the peaks 2, 4, 10 and the three isomers 13, 14 and 15 (Table 2): spectra permitted to localise the former double bond position and the compounds were 3-(8-tridecenyl)-phenol, 3-(8-tetradecenyl)-phenol, 3-(10-hexadecenyl)-phenol, 3-(8-heptadecenyl)-phenol, 3-(10-heptadecenyl)-phenol and 3-(12-heptadecenyl)-phenol, respectively. Thus, all monounsaturated alkylphenols were characterised (Table 3); these compounds presumably have the same configuration (Z) of the principal component, 3-(8-pentadecenyl)-phenol: this hypothesis was supported by the literature data (Alen et al., 2000; Paramashivappa et al., 2001 ; Phani Kumar et al., 2002; Tan et al., 2001) and also by our chromatograms, in which only one geometric isomer for each different unsaturated compound was visible.

Unfortunately, no informations were available for the double bond positions of the two diunsaturated compounds; it is possible

Table 2
GC–MS data of the dimethyldisulphide–trimethylsilyl derivatives of the monounsaturated cardanols in pistachio oil.

Peak	Retention time (min)	$[M]^{+\cdot}$, *m/z* (%)	Major fragment ions, *m/z* (%)
(2)	33:03	440 (33)	323 (100), 275 (48), 219 (25), 205 (20), 179 (53), 117 (36)
(4)	34:46	454 (42)	323 (100), 275 (42), 219 (30), 205 (19), 179 (47), 131 (32)
(6)	37:06	468 (40)	323 (100), 275 (40), 219 (27), 205 (21), 179 (52), 145 (43)
(7)	37:40	468 (48)	351 (100), 303 (40), 219 (32), 205 (20), 179 (96), 117 (32)
(10)	40:02	482 (35)	351 (100), 303 (46), 219 (31), 205 (22), 179 (83), 131 (36)
(13)	42:44	496 (29)	323 (100), 275 (43), 219 (28), 205 (19), 179 (48), 173 (28)
(14)	43:15	496 (37)	351 (100), 303 (48), 219 (29), 205 (25), 179 (86), 145 (54)
(15)	44:02	496 (24)	379 (81), 331 (100), 219 (17), 205 (20), 179 (82), 117 (35)

to hypothesize that peak 9 could be 3-(8,11-pentadecadienyl) phenol (Paramashivappa et al., 2001; Phani Kumar et al., 2002; Strocchi & Lercker, 1979) and peak 12 could be 3-(10,14-heptadecadienyl) phenol.

The composition of the pistachio cardanols is very different from other plant species: usually, only four compounds are found in cashew, all with a C-15 alkyl chain (Paramashivappa et al., 2001; Phani Kumar et al., 2002; Strocchi & Lercker, 1979; Tyman et al., 1981); two C-15 cardanols are reported in *Semecarpus vitiensis* (Pramono et al., 1985), C-15 and C-17 in *Rhus vernicifera,* an Anacardiacea (Du, 1990), a C-11 and a C-13 in *Knema hookeriana* (Alen et al., 2000), a C-15 and two C-17 in *Ginkgo biloba* (Tan et al., 2001) and nine different compounds in another Anacardiacea, *Rhus thyrsiflora* (Franke et al., 2001).

To our knowledge, 3-(8-tetradecenyl) phenol (peak 4), 3-tetradecyl phenol (peak 5) and 3-(10-hexadecenyl) phenol (peak 10) are new natural compounds.

Total cardanols content was roughly estimated from the weight of the purified TLC bands: the mean amount calculated on the five oil samples was 440 ± 95 mg kg^{-1}.

Quantitative data for each compound were calculated with the internal normalisation method (Table 3). The most abundant compounds were the C-15 cardanols (about 87% of total cardanols); monounsaturated compounds were predominant (87.89%) respect to saturated ones (10.95%). The double bond was in the position 8 in the C-13 and C-14 phenols and in 8 (prevalent isomer) and 10 in the C-15 cardanols; from the C-16 alkyl chain, the predominant isomer had the double bond in the position 10. Cardanols with an even alkyl chain were below 0.1%.

4. Conclusions

Sixteen different 3-alkylphenols from pistachio kernels, mainly monounsaturated, were recovered; fourteen were characterised with the aim of Nuclear Magnetic Resonance and Gas Chromatography–Mass Spectrometry. Dimethyldisulphide–trimethylsilyl derivatives were very useful to determine the double bond position of the monounsaturated alkylphenols. This is the first time that 3-alkylphenols are reported in pistachio kernels.

Acknowledgement

This work was supported by "PRA 2004: Analisi di composti fenolici in drupe e semi oleosi".

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Food Chemistry 117 (2009) 456–460

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Short communication

Rapid screening of volatile compounds in edible plants by direct chromatographic analysis

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ARTICLE INFO

Article history:
Received 2 June 2008
Received in revised form 9 January 2009
Accepted 12 April 2009

Keywords:
Aroma
Edible plants
GC-analysis
On-line coupling PTV–PTV

ABSTRACT

A method based on the use of the on-line coupling of two programmed temperature vaporizers (PTVs) is proposed to perform the concentration step required prior to the gas chromatographic determination of volatile compounds in edible plants. The reported procedure does not demand a previous sample handling stage whilst allowing the rapid and reliable screening of plant materials to be performed. The matrix to be analysed is directly introduced into the PTV of a gas chromatograph, thus avoiding the risk of losses and contamination typically observed when external concentration techniques are applied. The analytes in the vapour phase are swept from the matrix and then retained onto a suitable adsorbent material placed inside the second PTV injector. The described set-up allows the simple and rapid performance of the experimental work as the overall analysis of naturally occurring compounds with potential application as food ingredients can be performed in less than 1 h.

1. Introduction

The reliable determination of volatile components is of special relevance with respect to flavour and fragrance studies and, in general, to foodstuffs. As the aroma is considered to be a key issue to evaluate the consumer acceptance or rejection of a product, the volatile profile is often used to assess a number of valuable properties namely purity, quality and origin (Maarse & Belz, 1985; Marsili, 1997, 2002; Schreier, 1984; Surburg & Panten, 2006; Teranishi, Wick, & Hornstein, 1999).

On the other hand, the use of bioactive compounds extracted from natural sources has been lately considered as an interesting alternative in either the design of new functional foods or in the development of food additives. In this way, natural occurring compounds can be eventually used as substitutes of synthetic antioxidants and antimicrobial agents which might have negative effects on human health. As a consequence, further research on rapid and reliable screening of plant materials is highly demanded not only to identify new compounds but also to select the natural source most adequate for the isolation of the compounds of importance for specific applications.

Conventional sample preparation usually starts with an extraction step. A concentration procedure is then normally required to allow the detection of compounds by the chosen analytical method. Sample preparation comprises from conventional extraction technologies (e.g., liquid–liquid extraction, steam distillation, as well as static and dynamic headspace sampling) to modern techniques (e.g., high pressure solvent extraction methods, supercritical fluid extraction, as well as solvent-free and fast techniques, namely solid phase microextraction and stir bar sorptive extraction) (David & Sandra, 2007; Durán, Natera, Castro, & García-Barroso, 2006; Díaz-Maroto, Pérez-Coello, & Cabezudo, 2002; Salinas, Zalacain, Pardo, & Alonso, 2004). Advantages and disadvantages of the above mentioned procedures have also been described and the convenience of new developments to overcome their limits and, consequently, to enlarge their field of application has been emphasised very recently (Bicchi, Cordero, Liberto, Sgorbini, & Rubiolo, 2008).

In this context, the usefulness of a programmed temperature vaporizer (PTV) (Vogt, Jacob, Ohnesorge, & Obwexer, 1979) for the determination of volatile compounds in complex mixtures has already been demonstrated. Most methods previously reported have mainly focussed on large volume sampling introduction into the injector and the retention of the target compounds in a suitable material placed inside. The elimination of the solvent and the gas chromatographic analysis of the thermally desorbed compounds is subsequently performed. Specifically, the optimisation of a number of variables involved in the retention of the target solutes in an adsorbent kept inside the PTV injector body has allowed the determination of trace compounds in very different matrices (Mol, Janssen, Cramers, Vreuls, & Brinkman, 1995; Staniewski, Janssen, Cramers, & Rijks, 1992; Villén, Señoráns, Reglero, & Herraiz, 1995, 1996).

An interesting feasibility of the PTV injector involves its use for thermal desorption of volatiles from a solid sample material

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.04.046

directly introduced into the glass liner of the injector body (Reglero, Herraiz, Herraiz, & Sanz, 1989) although the risk of column overloading limits this application. There has also been reported the use of adsorbent materials to trap the compounds of interest (Tabera, Reglero, Herraiz, & Blanch, 1991), but in this case a concentration step must be performed externally to the chromatographic analysis itself. Results obtained with both procedures showed a potential which suggests the interest of combining their advantages, whilst overcoming their respective drawbacks, to integrate, in a unique analysis, both the extraction and concentration of the target compounds, as well as its subsequent sampling into a gas-chromatographic system.

The goal of this work was to evaluate the possibility of using the on-line coupling of two PTVs to achieve the internal concentration of the volatiles present in a solid sample material directly placed into the system. A further aim was to significantly reduce sample handling thus avoiding the risk of artefacts formation, as well as that of decomposition of thermally labile components which may distort the volatile profile of the sample to be analysed.

As an example of the performance of the proposed sampling-assembly for the determination of naturally occurring bioactive compounds in plant materials, in the present work we report the direct analysis of *Mentha piperita* because of its extensive use in foods and beverages. This specie is commonly employed in the liquor and confectionery industries, as well as in herbal teas or as ingredients of commercial spice mixtures used for many foods.

2. Materials and methods

2.1. Samples and materials

A standard solution containing α-phellandrene, limonene, menthone, dihydrocarvone and pulegone (1 mg/L of each compound in diethyl ether) was used to establish the experimental conditions more convenient for the analysis of plant material using the set-up proposed in this work. All standards were obtained from Sigma–Aldrich (Dorset, UK) whilst diethyl ether was supplied by Scharlau (Barcelona, Spain). *M. piperita* was acquired in the commercial market and was placed into the PTV-1 (see Fig. 1) without any kind of pre-treatment. After grinding the dried plant material, it was directly introduced into the silylated glass liner (54 × 3.4 mm i.d. × 5 mm o.d.) of the injector. A 4-cm length plug of Tenax TA (80–100 mesh Chrompack, Middelburg, The Netherlands) was used as packing material in the silylated glass liner (54 × 3.4 mm i.d. × 5 mm o.d.) of the PTV-2 (see Fig. 1). Tenax TA was kept in place at both ends with silanized glass wool (Phase Separations, Norwalk, CT, USA). Prior to its use, Tenax TA was conditioned under a stream of helium for 120 min at 300 °C. Although it was intended to analyse the standard solution and the plant material under the same conditions, its different nature eventually demanded the use of different conditions for specific variables (i.e., the amount of sample introduced into the system, the time interval during which helium is passed through the injector and the split ratio).

2.2. On-line coupling PTV–PTV

Sampling introduction and volatile concentration were accomplished using two Varian (Palo Alto, CA, USA) PTV injectors, Model 1079 (PTV-1 and PTV-2 in Fig. 1) linked through a fused-silica tube. In all cases the sample (i.e., a 0.4-μL volume of the standard solution or a 5.5-mg weight of the plant material) was introduced into the PTV-1 operated in the splitless mode and maintained at 50 °C under a helium stream (1.4 bars). Subsequently, a dynamic headspace was performed by stripping the target analytes from the sample in the vapour phase, 20 and 10 min being the times applied depending upon whether the standard solution or the plant material, respectively, was analysed. Finally, the volatile compounds were concentrated into the adsorbent material placed into the PTV-2 (kept at 10 °C) which was also operated in the splitless mode.

During the sweeping procedure, the chromatographic column was disconnected from the injector to concentrate the sample (Fig. 1). The PTV-2 was maintained at 10 °C to transfer solutes whilst avoiding their possible loss. Upon completion of this step, the column was connected again and the solutes retained in the packing material placed inside the glass liner of the PTV-2 were then transferred to the GC column by increasing the PTV temperature from 10 to 250 °C at 200 °C/min. The final temperature was kept for 10 min. The subsequent GC analysis was accomplished under the experimental conditions detailed below.

2.3. GC-analysis

GC-analysis was carried out using a CP-3800 Varian chromatograph and a 30 m × 0.25 mm i.d. fused-silica column coated with a 0.25-μm layer of poly (5% phenyl: 95% dimethylsiloxane) (VF-5 ms, Varian) using helium (2 mL/min) as the carrier gas. The oven temperature was increased from 60 °C (10 min) up to 100 °C (10 min) at 5 °C/min and up to 220 °C at 5 °C/min and the final temperature

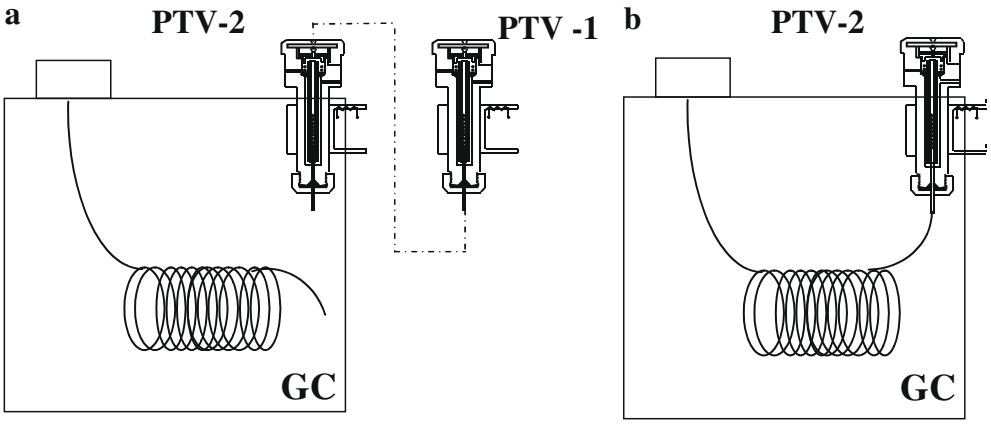

Fig. 1. Experimental set-up showing the on-line coupling of two programmed temperature vaporizers (PTV-1 and PTV-2). The analysis is performed in two steps which include the sample introduction into the PTV-1 whilst applying an helium flow to strip the volatile compounds which are retained inside the PTV-2 (a) and the thermal desorption of the solutes retained in the PTV-2 and its subsequent transfer to the chromatographic column (b). (See text for further details).

was maintained for 10 min. The split mode (split ratio; 20:1 and 60:1 for the standard solution and the plant material, respectively) was used in all cases. The flame ionisation detector (FID) used in the chromatographic analysis was set at 250 °C throughout the experimentation. Data acquisition was performed using a Star Toolbar system (Varian). Under the experimental conditions applied in the overall analysis, satisfactory blanks between consecutive runs were obtained for the complete procedure.

Compounds were identified by matching their GC retention times with those obtained from authentic standards analysed under the same experimental conditions. Moreover, for identification purposes, compounds retained on Tenax TA were thermally desorbed on the above mentioned GC column coupled to a Saturn 2000 ion-trap Varian mass spectrometer (MS). The recorded mass spectra were then compared with those of the US National Institute of Standards and Technology (NIST) library. For the MS, ionisation was accomplished by electron impact (EI). The transfer line temperature was set at 280 °C whilst 200 and 60 °C were the temperatures used for the trap and the manifold, respectively.

3. Results and discussion

A starting point of our research was the generally admitted statement that the less sample manipulation (and, therefore, the fewer variables affecting the experiment) the more likely the finally obtained results will be reliable. For that reason, we decided to reduce drastically the usually laborious and time consuming handling of the sample prior to the chromatographic analysis in such an extension that, in the method reported in the present work, it is actually limited to the simple introduction of the milled sample into the chromatographic system (i.e., an external fractionation step is not required prior to the chromatographic analysis).

Due to the relatively low concentrations in which several relevant volatile compounds may occur in plant material, it is evident that an effective concentration step is always convenient, or even essential, to produce a chromatographic profile that can be considered as truly representative of the investigated sample. Consequently, a method to concentrate the sample should minimize, or even prevent, possible sources of errors such as the generation of unknown peaks or the decomposition of thermally labile components in heated zones close to the column in which the chromatographic separation will be performed. Thus, with this specific objective, we formulated the hypothesis that the isolation of the part of the system in which the sample is placed, and also the dynamic headspace is performed, (step a in Fig. 1) from the part in which the stripped volatiles are retained in an adsorbent material, and subsequently are directly transferred onto the head of the chromatographic column (step b in Fig. 1), would result in a higher reliability of the overall on-line procedure investigated. When performing the experimentation under the conditions detailed in Section 2, the flow of helium passing through PTV-1, as well as the initial temperature of this injector, were established to facilitate the extraction of the volatile compounds from the sample. It should be underlined that the initial temperature of PTV-2 was fixed at a value substantially lower than that of PTV-1 (in this case 10 *vs.* 50 °C) to achieve the focusing of the analytes at the head of the column for improving chromatographic peak shape. Then, direct thermal desorption was applied to remove the volatiles from the adsorbent material in which they had been previously retained. This was achieved by increasing the PTV-2 temperature up to 250 °C.

Figs. 2 and 3 depict the chromatograms resulting from the PTV–PTV–GC-analysis of the standard solution and the *M. piperita*, respectively, under the experimental conditions detailed above. Table 1 gives the RSD values (calculated from a minimum of three replicates) and the recovery values obtained for all the compounds included in the mentioned standard solution. As can be seen, RSD values ranging from 3.7% to 14.4% were observed whilst recovery values close to 80% were estimated for some compounds (specifically for dihydrocarvone and pulegone).

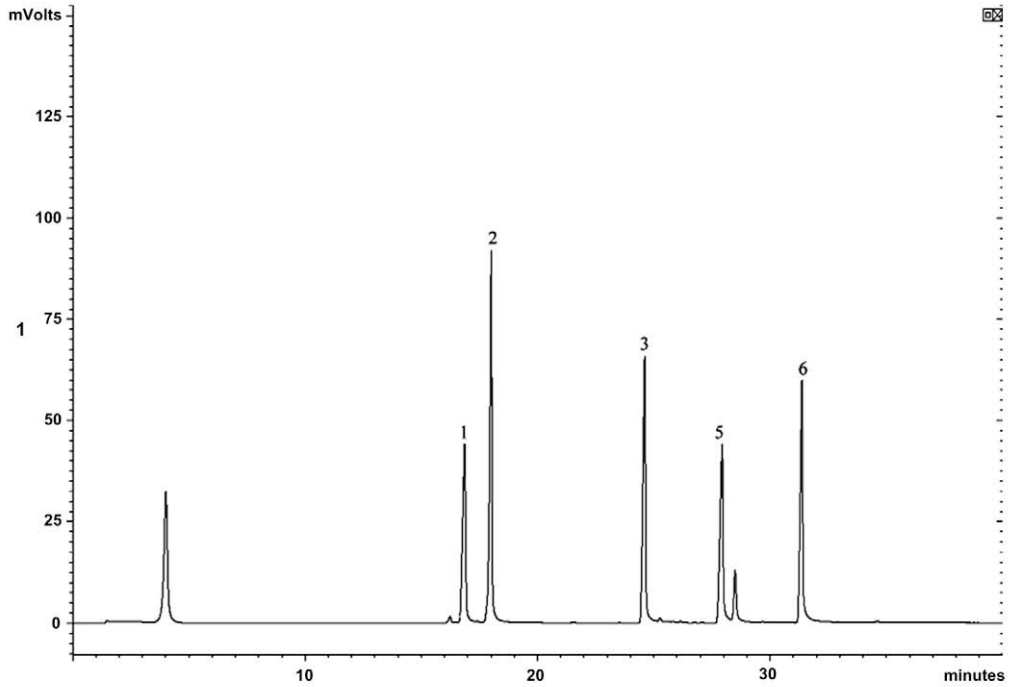

Fig. 2. PTV–PTV–GC-analysis (split ratio, 20:1) of the standard solution using the experimental set-up shown in Fig. 1 under the experimental conditions detailed in the text. Peak identification: 1. α-phellandrene, 2. limonene, 3. menthone, 5. dihydrocarvone, 6. pulegone.

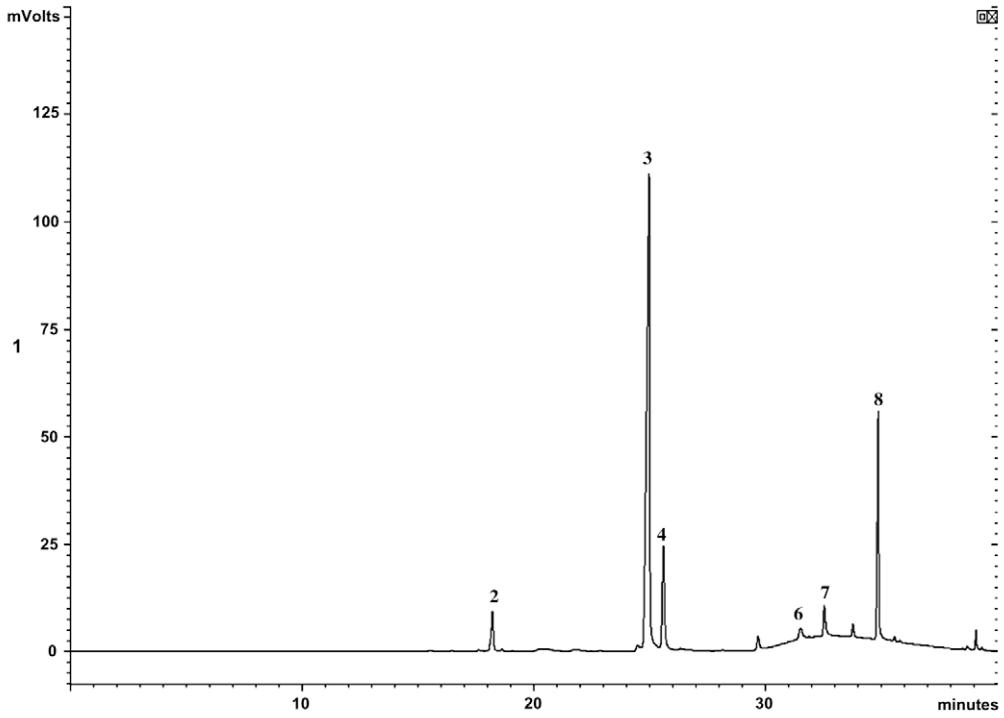

Fig. 3. PTV–PTV–GC-analysis (split ratio, 60:1) of a commercial sample of *M. piperita* using the experimental set-up shown in Fig. 1 under the experimental conditions detailed in the text. Peak identification: 2. limonene, 3. menthone, 4. isomenthone, 6. pulegone, 7. piperitone, 8. menthyl acetate.

Table 1
Relative standard deviation (RSD, $n = 3$) and recoveries obtained from the PTV–PTV–GC-analysis of the standard solution using the experimental set-up shown in Fig. 1.

Compounds	RSD (%)	Recovery (%)
α-Phellandrene	14.4	56.2
Limonene	9.7	44.7
Menthone	11.5	33.9
Dihydrocarvone	5.9	79.5
Pulegone	3.7	79.9

Interestingly, the PTV–PTV–GC-analysis of *M. piperita* demonstrated the feasibility of the proposed method for the analysis of real-life samples as the occurrence of characteristic compounds (i.e., menthone and isomenthone) could be reliably identified is less than 1 h. Moreover, the presence of other compounds, like limonene, pulegone, and piperitone occurring at quite lower concentrations with respect to that of menthone could also be determined (see Fig. 3). The RSD ($n = 3$) calculated for the compounds identified in the *M. piperita* sample, namely limonene, menthone, isomenthone, pulegone, piperitone and menthyl acetate resulted to be 11%, 16%, 14%, 14%, 11% and 11%, respectively.

It should be emphasised that although regarding the sensitivity achievable in the overall analysis it would have been more convenient to operate in the splitless mode during the GC separation of the *M. piperita* sample, we decided to use less favourable conditions (i.e., split ratio of 60:1). In this way, we intended to evaluate the performance of the experimental set-up in those cases in which the target analytes may occur in very different concentrations and, consequently, a split ratio must be applied to separate, with acceptable chromatographic resolutions, minor compounds eluting next to major compounds. Generally speaking, when analysing different plant materials the concentration range of specific sample components will determine the split ratio to be applied in each particular case.

The reported procedure involves a type of internal sample fractionation because the experimental values of the purge flow, the purge time and the temperature under which the sampling is performed obviously determine the amount of the different volatiles stripped from the sample. Thus, exclusively these compounds are transferred to the second PTV and, subsequently, are thermally desorbed onto the column. As a result, clean chromatograms can be eventually obtained which facilitates the optimization of chromatographic resolutions and, at last, the reliable determination of compounds occurring at both high and low levels.

On the contrary, the earlier reported one-step analysis by thermal desorption of a plant material directly introduced into a PTV injector does not include the previous stripping of the target compounds from the sample (Reglero et al., 1989). Consequently, rather complex chromatograms are usually obtained in which column overloading and overlapped peaks, as well as non-volatiles or other disturbing matrix components, ultimately may avoid the reliable identification and quantification of specific target compounds.

It is interesting to point out that in the present work the sampling step can be performed whilst keeping during transfer the GC column either connected or disconnected depending upon the chemical nature of the sample and the concentration range in which the target compounds occur. Likewise the split ratio must be optimised in each particular case either to avoid column overloading due to compounds present at high concentrations or to increase the sensitivity achievable in the analysis of compounds in minor quantities.

A further aspect of special interest concerning the potential field of application of the proposed design is the possibility of using the detailed PTV–PTV procedure to perform a multidimensional separation in the chromatographic analysis. To this aim, the only modification required with respect to the design depicted in Fig. 1 is the employment of an additional separation column just to make it possible the transfer, from one column into another, of different selected cuts. An important practical consequence of this fact will be

the use of this method for especially difficult separations in which both a pre-column and a main column are required to avoid overlapping of otherwise unresolved compounds in a GC-unidimensional system.

4. Conclusions

The preliminary results obtained with the proposed procedure (i.e., the on-line coupling PTV–PTV–GC) show its usefulness for the extraction and concentration of substances occurring in edible plants. The GC-analysis of volatile compounds using the mentioned sampling assembly offers clear advantages over other analytical techniques as its performance allows the use of mild conditions whilst stripping the target compounds from the sample matrix. Consequently, the risk of artefacts generation, with respect to conventional sample preparation methods, is basically avoided whereby volatile fraction profiles truly representative of the plant material can be performed in just one step, the overall analysis time being less than 1 h. A further interesting feature is the fact that the use of two PTV injectors allows the transfer to the chromatographic column of only those compounds previously purged off from the plant in the first PTV. Then, clean chromatograms can be obtained in which the identification of naturally occurring bioactive compounds with potential application as food ingredients can be easily and reliably accomplished.

Acknowledgements

Financial assistance from the Ministerio de Educación y Cultura, Project CTQ2006–01687 is gratefully acknowledged. María del Mar Caja thanks the Consejo Superior de Investigaciones Científicas (CSIC) for its financial support (Project I3P).

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Food Chemistry 117 (2009) 461–465

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Short communication

Analytical application of food dye Sunset Yellow for the rapid kinetic determination of traces of copper(II) by spectrophotometry

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ARTICLE INFO

Article history:
Received 13 August 2008
Received in revised form 12 April 2009
Accepted 13 April 2009

Keywords:
Copper determination
Kinetic method
Food samples
Sunset Yellow

ABSTRACT

A sensitive and rapid kinetic method for trace determination of Cu(II) was developed and validated, based on its catalytic effect on the oxidation of disodium-6-hydroxy-5-[(4-sulphophenyl)azo]-2-naphtalenesulphonic acid (wide used, food colour "Sunset Yellow FCF", E110, in text selected as SY) by hydrogen peroxide in borate buffer at pH 10.5. The reaction was monitored spectrophotometrically by measuring the decrease in the absorbance of SY at 478.4 nm. The optimum operating conditions regarding concentration of reagents, pH and temperature were established. The calibration curve was linear up to 318 ng mL^{-1} of Cu(II) and the limit of detection ($3\sigma/S$) is 5.0 ng mL^{-1}, and limit of quantification ($10\sigma/S$) is 16.67 ng mL^{-1}. The proposed kinetic procedure was successfully applied to monitoring of the concentration of Cu(II) in fruit, wine and milk samples from different areas. The results obtained by the proposed kinetic procedure were compared by those obtained by ICP-OES method, and shown good agreement. The proposed kinetic method could be used for monitoring of quality of drinks or fruit depending on Cu(II) concentration, because of its important role as nutritional element.

1. Introduction

Since copper occurs at low concentration in many real samples, such as natural, tap, waste waters, food, wine, drinks and soil, a sensitive, cost-effective, selective and rapid method is required for its determination in those samples. Copper is essential to human health, but only in limited amounts (Prasad & Halafihi, 2003).

In many food samples and drinks (fruit, juices, milk and wines) copper and iron are widely present. The high concentration of Cu, Fe, Mn and Zn may cause instability and turbidity of wine (Liskovskii & Skurikhin, 1976). Typical wines contain average copper levels at about 0.1–0.3 mg L^{-1} (Li, Guo, & Wang, 2008). In wines, the main sources of copper are equipments used in the wine production, addition of copper salts ($CuSO_4$) and pesticides employed during growth.

Hydrogen peroxide may be formed during the oxidation of phenols in wine, which has been widely accepted (Li et al., 2008). H_2O_2 in association with iron and copper tends to generate reactive oxygen species such as hydroxyl radical (·OH), which is known as the Fenton reaction (Li et al., 2008). In that way, most of the ·OH radicals generated in wine and fruit juice samples come from the metal-catalysed decomposition of hydrogen peroxide according to the Fenton-type reaction.

In recently reported references, Cu(II) was determined in different samples by using graphite furnace atomic absorption spectrometry (GF-AAS), (Ajtony et al., 2008; Karadjova, Izgi, & Gucer, 2002), inductively coupled plasma optical emission spectrometry (ICP-OES), (Lara, Cerutti, Sajonia, Olsina, & Martinez, 2005; Moreno et al., 2008). High performance liquid chromatography (Bermejo et al., 2001; Katerina, Christian, Ilse, Gregory, & Josef, 1999), and anodic stripping voltammetry (Cesar, Joanna, Jon, & Richard, 1999; Zeng, Liu, Tan, Zhang, & Gao, 2002) have also been used for the determination of copper with high sensitivity and selectivity, but suffer from more or less time consuming procedures and complicated instrumentation.

Despite of the instrumental improvements of the last decades, spectrophotometric techniques (Reddy, Reddy, Narayana, & Reddy, 2008) are still attractive from an analytical point of view since they are cheap, sensitive and simple to implement in laboratories. Among them, the kinetic-catalytic methods give high sensitivity and accuracy without the need for expensive and special equipment, by using different masking reagents or extraction to improve their selectivity (Crespo, Andrade, Inon, & Tudino, 2005; Crouch, Cullen, Schelline, & Kirkor, 1998; Crouch, Schelline, & Kirkor, 2000; Ghasemi, Kiaee, Abdolmaleki, & Semnani, 2008; Mitic, Miletic, & Obradovic, 2004; Mottola & Perez-Bendito, 1996; Prasad & Halafihi, 2003; Tomiyasu, Aikou, Anazawa, & Sakamoto, 2005; Tosic, Mitic, Obradovic, & Sunaric, 2004). Their sensitivity is at least 2–3 orders of magnitude higher than that of the ordinary

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doi:10.1016/j.foodchem.2009.04.042

spectrophotometric methods. Catalytic-kinetic methods have been widely used in determining many species at traces levels.

Some of them have low sensitivity (Ghasemi et al., 2008; Tosic et al., 2004), or poor selectivity, which makes difficulties in their application in complex samples. Compared to other kinetic methods for the determination of Cu(II), the kinetic method developed in this work falls to order of more sensitive methods (nanogram levels). Among its high sensitivity, the method has also advantage in view of its simple and rapid applicability to different real samples, using easy available masking agents for elimination main interfering effects (Fe^{3+}, Mn^{2+}, Ni^{2+} and Co^{2+}), without complicated pretreatments, with satisfactory accuracy which was confirmed by ICP-OES method.

In this study, we found that food dye SY is oxidised by hydrogen peroxide in borate buffer; this oxidation reaction is significantly catalysed by trace amounts of Cu(II). Consequently, the main aim of this study was the development of a new sensitive kinetic-spectrophotometric method for the determination of Cu(II), based on its catalytic effect, and optimisation of the procedure for its simple and direct application in the presence of other species in real samples. Since this element is usually present at low levels in many environmental (natural and waste waters, soil and air), food, wines, drinks or biologically samples (blood, serum, urine), sensitive and selective analytical techniques are required for its determination. The developed kinetic procedure is sensitive, rapid, low-cost and applicable. The method was successfully applied to determination of traces amounts of Cu(II) in fruit, wine and milk samples. An ICP-OES method was also used for determination of Cu(II) in these samples, in order to validate the proposed kinetic-spectrophotometric method. The obtained results have shown good agreement. Also, based on the results of this work it can be seen that oxidation of Sunset Yellow, as widely used artificial dye for food and drinks, is catalysed by traces of metal ions, in presence of oxidative compounds (such as H_2O_2) in alkaline media, at about 25 °C, which is followed by decolourisation of such a kind of food and drink samples.

2. Materials and methods

2.1. Apparatus

Spectrophotometric measurements were performed on Perkin–Elmer Lambda 15 UV–vis spectrophotometer (Perkin–Elmer Instruments, Shelton, USA) using cylindrical cell thermostated at about 25.0 °C. For the pH measurements, Radiometer PHM 29Bb pH metre, (MeterLab, USA) and a combined glass–calomel electrode, GK2311C were used. All solutions were kept in a thermostatic water-bath at 25.0 ± 0.1 °C before the beginning of the reaction. ICP-OES measurements were done by means of an atomic-emission spectrometer SPECTRO CIROS^{CCD} (Spectro Analytical Instruments, Kleve, Germany). High precision measuring for laboratory applications was performed by using an analytical balance METTLER TOLEDO, AB204-S, Switzerland.

2.2. Reagents and solutions

Analytical grade chemicals and deionised water (MicroMed high purity water system, TKA Wasseraufbereitungssysteme GmbH) were used for the preparation of all solutions.

All the stock solutions were stored in polyethylene containers. All the polyethylene containers and the glassware used were cleaned in aqueous HCl (1:1) and then thoroughly rinsed with deionised water. A 1.000 g L^{-1} Cu(II) (nitrate salt, Merck®, KGaA, Darmstadt, Germany) was used as a stock solution. Cu(II) working solutions were made by suitable dilutions of the stock solution. A solution of SY (disodium-6-hydroxy-5-[(4-sulphophenyl)azo]-2-naphtalenesulphonic acid), Fluka (Bushs, Switzerland) of 1×10^{-3} mol L^{-1} was prepared by weighing 0.0226 g of substance of analytical grade, using an analytical balance, and dissolving with deionised water in a calibrated volumetric flask of 50 mL. A 2 mol L^{-1} solution of hydrogen peroxide (Merck®) was prepared by appropriate dilution of 30% of reagent in volumetric flask of 50 mL with deionised water. The solution of borate buffer was prepared by appropriate mixing a solution of $Na_2B_4O_7{\cdot}10H_2O$ (Merck®) of (0.05 mol L^{-1}) and 0.1 mol L^{-1} solution of NaOH. Sigma buffers, pH of 7.00 ± 0.01 and pH of 4.00 ± 0.01, were used to calibrate the pH metre.

2.3. Kinetic-spectrophotometric procedure

A suitable aliquot of Cu(II) solution, specified below for each sample, was transferred into a 10 mL standard flask with a glass stopper. Then, 0.2 mL of 10^{-3} mol L^{-1} solution of SY, and 2 mL borate buffer pH of 10.5 were added and the solution was diluted up to 4 mL with deionised water. The solution was kept at 25.0 ± 0.1 °C in the thermostated bath. After the temperature of the solution had reached 25.0 ± 0.1 °C, 1 mL of hydrogen peroxide solution of 2 mol L^{-1} was added in reaction mixture to initiate the reaction. The reaction mixture was transferred immediately into the spectrophotometric cell and the absorbance at 478.4 nm was measured every 30 s during the first period of 6 min from the beginning of the reaction. The method of tangents was used and the slope of the absorbance–time curve, $dA/dt = tg\alpha$, was used as a measure of the reaction rate.

2.4. ICP-OES conditions

The following ICP-OES conditions were applied: plasma power 1400 W, coolant flow 14.00 L/min, auxiliary flow 1.00 L/min and nebuliser flow 0.90 L/min. It is simultaneous spectrometer with radial viewed plasma, Paschen–Runge mount optical system and 22 linear CCDs placed detectors arranged on a Rowland's circle, enabling a spectral range 125–770 nm, and with a Scott-type spray chamber. Energy for the plasma was achieved with a free-running 27.12 MHz generator. Emission intensities were axially measured at 324.754 nm, as these conditions provided the highest sensitivity. Working parameters were controlled and altered using software package Smart Analyser Vision®. Calibration was performed using external standard prepared from 1000 g L^{-1} stock of Cu(II), made up as appropriate with 2% nitric acid.

2.5. Sampling plan and preparation of samples

The proposed kinetic method was applied for comparative determination of traces amounts of Cu(II) in samples of wine, grapes and milk, at different locations in South Serbia (Porecje, Vucje) and Kosovo (Mitrovica, Novo Brdo), in order to monitor the influence of different environmental and geo-morphological conditions on its contain in mentioned samples. The proposed kinetic method was also applied for determination of traces amounts of Cu(II) in fruit samples (lemon and orange – Tivat, Montenegro) and vineyard leaf.

The grape, wine (white, red) and milk samples (randomly selected, during 2007 autumn season) are collected at the same time from following area: (grape, red and white wine, vineyard leaf) – Porecje, Serbia; (red grape) – Mitrovica, Kosovo; (cow milk) – Porecje, Serbia and Novo Brdo, Kosovo; (lemon, orange) – Tivat, Montenegro. For the determination of trace levels of elements, contamination or loss are of prime consideration. Examined samples (lemon, orange and grape) are collected in plastic containers, and maintained at 4 °C during transport, protecting them from

all sources of mineral contamination. Fresh milk and wine samples are taken in polyethylene bottles, that are pre-washed with detergent, followed by 10% HNO_3 and deionised water. Samples are maintained at 4 °C during transport to the laboratory and stored at 4 °C. Samples prepared by this way have been analysed immediately under procedure mentioned below, for each type of samples.

A 1 g of samples red grapes were accurately weighed and carefully heated with 10 mL nitric acid (65%) followed by gentle heating with the addition of 8 mL perchloric acid (70%) until colourless. After cooling 30 mL deionised water was added and heating resumed for 10 min. Finally, the solutions were cooled, then made up to 50 mL with deionised water. A 100 mL of wine samples and fresh squeesed juice of orange and lemon using a water bath was boiled dry and the residue was burned at a temperature of 450–500 °C for 12 h. After that, 5 mL of concentrated HNO_3 was added and the sample was boiled dry again. The residue was kept for about 2–3 h at the same temperature. After that the residue was dissolved in five drops of concentrated HNO_3 and diluted up to 50 mL with deionised water. In 10 mL of milk, three drops of CH_3COOH were added. Using a water bath the samples were boiled dry and the residue was burned at a temperature of 400–450 °C for 12 h. After that 1 mL of concentrated HNO_3 was added and the sample was boiled dry again. The residue was kept about 2–3 h at the same temperature as before. After that the residue was dissolved in two drops of concentrated HNO_3 and diluted up to 25 mL with deionised water.

For kinetic application in that way prepared samples of fruit, wine and milk are treated as following: to 0.2 mL of prepared sample solution was added 0.8 mL of 2 mol L^{-1} NaOH in order to adjust of working pH. After that, 1 mL of prepared sample was used for the recommended procedure for kinetic measurements.

In order to eliminate the main interference effects from the presence of Fe^{3+}, Mn^{2+}, a volume of 0.1 mL of 0.01 mol L^{-1} of F^- was added and of Co^{2+}, Ni^{2+} a volume of 0.5 mL of 0.01 mol L^{-1} of citrate solution, and recommended kinetic procedure was successfully applied on these prepared samples (Piatnickii & Suhan, 1990).

3. Results and discussion

3.1. Optimisation of chemical variables

The catalytic effect of Cu(II) on the oxidation reaction of 6-hydroxy-5-[(4-sulphophenyl)azo]-2-naphtalenesulphonic acid (SY), by hydrogen peroxide in borate buffer was observed (Fig. 1). Absorbance measurements have been performed at the wavelength of the absorption maximum of SY at 478.4 nm, at pH of 10.5 and at 25 ± 0.1 °C. In order to establish the optimum conditions for the trace analysis of Cu(II), the influence of the reaction variables were studied for both, non-catalytic and catalytic reactions.

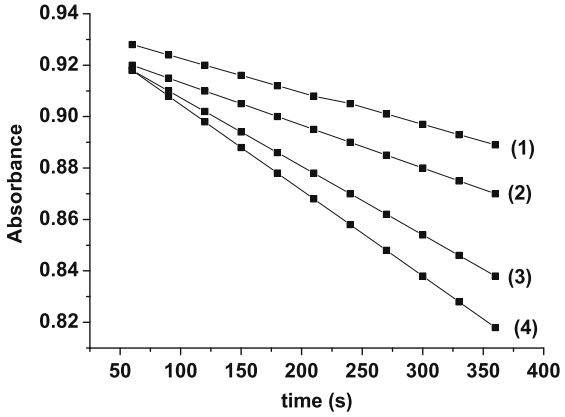

Fig. 1. Dependence of the reaction rate on the Cu(II) concentration. Experimental conditions: $C_{SY} = 4 \times 10^{-5}$ mol L^{-1}; $C_{H_2O_2} = 0.4$ mol L^{-1}; pH 10.5; $t = 25.0 \pm 0.1$ °C, $\lambda_{max} = 478.4$ nm; (1) $C_{Cu(II)} = 0$ ng mL^{-1}; (2) $C_{Cu(II)} = 32$ ng mL^{-1}; (3) $C_{Cu(II)} = 127$ ng mL^{-1}; (4) $C_{Cu(II)} = 254$ ng mL^{-1}.

3.2. Effect of pH

Keeping all other experimental parameter constant, the effect of pH was studied the first, to obtain information about the highest difference between the rate of the reactions. The dependence of the reaction rate on pH in borate buffer in the range of 9.2–11.5 has been studied. The optimal difference between the rates of non-catalytic and catalytic reactions has occurred at pH 10.5 and for further work a pH of 10.5 has been selected, because at higher value of pH rate of both reactions significantly increased and thus, enlarged errors during measurements. The dependence of the reaction rate on the volume of borate buffer was investigated. The maximum difference between the rate of the non-catalytic and catalytic reactions occurred for volume of 2.0 mL. For further work, a volume of 2.0 mL of borate buffer pH of 10.5 has selected as the optimal.

3.3. Effect of reagent concentration and temperature

The dependence of reaction rate on the concentration of SY was investigated over the range from 2×10^{-5} to 6×10^{-5} mol L^{-1}. For further work, a concentration of SY of 4.0×10^{-5} mol L^{-1} was selected as the optimal value, because at higher concentrations absorbance significantly increases ($A > 1$), which also increases the error of the spectrophotometric determination.

The dependence of the reaction rate on the concentration of H_2O_2 was investigated in the range 0.1–0.8 mol L^{-1}. The order for both reactions, catalytic and non-catalytic is zero, with respect to the concentration of H_2O_2 in the investigated interval. For further work, a concentration of H_2O_2 of 0.4 mol L^{-1} was selected as the suitable value.

The influence of temperature on the reaction rate was studied in the range 22–34 °C. The reaction rate increased as the temperature increased. However, although higher sensitivity could be obtained at the higher reaction temperature, it was troublesome to control the temperature precisely at the evaluated temperature; hence, a reaction temperature of 25 °C was chosen.

3.4. Validation of the proposed method

The calibration graph has been constructed under the following working conditions: $C_{SY} = 4 \times 10^{-5}$ mol L^{-1}; $C_{H_2O_2} = 0.4$ mol L^{-1}, pH 10.5; $t = 25.0 \pm 0.1$ °C, $\lambda = 478.4$ nm.

Under these conditions the change of the reaction rate was quite reproductive providing additional quality of the method in routine laboratory applications. The calibration curve was linear up to 318 ng mL^{-1} of Cu(II). The equation of the calibration curve was found to be:

Table 1
Effect of other ions on the determination of 191 ng mL^{-1} of Cu(II).

Tolerance limit Foreign ion/Cu(II)	Ion added
1000	Na^+, K^+, NH_4^+, Li^+, W^{6+}, Ca^{2+}, Cl^-, I^- CH_3COO^-
100	Zn^{2+}, Al^{3+}, Mg^{2+}, Cd^{2+}, V^{5+}, As^{3+}, As^{5+}, F^- NO_3^-, SO_4^{2-}, SO_3^{2-}, NH_4^+
10	Mo^{6+}, Hg^{2+}, Sn^{2+}, Sr^{2+}, Cr^{6+}, Ba^{2+}, $C_2O_4^{2-}$
1	Mn^{2+}, Fe^{3+}, Ni^{2+}, Co^{2+}, Au^{3+}

Table 2
Determination of Cu(II) in wine, fruit and milk samples.

Samples	Proposed method Found[a], ng mL^{-1}	RSD[c], %	R[d], %	ICP-OES method Found[a], ng mL^{-1}	RSD[c], %	R[d], %
Red grape (Kosovo)	271.0 ± 1.80	0.672	98.7	267.1 ± 1.22	0.457	98.9
Red grape (Porecje)	255.1 ± 3.63	1.421	101.2	252.7 ± 1.56	0.617	100.3
Red grape (Metohija)	277.4 ± 3.19	1.150	97.5	272.3 ± 1.33	0.488	98.9
Orange (Montenegro)	31.3 ± 1.72	5.486	98.4	30.9 ± 1.21	3.916	99.5
Lemon (Montenegro)	25.1 ± 0.95	3.783	100.5	24.9 ± 0.72	2.892	100.4
Red wine (Porecje)	313.5 ± 1.81	0.577	99.3	310.5 ± 1.95	0.628	99.5
White wine (Porecje)	278.5 ± 1.95	0.700	98.7	275.6 ± 1.80	0.270	99.5
Wineyard[b] Leaf (Porecje)	279.3 ± 2.05	0.734	97.4	275.9 ± 1.34	0.362	98.7
Milk (Novo Brdo, Kosovo)	156.1 ± 1.95	1.249	96.5	155.2 ± 1.05	0.676	97.7
Milk (Porecje, Serbia)	72.1 ± 1.10	1.525	100.3	70.8 ± 0.94	1.328	99.0

[a] Mean value ± standard deviation (ng mL^{-1}) (number of replicate measurements, n = 5).
[b] ng/g.
[c] Relative standard deviation.
[d] Recovery was calculated as: 100 − [(added value − found value) × 100]/added value.

$$tg\alpha \times 10^2 = 0.00262c + 0.8188,$$

where c is concentration of Cu(II) in ng mL^{-1}.

The limit of detection was determined as the signal-to-noise ratio ($3\sigma/S$) and it was 5 ng mL^{-1} and limit of quantification ($10\sigma/S$) was 16.67 ng mL^{-1}, where σ is the standard deviation of the blank, and S is the slope of the calibration curve.

In order to estimate the precision and accuracy of the proposed kinetic method, reaction rates were determined in five replicate determinations at each of three different Cu(II) concentrations 25, 191 and 318 ng mL^{-1} with RSD (%), respectively, 8.80%, 6.12% and 2.22%.

The method ruggedness was evaluated by five replicate determinations at different concentrations of Cu(II) (25, 191 and 318 ng mL^{-1}) over a period of 5 days. The low values of between-day RSD for different concentration of Cu(II) (less than 5%) indicate that solutions of SY, hydrogen peroxide and borate buffer have a very good stability.

The robustness of proposed method is a measure of its capacity to remain unaffected by small, but deliberate variations in the method parameters and provides an indication of its reliability during usage. The most significant variables of the system (temperature and the concentration of SY and hydrogen peroxide) were modified in the range ±10% (excluding pH, which was modified in the range ±5%) from their optimum values. Errors lower than 5% were observed in all cases. Thus, the proposed method was found to be robust for routine determination of Cu(II) in different real samples.

To access the selectivity of the method, the influence of some foreign ions on the catalytic reaction rate was studied, under the reaction conditions mentioned above, at a constant Cu(II) concentration of 191 ng mL^{-1}. The results are summarised in Table 1.

4. Application of the developed method

To evaluate the proposed kinetic method, the amounts of Cu(II) in wine, fruit and milk samples were determined. The results of proposed kinetic method have shown good agreement with results obtained by ICP-OES measurements (Table 2).

The obtained results confirm that the spectrophotometric kinetic method presented in this paper, under established working conditions, was successfully applied for Cu(II) determination in fruit, wine and milk samples, from different locations. The values of Cu(II) in milk, wine and grape samples from this area are also higher in comparing to values of Cu(II) in samples taken from other location without mines or plants (Porecje, Vucje, Serbia).

5. Conclusion

This work presents development and application of a rapid, simple, low-cost and sensitive kinetic method for the determination of trace amounts of Cu(II). The proposed method was applied in fruit, wine and milk samples. Validation of the method was made by determination of Cu(II) in these samples by using ICP-OES method, and results obtained using both methods have shown good agreement. The method has advantage in view of its sensitivity, low-cost, easy available chemicals and instrumentation, simple and direct application to many real samples.

Acknowledgements

This paper was supported by the Ministry of Science, Republic of Serbia (Project Nos. 142015 and 142079).

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Food Chemistry 117 (2009) 466–469

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Short communication

Analytical nutritional characteristics of seed proteins in six wild *Lupinus* species from Southern Spain

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ARTICLE INFO

Article history:
Received 3 December 2008
Received in revised form 14 April 2009
Accepted 15 April 2009

Keywords:
Lupinus
Seed proteins
Amino acids
Nutritional quality

ABSTRACT

The nutritional characteristics of seed proteins of Spanish wild populations of *Lupinus angustifolius*, *L. cosentinii*, *L. gredensis*, *L. hispanicus*, *L. luteus* and *L. micranthus* have been studied. Protein contents in this genus ranged from 23.8% in *L. gredensis* to 33.6% in *L. luteus*. On the one hand, *L. cosentinii* showed the most balanced amino acid composition, being only deficient in lysine. On the other hand, *L. gredensis* showed the worst amino acid composition. The *in vitro* protein digestibility (IVPD) was high in all species examined, ranging from 82.3% in *L. gredensis* to 89.0% in *L. cosentinii*. In addition to the amino acid composition and IVPD, other nutritional parameters, such as amino acid score, calculated biological value, predicted protein efficiency ratio or protein digestibility corrected amino acid score, were studied. These data yielded *L. luteus*, *L. hispanicus* and *L. cosentinii* as the species with seed proteins with the best nutritional properties, similar to those observed in other legumes with recognised high quality proteins, such as soybean. Results confirm the importance of studying wild populations of cultivated and non-cultivated *Lupinus* species as sources of seeds with good nutritional characteristics.

1. Introduction

Legumes represent, together with cereals, the main plant source of proteins in human diet. Beans are generally rich in high quality proteins, dietary fibre and carbohydrates and have a low content of saturated fats. For these reasons, beneficial health effects derived from legume consumption have been recognised and related to legume components, such as fibre or proteins and other minor compounds, such as certain lipids, polyphenols or bioactive peptides (Rochfort & Panozzo, 2007). These health promoting effects have been related to the prevention of diseases like diabetes mellitus, coronary heart diseases, or colon cancer (Duranti, 2006). However, legume consumption has decreased in recent decades in many Western countries, although the chemical and nutritional composition of beans show that they could play a much more important role in human nutrition. The genus *Lupinus* is not an exception and many of the cultivated species have seen their areas of cultivation reduced in the last century. However, the conservation of biodiversity may be, especially in developing countries, an important factor for their development. In order to recover and maintain this biodiversity the diversification of crops is necessary and this can be achieved by increasing our knowledge of local plants. Legume seeds are one of the most promising alternative sources of protein for human and animal nutrition. Among the main cultivated legumes, soybean is by far the most important. Other legumes, such as lupins, are being studied as potential alternative sources of protein in order to reduce the need to import soybean. *Lupinus* is an ancient crop belonging to the tribe *Genisteae* that was widely cultivated in the Mediterranean Region and South American Andes. The major cultivated species of lupins are *L. albus* (white lupin), *L. angustifolius* (blue lupin), *L. luteus* (yellow lupin) and *L. mutabilis* (pearl lupin). On average, lupin seeds have total protein content similar to soybean and acceptable amounts of essential amino acids. The chemical, functional and nutritional properties of lupin proteins mean that lupin seeds can be considered as a source of high quality protein (Lampart-Szczapa, Obuchowski, Czaczyk, Pastuszewska, & Buraczewska, 1997), with the protein content of cultivated lupin seeds ranging from 33.8% in *L. angustifolius* (Lqari, Vioque, Pedroche, & Millán, 2002) to 44% in *L. albus* (Duranti, Restani, Poniatowska, & Cerletti, 1981). Another interesting aspect of lupins as a new source of protein is that they can be grown in soils and in climates where soybean cannot grow. It has been suggested that lupin proteins supplemented with methionine could replace soy concentrate in countries where soybean must be imported (Ruiz & Hove, 1976). Moreover, lupin seeds have a lower content of the main anti-nutritional components often contained in legumes, such as phytates, oligosaccharides, trypsin inhibitors and lectins (El-Adawy, Rahma, El-Bedawey, & Gafar, 2001). Furthermore, *Lupinus* seed flours have been used for the production of

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doi:10.1016/j.foodchem.2009.04.039

protein isolates with good functional and nutritional properties (Lqari et al., 2002). The aims of this study were to evaluate the nutritional characteristics of seed proteins of six lupins species which grow in Southern Spain.

2. Materials and methods

2.1. Materials

Trypsin, chymotrypsin and peptidase were acquired from Sigma (Tres Cantos, Madrid, Spain). Diethyl ethoxymethylenemanolate was purchased from Fluka. All other chemicals were of analytical grade. The samples of *Lupinus* seeds were collected from wild populations in Spain.

2.2. Amino acid analysis

Duplicate samples (10 mg) were hydrolysed with 4 ml of 6 N HCl. The solutions were sealed in tubes under nitrogen and incubated in an oven at 110 °C for 24 h. Amino acids were determined after derivatisation with diethyl ethoxymethylenemalonate by high-performance liquid chromatography (HPLC), according to the method of Alaiz, Navarro, Giron, and Vioque (1992), using D,L-α-aminobutyric acid as an internal standard. Tryptophan was analysed by HPLC after basic hydrolysis according to Yust et al. (2004).

2.3. In vitro protein digestibility (IVPD)

In vitro protein digestibility was determined according to the method of Hsu, Vavak, Satterlee, and Miller (1977).

2.4. Determination of nutritional parameters

The amino acid composition of studied lupins was used for the determination of several nutritional parameters of lupin seed proteins:

- Amino acid score (chemical score) was calculated as:
  % sample essential amino acids contents/% recommended essential amino acids (FAO/WHO/UNU, 1985).
- Protein efficiency ratio values (PER) were calculated from the amino acid composition of lupin seeds based on the following three equations (Alsmeyer, Cunningham, & Happich, 1974):

$$\mathrm{PER}_1 = -0.684 + 0.456 \times \mathrm{Leu} - 0.047 \times \mathrm{Pro}$$

$$\mathrm{PER}_2 = -0.468 + 0.454 \times \mathrm{Leu} - 0.105 \times \mathrm{Tyr}$$

$$\mathrm{PER}_3 = -1.816 + 0.435 \times \mathrm{Met} + 0.78 \times \mathrm{Leu} + 0.211 \times \mathrm{His} - 0.944 \times \mathrm{Tyr}$$

- Protein digestibility corrected amino acid score (PDCAAS) (FAO/WHO, 1989) was calculated as:
  The lowest individual amino acid score × IVPD.
- Predicted biological value (BV) was calculated according to Morup and Olesen (1976) using the following equation:

$$\mathrm{BV} = 10^{2.15} \times \mathrm{Lys}^{0.41} \times (\mathrm{Phe} + \mathrm{Tyr})^{0.60} \times (\mathrm{Met} + \mathrm{Cys})^{0.77} \times \mathrm{Thr}^{2.4} \times \mathrm{Trp}^{0.21}$$

where each amino acid symbol represents:

% amino acid/% amino acid FAO pattern (1985), if % amino acid ⩽ % amino acid FAO pattern or:
% amino acid FAO pattern (1985)/% amino acid, if % amino acid ⩾ % amino acid FAO pattern.

2.5. Cluster analysis

Cluster analysis of different taxa was performed using PRIMER-pc program, employing the Bray–Curtis index of dissimilarity (Bray & Curtis, 1957). The dissimilarity index was transformed to the index of similarity (1 − dissimilarity index × 100).

3. Results and discussion

Seed protein contents in studied lupins ranged from 23.8% in *L. gredensis* to 33.6% in *L. luteus* (Table 1). The percentages observed in wild populations are lower than those observed in seeds belonging to cultivated samples from the same species. Hence, *L. angustifolius* samples studied contained as an average 26.6% protein in contrast to 33.8% in commercial samples (Lqari et al., 2002). However, protein contents of the studied lupins were higher than those observed in other commercial legumes such as chickpea (24.7%) (Sánchez-Vioque, Clemente, Vioque, Bautista, & Millán, 1999).

The amino acid composition of studied wild lupin populations is shown in Table 1. All species show an amino acid pattern characteristic of legumes. Thus, except *L. cosentinii*, all taxa are deficient in sulphur amino acids and tryptophan according to FAO recommendations (FAO/WHO/UNU, 1985). Also, some species were deficient in aromatic amino acids and valine. Besides, all taxa, except *L. hispanicus*, were deficient in lysine, while in legumes the lysine content is usually above that suggested by FAO (1985). On the one hand, from the amino acid composition point of view, the most equilibrated species are *L. cosentinii* which is only deficient in lysine, and *L. hispanicus* which is also deficient in sulphur amino acids and tryptophan. On the other hand, *L. angustifolius* and *L. luteus* appear as the species which have the worst amino acid composition. This deficiency of lysine, tryptophan and valine has also been reported for commercial samples of *L. luteus, L. angustifolius* and *L. albus* (Sujak, Kotlarz, & Strobel, 2006).

The importance of protein composition in the diet has been well known for many years. Less clear is how to determine the nutritional quality of protein intake, which depends mainly on the amino acid contents. The first step is to determine the amino acid composition of proteins. To make use of these amino acids proteins must be digested in order to release them. Many different factors may impair digestibility, which would result in lower protein quality. Hence, in addition to amino acid composition, protein digestibility has been proposed as another protein characteristic which determines the protein quality. *In vitro* protein digestibility (IVPD) in studied *Lupinus* ranged from 82.3% in *L. gredensis* to 89.0% in *L. cosentinii.* This is a high IVPD, similar to those observed in other lupins, such as *L. albus* (86.9–88.8%) (El-Adawy et al., 2001), or to soybean (85.8%), rice (84.8%), or wheat (83.7%) (Wolzak, Elias, & Bressani, 1981). Moreover, it is higher than those observed in other legumes, such as chickpea (76.2%) (Sánchez-Vioque et al., 1999).

The highest predicted biological value was in *L. cosentinii* and *L. luteus* with values of 85.9 and 90.2, respectively (Table 2). The lowest predicted biological values were observed in *L. hispanicus* with 42.3. The best predicted biological values observed in studied *Lupinus* were lower than those described for lactoalbumin, with 97%, but higher than those reported for other crops, such as triticale (65.3) or wheat (61.6) (Friedman, 1996).

All *Lupinus* species studied here showed PER theoretical values over 2 and many even close to 3, corresponding to proteins of high nutritional value. Better calculated PER values were observed in *L. luteus* with indexes around 3. The lowest values were calculated for *L. gredensis* and *L. angustifolius*. Calculated PER values in wild populations of *L. Luteus* studied (Table 2) were higher than those previously observed in cultivated *L. luteus* (between 2.17 and 2.67) (Sujak et al., 2006), and those in many meat-containing foods

Table 1
Seed protein amino acids composition of studied lupins. Data expressed as g/100 g protein are the average ± standard deviation of indicated number of populations studied.

	L. angustifolius	*L. cosentinii*	*L. gredensis*	*L. hispanicus*	*L. luteus*	*L. micranthus*	FAO[a]
n[b]	4	1	1	3	3	3	
Asp[c]	11.8 ± 0.6	11.7	16.0	12.0 ± 0.1	11.6 ± 0.3	12.1 ± 0.1	
Glu[d]	22.9 ± 0.2	24.2	22.4	22.0 ± 0.7	23.7 ± 0.7	22.4 ± 0.6	
Ser	6.4 ± 0.3	5.2	7.8	6.4 ± 0.0	6.5 ± 0.0	5.7 ± 0.1	
His	2.9 ± 0.2	2.6	2.7	2.8 ± 0.1	2.8 ± 0.1	2.6 ± 0.0	1.9
Gly	4.6 ± 0.1	4.4	6.0	4.6 ± 0.1	4.5 ± 0.1	4.4 ± 0.1	
Thr	4.1 ± 0.1	4.1	3.0	4.3 ± 0.2	3.8 ± 0.2	4.2 ± 0.1	3.4
Arg	11.4 ± 0.3	10.3	11.2	11.2 ± 0.4	11.5 ± 0.7	13.0 ± 0.9	
Ala	3.9 ± 0.0	3.9	4.5	3.8 ± 0.1	3.6 ± 0.2	4.0 ± 0.1	
Pro	3.1 ± 0.3	3.4	2.1	2.3 ± 0.2	2.3 ± 0.2	2.2 ± 0.2	
Tyr	2.9 ± 0.0	2.5	2.4	2.7 ± 0.1	2.3 ± 0.1	2.8 ± 0.1	6.3[e]
Val	3.4 ± 0.0	3.8	1.9	4.0 ± 0.1	3.3 ± 0.1	3.7 ± 0.1	3.5
Met	0.5 ± 0.1	0.7	0.7	0.1 ± 0.2	0.2 ± 0.1	0.6 ± 0.0	2.5[f]
Cys	1.0 ± 0.1	1.8	1.3	1.2 ± 0.1	2.0 ± 0.3	1.2 ± 0.3	
Ile	3.3 ± 0.0	3.6	2.2	3.7 ± 0.0	3.1 ± 0.0	4.0 ± 0.0	2.8
Trp	1.0 ± 0.1	1.1	0.7	0.8 ± 0.2	0.8 ± 0.2	0.5 ± 0.1	1.1
Leu	7.4 ± 0.2	7.8	6.9	7.9 ± 0.1	8.2 ± 0.0	7.8 ± 0.1	6.6
Phe	4.0 ± 0.0	3.9	3.4	4.3 ± 0.1	4.0 ± 0.1	3.9 ± 0.1	
Lys	5.2 ± 0.1	5.2	4.9	5.9 ± 0.2	5.4 ± 0.1	4.9 ± 0.1	5.8
Protein	26.6 ± 1.6	24.7	23.8	33.2 ± 1.2	33.6 ± 1.8	31.1 ± 1.4	

[a] Suggested pattern of amino acid requirements (FAO/WHO/UNU, 1985).
[b] Number of populations studied.
[c] Asp + Asn.
[d] Glu + Gln.
[e] Tyr + Phe.
[f] Met + Cys.

Table 2
Nutritional characteristics of studied lupin seed proteins. Results are the average of populations indicated in Table 2 for each taxa.

	L. angustifolius	*L. cosentinii*	*L. gredensis*	*L. hispanicus*	*L. luteus*	*L. micranthus*
IVPD[a]	87.6	89.0	82.3	88.5	88.5	87.5
% EAA/TAA[b]	34.7	35.3	29.3	36.5	33.9	35.0
AAS[c]	102.4	104.1	86.4	107.7	100	103.2
BV[d]	47.3	85.92	67.9	42.3	90.2	50.2
PER_1[e]	2.54	2.72	2.36	2.81	2.95	2.77
PER_2	2.60	2.82	2.39	2.85	3.03	2.80
PER_3	2.05	2.81	2.15	2.51	3.09	2.43
PDCAAS[f]	0.53	0.80	0.45	0.46	0.64	0.40

[a] *In vitro* protein digestibility.
[b] % Essential amino acids/total amino acids.
[c] Amino acids score.
[d] Biological value.
[e] Protein efficiency ratio.
[f] Protein digestibility corrected amino acid score.

(Alsmeyer et al., 1974). The *Lupinus angustifolius* calculated PER reported here were similar to those calculated for cultivated samples (between 2.10 and 2.77) (Sujak et al., 2006). Similar values to those reported here for *Lupinus* have been described for other legumes, such as soybean (2.57) (Wolzak et al., 1981) or chickpea (2.8) (Newman, Roth, Newman, & Lockerman, 1987). Lower PER values were observed in other legumes such as peanut (between 1.45 and 1.76) (Ghuman, Mann, & Hira, 1990), or *Vigna radiata* (between 1.6 and 2.1) (Savage & Deo, 1989).

The use of amino acid scores has been proposed as a more accurate alternative to PER parameters (Sarwar et al., 1984). The amino acids score was the highest in *L. hispanicus* and *L. cosentinii* with average values of 107.7 and 104.1, respectively (Table 2). The lowest value was observed in *L. gredensis* with average values of 86.4. Similarly, the% essential amino acids / total amino acids ratio was the highest in *L. hispanicus* and *L. cosentinii* with average values of 36.5 and 35.3, respectively.

The PDCAAS is nowadays the most recommended theoretical parameter for evaluating the nutritional quality of food proteins. It is based on FAO recommendations of amino acid requirements (FAO/WHO/UNU, 1985) and *in vitro* protein digestibility. The highest PDCAAS value for a given protein is 1.0. The lowest PDCAAS was observed in *L. micranthus* with 0.4, while the highest PDCAAS was observed in *L. cosentinii* with 0.80. This index was higher than that observed in other legumes, such as peas (0.69), beans (0.68) or lentils (0.52).

The analysis of similarity based on a profile of seed protein amino acid composition shows two major groups (A and B, Fig. 1) with 91.7% affinity. The first includes only *L. gredensis,* while the remaining species studied form the other group (group B). Within group B, two groups (C and D) are distinguished with 95.8% similarity. Group C includes only *L. cosentinii*, while the remaining species form group D. In the latter group two other groups are recognised (E and F) with 96% affinity. Not considering *L. gredensis*, the separation of *L. cosentinii* (group C) from the other species (group D) supports the delimitation of the *Lupinus* species on the basis of the seed coat texture into two distinct groups: the rough-seeded (*L. cosentinii*) and the smooth-seeded species (the remaining species) (Naganowska, Wolko, Sliwinska, & Kaczmarek, 2003; Przybylska & Zimmniak-Przybylska, 1995). With respect to group D the subdivision (with 96% affinity) in group E (*L. micranthus*) and group F is in accordance with molecular data (Naganowska et al., 2003). According to the amino acid composition *L. gredensis* is the most divergent specie. In conclusion, the analysis of similarity of studied *Lupinus* based on the amino acid composition shows some discrepancy with the established interspecific taxonomical

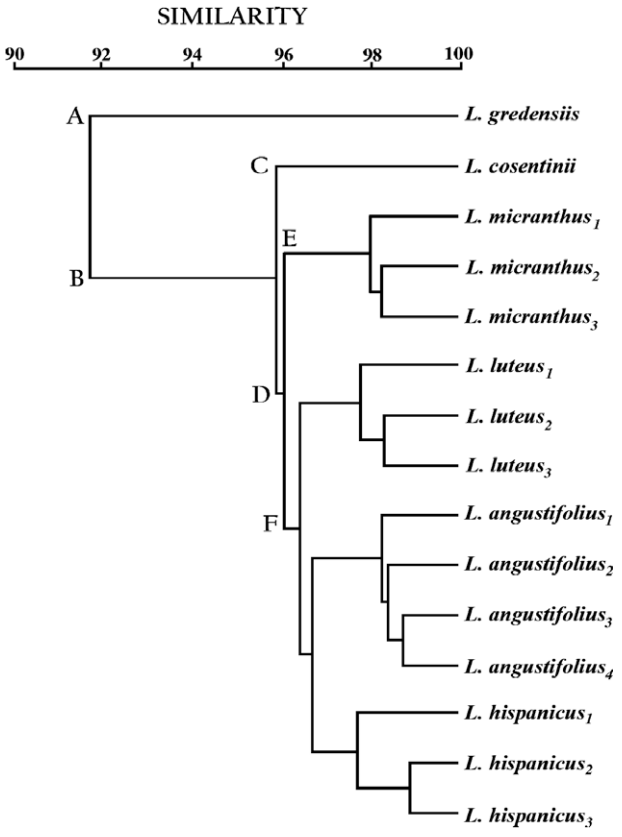

Fig. 1. Clustering based on the amino acid composition of studied *Lupinus* species, according to the Bray–Curtis similarity index (1 − dissimilarity index × 100).

relationships. However, amino acid composition was useful to group in single clusters populations belonging to the same specie.

From the studied species *L. cosentinii* appears as the one with the most equilibrated amino acid composition. It was only deficient in lysine. It is used for livestock feeding and soil fertilisation and has been used in breeding programmes in Australia since 1954 (Weder, Salmanowicz, & Köhler, 1997) due to its successful adaptation to alkaline soils.

With respect to the nutritional parameters, *L. luteus, L. hispanicus* and *L. cosentinii* showed the best results. On the contrary, *L. gredensis* showed the worst nutritional characteristics.

Results confirm the interest in studying wild populations of cultivated and non-cultivated *Lupinus* species as a source of seeds with good nutritional characteristics. This may help in the domestication of new species or the use of wild populations in breeding programmes, favouring the bio-conservation of *Lupinus*.

Acknowledgement

This work was financed by grant AGR-711 from Junta de Andalucía (Spain). Thanks are due to María Dolores García-Contreras for technical assistance.

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Food Chemistry 117 (2009) 470–472

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Short communication

Production and analysis of ochratoxin A produced by *Aspergillus ochraceus* ITEM 5137 in submerged culture

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ARTICLE INFO

Article history:
Received 2 December 2008
Received in revised form 18 February 2009
Accepted 12 April 2009

Keywords:
Ochratoxins
Aspergillus ochraceus
Biofermenter
HPLC
Submerged culture
Filamentous fungi

ABSTRACT

A rapid simple and economical method was described for the determination of ochratoxin A produced by *Aspergillus ochraceus* ITEM 5117 grown in a biofermenter in submerged culture. The ochratoxin A was determinate with RP-HPLC-FLD (reverse phase high performance liquid chromatography) with direct injection in the HPLC apparatus using a C18 column. The mycotoxin was completely resolved by using the mixture acetonitrile, water and acetic acid (49:49:2 v/v) as the mobile phase with a flow rate of 1.0 mL/min. Mean recoveries of ochratoxin A ranged from 95.36% to 103.15%. The limit of detection for ochratoxin A in medium was found to be 1 $\mu g\,L^{-1}$.

1. Introduction

Mycotoxins, a structurally diverse group of mostly small molecular weight compounds produced by the secondary metabolism of fungi, are ubiquitous in a broad range of commodities and feeds (Steyn, 1995). They may cause a toxic response, known as mycotoxicosis, when ingested by higher vertebrates and other animals. The mycotoxigenic fungi involved with the human food chain belong mainly to three genera: *Aspergillus*, *Fusarium* and *Penicillium* (Sweeney & Dobson, 1998).

Among the mycotoxins that have a substantial importance in the medical field and in foods, further at the aflatoxins and trichotecenes, there are OTA, beauvericin and the enniatins belonging to classes A and B (Sweeney & Dobson, 1998).

The mycotoxin OTA derives its name from *Aspergillus ochraceus*, the first mould from which it was isolated. It is the main toxic component in cultures of this mould, but it is also produced by other ubiquitous moulds such as various other strains of *Aspergillus* and *Penicillium*. OTA consists of a dihydroisocoumarin moiety linked through its 7-carboxy group by an amide bond to L-phenylalanine, as showed in Fig. 1 (Rohr, Dietrich, Schlatter, & Schlatter, 1995).

Several toxic effect of OTA have been described, such as inhibition of protein synthesis, impairment of calcium homeostasis, induction of lipid peroxidation, oxidative stress and DNA damage, with species and tissue specific differences (Almela et al., 2007). Furthermore, this mycotoxin is known/considered to be nephrotoxic, cytotoxic (Rinaldi, Mancini, Ferruzza, Sambuy, & Peruzzi, 2007), carcinogenic, teratogenic and immunosuppressive. It may also induce gene mutation, although the mechanism of genotoxicity is not clear (Rinaldi et al., 2007).

Human exposure to OTA has been related to Balkan Endemic Nephropathy (BEN) syndrome, but available clinical and epidemiological data do not confirm this hypothesis because similar concentrations of this mycotoxin do not cause the disease in other European areas (Abouzied et al., 2002; Blank et al., 2003; Creppy, Baudrimont, & Betleder, 1998; Jimmer, Lopez de Cerain, Gonzales-Penas, & Bello, 1999). On the other hand, BEN syndrome has been associated with other nephrotoxic agents (Pfohl-Leszkowicz, Petkova-Bocharoma, Chernozensky, & Castegnaro, 2002).

The underlying mechanism of OTA-mediated pathologies has not been fully elucidated (O'Brien & Dietrich, 2006; Ringot, Chango, Schneider, & Larondelle, 2006).

Various analytical methods have been developed to evaluate OTA, in different matrix such as foods and biological fluids with HPLC or thin-layer chromatography (TLC) (Assaf, Betbeder, Creppy, Pallardy, & Azouri, 2004; Munoz, Vega, Rios, Munoz, & Madariaga, 2006; Pittet & Royer, 2002; Santos & Vargas, 2002). We propose a new HPLC method for the evaluation of OTA produced by *A. ochraceus* ITEM 5117 in submerged culture with a biofermenter without any sample preparation. The HPLC analysis was performed by a direct injection in a C18 monolithic column, which allows to directly analyse liquid matrix with any previous treatment.

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doi:10.1016/j.foodchem.2009.04.041

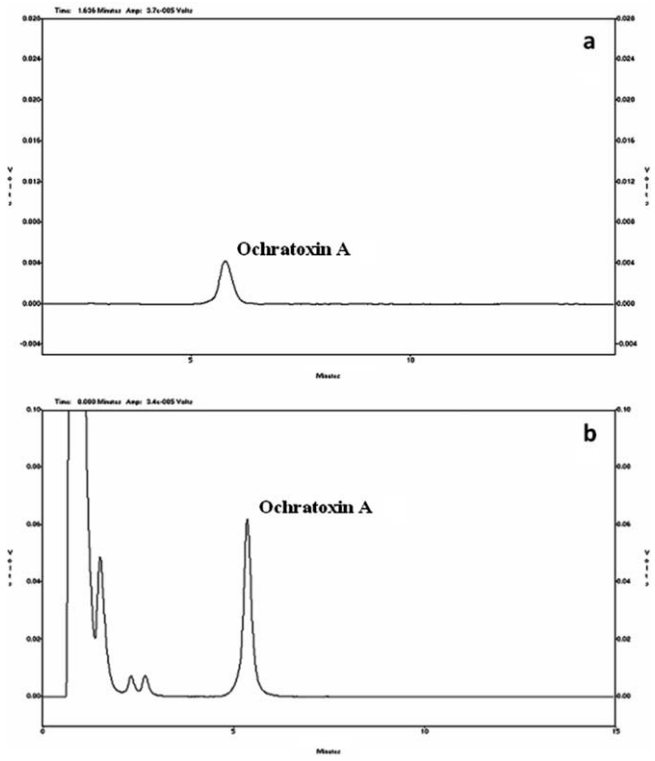

Fig. 1. (a) Chromatogram related to the ochratoxin A standard (10 μg L^{-1}) analysed with monolithic C18 column and (b) separation of ochratoxin A detected in PDB inoculated with *Aspergillus ochraceus* ITEM 5117 at ninth fermentation day, on the monolithic C18 column.

2. Materials and methods

2.1. Microorganism and inoculum preparation

A commercial cultural broth, potato dextrose broth (PDB), was used for this study, obtained from Oxoid (Basingstoke, UK). A suspension of conidia in concentration of 10^6 conidia mL^{-1} of *A. ochraceus* ITEM 5137 was used for inoculation. Conidial concentration was measured by optical density at 600 nm and adjusted to 10^6 conidia mL^{-1} in the biofermenter (Kelly, Grimm, Bendig, Hempel, & Krull, 2006). The strain used is deposited in culture collections at the Istituto delle scienze delle Produzioni Alimentari del Consiglio Nazionale delle Ricerche (ISPA-CNR) (Bari, Italy), in sterile 18% glycerol.

2.2. Cultivation system

The cultivations are accomplished by triplicate in 5 L bioreactors Inforce (Bottmingen, Switzerland) with a working volume of 4 L. For agitation, two rushton turbines (Ø 45 mm) are used and aeration related to the working volume is set to a constant value of 2 L min^{-1}.

The agitation rate was set at 180 rpm. Temperature and pH are maintained at 30 °C and 7.0, respectively. The pH is controlled by the addition of sodium hydroxide (0.5 M).

The biofermenter was inoculated with conidia produced in preculture for 24 h at low agitation rate of 50 rpm.

In order to evaluate OTA production during the mould growth, a volume of 2 mL of the broth containing *A. ochraceus* ITEM 5117 was sampled every three days during one month, it was filtered through 0.22 μm regenerated cellulose membrane and injected in triplicate directly in the HPLC for OTA determination.

2.3. Apparatus and column

Direct HPLC analysis of OTA was performed using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with two binary pumps (mod. LC-10ACvp), a fluorometre detector (mod. RF-10Axl), a system controller (mod. SCL-10Avp) and a valve inject mod. 7725 Rheodyne (Park Court Rohnert Park, USA). The fluorometre detector was set up at λ_{ex} of 333 nm and λ_{em} of 460 nm. The instrument was fitted with a 20 μL peek loop and a 100 μL precision syringe Hamilton (Reno, USA) was used for this injection. The chromatograms were stored and processed with a Shimadzu software Class-VP v.4.3 (Shimadzu, Kyoto, Japan). The mobile phase was a mixture of acetonitrile, water and acetic acid (49:49:2 v/v/v, respectively) at a constant flow of 1 mL min^{-1} under/using isocratic conditions and an Onyx Monolithic C18 100 × 3 mm ID 5 μm, Phenomenex (Torrance, USA) analytical column was used. The OTA retention time was 5.8 min (Fig. 1) with a retention factor of $k' \cong 6.56$.

2.4. Chemicals

A stock standard solution of OTA Sigma–Aldrich (St. Louis, USA) was prepared by dissolving 1 mg of standard in 1 mL of pure methanol, obtaining a stock solution of 1000 mg L^{-1}. This stock solution was used to prepare the work solutions (from 1 to 100 μg L^{-1}) by preparing appropriate dilutions with pure methanol. Acetonitrile, methanol, water (all HPLC grade) and acetic acid were purchased from Merck (Whitehouse Station, USA). The 0.2 μm filter used were the type RC25 Phenomenex (Torrance, USA).

2.5. Calculation of results

The amount of OTA, in the mediums, analysed by HPLC was determined by using a calibration graph of concentration with a linear range from 1 to 100 μg L^{-1}. The linear fit was: $y \cong 8500.7 \cdot x$ (with a $r^2 \cong 0.9998$). The limit of quantification (LOQ) was 1 μg L^{-1} and the limit of detection (LOD) was 0.1 μg L^{-1}, calculated as 3 and 10 times the height of the noise level, respectively.

2.6. Recovery

The mediums without amounts of OTA were supplemented with different levels of ochratoxin A, in particular in the concentration of 1, 5, 10, 50 and 100 μg L^{-1} to determine the recovery of the procedure analysis.

3. Results and discussion

3.1. Recovery analysis

The recovery analysis showed very satisfying values (Table 1). The recovery of the ochratoxin A was 95.36% or higher for added levels of 1, 5, 10 and 100 μg L^{-1}. The % SD ranged from 0.35% in the medium containing 50 μg L^{-1}, and 2.02% in the medium containing 5 μg L^{-1} of the toxin.

Table 1
Recovery of ochratoxin A from medium supplemented with different toxin's concentration.

Spiking level (μg L^{-1})	Recovered (μg L^{-1})	Recovery (%)	Standard deviation (%) (n = 3)
1	0.99	99.00	0.56
5	5.15	103.15	2.02
10	10.17	101.70	1.05
50	47.68	95.36	0.35
100	98.62	98.62	2.48

Table 2
Monitoring of ochratoxin A concentration produced by *Aspergillus ochraceus* in PDB during 30 days of fermentation.

Days	Ochratoxin A produced by *Aspergillus ochraceus* ITEM 5117 in PDB ($\mu g\ L^{-1}$)
0	20.05
3	22.26
6	19.02
9	121.89
12	147.33
15	161.53
18	162.96
21	146.57
24	144.71
27	126.94
30	122.30

3.2. Separation efficiency

The standard of ochratoxin A was subjected to a series of chromatographic runs in order to establish optimum condition for separation in C18 monolithic column (Fig. 1). The water used alone as the mobile phase was not found sufficient to resolve the ochratoxin A. Including acetonitrile in the mobile phase mixture 50% resulted a better separation owing to faster elution. Fig. 1 illustrates the separation of pure ochratoxin A on a monolithic C18 column using a mixture acetonitrile, water and acetic acid (49:49:2 v/v/v) as the mobile phase at a flow rate of 1.0 mL/min.

Fig. 1 also illustrates the medium chromatogram in which all co-extractives were resolved from each other under the same chromatographic condition. Retention time of ochratoxin A was approximately 6.0 min. RSDs was 0.74%, indicating good retention time reproducibility.

3.3. Applicability

The method was used to evaluate the amount of ochratoxin A produced by *A. ochraceus* ITEM 5117 in submerged culture, using PDB as growth medium, in biofermenter during 30 days. Results are shown in the Table 2. The initial OTA concentration detected when the *Aspergillus* was inoculated in PDB was 20 $\mu g\ L^{-1}$, because the mould had already activated its secondary metabolism in the preinocul culture.

However, OTA's amount experienced a great increment at the nine fermentation's day, rising from 19 to 121 $\mu g\ L^{-1}$. The biggest OTA concentration was reached at the 18th day of growth, corresponding to 162 $\mu g\ L^{-1}$ of the toxin (Harris & Mantle, 2001).

Between the 21st and the 30th day the toxin's concentration decreased from 146 to 122 $\mu g\ L^{-1}$, because in this phase the microbial death starts occurring, and the microorganism use the toxin as a carbon source (Munoz et al., 2006).

4. Conclusion

The OTA is one of most studied mycotoxins because results to be involved in different toxicological and medical fields. Considering that the study of secondary metabolism products in submerged culture is fundamental for understanding the secondary moulds metabolism, a method to analyse OTA in the medium would be rapid, accurately and possibly economic. In this study, a simple, a precise and a sensitive HPLC method which requires less chemicals and time for the determination of ochratoxin A is described.

The method passed a series of validation tests including separation efficiency, reproducibility and applicability and found useful for routine control of ochratoxin A production in submerged culture fermentation which involved many ochratoxigenic moulds.

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Food Chemistry 117 (2009) 473–484

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Quantitative determination and characterisation of the main odourants of Mencía monovarietal red wines

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ARTICLE INFO

Article history:
Received 18 February 2009
Received in revised form 4 April 2009
Accepted 7 April 2009

Keywords:
Solid-phase microextraction
Mencía wine
Volatile compounds
Odourant
Odour activity value
Factorial design
Gas chromatography–electron trap mass spectrometry

ABSTRACT

This study focus on the development of a headspace solid-phase microextraction (HS-SPME) method coupled to gas chromatography–ion trap mass spectrometry (GC–ITMS) to identify and quantify simultaneously 32 characteristic volatile compounds. A mixed level factorial 3×2^3 design was performed to asses the influence of several factors and their interactions (extraction temperature, fibre coating, salt addition and sample volume) on the extraction efficiency. The evaluation of the design showed that temperature and fibre coating were the most significant variables. The method showed good linearity ($r^2 > 0.990$) and repeatability (RSD $\leqslant$ 16%) for all compounds, with detection limits ranging from 0.0024 μg/L for limonene to 238 μg/L for ethyl 3-hydroxybutyrate, in general below their odour threshold limits. Matrix effects were thoroughly studied. The method was after applied to the first aroma characterisation of the monovarietal young red wine Mencía from the Galician (NW Spain) designation of origin "Valdeorras DO". To achieve the identification of the major would-be impact odourants, the odour activity value (OAV) was also calculated.

1. Introduction

The wine is a complex matrix which contains volatile compounds, as minor basic components, which play an important role in the organoleptic quality to their account for wine flavour, basically. These odourants are produced through metabolic pathways during ripening and harvest of grapes (primary or pre-fermentative aroma), during their fermentation (secondary or fermentative aroma) and/or also during the storage of wines (post-fermentative aroma or bouquet) (Etiévant, 1991); then, factors such as grape variety, environmental conditions (climate and soils), fermentation conditions (yeast flora, pH and temperatures), wine-making process and ageing conditions are primordial in the final composition of these odourants. As a final result, wine aroma contains over 1000 compounds that belong to heterogeneous chemical groups such as acids, alcohols, aldehydes, esters, ethers, hydrocarbons, ketones, lactones, nitrogen compounds, sulphur compounds and/or terpene alcohols (Bonino et al., 2003; Cabredo-Pinillos, Cedrón-Fernández, & Sáenz-Barrio, 2008; Martí, Mestres, Sala, Busto, & Guasch, 2003; Tao, Li, Wang, & Zhang, 2008).

The concentration levels of the volatile odourants are variable, ranging from several mg/L to a few ng/L. Due to this complexity and these low levels, the use of extraction/concentration techniques are essential. There are several extraction techniques for the gas chromatographic (GC) analysis of volatile aroma components of wine. Liquid–liquid extraction (LLE) technique was the most widely used to extract volatile compounds in wine samples; however, LLE requires large volumes of organic solvents, it is time-consuming and low recoveries are registered (Cutzach, Chatonnet, & Dubourdieu, 2000; Lee & Noble, 2003; Silva Ferreira & Guedes de Pinho, 2003). Headspace techniques (static and dynamic) require specific instrumentation coupled with the GC instrument (Aznar & Arroyo, 2007). Other alternative extraction techniques include simultaneous extraction, distillation and solid-phase extraction (Campo, Cacho, & Ferreira, 2007; Castro, Natera, Durán, & García-Barroso, 2008; Hernanz, Gallo, Recamales, Meléndez-Martínez, & Heredia 2008), supercritical fluid extraction (Carro, García, & Cela, 1996; Karásek et al., 2003), liquid–liquid microextraction (Farina, Boido, Carrau, & Dellacassa, 2007; Ferreira, Escudero, López, & Cacho, 1998), and stir-bar sorptive extraction (Alves, Nascimento, & Nogueira, 2005; Díez, Dominguez, Guillén, Veas, & Barroso, 2004; Zalacaín, Marín, Alonso, & Salinas, 2007); they offer specified advantages but also they can present some drawbacks such as the possibility of contamination with solvents, artefact formation and insufficient selectivity.

Solid-phase microextraction (SPME) appears like an excellent alternative to conventional extraction techniques due to their advantages: simple, fast, inexpensive, sensitive and no-solvent required, among others. Headspace SPME (HS-SPME) has been widely applied in wine analysis to identify key primary, secondary and bouquet aroma compounds (Alves et al., 2005; Bonino et al.,

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.04.014

2003; Bosch-Fusté et al., 2007; De la Calle García et al., 1997; Martí et al., 2003; Perestrelo, Caldeira, Rodrigues, & Câmara, 2008; Tat, Comuzzo, Stolfo, & Battistutta, 2005; Torrens, Riu-Aumatell, López-Tamames, & Buxaderas, 2004); to identify the geographical origin of the product, the authenticity preventing product adulteration (Cabredo-Pinillos et al., 2008; Jurado et al., 2008) and/or to determine fungicide residues coming from the phytosanitary treatments of the grapes (Rial-Otero, Yagüe-Ruiz, Cancho-Grande, & Simal-Gándara, 2002).

This work is focussed on primary and secondary odourants of Mencía red wine. Mencía wine is a monovarietal young red wine produced under the Galician designations of origin "Valdeorras DO", "Ribeira Sacra DO" and "Monterrei DO" (Ourense, NW Spain). Only a previous work was published to establish an aroma pattern for Mencía variety from "Monterrei DO" and "Ribeira Sacra DO" using time-consuming LLE with different extraction solvents depending of the chemical family (Calleja & Falqué, 2005), but it is known that new trace aroma compounds are not detectable by conventional LLE technique (Bonino et al., 2003). The main purposes of this work were basically two:

1. To present a unique method based on HS-SPME, sampling with DVB-CAR-PDMS fibre, and subsequent GC–ITMS for the identification and determination of the most representative odourants of Mencía wines from the "Valdeorras DO".
2. To define the first profile of the major volatile compounds and impact odourants in Mencía wines from this region.

2. Experimental

2.1. Volatile standards

Volatile compounds tested in Mencía red wines were purchased from Fluka (St. Louis, MO, USA), Sigma–Aldrich (St. Louis, MO, USA) and Safc (St. Louis, MO, USA).

2.2. Standard and working solutions

Individual standard solutions for the volatile compounds were prepared in EtOH. Secondary standard solutions were also prepared by dilution in EtOH of the individual standard solutions. All the standards were stored in the darkness at 4 °C.

Working solutions, containing target analytes, were prepared by mixing and diluting different amounts of secondary standard solutions in a synthetic wine solution previously prepared by dissolving 130 mL of EtOH, 7.2 mL of glycerol, 3 g of tartaric acid, 0.7 g of citric acid, 0.9 mL of lactic acid, 0.5 mL of acetic acid, 1.5 g of glucose:fructose (1:1), 35 μL of HSO_3^- (5% in water) and a suitable amount of sodium hydroxide in deionised water (1 L of total volume); the percent of EtOH and pH value of the synthetic model wine was 13% (v/v) and 3.3, respectively, to reproduce the properties of the young Mencía wine studied. 2-octanol and methyl nonanoate were employed in working solutions as internal standards because of their chemical structure (belonging to alcohol and ester families, respectively) and their absence as natural components in wine.

2.3. Wine samples

Red wines of the 2007 vintage, originating from Mencía grape variety from the "Valdeorras DO" (Ourense, NW Spain) were elaborated in the winery of the Regulatory Council of the Valdeorras DO. All samples were taken from bottled wine (750 mL) ready for sale and were stored at 4 °C until analysis.

2.4. SPME coating

The commercially available SPME device and fibres were purchased from Supelco (Bellefonte, PA, USA). An 85 μm polyacrylate (PA) and 50/30 μm divinylbenzene-carboxen-polydimethylsiloxane (DVB-CAR-PDMS) fibres were initially conditioned according to the instructions of the manufacturer in order to remove contaminants and to stabilize the phase. Conditioning was carried out in an extra split/splitless port (split open) with helium carrier gas prior to each extraction. This procedure prevents the passive extraction of interfering analytes from room air.

2.5. Experimental design

The influence of the main variables potentially affecting the efficiency of SPME was evaluated by using an experimental design approach, which allowed the simultaneous variation of all variables selected and the detection of probable interactions among them that could not be found in classical one-at-a-time optimisation. Factors included in the factorial design were: temperature (A) at three levels (25, 42.5 and 60 °C); fibre coating (B) at two levels (PA, DVB-CAR-PDMS); salt addition (NaCl) (C) at two levels (0% and 30%) and sample volume (D) at two levels (15 and 30 mL).

A mixed level factorial 3×2^3 design, which involved 24 experiments, was selected. The design had resolution *V*, which means that it was capable of evaluating all main effects and all two-factor interactions, allowing 12 degrees of freedom to estimate the experimental error.

Experiments were performed in headspace mode with aliquots of synthetic wine spiked at 1 mg/L of 2-methyl-1-propanol (**9**), 1-butanol (**10**), 3-methyl-1-butanol (**11**) and ethyl 3-hydroxybutyrate (**24**); 500 μg/L of terpineol (**3**), *trans*-3-hexen-1-ol (**13**), benzyl alcohol (**16**), *cis*-3-hexen-1-ol (**14**), *trans*-2-hexen-1-ol (**15**), 2-phenylethyl acetate (**19**), ethyl 2-methylpropanoate (**20**), ethyl butanoate (**21**), 4-ethyl-2-methoxyphenol (**28**), 4-ethyl-phenol (**29**) and 2-methoxy-4-vinyl phenol (**30**); 150 μg/L of linalool (**2**), citronellol (**4**), nerol (**5**), β-damascone (**6**), α-ionone (**7**), β-ionone (**8**), 1-hexanol (**12**), 3-methyl-1-butyl acetate (**17**), diethyl succinate (**26**), 2-furfuraldehyde (**31**) and benzaldehyde (**32**); 30 μg/L of limonene (**1**), hexyl acetate (**18**), ethyl hexanoate (**22**), ethyl octanoate (**23**), ethyl decanoate (**25**) and ethyl dodecanoate (**27**). Samples were magnetically stirred and the sampling time was set at 30 min.

The experimental design matrix and data analysis resulting from the experimental design were performed with the statistical software package Statgraphics Plus 5.1 (Manugistics, Rockville, MD, USA).

2.6. Final analytical procedure

2.6.1. HS-SPME

Wine samples (30 mL) were placed in a 40 mL glass sample vial. To each sample, 2-octanol and methyl nonanoate were used as internal standards (50 μL of a mix solution of 30 mg/L) and 9.0 g of Nacl was added. The vial was sealed with a Teflon-faced septum cap. Pre-conditioning of the sample at the extraction temperature was established between 10 and 15 min; some authors found this could be an important factor in the yield of the flavour extraction (Carrillo, Garrido-López, & Tena, 2006). After pre-conditioning, the SPME fibre was exposed to the headspace for 45 min. The sample was shaken with a magnetic stirring bar at 1100 rpm at controlled temperature (25 °C) during the extraction process and immediately the fibre was inserted during 10 min into the GC injector port (at 250 °C) for thermal desorption of the analytes.

2.6.2. GC-ITMS

GC analyses were carried out on a Trace GC Thermo Finnigan gas chromatograph (Rodano, Italy) equipped with a PolarisQ ion trap mass selective detector (ITMS), interfaced to a PC computer running the software XCalibur 1.4, from Thermo Electron Corporation (Italy). Chromatographic separations were done by using a Supelcowax fused-silica capillary column (60 m × 0.25 mm ID, 0.25 μm film thickness) from Supelco. The carrier gas, helium, operated at 1 mL/min in constant flow mode. Splitless mode was selected for 10 min. The oven temperature was programmed as follows: 40 °C for 2 min; 2 °C/min ramp to 225 °C where it was held for 15 min. The transfer line temperature was 260 °C, and the ion-trap manifold temperature was 250 °C. The ion energy for electron impact (EI) was always 70 eV. Mass detection was performed in the single ion range (SIR) mode; selected ions (m/z) used for qualitative and quantitative purposes are shown in Table 2.

3. Results and discussion

3.1. Chromatographic analysis

The first step consisted of developing experiments conducted to optimize the chromatographic separation of the 32 target analytes. Under the finally selected conditions, described in Section 2.6, an adequate separation of the compounds was achieved in 82 min, only 2-phenylethyl acetate (peak 19) and β-damascone (peak 6) were not completely resolved. Terpineol (peak 3) gave peak clusters corresponding to their α, β and γ isomers; in this study, the area of the α-isomer (the most abundant) was taken into account for determination.

3.2. Evaluation of the SPME process: factorial design

Extraction temperature is one of the most relevant parameters to evaluate in SPME since it affects both thermodynamics and kinetics of the extraction; temperature may enhance or worsen the extraction process depending on the volatility, vapour pressures and Henry's law constant values of the compounds (Pawliszyn, 1997). Two types of fibres (PA and DVB-CAR-PDMS), among those used most routinely for assaying wine volatiles, were also tested taking account of the physico-chemical characteristics of the target compounds under consideration. In agreement with published results, PA fibre showed an effective extraction for polar compounds such as alcohols of low molecular weight (Begala, Corda, Podda, Fedrigo, & Traldi, 2002; Martí et al., 2003; Rocha, Ramalheira, Barros, Delgadillo, & Coimbra, 2001; Vaz Freire, Costa Freitas, & Relva, 2001), higher alcohols, fatty acids (Perestrelo et al., 2008) and terpenes (De la Calle García et al., 1997; Rocha et al., 2001; Vaz Freire et al., 2001); on the other hand, previous works confirmed that DVB-CAR-PDMS is a versatile fibre, as it is highly sensitive for different chemical classes of analytes (esters, fatty acids, alcohols) (Carrillo et al., 2006; Howard, Mike, & Riesen, 2005; Martí et al., 2003; Setkova, Risticevic, & Pawliszyn, 2007; Tat et al., 2005; Torrens et al., 2004). The effect of ionic strength – no-addition and addition of 30% of NaCl (saturated condition) – was considered in the experimental design. Generally, the presence of electrolyte in an adsorption system could influence the adsorption in two ways: changing the properties of the phase boundary and decreasing the solubility of hydrophobic compounds (Yang & Peppard, 1994). Since the amount adsorbed in the SPME fibre is dependent on the sample volume, different volumes (15 and 30 mL) of the sample (subsequently different headspace volumes) were also tested. Although the presence of a gas phase should not change the distribution of an analyte between the liquid phase and the adsorption phase at equilibrium, the volume of the gas phase also influence the actual amount adsorbed on the fibre (Yang & Peppard, 1994).

The selection of some factors such as sampling mode and magnetic stirring were kept constant during the experimental design following the considerations cited in the bibliography (De la Calle García et al., 1997; Selli et al., 2004) in this way, HS-SPME mode should be more appropriated than direct sampling mode (D-SPME) for the analysis of aroma components of wines due to headspace mode showed better performance and higher sensitivity than direct immersion, since avoid the occurrence of interferences (Alves et al., 2005) and the direct contact of the coating with the relative high content of EtOH present in wine (De la Calle García et al., 1997). A magnetic stirring could be useful to accelerate the transfer of the analytes from the sample to the fibre coating; stirring facilitates the release of volatile compounds by increasing the surface of the liquid–vapour interface (Rocha et al., 2001).

Due to the selected design (3×2^3 mixed level factorial design) allowed 12 degrees of freedom, some statistic tests and graphic tools were evaluated in order to determine which factors had a statistically significant effect on the extraction process of each odourant, as well as which were the significant interactions between factors. The analysis of variance (ANOVA) was performed to evaluate and to asses the significance of the selected model. The most relevant graphical results of the design – Pareto charts and main effects plots – are showed in Fig. 1, respectively. One representative compound of each family is selected instead of the 32 aroma compounds studied. Pareto charts are useful graphs to illustrate the effect of the variables and their interactions. The bar length is proportional to the influence of each investigated variable or their interactions on the response. These charts include a vertical line corresponding to the 95% confidence interval; the main effects or interactions that exceed this reference line may be considered statistically significant (Fernández-Álvarez et al., 2008). The main effect plots are other graphical valuable tools for the interpretation of experimental design outcomes. This kind of plots shows the main effects with a line drawn between the low and the high level of the corresponding factors. The slope and the length of the depicted curves represent the variation in the response measured for each compound when the associated factor changes from the low to the high level considered in the domain of the design. The sign (+ or −) of the slope is related to the positive or negative exerted influence (Carpinteiro, Rodríguez, Cela, & Ramil, 2009).

Table 1 summarises the most favourable conditions evaluated for the extraction of the target compounds, including the significant interaction effects. As can be seen, the fibre coating (B) was the most important factor with a significant effect for 26 of the 32 studied compounds (Fig. 1, Table 1); the DVB-CAR-PDMS fibre exhibited the maxima responses in all cases, including those aromas where the fibre coating is not statistically significant (linalool, citronellol, nerol, 2-methyl-1-propanol, 1-butanol and ethyl hexanoate). The second important factor on the aroma response was the extraction temperature (A), presenting statistically significance for 18 compounds. Main effects plots of one of these 18 flavours (2-phenylethyl acetate) showed a slightly improvement at 42.5 °C (medium level), for four compounds the response is indistinct at 25 °C or 42.5 °C (benzyl alcohol, 4-ethylphenol, 2-furfuraldehyde and benzaldehyde) and the remaining compounds registered higher peak areas at room temperature (25 °C). Just in the case of citronellol, β-damascone, α-ionone, β-ionone, 1-butanol, ethyl hexanoate, ethyl decanoate, ethyl dodecanoate, 4-ethyl-2-methoxyphenol and 2-methoxy-4-vinyl phenol, 42.5 °C led also to higher chromatographic responses, but, for these compounds, the extraction temperature was not a significant variable. Salt addition was a significant factor for the extraction of only three of the investigated flavours (limo-

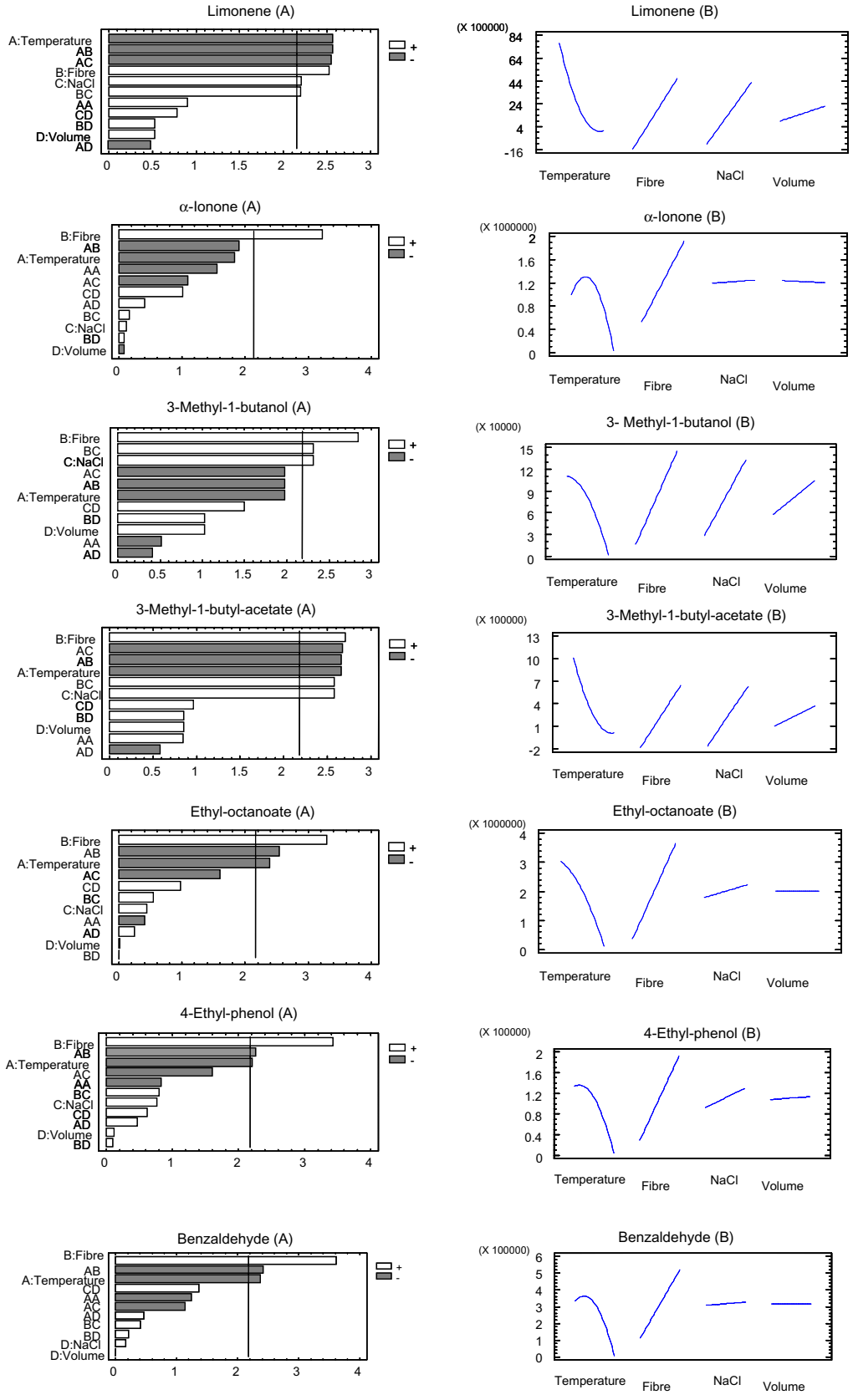

Fig. 1. Pareto charts (A), main effects plot (B) and interaction plots (C) for some selected aroma compounds.

nene, 3-methyl-1-butanol and 3-methyl-1-butyl acetate). For most of the remaining compounds, according to the interpretation of the main effects charts, the "salting-out" effect was shown to enhance the extraction. Finally, the volume was not

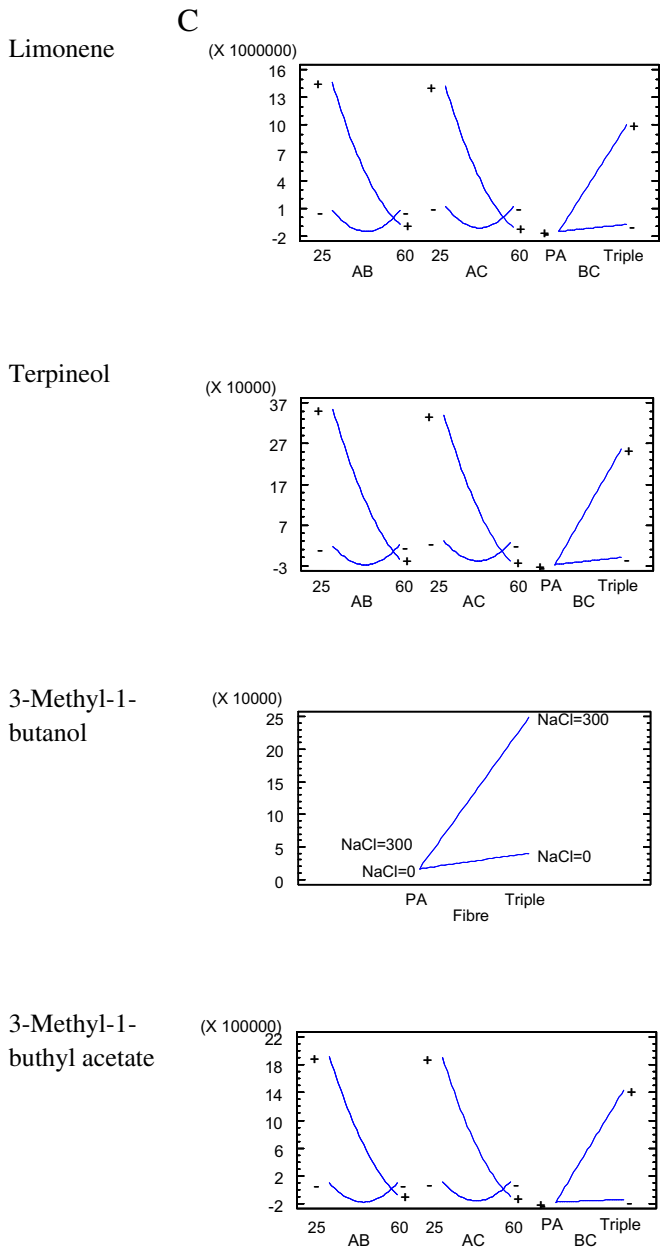

Fig. 1 *(continued)*

statistically significant for any of the studied compounds; nevertheless, in many cases the response achieved was higher with 30 mL of sample volume.

Other important graphical tool in experimental design analysis is the interaction plots, where the effect of two factors is simultaneously analyzed. Several interaction effects were significant for various analytes and some of them are depicted in Fig. 1. The interaction temperature-fibre coating (AB) was significant for 17 compounds. Working with triple fibre at 25 °C led to better responses than working at 60 °C with both fibres. Another significant interaction was found between temperature and salt addition (AC) for limonene, terpineol and 3-methyl-1-butyl acetate (Fig. 1). In this case, slight differences between no-addition and addition of 30% of NaCl were observed at 60 °C, however, at 25 °C, these differences were important and better results were obtained with salt addition. The combined effect between fibre coating and salt addition (interaction BC) is statistically significant in the case of limonene, 3-methyl-1-butanol and 3-methyl-1-butyl acetate (Fig. 1). The influence of the salt addition is much higher for triple fibre than PA fibre.

Taking into account both main and second order effects, the final proposed method for the simultaneous extraction of the 32 investigated wine volatile compounds was as follows: DVB-CAR-PDMS fibre coating, 30% NaCl, 30 mL of sample and 25 °C (Table 1).

3.3. HS-SPME adsorption and desorption processes

The extraction of volatile compounds with the DVB-CAR-PDMS fibre using HS-SPME is basically an adsorption process due to the presence of the DVB and CAR sorbents immobilized on the fibre by using PDMS. Optimum sorption time is defined as the time after which the amount of extracted analyte remains constant. Duplicate synthetic wine samples, fortified at the same levels described in the Section 2.5, were analyzed under the experimental conditions obtained by means of the experimental design. The influence of EtOH content during the extraction process was not previously checked (the final EtOH percentage in the samples was 15%) due to their concentration in wine has only a small influence on the peak intensities of aroma compounds in HS-SPME (De la Calle García et al., 1997); in the same sense, Jurado et al. (2008) did not observed significant differences in the responses with the variation of the EtOH content between 9.5% and 16.5%.

In general, longer sampling time than 45 min did not increase the total amount of volatile compounds. Of more detailed form, three different behaviours were registered. For a first group of volatile compounds (e.g. linalool, limonene 1-butanol, 3-methylbutanol, 1-hexanol, *trans*-2-hexen-1-ol *cis*-3-hexen-1-ol, *trans*-3-hexen-1-ol, isopentyl acetate, hexyl acetate, ethyl hexanoate or benzaldehyde), the most volatile ones (boiling points lower than 200 °C), a decrease in the corresponding area to 90 min was registered respect to 45 min. For a second group of volatile compounds (e.g. citronellol, nerol, terpineol, β-damascone, α-ionone, benzyl alcohol, 2-phenylethyl acetate, ethyl octanoate, ethyl decanoate, 4-ethylguaiacol or 4-vinylguaiacol), with a intermediate volatility (boiling points between 200 and 260 °C), no significant differences were observed in ITMS areas registered at 45 and 90 min which indicates that the equilibrium process has been reached. For the third minority group (e.g. β-ionone), with a low volatility, it was observed that 45 min was not enough to reach the equilibrium; according to Ai (1997), SPME determination is feasible at non-equilibrium conditions and then, sorption time can be shortened. As a conclusion, 45 min was considered as the optimized exposure time for all the volatile compounds studied.

The GC injector temperature was optimized to ensure that all the volatile compounds are completely desorbed from the fibre and to avoid carryover. For the DVB-CAR-PDMS fibre, three temperatures (230, 250 and 270 °C) were tested. Finally, 250 °C was selected as the optimum value. Desorption profiles showed that 8 min was enough to ensure total desorption for the majority of the compounds, except to some norisoprenoids (β-ionone), higher alcohols (benzyl alcohol) or volatile phenols (4-ethylguaiacol or 4 ethyl-phenol); moreover, carryover on SPME fibres due to incomplete desorption of analytes during the desorption process was attributed to strong adsorption in the coating. This fact was resolved with a desorption time of 10 min; then, no aromas or aromas lower than limits of detection (LODs) appeared in the chromatogram corresponding to the analysis of the fibre prior to re-exposure.

3.4. Quality parameters in synthetic wine

With the selected conditions for the SPME procedure, analytical quality parameters were evaluated in order to asses the performance of the HS-SPME/GC–ITMS method.

Table 1
Optimal experimental conditions and significant interactions given for each compound by the mixed level factorial 3×2^3 design (significant factors are emphasised in bold and italics).

Peak code	Odourants	Extraction temperature (°C)	Fibre coating	NaCl (%, w/v)	Volume (mL)	AB[a]	AC[a]	BC[a]
Terpenes								
1	Limonene	***25***	***DVB-CAR-PDMS***	***30***	30	*x*	*x*	*x*
2	Linalool	25	DVB-CAR-PDMS	30	30			
3	Terpineol	***25***	***DVB-CAR-PDMS***	30	30	*x*	*x*	
4	Citronellol	42.5	DVB-CAR-PDMS	0–30[b]	15			
5	Nerol	25	DVB-CAR-PDMS	30	30			
Norisoprenoids								
6	β-Damascone	42.5	***DVB-CAR-PDMS***	30	15–30[b]			
7	α-Ionone	42.5	***DVB-CAR-PDMS***	0–30[b]	15–30[b]			
8	β-Ionone	42.5	***DVB-CAR-PDMS***	0	15–30[b]			
Higher alcohols								
9	2-Methyl-1-propanol	25	DVB-CAR-PDMS	30	15			
10	1-Butanol	42.5	DVB-CAR-PDMS	0	15			
11	3-Methyl-1-butanol	25	***DVB-CAR-PDMS***	***30***	30			*x*
12	1-Hexanol	***25***	***DVB-CAR-PDMS***	30	15	*x*		
13	*trans*-3-hexen-1-ol	***25***	***DVB-CAR-PDMS***	30	30	*x*		
14	*cis*-3-hexen-1-ol	***25***	***DVB-CAR-PDMS***	30	30	*x*		
15	*trans*-2-hexen-1-ol	***25***	***DVB-CAR-PDMS***	30	30	*x*		
16	Benzyl alcohol	***25***	***DVB-CAR-PDMS***	30	30	*x*		
Acetates								
17	3-Methyl-1-butyl acetate	***25***	***DVB-CAR-PDMS***	**30**	30	*x*	*x*	*x*
18	Hexyl acetate	***25***	***DVB-CAR-PDMS***	30	30	*x*		
19	2-Phenylethyl acetate	***42.5***	**DVB-CAR-PDMS**	30	15–30[b]			
Ethyl esters								
20	Ethyl 2-methylpropanoate	***25***	***DVB-CAR-PDMS***	30	30	*x*		
21	Ethyl butanoate	***25***	***DVB-CAR-PDMS***	30	30	*x*		
22	Ethyl hexanoate	42.5	DVB-CAR-PDMS	0	30			
23	Ethyl octanoate	***25***	***DVB-CAR-PDMS***	30	15–30[b]	*x*		
24	Ethyl 3-hydroxybutyrate	***25***	***DVB-CAR-PDMS***	30	30	*x*		
25	Ethyl decanoate	42.5	***DVB-CAR-PDMS***	0	15			
26	Diethyl succinate	***25***	***DVB-CAR-PDMS***	30	30	*x*		
27	Ethyl dodecanoate	42.5	***DVB-CAR-PDMS***	0	15			
Volatile phenols								
28	4-Ethyl-2-methoxyphenol	42.5	***DVB-CAR-PDMS***	0–30[b]	15			
29	4-Ethyl-phenol	***25***	***DVB-CAR-PDMS***	30	30	*x*		
30	2-Methoxy-4-vinyl phenol	42.5	***DVB-CAR-PDMS***	30	15			
Aldehydes								
31	2-Furfuraldehyde	***25***	***DVB-CAR-PDMS***	30	15	*x*		
32	Benzaldehyde	***25***	***DVB-CAR-PDMS***	30	15–30[b]	*x*		

[a] *x*: Denotes a significant second-order interaction.
[b] When two values appear, it means that this factor did not affect the response, it does not matter which.

The repeatability (intra-day) and reproducibility (inter-day) of the method were calculated by analyzing five spiked synthetic wine samples in the same day and in two different not consecutive days, respectively. Results are reported in Table 2. As can be seen, the relative standard deviation (RSD) for repeatability was about 10% for all compounds; and for reproducibility, RSD% values increased around 2% but they continued being lower than 15%, except for ethyl butanoate (22.9%). These values allow confirming the good precision of the method and they agree with values contributed by other authors for the same fibre (Tat et al., 2005; Torrens et al., 2004).

Linearity of the method was evaluated by plotting 5-point calibration curves of the analyte area relative to that of the internal standard (internal standard selected for each compound is specified in Table 2). Linear ranges and determination coefficients (r^2) obtained for each volatile compound are given in Table 2. The method exhibited good linearity in the examined concentration range for all compounds with correlation coefficients (r^2) higher than 0.99.

Finally, limits of detection (LODs) and quantification (LOQs) were evaluated on the basis of the signal background obtained with the analysis of unfortified synthetic wine samples ($n = 7$) (Table 2). The LOD and LOQ were defined as the concentrations of analyte that provide signal-to-noise ratios of 3 and 10, respectively. The LODs, which ranged from 0.0024 μg/L for limonene to 238 for ethyl 3-hydroxybutyrate, were below the odour threshold limits reported in the literature for the studied compounds (Ferreira, López, & Cacho, 2000).

3.5. Matrix effects study

Wine is a very complex matrix; besides EtOH content there are others wine compounds, such as protein, amino acids, sugar and polyphenols, that make each wine different from the others (Pizarro, Pérez-del-Notario, & González-Sáiz, 2007). In HS-SPME, the distribution constants between the liquid phase, the headspace and the coating are strongly dependent on the matrix. In this respect, Dufour and Bayonove (1999) have suggested that wine polyphenols may interact with aroma compounds, reducing vapour pressure in some cases; Rocha et al. (2001) revealed the extent of the changes in the concentration of one matrix component in the SPME absorption of the other liquid matrix components, in general the change in the concentration of one matrix component affects the GC peak areas of all the others. For this reason, it is imperative in aroma analyses to check the matrix interferences in detail to avoid fatal quantification errors.

Table 2
Quality parameters of the optimized HS-SPME/GC-ITMS method using the DVB-CAR-PDMS fibre.

No		Target ions (m/z)	Repeatability (RSD %)[c]	Reproducibility (RSD %)[c]	Linearity range[d]	Determination coefficient (r^2)	LOD[e] (μg/L)	LOQ[e] (μg/L)
		Synthetic wine						
Terpenes								
1	Limonene[b]	67 + 69	11.5	12.2	0.1–50 μg/L	0.997	0.0024	0.0051
2	Linalool[a]	43 + 79 + 91	6.4	6.3	0.5–100 μg/L	0.995	0.035	0.064
3	Terpineol (α− + β− + γ−)[b]	63 + 93	7.9	10.2	5–100 μg/L	0.995	0.051	0.12
4	Citronellol[b]	67 + 81	10.7	12.7	0.5–100 μg/L	0.995	0.15	0.31
5	Nerol[b]	41 + 67 + 91 + 121	15.6	13.3	0.5–100 μg/L	0.997	0.15	0.31
Norisoprenoids								
6	β-Damascone[b]	121 + 167	9.6	8.6	0.1–50 μg/L	0.999	0.0080	0.017
7	α-Ionone[b]	93 + 121	11.3	10.5	0.1–50 μg/L	0.999	0.010	0.025
8	β-Ionone[b]	91 + 177	11.1	9.8	0.1–50 μg/L	0.999	0.014	0.030
Higher alcohols								
9	2-Methyl-1-propanol (or isobutanol)[a]	41 + 59	3.7	13.8	1–100 mg/L	0.999	57	108
10	1-Butanol[a]	41 + 43 + 55	10.5	13.3	1–100 mg/L	0.999	69	165
11	3-Methyl-1-butanol (or isoamyl alcohol)[a]	41 + 43 + 55	9.2	8.4	1–100 mg/L	0.999	1.8	3.3
12	1-Hexanol[a]	41 + 56 + 69	7.5	7.4	50–1000 μg/L	0.993	0.17	0.28
13	*trans*-3-hexen-1-ol[a]	41 + 67	7.4	9.7	50–1000 μg/L	0.995	0.21	0.39
14	*cis*-3-hexen-1-ol[a]	41 + 67	6.8	9.3	50–1000 μg/L	0.995	0.077	0.16
15	*trans*-2-hexen-1-ol[a]	41 + 67	9.1	9.7	50–1000 μg/L	0.993	0.65	1.44
16	Benzyl alcohol[b]	79 + 108	12.5	12.3	50–1000 μg/L	0.996	3.9	7.0
Acetates								
17	3-Methyl-1-butyl acetate (or isopentyl acetate or isoamyl acetate)[a]	41 + 43 + 55	9.8	11.7	1–500 μg/L	0.999	0.055	0.098
18	Hexyl acetate[a]	41 + 56	7.5	5.4	0.1–50 μg/L	0.999	0.012	0.019
19	2-Phenylethyl acetate[b]	78 + 104	13.4	7.1	0.5–100 μg/L	0.999	0.026	0.057
Ethyl esters								
20	Ethyl 2-methylpropanoate (or ethyl isobutyrate)[a]	41 + 73 + 88	10.7	9.1	50–1000 μg/L	0.990	0.99	1.5
21	Ethyl butanoate (or ethyl butyrate)[a]	41 + 43 + 55	10.9	22.9	1–500 μg/L	0.999	0.58	0.88
22	Ethyl hexanoate (or ethyl caproate)[a]	41 + 43 + 55	7.0	6.0	0.5–100 μg/L	0.994	0.033	0.072
23	Ethyl octanoate (or ethyl caprylate)[b]	41 + 55	15.8	13.1	0.1–50 μg/L	0.999	0.0049	0.010
24	Ethyl 3-hidroxybutyrate[a]	41 + 45	9.8	15.0	1–100 mg/L		238	371
25	Ethyl decanoate[a]	55 + 73	12.1	13.4	0.1–50 μg/L	0.996	0.016	0.023
26	Diethyl succinate[b]	55 + 101	15.3	10.3	50–1000 μg/L	0.991	0.32	0.58
27	Ethyl dodecanoate (or ethyl laurate)[a]	55 + 107	13.8	13.6	0.1–50 mg/L	0.999	0.046	0.11
Volatile phenols								
28	4-Ethyl-2-methoxyphenol (or 4-ethylguaiacol)[b]	137 + 152	11.9	11.7	0.5–100 μg/L	0.998	0.044	0.081
29	4-Ethyl-phenol[b]	77 + 107	10.1	4.5	25–500 μg/L	0.997	0.076	0.15
30	2-Methoxy-4-vinyl phenol (or 4-vinylguaiacol)[b]	77 + 150	16.1	6.9	25–500 μg/L	0.995	1.4	2.8
Aldehydes								
31	2-Furfuraldehyde (or 2-furfural)[a]	69 + 97 + 125	10.6	14.9	10–250 μg/L	0.992	0.037	0.068
32	Benzaldehyde[a]	77 + 105	6.8	8.5	0.5–100 μg/L	0.994	0.10	0.17
		"Non-flavour" wine						
Terpenes								
1	Limonene	67 + 69	13.3	11.8	0.1–50 μg/L	0.995[b]	0.041	0.077
2	Linalool	43 + 79 + 91	4.2	5.4	2.5–50 μg/L	0.998[a]/0.996[b]	1.0	2.5
3	Terpineol	63 + 93	6.6	7.9	2.5–30 μg/L	0.986[a]/0.994[b]	1.4	3.1
4	Citronellol	67 + 81	7.9	9.0	2.5–50 μg/L	0.995[a]/0.998[b]	0.29	0.72
Higher alcohols								
10	1-Butanol	41 + 43 + 55	5.8	5.4	2–40 mg/L	0.998[a]/0.992[b]	208	457

(*continued on next page*)

Table 2 (*continued*)

No		Target ions (*m*/*z*)	Repeatability (RSD %)[c]	Reproducibility (RSD %)[c]	Linearity range[d]	Determination coefficient (r^2)	LOD[e] (μg/L)	LOQ[e] (μg/L)
		"Non-flavour" wine						
Ethyl esters								
20	Ethyl 2-methylpropanoate	41 + 73 + 88	10.8	11.4	10–250 μg/L	0.994[b]	10	16
24	Ethyl 3-hidroxybutyrate	41 + 45	17.8	16.8	2–40 mg/L	0.993[a]/0.987[b]	74	185
Volatile phenols								
29	4-Ethyl-phenol	77 + 107	7.1	10.0	2.5–500 μg/L	0.994[a]/0.990[b]	0.7	1.3

Synthetic wine. Concentration level: 30 μg/L for compounds no. **1**, **18**, **22**, **23**, **25**, **27**; 50 μg/L for compounds no. **6**, **7**, **8**; 100 μg/L for compounds no. **2**, **3**, **4**, **5**, **28**; 150 μg/L for compounds no. **2**, **17**, **26**, **31**, **32**; 500 μg/L for compounds no. **13**, **14**, **15**, **16**, **19**, **20**, **21**, **29**, **30**; and 1 mg/L for compounds no. **9**, **10**, **11**, **24**.
Non-flavour wine. Concentration level: 0.5 μg/L for compound no. **1**; 5 μg/L for compounds no. **2**, **3**, **4**; 25 μg/L for compound no. **29**; 100 μg/L for compound no. **20**; and 10 mg/L for compounds no. **10**, **24**.
[a] IS 1: 2-octanol.
[b] IS 2: methyl nonanoate.
[c] $n = 5$, mean of determinations.
[d] 5 calibration levels (by duplicate).
[e] $n = 7$, mean of determinations.

Table 3 shows relative percentage of peak areas of volatile compounds in a real wine with regard to synthetic wine. Both wines were spiked at the same concentration level and analyzed twice. Results from the table revealed that peak areas of analytes decreased significantly, demonstrating a remarkably matrix effect, that means some kind of competition between interfering substances and analytes occurred. This phenomenon, in spite of its importance, has not been commonly studied (Begala et al., 2002; Bonino et al., 2003; Cabredo-Pinillos et al., 2008; Perestrelo et al., 2008; Tao et al., 2008; Torrens et al., 2004) which has not guaranteed appropriate quantification and consequently reliable results.

Different actions could be taken to overcome such problems: (1) the use of isotopically-labelled analytes as IS in isotope dilution based methods; (2) the use of multiple headspace SPME (MHS-SPME) procedure, which leads to a complete extraction of the analytes performing several consecutive extractions (Martínez-Uruñuela, González-Saiz, & Pizarro, 2005); (3) the use of appropriate ISs, which are affected by the matrix effect in the same way as the analytes (structurally similar); (4) a dilution of

Table 3
Matrix effects in Mencía wine.

Peak code	Odourants	Matrix effect[a] (%)
Terpenes		
1	Limonene	41
2	Linalool	9
3	Terpineol	13
4	Citronellol	8
5	Nerol	6
Norisoprenoids		
6	β-Damascone	7
7	α-Ionone	6
8	β-Ionone	5
Higher alcohols		
9	2-Methyl-1-propanol	33
10	1-Butanol	33
11	3-Methyl-1-butanol	14
12	1-Hexanol	9
13	*trans*-3-Hexen-1-ol	19
14	*cis*-3-Hexen-1-ol	20
15	*trans*-2-Hexen-1-ol	15
16	Benzyl alcohol	29
Acetates		
17	3-Methyl-1-butyl acetate	7
18	Hexyl acetate	5
19	2-Phenylethyl acetate	12
Ethyl esters		
20	Ethyl 2-methylpropanoate	20
21	Ethyl butanoate	−11[b]
22	Ethyl hexanoate	9
23	Ethyl octanoate	−26[b]
24	Ethyl 3-hydroxybutyrate	25
25	Ethyl decanoate	−39[b]
26	Diethyl succinate	−43[b]
27	Ethyl dodecanoate	33
Volatile phenols		
28	4-Ethyl-2-methoxyphenol	13
29	4-Ethyl-phenol	84
30	2-Methoxy-4-vinyl phenol	78
Aldehydes		
31	2-Furfuraldehyde	−28[b]
32	Benzaldehyde	15

Concentration level: 1 μg/L for compounds no. **1**, **6**, **7**, **8**, **18**, **23**, **25**, **27**; 20 μg/L for compounds no. **2**, **3**, **4**, **5**, **19**, **22**, **28**, **32**; 100 μg/L for compounds no. **17**, **21**, **29**, **30**; 250 μg/L for compounds no. **12**, **13**, **14**, **15**, **16**, **20**, **26**; and 20 mg/L for compounds no. **9**, **10**, **11**, **24**.

[a] Matrix effect (%) = 100 × (peak area obtained in a spiked Mencía wine sample − peak area obtained in a Mencía wine sample)/peak area obtained in synthetic wine at the same spiked level.

[b] Negative values due to high response of these aromas in Mencía wines, the addition is not noticeable.

the sample; (5) the generation of a matrix similar to the real wine but free of volatiles, a "non-flavour" wine. The non-volatile components remain unchanged and the concentration of the ethanol and pH value of the "non-flavour" wine was equal to that of the real wine (13% (v/v) EtOH, pH 3.3) (Liu, Zeng, & Tian, 2005); and (6) quantification by standard addition into each sample and with each analyte investigated.

In this work two of these different strategies (4 and 5) were tested for their ability to compensate or minimize the influence of matrix effects in wine analysis. They were evaluated by comparison with those results obtained by strategy number 6 (standard addition; four concentration levels performed in duplicate), assuming that the standard addition approach provides the true analyte concentrations. The main conclusions of these evaluations are as follows:

1. For aroma compounds with high analytical responses, a 15-fold dilution of the sample with synthetic wine and internal calibration in synthetic wine with appropriate ISs was considered. The criterion followed to optimize wine dilution was the maximum dilution at which was possible to obtain a quantifiable response of those analytes that overloaded the fibre in Mencía wine (i.e. 3-methylbutanol, 2-methyl-1-propanol, benzyl alcohol, 1-hexanol, diethyl succinate, ethyl butanoate, 2-furfuraldehyde, 3-methyl-1-butyl acetate, 2-phenylethyl acetate, benzaldehyde, ethyl hexanoate, ethyl octanoate, ethyl decanoate, hexyl acetate, ethyl dodecanoate). As can be seen in Table 4, standards prepared in synthetic wine could be used for most of the fibre overloading compounds. Only for 2-phenylethyl acetate, diethyl succinate and ethyl dodecanoate, significant differences (Students *t*-test, at a 95% confidence level) were observed between concentrations calculated by standard addition and IS calibration. In this case, quantification of analytes must be performed by matrix-matched standards.
2. For minor aroma compounds quantification was performed using internal standard method preparing the calibration standards in "non-flavour" wine. Previously, quality parameters were calculated as it was described for synthetic wine (Table 2), the degree of agreement between the real amount added and that determined by interpolation (relative recovery %) was satisfactory, close to 100%. IS calibration with 'non-flavour' wine was possible for 8 of the 13 remaining compounds. From the statistical data analysis (Students *t*-test, at a 95% confidence level), no significant

Table 4
Comparison of concentrations obtained by: (1) ISTD calibration in synthetic wine and standard addition calibration in 15-fold diluted Mencía wine. (2) ISTD calibration in "non-flavour" wine and standard addition calibration in Mencía wine.

Peak code	Odourants	Concentration ± SD[c] Standard addition calibration in 15-fold diluted Mencía wine	Concentration ± SD[c] ISTD calibration in synthetic wine
Higher alcohols			
9	2-Methyl-1-propanol	1.81 ± 0.81 mg/L	2.76 ± 0.14[a]/1.73 ± 0.06[b] mg/L
11	3-Methyl-1-butanol	14.06 ± 0.91 mg/L	16.65 ± 0.67[b] mg/L
12	1-Hexanol	105.40 ± 8.07 μg/L	119.34 ± 9.98[b] μg/L
16	Benzyl alcohol	35.94 ± 0.36 μg/L	36.30 ± 2.36[b] μg/L
Acetates			
17	3-Methyl-1-butyl acetate	46.64 ± 3.00 μg/L	61.99 ± 4.97[a] μg/L
18	Hexyl acetate	0.25 ± 0.08 μg/L	0.63 ± 0.26[b] μg/L
19	2-Phenylethyl acetate	2.48 ± 0.10 μg/L	
Ethyl esters			
21	Ethyl butanoate	13.66 ± 2.37 μg/L	18.24 ± 4.82[a]/23.16 ± 5.12[b] μg/L
22	Ethyl hexanoate	19.28 ± 0.09 μg/L	21.98 ± 1.45[a]/20.53 ± 1.40[b] μg/L
23	Ethyl octanoate	23.73 ± 0.10 μg/L	26.89 ± 2.31[a]/26.49 ± 2.56[b] μg/L
25	Ethyl decanoate	2.75 ± 0.28 μg/L	3.46 ± 0.89[a] μg/L
26	Diethyl succinate	328.52 ± 3.47 μg/L	
27	Ethyl dodecanoate	0.70 ± 0.01 μg/L	
Aldehydes			
31	2-Furfuraldehyde	216.10 ± 1.35 μg/L	207.41 ± 16.95[a] μg/L
32	Benzaldehyde	15.03 ± 0.05 μg/L	
Peak code	Odourants	Concentration ± SD[c] Standard addition calibration in Mencía wine	Concentration ± SD[c] ISTD calibration in "non-flavour" wine
Terpenes			
1	Limonene	0.09 ± 0.03 μg/L	0.12 ± 0.01[b] μg/L
2	Linalool	7.95 ± 1.00 μg/L	6.43 ± 0.24[a]/6.44 ± 0.42[b] μg/L
3	Terpineol	4.61 ± 0.32 μg/L	4.70 ± 0.35[a]/4.40 ± 0.28[b] μg/L
4	Citronellol	8.37 ± 2.02 μg/L	6.20 ± 0.10[a]/6.26 ± 0.63[b] μg/L
Norisoprenoids			
7	α-Ionone	0.12 ± 0.02 μg/L	
8	β-Ionone	0.45 ± 0.02 μg/L	
Higher alcohols			
10	1-Butanol	1.57 ± 0.43 mg/L	1.78 ± 0.11[a]/1.52 ± 0.22[b] mg/L
13	*trans*-3-hexen-1-ol	27.49 ± 6.21 μg/L	
14	*cis*-3-hexen-1-ol	28.24 ± 7.99 μg/L	
15	*trans*-2-hexen-1-ol	4.21 ± 2.00 μg/L	
Ethyl esters			
20	Ethyl 2-methylpropanoate	20.64 ± 6.99 μg/L	19.01 ± 5.2[b] μg/L
24	Ethyl 3-hidroxybutyrate	2.89 ± 0.16 mg/L	2.73 ± 0.01[a]/2.52 ± 0.31[b] mg/L
Volatile phenols			
29	4-Ethyl-phenol	2.49 ± 1.54 μg/L	4.84 ± 0.40[a]/3.14 ± 0.59[b] μg/L

[a] IS 1: 2-octanol.
[b] IS 2: methyl nonanoate.
[c] $n = 2$, mean of determinations.

differences between both calibration methods (standard addition and IS calibration) could be concluded. The quantification process (Table 4) confirmed that for α-ionone, β-ionone, *trans*-3-hexen-1-ol, *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol, matrix effects can not be compensated for by using IS method and selecting the "non-flavour" wine as working standard matrix.

3.6. Quantitative composition and odour active values

The first characterisation of the main odourants of Mencía red wines from the "Valdeorras DO" is given in Table 5. Concentration data indicated that 28 out of the 32 studied compounds could be found in the volatile fraction of the wine; higher alcohols were quantitatively the largest group of the volatiles in Mencía wine followed by acetates from higher alcohols. To evaluate the contribution of a volatile compound to the aroma of a wine, the odour activity value (OAV), known also as aroma index, was calculated as the ratio between the concentration of the compound and the odour threshold found in the literature (Ferreira, Rapp, Cacho, Hastrich, & Yavas, 1993; Ferreira et al., 2000; Guth (1997); Perestrelo, Fernandes, Albuquerque, Marques, & Câmara, 2006). This value constitutes a preliminary step to achieve the identification of the potentially most important wine odourants in a wine.

3.6.1. Terpenes

Terpenes play a significant role in the varietal odourants of wines; they are located in grape skin and linked to sugars,

Table 5
Characterisation of the main odourants of Mencía red wine from "Valdeorras DO".

Odourants	Concentration (μg/L)	Odour threshold (μg/L)[a]	Aroma index (OAV)	Odour descriptor[a]
Terpenes				
Limonene	0.1	15	0.01	Fruity, lemon
Linalool	6.4	25	0.3	Flowery; fruity, muscat
Terpineol (α− + β− + γ−)	4.7	250	0.02	Piney, iris, teil
Citronellol	6.3	18	0.3	Spicy, clove
Sub-total concentration	17.5			
%	0.006			
Norisoprenoids				
α-Ionone	0.1	2.6	<0.01	Fruity, raspberry; Floral, violet
β-Ionone	0.5	0.09	5.0	Floral, violet
Sub-total concentration	0.6			
%	<0.001			
Higher alcohols				
2-Methyl-1-propanol (or isobutanol)	25,950	40000	0.6	Green
1-Butanol	1520	150000	0.01	Sweet, medicinal
3-Methyl-1-butanol (or isoamyl alcohol)	249,750	30000	8.3	Chemical, fusel alcohol
1-Hexanol	1790	800	2.2	Vegetative, grass/cut
Trans-3-hexen-1-ol	27.5	n.f.[b]	n.d.[c]	Vegetative, grass/cut
Cis-3-hexen-1-ol	28.2	400	0.07	Vegetative, grass/cut
Trans-2-hexen-1-ol	4.2	15000	<0.01	Fruity, unripe banana
Benzyl alcohol	544	620	0.9	Fruity blackberry
Sub-total concentration	279,615			
%	96.4			
Acetates				
3-Methyl-1-butyl acetate (or isoamyl acetate)	930	30	31	Fruity, banana
Hexyl acetate	9.5	1500	0.01	Fruity, apple, pear
2-Phenylethyl acetate	37.2	250	0.1	Floral, roses
Sub-total concentration	976			
%	0.3			
Ethyl esters				
Ethyl 2-methylpropanoate (or ethyl isobutyrate)	19.0	15	1.3	Fruity, banana
Ethyl butanoate (or ethyl butyrate)	274	20	13.7	Fruity, strawberry
Ethyl hexanoate (or ethyl caproate)	308	14	22	Fruity, green apple; floral, violet; spicy, anise
Ethyl octanoate (or ethyl caprylate)	397	5	80	Fruity, pineapple, pear; floral
Ethyl 3-hidroxybutyrate	2.5	20,000	<0.01	Fruity, grape green
Ethyl decanoate	52	200	0.3	Chemical, soapy
Diethyl succinate	4928	6000	0.8	Wine
Ethyl dodecanoate (or ethtyl laurate)	10.5	800	0.01	Sweet, waxy, floral, soapy
Sub-total concentration	5991			
%	2.1			
Volatile phenols				
4-ethyl-phenol	3.1	440	<0.01	Other, stall, animal
Sub-total concentration	3.1			
%	<0.001			
Aldehydes				
2-Furfuraldehyde (or 2-furfural)	3111	14,100	0.2	Caramelized, sweet, caramel
Benzaldehyde	226	2000	0.1	Bitter, cherry
Sub-total concentration	3337			
%	1.1			
Total odourant concentration	289,940			

[a] Ferreira et al. (1993), Ferreira et al. (2000), Guth (1997) and Perestrelo et al. (2006).
[b] n.f.: Not found.
[c] n.d.: Not determined.

constituting an important part of the grape bouquet. Terpenes are not changed by the metabolism of yeast during fermentation (Mateo & Jiménez, 2000).

Four terpenes were detected al low concentrations in the sample wine: linalool, citronellol, terpineol and limonene (6.4, 6.3, 4.7 and 0.1 μg/L, respectively). They made up of 0.006% of the total volatile analyzed compounds. In all cases, the OAVs were lower than 1. It seems that these terpenoid compounds could not contribute substantially to the aroma of the Mencía wines.

3.6.2. Norisoprenoids

The presence of norisoprenoids is considered to be a quality factor and typical from each grape variety. α-Ionone and β-ionone were detected at concentrations of 0.1 and 0.5 μg/L, respectively. The content of β-ionone is higher than its odour threshold with an OAV of 5. This compound supplies a characteristic aroma of "violets" to the Mencía wine.

3.6.3. Higher alcohols

This volatile fraction is composed mainly by *n*-alcohols of C_6 chain length and aroma compounds such as benzyl alcohol. The higher alcohols could be synthesized by yeast through either the anabolic pathway from glucose, or the catabolic pathway from their corresponding amino acids (valine, leucine, *iso*-leucine and phenylalanine). Consequently, they are released to the medium as secondary products of metabolism of yeasts being responsible for the secondary or 'fermentative' aroma of wines.

The higher alcohols are quantitatively the largest group of the volatile compounds found in Mencía wines (96.4% of the total volatiles analyzed). This volatile fraction was mainly composed of 2-methyl-1-propanol (or isobutanol) (25,950 μg/L), 1-butanol (1520 μg/L), 3-methyl-1-butanol (or isoamyl alcohol) (249,750 μg/L), 1-hexanol (1790 μg/L) and benzyl alcohol (545 μg/L).

3-Methyl-1-butanol was markedly the most abundant higher alcohol, being present at levels higher than its odour threshold (30,000 μg/L), thus its sensorial contribution with "alcohol, fusel" odour was expected. The next higher alcohol with a significant role in the aroma of Mencía wine was 1-hexanol that contributed with "herbaceous" and "vegetal" odour. Benzyl alcohol, with an OAV very close to unity, supplied an agreeable scent of "blackberry fruits".

3.6.4. Acetate esters

Acetate esters were the result of the reaction of acetyl-CoA with higher alcohols formed by degradation of amino acids or carbohydrates (Perestrelo et al., 2006). 3-Methyl-1-butyl acetate with a fruity odour of "banana" was found in a concentration of 930 μg/L, 31 times over its odour threshold.

3.6.5. Ethyl esters

The ethyl esters of the fatty acids are one of the most relevant groups of compounds in the wine aroma. They are produced enzymatically during yeast fermentation and from ethanolysis of acyl-CoA that is formed during fatty acids synthesis or degradation. Their concentration is dependent on several factors mainly: yeast strain, fermentation temperature, aeration degree and sugar contents (Perestrelo et al., 2006). The nuances of ethyl esters coincide with the fruity and floral descriptors of the wines and play a modulating role in the quality (Ferreira, Fernández, Peña, Escudero, & Cacho, 1995).

The ethyl ester concentration was 5991 μg/L, being 2.1% of the total volatiles analyzed. The contribution of some of them to wine flavour and aroma was clearly important, thus ethyl octanoate ("pineapple, pear") and ethyl hexanoate ("green apple") showed the highest OAVs of the studied volatile compounds (80 and 22, respectively), consequently the strongest odour impact. Ethyl butanoate ("strawberry") with an OAV of 13.7, ethyl 2-methylpropanoate ("banana") with 1.3 and diethyl succinate ("wine") with an OAV near unity (0.8) also contributed to a great extent to the aroma of Mencía wines.

3.6.6. Volatile phenols

These compounds could originate from *p*-coumaric and ferulic acids by decarboxylation (Perestrelo et al., 2006). Within the family of volatile phenols only 4-ethyl-phenol was found at a low concentration of 3.1 μg/L. No contribution of this compound could be appreciated in the aroma of the wine because of its low OAV value (0.007).

3.6.7. Aldehydes

Aldehydes are formed from unsaturated fatty acids, such as linoleic and linolenic acids. Also, they can be considered as products of lipoxygenase catalysis (Perestrelo et al., 2006). The contents of the two aldehydes analyzed, 2-furfuraldehyde and benzaldehyde, although high (3111 and 226 μg/L, respectively), showed values lower than their odour thresholds (OAVs: 0.2 and 0.1, respectively).

4. Conclusions

A mixed level factorial design was performed to obtain the appropriated extraction conditions for the determination of primary and secondary aroma compounds in Mencía red wine by HS-SPME. The proposal analytical procedure was: 30 mL of sample with addition of 30% of NaCl, DVB-CAR-PDMS fibre, 25 °C of extraction temperature, 45 min of extraction time with agitation, 250 °C of desorption temperature and 10 min of desorption time. A marked matrix effect was registered; to overcome this negative phenomenon two suitable approaches were considered for 24 aroma compounds: a 15-fold dilution of the sample and IS calibration in synthetic wine for determining major aroma compounds and IS calibration in "non-flavour" wine for minor compounds. The remaining 8 analytes – 2-phenylethyl acetate, diethyl succinate, ethyl dodecanoate, α-ionone, β-ionone, *trans*-3-hexen-1-ol, *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol – were easily quantified by standard addition quantification to guarantee reliable results.

With regard to aroma profile of Mencía wine from "Valdeorras DO", this wine is characterized by the presence of high levels of higher alcohols (96.4%). Considering all the odourants detected with OAVs > 1, ethyl esters and acetates provided "banana", "strawberry", "green apple", "pineapple" and "pear" nuances to the aroma; higher alcohols conferred "fused alcohol" and "vegetative" nuances; and, finally, β-ionone gave a pleasant odour of "violet" to the wine, the typical aroma distinctive of this red young wines.

Acknowledgements

This work was funded by the AGL2007–62075 (Spanish Ministry of Education and Science) and the PGIDT07TAL016383PR (Autonomous Community Government in N.W. Spain) contracts. C. González-Barreiro was funded by a Parga Pondal contract from the Galician Council of Innovation and Industry. The authors thank to Dr. Elena Martínez-Carballo for their assistance with the statistical analyses.

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Food Chemistry 117 (2009) 485–490

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Determination of three dyes in commercial soft drinks using HLA/GO and liquid chromatography

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ARTICLE INFO

Article history:
Received 16 June 2008
Received in revised form 20 January 2009
Accepted 12 April 2009

Keywords:
Food dyes
Soft drinks
Net analyte signal
HLA/GO
HPLC

ABSTRACT

Allura Red, Sunset Yellow, and Tartrazine usually present in commercial soft drinks were accurately quantified using HLA/GO method, a net analyte signal-based method, without the need for solutes separation. The obtained percent recoveries (R.S.D.) of dyes were 99.8 (2.8), 102 (3.3), and 100 (4.5) for Allura Red, Sunset Yellow, and Tartrazine, respectively. The calibration method was applied for analysis of food dyes in powdered soft drinks with minimum sample preparation measures. The proposed HLA/GO method was validated against a standard HPLC method. Statistical analyses showed insignificant differences between the results of two methods. The developed HLA/GO method offers a simple and rapid procedure for determination of food dyes in powdered soft drinks in comparison with HPLC methodology.

1. Introduction

Allura Red (E-129), Sunset Yellow (E-110), and Tartrazine (E-102) are three highly used synthetic dyes which are added to many food products (López-de-Alba, Wróbel-Kaczmarczyk, Wróbel, López-Martínez, & Hernández, 1996; Nevado, Cabanillas, & Salcedo 1995). Allura Red, Sunset Yellow, and Tartrazine are used as additives in pharmaceuticals and cosmetics because they can be easily mixed to give favourable shades and because of their low price compared with natural colourants (López-de-Alba, López-Martínez, Cerdá, & De-León-Rodríguez, 2001). Generally, synthetic dyes contain azo (N=N) functional groups and aromatic ring structures, so they are harmful to human health (López-de-Alba et al., 2001). An extensive review on the genotoxicity of food, drug, and cosmetic azo dyes and other dyes had been presented in the literature (Combes & Haveland-Smith, 1982). Accordingly, monitoring of synthetic food dyes in high consumption products such as beverages becomes an essential task. The European Union (Directive 94/36//EC, 1994) has issued comprehensive schemes that regulating the use of food colours and their allowed levels in all food products. Such regulations will guide the analytical chemists for monitoring synthetic dyes in food products. Allura Red, Sunset Yellow, and Tartrazine are probably the most common dyes that applied (individually or together) to colour soft drink powders. According to the legislations of the European Union for food dyes (which is the same legislations adopted by the official food control laboratories in Jordan), the maximum level of Allura Red, Sunset Yellow, and Tartrazine dyes should not be more than 100 μg ml^{-1} (individually or in combination) in non-alcoholic beverages with added juices and/or flavours (Directive 94/36//EC, 1994). The task of monitoring synthetic dyes in food products should be the responsibility of official food control laboratories and the food manufacturers to ensure safety for the consumers.

In fact, many analytical methods were provided for analysis of synthetic colours in beverages. The direct determination of food dyes is generally impossible. The colours must be separated from foodstuffs and purified, as co-extractives interfere with analysis of the colours. Moreover, the extract must be also concentrated because many food colours are used at low concentrations. The common analytical techniques that frequently used for determination of colours include visible spectrophotometry (Berzas, Rodríguez Flores, Villaseñor Llerena, & Rodríguez Fariñas, 1999), thin-layer chromatography (Oka et al., 1987), and mostly high performance liquid chromatography (Alves, Brum, de Andrade, & Netto, 2008; Chen, Mou, Hou, Riviello, & Ni, 1998). A typical example on using liquid chromatography for food dyes determination is the work of (García-Falcón & Simal-Gándara 2005), the authors provided a simple chromatographic method for analysing five synthetic food dyes in commercial soft drinks within minimal sample clean-up. A recommendation to adopt the earlier method for a serious control of dyes in food products was suggested by the authors.

Recently, multivariate calibration techniques have been employed for simultaneous determination of several analytes in a given sample without the need for analytes separation or even matrix clean-up in some instances (Adams, 2004; Martens & Naes,

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doi:10.1016/j.foodchem.2009.04.097

1992; Otto, 1999). Classical least squares (CLS), principal component regression (PCR), and partial least squares (PLS) are three multivariate calibration methods that have received considerable attention in the chemometric literature (Adams, 2004; Geladi & Kowalski, 1986; Martens & Naes, 1992; Otto, 1999). Beside the mentioned multivariate calibration methods, newly developed calibration methods based hybrid linear analysis (HLA) were applied in chemical analysis with satisfactory results (Goicoechea & Olivieri, 1999).

The aim of this work is to apply HLA/GO calibration method, for the first time, for simultaneous determination of Allura Red, Sunset Yellow, and Tartrazine in four powdered soft drinks without running expensive chromatographic separation for the samples. The figures of merit of the proposed HLA/GO method for dyes determination are reported. The proposed method is validated against a standard liquid chromatographic method.

2. Materials and Methods

2.1. Instruments and software

A Cary 3E UV–Vis double beam spectrophotometer (Varian company, Australia) connected to a PC for data acquisition was used for all spectral measurements. A PerkinElmer instrument (Series 200 Pump, Germany) with UV/Vis detector was used for chromatographic analysis of food dyes. The pH measurements were carried out using a WTW-Inolab (Germany) pH-meter using a companied glass electrode. Data treatment and numerical calculations were carried using MATLAB® (version 6.1). All programs were run on a PC (Pentium IV 3.00 GHZ) with Microsoft windows XP operating system.

2.2. Materials, reagents, and solutions

The dyes of purity higher than 99% were purchased from the Aldrich Company (USA). The employed dyes were Allura Red (E-129 and colour index: 16035), Sunset Yellow (E-110 and colour index: 15985), and Tartrazine (E-102 and colour index: 19140). Throughout the text, Allura Red, Sunset Yellow, and Tartrazine were referred to as AR, SY, and T, respectively. All other chemicals were obtained from Riedel-de Haën (Germany) and were of analytical reagent grade. Standard solutions (1000 μg ml^{-1}) of dyes were prepared using pure water. Acetic acid/sodium acetate (0.1 M, pH = 5.0) was used as buffer solution. The solutions employed in chromatographic analyses were: methanol (HPLC grade) and NaH_2PO_4/Na_2HPO_4 buffer (0.10 M, pH = 7.0). Dyes standard solutions (100 μg ml^{-1}) and food solutions were prepared with dilution with methanol–water mixture (v/v, 50/50) for HPLC method.

2.3. Preparation of soft drink solutions

Four types of powdered soft drinks of mango and orange flavours were collected from different local stores: TANG®/Mango & TANG®/Orange (Kraft Foods Incorporation, USA) and SQUEEZE®/Mango & SQUEEZE®/Orange (Confectionary Development Industrial Company, Syria) were selected for this study. Solutions of powdered drinks were prepared by dissolving 2.0 g in 100 ml water with good mixing. The mixture was heated on a water bath for 10 min. The obtained mixture was centrifuged for 30 min at 3500 rpm and filtered. Different aliquots of the filtrate were diluted with acetate buffer for chemometric analysis or with methanol–water mixture for HPLC analysis. Qualitative assays were done for the detection of food dyes in commercial samples by paper chromatography. Water-dissolved samples and standard dyes were applied to normal-phase chromatographic papers and developed in the mobile phase of *n*-butyl alcohol–ethyl alcohol–water (1:1:1). R_f values, the ratio between the distance travelled by the dye and the distance travelled by the solvent, were estimated for the samples and the standards.

2.4. Procedures

2.4.1. HLA/GO method

In 25 ml volumetric flasks, aliquots of the stock dyes solutions were added to get concentration range between 0–10 for AR and T and 0–7 μg ml^{-1} for SY. Before final dilution with water, 5.0 ml of acetate buffer solution (pH 5) was added. The spectral measurements for all solutions were carried out over the spectral range 200–800 nm against a blank. The design of the calibration and validation sets is presented as 3D plot (Fig. 1). The design of calibration set was based on four-level fractional factorial design according to Brereton's procedure (Brereton, 1997). The validity of HLA/GO method was further tested on an independent validation set. Prior analysis of dyes by HLA/GO method, the optimum number of wavelengths was carefully selected as will be shown later. Analysis of dyes in standard solutions and food samples was carried out separately for each dye as following: (a) an average spectrum of all calibration samples (37 spectra) was obtained, (b) A_k matrix was obtained for the dye of interest, (c) NAS projection matrix of this dye was obtained as $\mathbf{P}_{NAS,k} = [\mathbf{I} - \mathbf{A}_k^T(\mathbf{A}_k^T)^+]$, (d) $\mathbf{s}_k^*$, the net sensitivity vector, was then estimated using HLA/GO algorithm, and (e) the prediction of dye concentration in validation or food samples was obtained from the corresponding sample spectrum ($\mathbf{r}$) as: $c_{un,k} = \frac{(\mathbf{s}_k^*)^T \mathbf{r}_k^*}{\|\mathbf{s}_k^*\|^2}$, where $\mathbf{r}_k^*$ was estimated as: $[\mathbf{I} - \mathbf{A}_k^T(\mathbf{A}_k^T)^+]\mathbf{r}$. In the earlier formulae, **T**, || ||, and + are stand for matrix or vector transpose, Euclidean norm, and pseudoinverse, respectively. **I** is the unity matrix of $n \times n$ order. The adopted algorithm is outlined in the literature (Goicoechea & Olivieri, 1999, 2000).

2.4.2. Chromatographic procedure

Analytical separation and quantification of dyes was achieved at 1.3 ml min^{-1} and 22.6 °C with a 0.1 M phosphate buffer (pH 7) – methanol gradient. Solvent programme was started with 14% methanol, held for 0.5 min, followed by a linear gradient to 25% methanol in 7.0 min, and another linear gradient to 31% methanol in 10 min. Dyes eluting from the column were detected by UV-spectrophotometer set at 475 nm. A 10 μl sample volume was injected in all experiments.

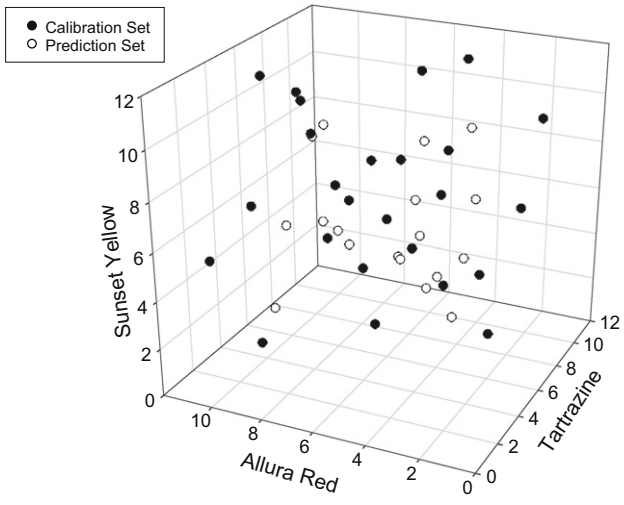

Fig. 1. A 3D graphical presentation of calibration and prediction sets.

2.4.3. One-component calibration

A series of standard dye solutions in the concentration range from 1 to 40 ppm was prepared for one-component calibration. An adequate volume of the stock dye solution and 5.0 ml of buffer solution were placed in a 25 ml volumetric flask and the flask was filled with pure water. The absorbance was recorded at the wavelength corresponding to maximum of the absorption band of each dye. The calibration equations and the linear dynamic ranges were calculated for each dye.

3. Results and discussion

3.1. Spectral overlap and application of HLA/GO calibration for dyes determination

The absorption spectra of dyes recorded at pH 5.0 are presented in Fig. 2. As indicated in Fig. 2, the three dyes were active in ultraviolet and visible regions and this is attributed to their chemical structure which contains many active functional groups. Generally speaking, the spectral overlap between dyes is high over the entire spectral range. Within the spectral region 350–650 nm, the spectra of AR and SY were highly overlapped, while, a low extent of spectral overlap between T and other dyes in that region was observed. The estimated degrees of spectral overlap between dyes, as obtained from Goicoechea and Olivieri method (Goicoechea & Olivieri, 1998), were 65%, 75%, and 95% between T–SY, T–AR, and SY–AR, respectively. The presence of such high overlapping (particularly between SY and AR) limits the analysis of these dyes in food products by conventional univariate calibration methods. Accordingly, the spectral resolution of food dyes is necessary and this could be established either by: (a) using chromatographic methods like HPLC or TLC or (b) applying multivariate calibration methods.

Recently, many applications of multivariate calibration methods have been reported in chemical (Galeano Diaz, Guiberteau Cabanillas, Alexandre Franco, Salinas, & Viré, 1998), food (López-de-Alba et al., 2001), and pharmaceutical (Goicoechea & Olivieri, 1998; Ni, Wang, & Kokot, 2001) analysis.

López-de-Alba and co-workers were effectively applied PLS (1 and 2 types) and PCR for analysis of R40, Y6, and Y5 in commercial soft drinks and compared their results with a standard HPLC method (López-de-Alba et al., 2001). Nevado, Flores, & Llerena, 1998 have proposed a simple, accurate and rapid PLS calibration method for analysis of Tartrazine, sunset Yellow, and Ponceau in three commercial soft drinks without the need for solutes separation or sample clean-up due to the simple nature of soft drink matrix (Nevado et al., 1998).

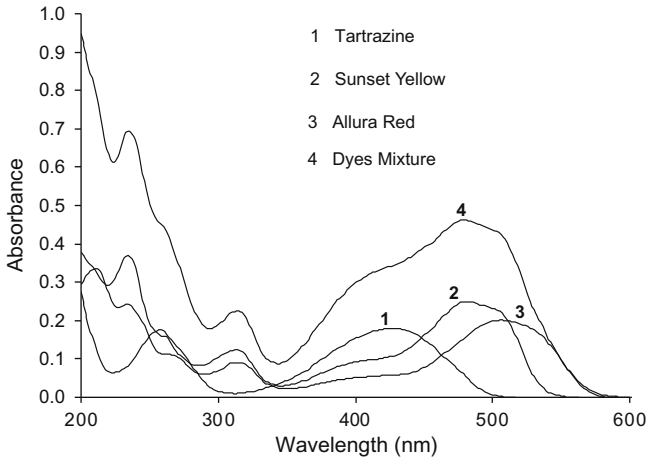

Fig. 2. UV–Vis spectra of pure and mixture of dyes recorded at pH 5.0. Dyes content: Allura Red and Sunset Yellow 5.0 μg ml^{-1}, and Tartrazine 6.0 μg ml^{-1}.

Lorber had proposed the common net analyte signal (NAS) concept that permits estimation figures of merit for multivariate data (Lorber, 1986). The estimation of those figures of merit was initially restricted to the CLS model (when pure spectra and concentrations of all components are known (Lorber, 1986)). After that, NAS concept was extended to inverse calibration methods that only need the concentration of the target analyte in the calibration set (Lorber, Faber, & Kowalski, 1997). Recently, new families of multivariate calibration methods have been proposed based on the concept of NAS which are know as NAS-based calibration methods (Berger, Koo, Itzkan, & Feld, 1998; Espinosa-Mansilla, Merás, Rodríguez Gómez, Muñoz de la Peña, & Salinas, 2002; Ferré & Faber, 2003; Goicoechea & Olivieri, 2000; Mirmohseni, Abdollahi, & Rostamizadeh, 2007; Moore, Cogdill, Short, Hair, & Wildfong, 2008; Xu & Schechter, 1997).

These NAS-based calibration methods used suitable scaling procedures for eliminating the information of the analyte of interest during the calibration step. Removing the spectrum of the pure component from calibration data is the basic step in hybrid linear analysis (HLA) method (Berger et al., 1998). A number of calibration methods have been proposed based on earlier HLA method like HLA/XS (Xu & Schechter, 1997), HLA/GO (Goicoechea & Olivieri, 2000), and HLA/AS (Ferré & Faber, 2003). In fact, the earlier NAS-based methods have been applied for determination of many analytes in complex matrices. HLA method was used for determination of tetracycline in human serum using fluorescence data (Goicoechea & Olivieri, 1999). A good review on NAS-based calibration methods was provided by Ferré and Faber (2003). Beside their simplicity and ease of implementation, the HLA-based calibration methods can be applied for determination of an analyte in a complex matrix without the need for including the effect of other interferences in the calibration stage (Ferré & Faber, 2003). Particularly, in HLA/GO calibration method, there is no need to have the pure spectrum of analyte of interest like the case in CLS, HLA, and HLA/XS calibration methods (Ferré & Faber, 2003; Goicoechea & Olivieri, 2000; Lorber et al., 1997).

In this work, and for the first time, the application HLA/GO method is applied for determination of AR, SY, and T in their mixtures and in food containing these dyes. For sake of validation of HLA/GO method, an independent HPLC method is applied.

3.2. Effect of solution pH on dyes absorption and optimisation of calibration samples

One of most experimental variables that highly affect dyes absorption is solution pH. Therefore, dyes solutions should be maintained at certain pH so that high absorption intensities (for all dyes) should be observed at that pH. The influence of pH on the absorption intensity of food dyes was investigated over a wide pH range: 2–12. Dyes solutions of variable pH were prepared using buffer solutions. Generally speaking, the absorbance values of dyes (at their corresponding λ_{max}) were not changed over the pH range 2–8, after which a considerable reduction in absorbance readings was observed for all dyes. It is highly possible that dyes have been completely ionised at higher pH values and this will affect their light absorption. Based on that, pH 5 was selected as an optimum pH for measuring dyes absorbances. Moreover, dyes solutions were found stable over 30 days when placed in a cold dark place (5.0 °C).

3.3. Designing of calibration mixtures, outlier(s) detection and wavelength selection

The efficiency of multivariate calibration methods is highly dependant on the design of standard mixtures in the calibration

and validation sets and the presence or absence of outliers. Four important issues should be addressed when building calibration and validation sets: (a) the concentration of individual solute should be within its linear range obtained from univariate calibration, (b) calibration set should be orthogonally deigned to reduce the collinearity in calibration matrix and to ensure high prediction power for the method, (c) the absorbances of calibration and validation mixtures should not be higher than the maximum response of the instrument. Usually, in spectrophotometric measurements absorbances higher than 1.0 were not considered and dilution for these mixtures is suggested, and (d) the concentration of solutes in validation set should be within the range used in calibration set. These issues were taken into consideration when building calibration and validation sets for the current dyes system. The obtained dynamic ranges were: 0.3–32, 0.3–35, and 0.4–25 μg ml^{-1} for AR, SY, and T, respectively. To avoid high absorption intensities for standard mixtures (i.e., absorption values > 1.0), the concentration ranges of dyes were adjusted in away to achieve that aim.

Elimination of outlier(s) from calibration or validation samples is essential prior multivariate calibration. Usually, the detection of outlier(s) can be simply carried out using Mahalanobis distance method (López-de-Alba, López-Martínez, Cerdá, & Amador-Hernández, 2006) or principal component analysis (Adams, 2004; Hemmateenejad, Akhond, & Samari, 2007). Cluster plots (derived from principal component analysis PCA) represent a more systematic way to detect outlier(s). Usually, cluster plots obtained by plotting the first principal component (PC1) against the next component (PC2). In the absence of outliers, all samples should form a clear cluster of the data. PCA was carried out for spectral data of calibration and validation sets. For both sets, the variances (94%) in absorption data can be effectively presented using the first two principal factors. PC1–PC2 plot (not shown) clearly indicated that both sets were fall within the same domain and form one homogenous cluster. If an outlier sample is there, then it will be much away from the cluster of samples. Furthermore, cluster analysis indicated the high homogeneity between two sets and this ensures a high prediction power of HLA/GO multivariate calibration method for future samples.

Most of multivariate calibration methods, in fact, are considered as full-spectrum methods because, in some instances, the entire data in a given spectrum would be included in numerical analysis. Including all spectral data points is not necessary to improve the quality of analysis, moreover, it has been shown for many multicomponent systems that reduction of data points could improve the accuracy and precision of numerical analysis (Brown, 1992). Hence, various criteria have been developed to allow for effect wavelength selection prior multivariate calibration (Al-Degs, El-Sheikh, Al-Ghouti, Hemmateenejad, & Walker, 2008). In this work, the method proposed by López-de-Alba and co-workers is employed to select the optimum wavelength region(s) for HLA/GO calibration (López-de-Alba, López-Martínez, & De-León-Rodríguez, 2002). For each dye, the correlation coefficients between dye concentration (in calibration set) and the corresponding absorbance at each wavelength were estimated.

The correlation coefficient–wavelength plot of dyes (not presented) indicated that T and AR showed good correlation over the wavelength range 362–455 and 450–550 nm, respectively. On the other hand, SY dye exhibited a poor correlation with the entire spectral range. For HLA/GO calibration, six spectral regions (with 1.0 nm interval) were selected based on the correlation coefficient – wavelength plot: 200–800, 200–600, 200–350, 300–600, 350–550, and 350–800 nm. HLA/GO calibration was carried out separately for each dye for each selected wavelength range. The quality of calibration was assessed by calculating relative error of prediction (REP%) for dyes concentrations (Goicoechea & Olivieri, 1999 and Hemmateenejad et al., 2007).

The prediction power of HLA/GO was satisfactory (total REP < 1%) when the analysis was limited to 350–550 nm spectral range. However, the spectral range 300–600 nm was also useful for HLA/GO calibration where the total REP% value for dyes determination was about 2.0%. It is interesting to notice that including the entire spectral range (200–800 nm, 601 points/spectrum) has decreased the method's efficiency for prediction as inferred from the high total REP% value (7.32%). The spectral domain 200–350 nm is not recommended for calibration where the maximum values of REP% were reported for that range (13.61%). The modest prediction power of HLA/GO method over 200–350 nm range could be attributed to the high spectral overlap between dyes in that region (Fig. 2).

After checking out outliers and setting the suitable spectral range, HLA/GO calibration method is ready for dyes determination in validation set and food samples and this subject will be investigated next.

3.4. Determination of food dyes in synthetic mixtures by HLA/GO calibration

In this section, HLA/GO method is employed for prediction of dyes contents in calibration and validation sets at the optimum calibration factors (pH 5 and wavelength range 350–550 nm). This goal was achieved by estimating the net sensitivity vectors ($\mathbf{s}_k^*$) for dyes (separately) and then use these vectors for estimating dyes concentrations in calibration/validation sets and food samples. For each dye in both sets, PRESS (prediction error sum of squares), relative error of prediction (REP%), and square of correlation coefficients (r^2) were calculated and the results were summarised in Table 1.

As clearly indicated in Table 1, the HLA/GO method gave satisfactory results for dyes determination. In all cases, r^2 values were approached to unity which reflects the high closeness between real and predicted values. The average recoveries and R.S.D. values were also satisfactory. In both sets, the average recoveries were within the range: 99.5–102.3% and R.S.D. values were <5.0% in all cases. In summary, the proposed HLA/GO method offers a simple and rapid procedure for dyes determination in water with high precision and accuracy without the need for any previous separation step.

Figures of merit (like accuracy, precision, repeatability, and detection limits) were evaluated for the proposed calibration method as outlined in the literature (Lorber et al., 1997). Analytical intraday precision was determined by analysing a standard mixture of dyes (containing 5, 5, and 6 μg ml^{-1} of AR, SY, and T, respectively) in six replicates in one day. Interday precision was determined by measuring the same control mixture in duplicate for three days. The concentration of each dye in the control mixture was determined using HLA/GO method on the first day of the study. The standard solution of dyes was prepared and analysed six times within the same day to measure the repeatability of the method. The obtained accuracy and R.S.D. values were

Table 1
Assessment of HLA/GO method for dyes quantification in water.

Dye	Internal Validation[a]			External Validation[a]		
	PRESS	REP%	r^2	PRESS	REP%	r^2
Allura Red	0.24	0.62	0.9996	0.31	0.91	0.9995
Sunset yellow	0.17	0.84	0.9991	0.28	0.63	0.9992
Tartrazine	0.36	0.92	0.9998	0.18	0.75	0.9994

[a] PRESS, REP%, and r^2 values were all estimated for non-zero dye concentrations. The proposed method predicts a very close value to zero when no dye had been added (data not given).

99.2–101.2% and 1.13–2.51 for intraday analysis and 99.1–101.4% and 1.41–1.76 for interday analysis.

The earlier values were satisfactory and reflecting the high accuracy and precision of HLA/GO method for both intraday and interday analysis. The repeatability was also high with a random error of <3%. The obtained detection limits for AR, SY, and T were 0.66, 0.52, and 0.43 μg ml^{-1}, respectively.

3.5. Determination of food dyes in food drinks using HLA/GO method

There are a number of studies that discussed the application of multivariate calibration for food dyes analysis. For example, Nevado and co-workers have applied PLS-1, PLS-2, and PCR for determination of four food dyes in different commercial beverages (Nevado et al., 1995). López-de-Alba and co-workers have successfully applied PLS-1 and PLS-2 on zero and first order spectral data for sake of determination of three common food dyes in soft drinks (López-de-Alba et al., 2001). Moreover, the pervious authors have validated the proposed chemometric methods against independent chromatographic methods. According to our literature survey, HLA/GO calibration method has not been applied for food dyes analysis in any food type. The HLA/GO method was applied for determination of AR, SY, and T in four commercial soft drinks. The solutions of food samples were prepared as described earlier and scanned using the spectrophotometer. The spectral data were then subjected to HLA/GO calibration to find dyes contents in each sample. The results of analysis were provided in Table 2. The results obtained by HPLC method were given in the same table for sake of comparison.

As indicated in Table 2, food dyes were simultaneously quantified in commercial samples with a reasonable precision using HLA/GO method. For more validation to HLA/GO method, the same food samples were analysed using an independent analytical method. In such cases, one has two options to go through. The first option is to carry out standard addition techniques and the second one is to apply an independent standard method (like HPLC) for dyes determination in food samples. The second option was adopted here because of the high validity of HPLC method in food dyes analysis (Alves et al., 2008).

Under the conditions already outlined earlier for HPLC method, calibration graphs (peak area vs. dye content) were obtained from triplicate determination using five standard solutions of dyes. Table 3 summarised the chromatographic results for dyes separation. The results obtained for dyes determination by an independent HPLC method was also provided in Table 2. To decide whether the results of HLA/GO and HPLC methods are comparable or not, *t* and *F* statistical tests were carried out. For all cases, *t*-calculated values were lower than *t*-table values which indicated that no significant difference between HPLC and the proposed method for determination of dyes in food samples. Moreover, *F*-calculated values were also lower than *F*-table values which confirmed that both methods are of similar precision. The results in Table 2 indicated that HLA/GO method was able for dyes quantification in four commercial soft drinks without the need for running chromatographic separation. Relatively speaking, the proposed HPLC method has better precision than HLA/GO method as indicated from S.D. values and this was expected because dyes were totally separated before their quantification, while, in HLA/GO method the dyes were quantified directly in the sample matrix. Compare to HPLC method, HLA/GO method was rapid, easy to implement, and of low running cost for dyes quantification. The HLA/GO method could be used for food dyes quantification in cases where the chromatographic ones cannot be implemented owing to cost limitations or lack of analytical instrumentation. It is worth to mention that the levels of AR, SY, and T in soft drinks were below the limit set by European legislations (Directive 94/36//EC, 1994).

The results of HLA/GO and liquid chromatography were, qualitatively, confirmed by simple paper chromatography. The paper chromatographic tests indicated the absence of AR in TANG/Mango, TANG/Orange, and SQUEEZE/Orange and confirmed the existence of all dyes in SQUEEZE/Mango. The separated dyes from food samples were have well-resolved bands and identical R_f values to those obtained from the standards, 0.71, 0.45, and 0.37 for AR, SY, and T, respectively.

It is worth to mention here that the excipients that present in soft drink samples (like citric acid, sucrose, and synthetic flavouring materials) have a low interference with dyes determination. This conclusion was drawn when the spectrum of pure dyes mixture was matched with the spectra of food samples. Fig. 3 showed the spectra of dyes in water and in SQUEEZE/Mango sample. SQUEEZE/Mango sample was selected because of the presence of the three dyes in high amounts (see Table 2).

The similarity between the spectra depicted in Fig. 3 indicated that other food excipients do not interfere in dyes determination by HLA/GO or even HPLC method. It seems that other food excipients are active within the spectral range (250–305 nm) as indi-

Table 2
Determination of Allura Red, Sunset Yellow, and Tartrazine in four commercial soft drinks using HLA/GO and HPLC methods.[a]

Sample	Allura Red		Sunset Yellow		Tartrazine	
	HLA/GO	HPLC	HLA/GO	HPLC	HLA/GO	HPLC
TANG/Mango	ND[b]	ND	43.0(2.2)	44.7(1.3)	85.3(2.4)	82.7(1.6)
TANG/Orange	ND	ND	24.2(1.6)	22.0(1.8)	78.0(3.3)	76.3(1.6)
SQUEEZE/Mango	29.1(1.4)	31.0(1.6)	21.6(2.3)	19.8(0.7)	34.2(2.4)	36.6(1.0)
SQUEEZE/Orange	ND	ND	17.7(1.6)	15.8(1.4)	19.0(1.5)	21.3(1.3)

[a] Concentrations are in μg ml^{-1}. Results are average of four experiments, *t*-table(0.05, 6) = 2.45, *F*-table(0.05, 3, 3) = 15.44.
[b] Not detected in the sample.

Table 3
Chromatographic data for Tartrazine T, Sunset Yellow SY, and Allura Red AR separation.

Dye	t_R(min)[a]	Dynamic range (μg ml^{-1})	LOD (μg ml^{-1})[b]	Calibration equation (Peak Area vs. conc.)	Precision (R.S.D.)	Correlation coefficients
T	4.6	0.50–16.0	0.30	*PA = 51866 C + 104792*	2.2	0.9998
SY	10.1	0.10–13.0	0.06	*PA = 278186 C + 502351*	3.2	0.9979
AR	13.8	0.10–12.0	0.04	*PA = 302834 C + 420563*	2.8	0.9985

[a] Retention times of dyes.
[b] Limits of detection were estimated on the basis of a signal-to-noise ratio of 3 (3S/N method).

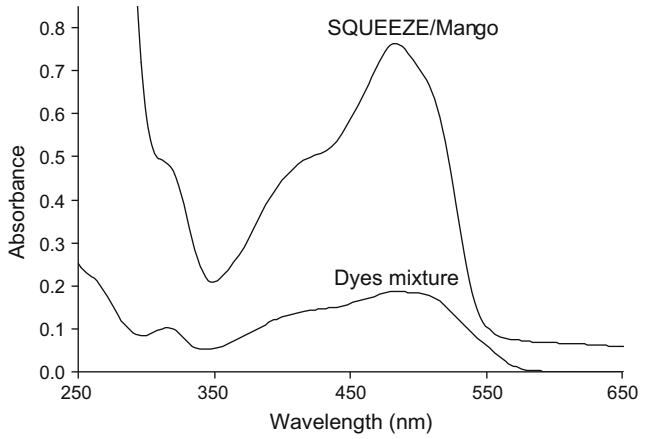

Fig. 3. Absorbance spectra of SQUEEZE/Mango solution (2.0 sample in 100 ml pure water) and synthetic dyes mixture (10.0 μg ml^{-1} for Tartrazine and Sunset yellow and 7.0 μg ml^{-1} for Allura Red) pH = 5.0.

cated from their high absorbance and, fortunately, this region was excluded from the chemometric analysis. In case where a high interference is present with target dyes, a suitable extraction and clean-up procedures are necessary to extract dyes prior applying HLA/GO or HPLC methods.

4. Conclusions

HLA/GO method, as a multivariate calibration method, was found useful method for simultaneous determination of AR, SY, and T dyes in pure solutions and in four soft drink samples. The percent recoveries and R.S.D. values for dyes determination in solution were 99.3(4.2), 101.3(3.8), and 102.4(4.6) for AR, SY, and T, respectively. The results obtained by HLA/GO method for determination of dyes were fairly comparable to those obtained by HPLC method which reflects the effectiveness of the former method. Fortunately, the components of soft drinks did not strongly interfere with dyes analysis. In case of presence a strong interference, dyes should be extracted and preconcentrated prior their analysis. HLA/GO calibration method is a reasonable substitute for expensive HPLC method for quantification of dyes in soft drinks.

Acknowledgements

The financial support from the Hashemite University/Deanship of Academic Research is gratefully acknowledged. The author thanks Mr. Khaled Akel and Mr. Mazin Musa for their technical assistant.

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Food Chemistry 117 (2009) 491–498

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Classification of brandies and wine distillates using front face fluorescence spectroscopy

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ARTICLE INFO

Article history:
Received 18 June 2008
Received in revised form 13 January 2009
Accepted 12 April 2009

Keywords:
Brandy
Fluorescence
Chemometry
Authentication

ABSTRACT

This study demonstrates the use of front face fluorescence spectroscopy and multivariate data analysis for differentiating brandies from wine distillates. Owing to the low price of the wine distillates, they are sometimes used for the counterfeiting brandies. For this reason, there is a need for a rapid method for drink authentication to reassure consumers and protect regional designations. Total luminescence and synchronous scanning fluorescence spectra were recorded followed by a classification of samples using principal component analysis (PCA) and hierarchical cluster analysis (HCA). Both PCA and HCA carried out on the emission spectra (360–650 nm) recorded at excitation wavelength 350 nm and synchronous fluorescence spectra (200–700 nm) collected at wavelength interval 90 nm provide very good differentiation between the two spirit classes. Less good classification was obtained using excitation spectra (225–425 nm) obtained at emission wavelength 440 nm. These results indicate that the front face fluorescence spectroscopy offers a promising approach for the authentication of brandies.

1. Introduction

Brandy is a spirit drink produced from wine spirit, whether or not blended with a wine distillate distilled at <94.8 vol.%, provided that that distillate does not exceed a maximum of 50% by volume of the finished product. This spirit is aged for at least one year in oak receptacles or for at least six months in oak casks. Wine spirit is a spirit drink produced by the distillation at <86 vol.% of wine or wine fortified for distillation or by the redistillation of a wine distillate at <86 vol.%. Wine spirit shall not contain added ethanol of agricultural origin (Regulation (EC) No 110/2008).

In Slovak Republic there are two types of these spirits: "wine distillates" are less expensive, and can be legally produced using wine distillates diluted with ethanol from other sources, whereas "brandy" is more expensive and should contain ethanol from grape. Brandy has to be aged for a certain period in oak casks to obtain characteristic taste, flavour and colour. Traditional techniques utilised to analysis of brandy, such as gas chromatography–mass spectrometry (Caldeira, Pereira, Climaco, Belchior, & Bruno de Sousa, 2004), high-performance liquid chromatography (Canas, Belchior, Spranger, & Bruno-de-Sousa, 2003) and capillary electrophoresis (Panossian, Mamikonyan, Torosyan, Gabrielyan, & Mkhitaryan, 2001), focus on the determination of a certain marker compounds in a test product followed by a comparison of the values obtained with those previously documented for authentic product. This approach is often time-consuming, expensive, requires highly trained staffs and the range of compounds, which must be quantified to ensure authenticity, is continuously increasing. In the last few years, there has been an increasing need to develop rapid, inexpensive and effective analytical methods, as well as requiring low sample manipulation. These demands lead to an approach based on food matrix characterisation as a whole and spectroscopic methods combined with chemometric techniques have been studied for this purpose.

While there has been a notable growth for near-infrared (Barboza & Poppi, 2003; Pontes et al., 2006) and Fourier transform infrared (Lachenmeier, 2007; Palma & Barroso, 2002) spectroscopy, there has been little research carried out using either UV–VIS absorption or fluorescence spectroscopy in spirit drink authentication applications. Recent work using UV–VIS absorption spectroscopy dealt with discriminating 100% agave tequilas from other types of tequila (Barbosa-García et al., 2007).

A review (Christensen, Norgaard, Bro, & Engelsen, 2006) has revealed that the application of fluorescence spectroscopy for direct analysis of food has increased during the last decade, probably due to the wide-spread use of multivariate data analysis tools.

Front face fluorescence spectroscopy has demonstrated its feasibility to classify Swiss honeys from seven different floral sources (Karoui, Dufour, Bosset, & De Baerdemaeker, 2007). This method has also been used for the determination of the geographical origin of milk (Karoui, Martin, & Dufour, 2005) and cheeses (Karoui, Bosset, Mazerolles, Kulmyrzaev, & Dufour, 2005; Karoui, Dufour, et al., 2005). Within the area of cheese ripening, the studies concluded

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doi:10.1016/j.foodchem.2009.04.053

that fluorescence spectroscopy is suitable to discriminate each ripening stage (Karoui & De Baerdemaeker, 2007).

The potential of front face fluorescence spectroscopy combined with principal component analysis (PCA) and factorial discriminant analysis (FDA) has been investigated for discriminating different wines according to variety, typicality and vintage. PCA performed on the whole collection of excitation spectra allowed a good discrimination between French and German wines. Using FDA, correct classification of typical and non-typical Beaujolais amounting to 95% was observed for the emission fluorescence data set (Dufour, Letort, Laguet, Lebecque, & Serra, 2006).

Synchronous scanning fluorescence spectra and multivariate data analysis (nearest neighbour method and linear discriminant analysis) have been used for classification of differently stored beer samples (Sikorska, Górecki, Khmelinskii, Sikorski, & De Keukeleire, 2006) and of different beers from different breweries (Sikorska, Górecki, Khmelinskii, Sikorski, & De Keukeleire 2004). Synchronous scanning fluorescence spectra are also useful for detection of adulteration of virgin olive oil (Poulli, Mousdis, & Georgiou, 2007).

The aim of this paper is to show that front face fluorescence spectroscopy and multivariate statistical methods (principal component analysis and hierarchical cluster analysis, HCA) can be used for distinguishing between commercial samples of brandies and wine distillates. It appears that front fluorescence spectroscopy may give a quick and non-destructive answer to the product authenticity as spectra can be recorded directly on samples.

2. Materials and methods

2.1. Samples

The studies were performed on 16 brandies (B) from three different producers (B_1, $n = 8$; B_2, $n = 4$; B_3, $n = 4$) and 30 wine distillates (D) from five different producers (D_1, $n = 12$; D_2, $n = 10$; D_4, $n = 4$; D_5, $n = 2$; D_6, $n = 2$). Four brandies were acquired directly from the manufacturers; other samples were purchased from the local supermarkets. Brandy B_1, a traditional Slovak product from the Small Carpathian viticultural region, is made of the grape using a classic method of ageing wine spirit in small 300 l oak barrels for a minimum 5 years. The wine spirit then goes to 20,000 l barrels for next 3 years. Brandy B_2 is made of the pure high quality foreign wine spirit matured by natural way in oak barrels. B_3 is made of the wine spirit from the East Slovak viticulture region matured by natural way in oak barrels.

Wine distillates are produced using wine spirits diluted with ethanol from other sources. They are frequently mixed with sugar, brandy aroma and caramel. Wine distillates D_1 contain honey and colourants (E 102, E 110, E 120, E 122, E 132 and E 151).

Samples were stored in the dark at room temperature until the day of analysis.

2.2. Fluorescence spectroscopy

Fluorescence spectra were recorded using a Perkin–Elmer LS 50 Luminescence spectrometer equipped with a Xenon lamp and a variable angle front-surface accessory. The incidence angle of the excitation radiation was set at 56°. Samples were placed in 10 mm × 10 mm × 45 mm quartz cell. Excitation and emission slits were both set at 5 nm.

Fluorescence excitation spectra were recorded between 200 and 500 nm (increment 0.5 nm), repeatedly, at emission wavelengths from 300 to 600 nm, spaced by 5 nm interval in the emission domain.

Fluorescence emission spectra were recorded from 250 to 700 nm (increment 0.5 nm), repeatedly, at excitation wavelengths from 200 to 500 nm, spaced by 5 nm interval in the excitation domain.

Synchronous fluorescence spectra were collected by simultaneously scanning the excitation and emission monochromator in the excitation wavelength range 200–700 nm, with constant wavelength differences $\Delta\lambda$ between them. Spectra were recorded for $\Delta\lambda$ interval from 10 to 100 nm, in steps of 5 nm. Fluorescence intensities were plotted as a function of the excitation wavelength.

Fluorescence measurements were done in triplicate for each sample.

The spectrometer was interfaced to a computer supplied with FL Data Manager Software (Perkin–Elmer) for spectral acquisition and data processing.

Contour maps of total luminescence and synchronous scan fluorescence spectra were plotted using Windows-based software OriginPro 7.5 (OriginLab, USA, 2002).

2.3. Mathematical analysis of data

PCA and HCA were applied to the fluorescence spectra to investigate differences between the samples. PCA is an unsupervised (we have no prior knowledge of the groups to be expected) pattern recognition method that reduces the dimensionality of the original data matrix while retaining the maximum amount of variability as well as recognising the data structure. PCA decomposes a data matrix with n rows (samples) and p columns (variables) into the product of a scores matrix, with n rows (samples) and $d < p$ columns (principal components, PCs), and a loadings matrix, with $d < p$ rows (PCs) and p columns (variables). The scores are the positions of the samples in the space of the principal components and the loadings are contributions of the original variables to the PCs. All PCs are mutually orthogonal, and each successive PC contains less of the total variability of the initial data set. Usually, only a limited number $d < p$ of PCs are retained, as the variability in the others is due to noise. This reduces the dimensionality of the data considerably, enabling effective visualisation, classification, and regression of multivariate data (Geladi, 2003).

HCA is an unsupervised pattern recognition method detecting natural grouping in data. Objects are grouped in clusters in terms of their similarity. The initial assumption is that the nearness of objects in the space defined by the variables reflects the similarity of their properties. There are diverse possibilities and rules used to measure distances (in various metrics in multidimensional space), form and linkages among individual clusters. We used hierarchical (agglomerative) cluster analysis, where similarity extent was measured by Manhattan (city-block) distances and cluster aggregation was based on Ward's method (Otto, 1999).

Microsoft Excel 2000 and Statistica software version 6.0 (StatSoft, USA, 2001) were used for statistical analysis.

3. Results and discussion

3.1. Total luminescence spectra

Recently, total luminescence spectroscopy (TLS) has been applied to obtain emission spectra over a range of excitation wavelengths in the form of Excitation–Emission matrices (EEM contour maps), which allowed to obtain more detailed information than that obtained using conventional mono dimensional fluorescence (Poulli, Mousdis, & Georgiou, 2005; Sikorska et al., 2006).

Total luminescence spectra of the brandy and wine distillate are shown in Fig. 1 as contour maps. In general, spectral features and fluorescence intensity values of all brandies are typical of brandies of similar origin and nature. Brandy total luminescence contour

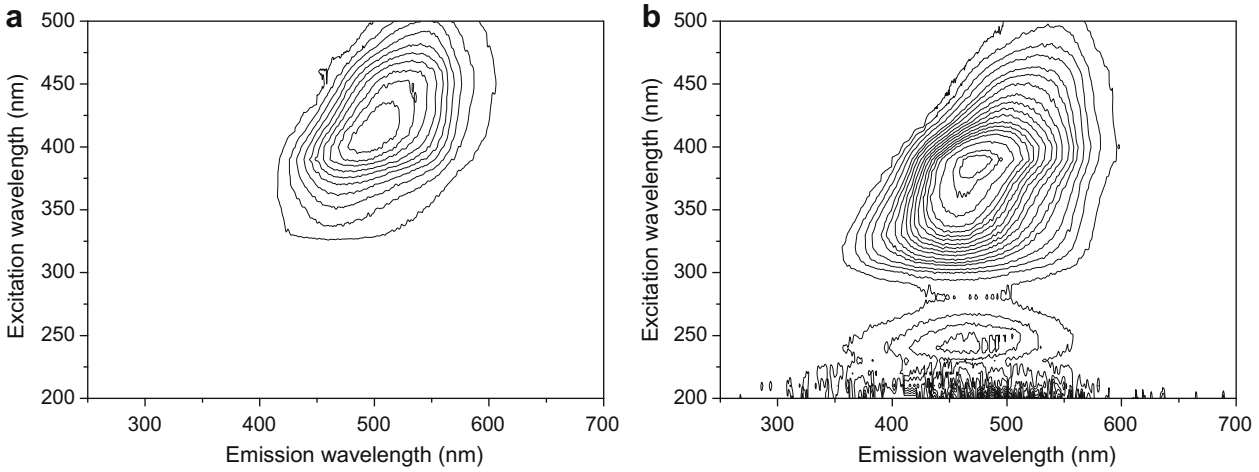

Fig. 1. Contour plots of total luminescence spectra of brandy (a) and wine distillate (b) samples. Contours join the points of equal fluorescence intensity.

map spreads in the excitation wavelength range higher than 320 nm, in contrast to wine distillate that spreads in the full excitation wavelength range. The contours for brandy are concentrated in the emission wavelength region 470–520 nm and excitation wavelength region 390–430 nm, while the contours for wine distillates are concentrated in the emission wavelength region 450–500 and 440–490 nm and the excitation wavelength region 360–390 and 230–250 nm, respectively.

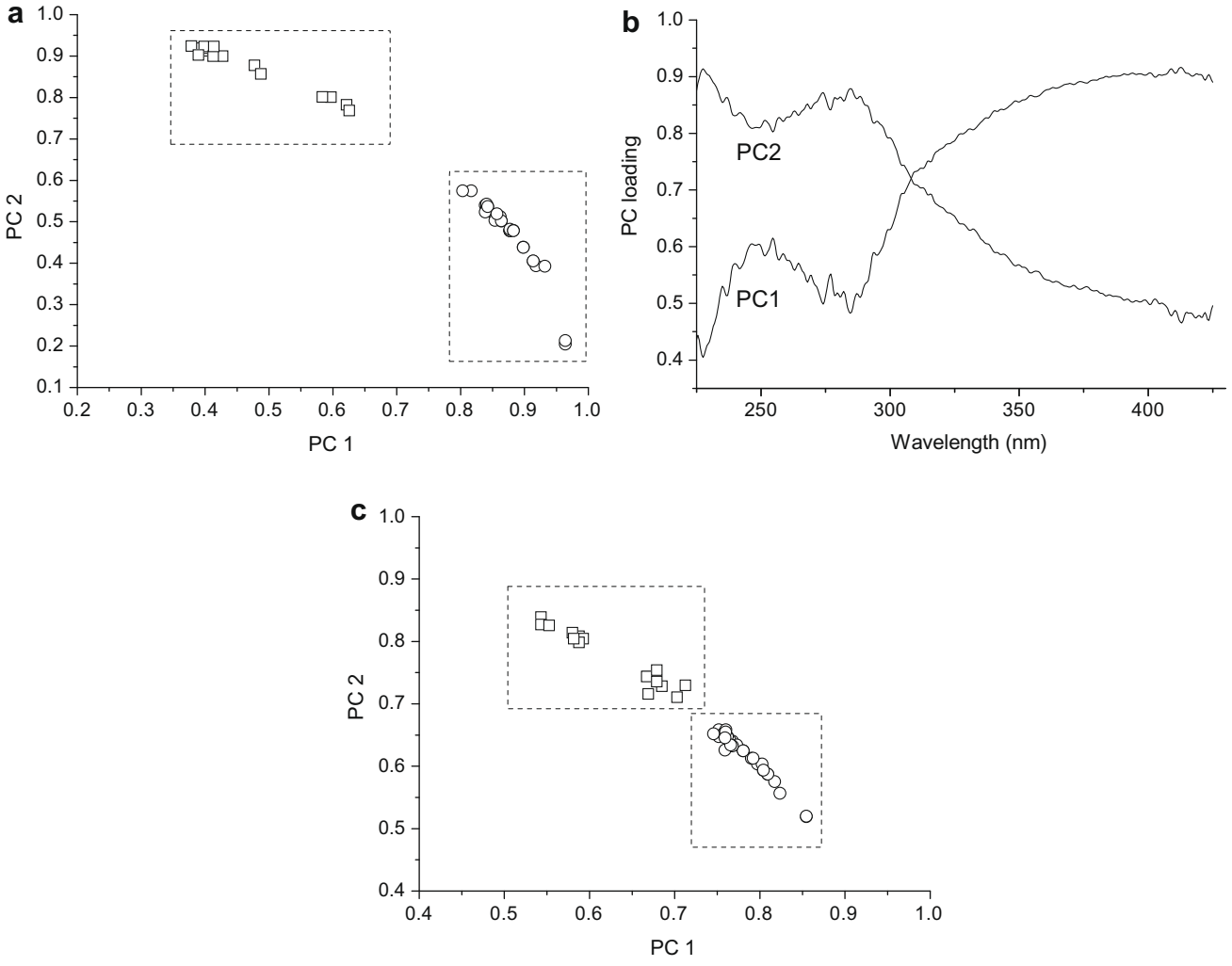

Fig. 2. Principal component analysis similarity map (score plot) determined by principal component 1 (PC1) and principal component 2 (PC2) (a) and spectral pattern (loading) corresponding to PC1 and PC2 (b) for excitation fluorescence spectra recorded at emission wavelength 440 nm (a and b) and principal component analysis similarity map determined by PC1 and PC2 for emission spectra recorded after excitation at 350 nm (c) on brandy (□) and wine distillate (○) samples.

The fluorescence spectra of brandies B_1 are characterised by the main fluorophores centred at an excitation/emission wavelength pair of 410/500 nm. The other brandies have the following excitation/emission maxima: B_2 – 396/485 nm, B_3 – 396/485 nm. However, the excitation/emission wavelength values of the major peaks of the brandies are generally longer than those usually measured for wine distillates, e.g., D_1 – 390/475 nm, D_2 – 388/472 nm. The spectra of the wine distillates reveal the presence of an additional band at 240–250 nm in excitation and 440–460 nm in emission. The shape and intensity of these intermediate emissions varies from one distillate to another. Further, another excitation band was observed for the distillates with excitation maxima at 330 and 395 and an emission region above 520 nm. Moreover, wine distillates show stronger fluorescence than brandies.

As it is clearly shown in Fig. 1 the shape and the fluorescence intensities are different between the two classes of beverages. Consequently, total luminescence spectroscopy can be used to characterise brandy and wine distillate samples using suitable wavelength regions.

3.2. Multivariate analysis of excitation and emission spectra

The PCA was applied separately on the excitation and emission spectra to investigate differences between samples. The best classification was achieved using emission spectra (360–650 nm) recorded at excitation wavelength 350 nm or excitation spectra (225–425 nm) recorded at emission wavelength 440 nm. The fluorescence spectra showed different shapes. The width of excitation spectra was larger for wine distillates than those for brandies. Wine distillates had higher fluorescence intensity regardless of wavelength but they were also more heterogeneous in this respect.

The similarity map defined by the PC1 and PC2 of the PCA performed on excitation spectra accounted for 99.6% of the total variance with PC1 accounting for 91.2% and allowed a good discrimination of beverages (Fig. 2a). Spectral pattern associated with the PCs provide the characteristic wavelengths that may be used to discriminate between spectra (Fig. 2b). The spectral pattern associated with the PC1 exhibited a positive peak around 250 nm and a broad positive peak between 320–420 nm corresponding to caramel. The spectral pattern associated with the PC2 exhibited positive peaks at 230 and 290 nm.

While a discrimination of the brandy and wine distillate samples was achieved with excitation data collection, a different trend was observed with emission spectra. Although the PCA similarity map defined by the PC1 and PC2 (98.5% and 1.3% of the total variance, respectively) did not lead to a clear discrimination of beverages, a general trend pointing out the brandies and wine distillates was observed on the map (Fig. 2c).

In a second step, HCA was carried out separately on the excitation and emission spectra in order to evaluate the number of subsets of similar samples appearing in the complete data set. For the sake of simplicity, a reduced number of samples were included in the figures, but from them we can clearly recognise the clustering common to all the samples. The evaluation of the above mentioned excitation spectral region provided two main clusters (Fig. 3a). All wine distillates are linked together in the first main cluster. This main cluster is heterogeneous enough but consists of various small groups of very similar products. For instance, most of the wine distillates from producer 1 (D_1) is found as such in one subgroup with 95% of similarity among them. All brandies are linked together in the second main cluster. One subcluster is constituted of B_2 (brandy from producer 2) with 98% of similarity. Another subcluster is constituted of B_1 and B_3 with 96% of similarity among them and 87% in relation to B_2.

Fig. 3b shows the results from HCA concerning the emission spectra. The first main cluster contains wine distillate samples, while the second one contains brandy samples. Wine distillates

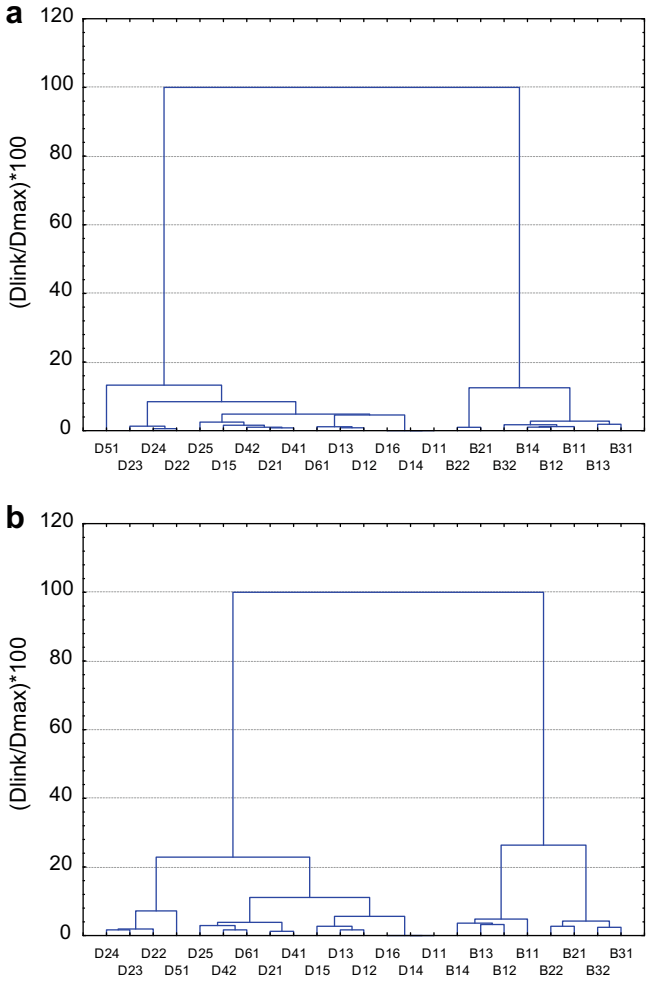

Fig. 3. Hierarchical cluster analysis dendrogram using Manhattan distance for excitation fluorescence spectra recorded at emission wavelength 440 nm (a) and emission spectra recorded after excitation at 350 nm (b) on brandies (*B*) and wine distillates (*D*).

form three smaller groups. One small group is constituted of samples D_2 with 98% of similarity among them and by sample D_5 with 92% of similarity with the samples D_2 of this group. Another group is constituted of D_2, D_4 and D_6 with 96% of similarity among them and 90% in relation to the group of D_1. Samples D_1 show a similarity of 94% to each other. Brandy samples form three small subclusters of the second main cluster corresponding to three different producers. One subcluster is constituted of B_1 with 96% of similarity. Another subcluster is constituted of B_2 and B_3 with 96 % of similarity among them and 74% in relation to B_1.

3.3. Total synchronous fluorescence spectra

The contour plots of total synchronous fluorescence (TSF) spectra were obtained by plotting the fluorescence intensity (z-axis) as a function of excitation wavelength (x-axis) and wavelength interval $\Delta\lambda$ (y-axis). The TSF spectra of a brandy sample are given in Fig. 4a. It shows that the TSF contour map spreads in the excitation wavelength 330–530 nm and in the wavelength interval 10–100 nm. The contours are concentrated in the excitation wavelength region 390–440 nm and wavelength interval 70–100 nm. The plot shows one fluorescence maximum only. For example, the maximum fluorescence intensity was observed at excitation wavelength 410 nm ($\Delta\lambda$ = 90 nm), 396 nm ($\Delta\lambda$ = 90) and 396 nm ($\Delta\lambda$ = 90 nm) for brandy B_1, B_2 and B_3, respectively.

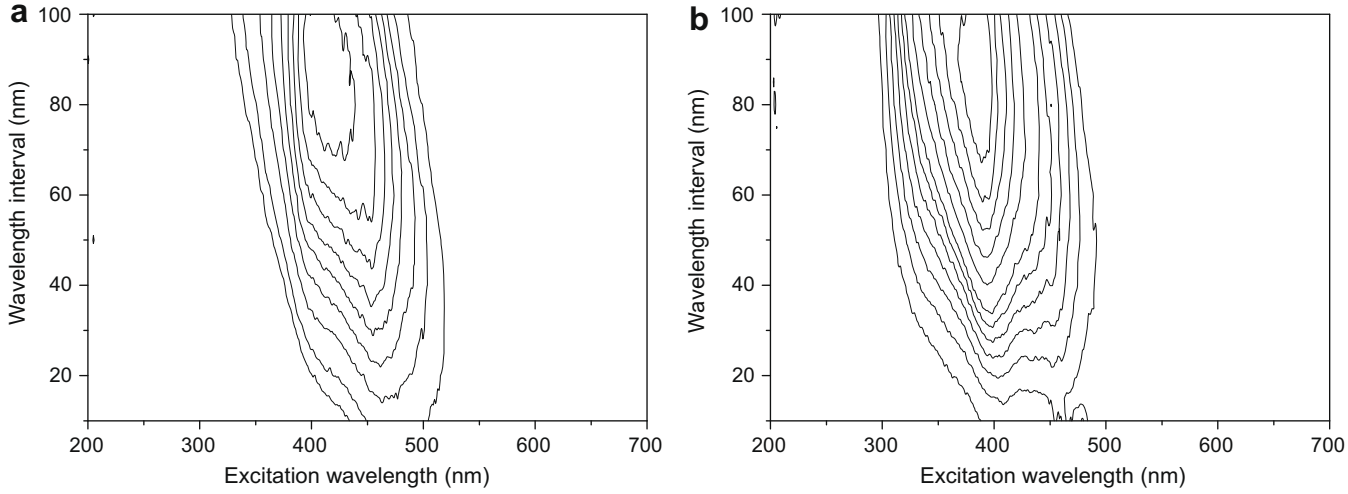

Fig. 4. Contour plots of total synchronous fluorescence spectra of brandy (a) and wine distillate (b) samples. Contours join the points of equal fluorescence intensity.

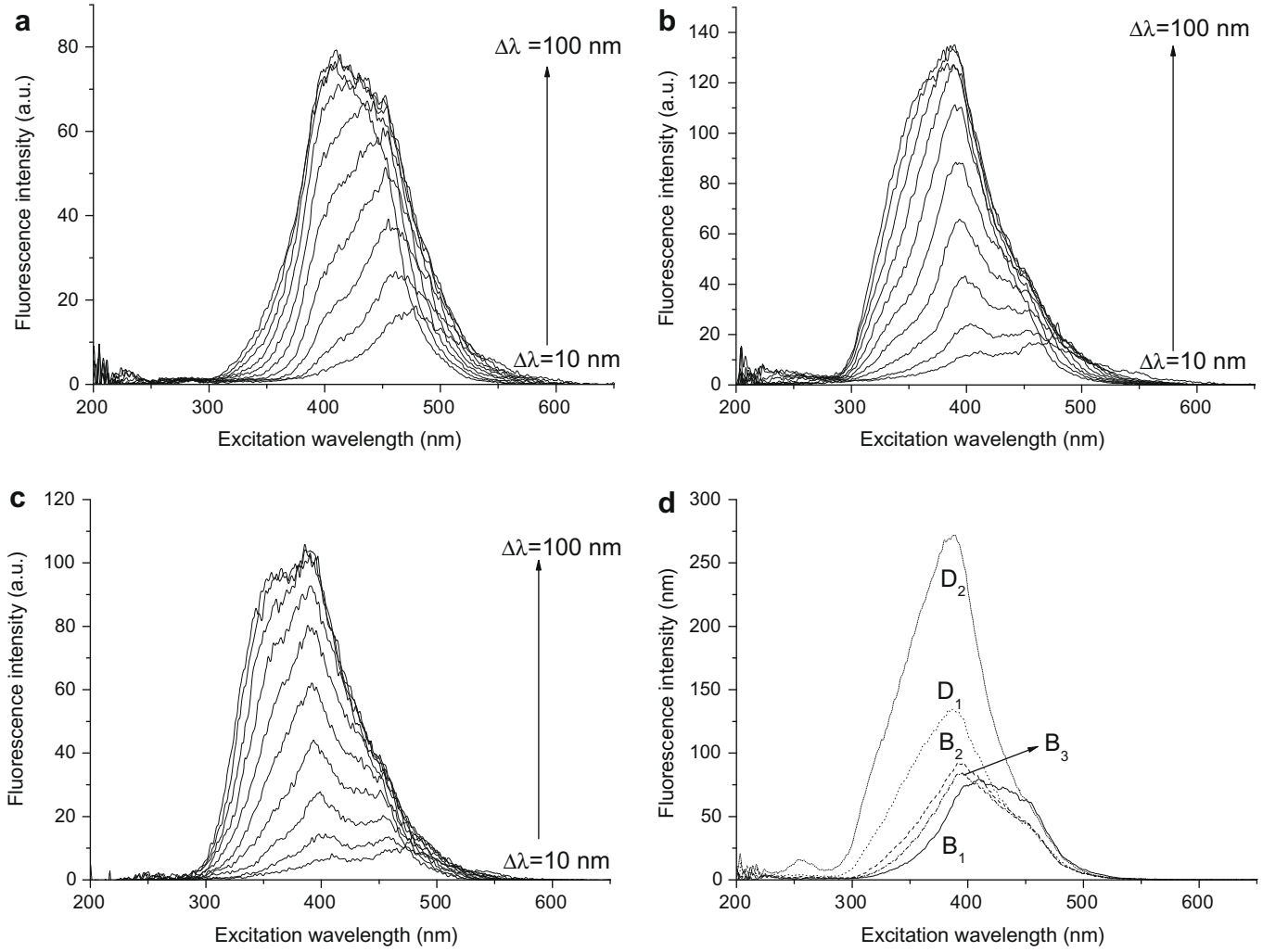

Fig. 5. Synchronous fluorescence spectra of brandy (a), wine distillate (b) and caramel (E 150a) (c) recorded at wavelength interval from 10 to 100 nm in steps of 10 nm. Synchronous fluorescence spectra of brandy (*B*) and wine distillate (*D*) samples from various producers (1–3) obtained at wavelength interval 90 nm (d).

The TSF spectra of a wine distillate sample are given in Fig. 4b. The contour map spreads in the excitation wavelength 300–510 nm and $\Delta\lambda$ 10–100 nm. The contours are concentrated in the excitation wavelength 370–410 nm and $\Delta\lambda$ 70–100 nm. The spec-

tra of wine distillates are characterised by two fluorescence maxima, one at ~390 nm and the other at ~440 nm, and a shoulder at about 330 nm. The maximum fluorescence intensity for distillate D_1 and D_2 was observed at excitation wavelength 390 nm ($\Delta\lambda$ = 85 nm) and 388 nm ($\Delta\lambda$ = 85), respectively.

Generally, the fluorescence maxima shift to shorter wavelengths with increasing $\Delta\lambda$ for both brandy and wine distillates. Wine distillates give a shorter wavelength high intensive fluorescence band and brandies give a longer less intensive band.

Fig. 5 shows the shift, simplification and amplification of synchronous fluorescence spectra of brandy, wine distillate sample and caramel (E 150a) using different wavelength intervals. For brandy, the plot shows one fluorescence maximum only blue-shifted from 480 to 400 nm with increasing $\Delta\lambda$ (Fig. 5a). For wine distillate, three overlapping bands with maxima at 412, 433 and 478 nm are apparent for λ = 10 nm (Fig. 5b). For $\Delta\lambda$ = 30 nm, the fluorescence intensity of bands increased, changes in their relative intensities were noted, and maxima were blue-shifted to 396, 435 and 453 nm, respectively. For $\Delta\lambda$ = 40 nm, one band with maxima at 395 and shoulder at 439 nm are observed. In addition, a short-wavelength shoulder appeared with a maximum at about 340 nm. The further increase of intensity and band broadening was apparent for $\Delta\lambda$ = 50 nm, resulting in only one band with maxima at 391 nm and shoulder at 336 nm. At higher $\Delta\lambda$ values, the maximum of the synchronous spectrum is shifted to the blue and broadened, with additional fluorescence intensity changes. The shape and intensity of the fluorescence maxima varied from one wine distillate to another. Moreover, the shape of the spectra changed with distillate samples, varying in the shoulder intensity and/or $\Delta\lambda$ where shoulder appear (30–50 nm)/disappear (60–80 nm).

An alcoholic beverage is a complex mixture consisting of a large variety of substances with different spectroscopic characteristics. Due to this complexity, an intrinsic fluorescence of the beverage is result of the overlapping numerous fluorescence bands. Although complete assignment of the fluorescence bands is beyond the scope of our work, some preliminary assignments could be made, based on comparison of the fluorescence spectra with the fluorescent characteristics of particular beverage constituents. The short-wavelength fluorescence, with excitation at 280 nm and emission maxima located at 370 and 450 nm, was clearly observed in diluted brandy samples, along with the longer-wavelength fluorescence, with excitation at 340 nm and emission at 450 nm (data not shown). The former band was preliminary attributed to the aromatic amino acids. Since phenolic acids exhibited emission between 360 and 420 nm after excitation set between 250 and 280 nm, they can also modify the shape of the fluorescence spectra after excitation at 280 nm. The latter band could be

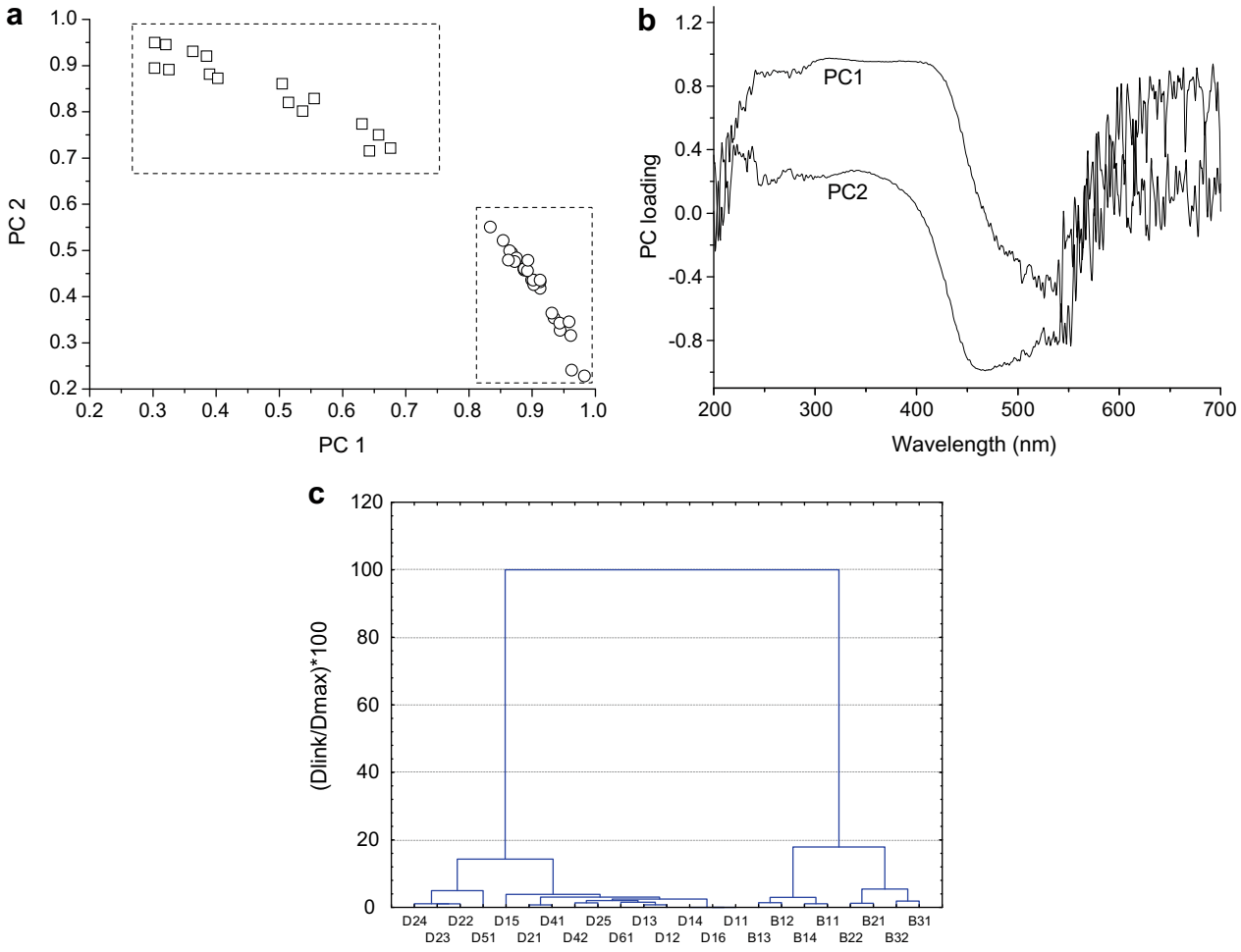

Fig. 6. Principal component analysis similarity map (score plot) determined by principal components 1 (PC1) and principal component 2 (PC2) (a) and spectral pattern (loading) corresponding to PC1 and PC2 (b) for synchronous fluorescence spectra recorded at wavelength interval 90 nm on brandy (□) and wine distillate (○) samples. Hierarchical cluster analysis dendrogram (c) using Manhattan distance for synchronous fluorescence spectra recorded at wavelength interval 90 nm on brandy (*B*) and wine distillate (*D*) samples.

due to the presence of fluorescent cinnamic acids, coumarins – namely scopoletin, Maillard reaction products, tannins and other unknown fluorescent compounds present in brandies.

The short-wavelength band could not be observed in the bulk brandy samples, while the longer-wavelength band was fairly intense. On the contrary, these two bands were clearly observed in the bulk wine distillate samples, showing higher intensity.

As mentioned, the wine distillates are frequently coloured with caramel (E 150a) to imitate the effect of long ageing in wooden casks. Therefore we assumed that the longer-wavelength bands originate from this colourant. To support this assumption, spectra of caramel in water were recorded. Emission spectra recorded after excitation at 390 nm showed a maximum located around 482 nm. The shape and intensity of the synchronous fluorescence spectra depend on the difference between excitation and emission wavelengths $\Delta\lambda$ (Fig. 5c). For $\Delta\lambda = 10$ nm, two bands with maxima at 411 and 477 nm are observed. At increased $\Delta\lambda$, the first band was shifted to 398 and 393 nm and the second one to 453 and 440 nm for $\Delta\lambda = 30$ and 40 nm, respectively. For $\Delta\lambda = 50$ nm, only one band with maxima at 391 nm was recorded. Comparison shows that the maxima observed for caramel at different $\Delta\lambda$-values are consistent with the respective maxima in wine distillates, so confirming a correct assignment of the longer-wavelength band to caramel.

3.4. Multivariate analysis of synchronous fluorescence spectra

PCA was applied separately on synchronous spectra measured at $\Delta\lambda$ 10–100 nm. The best classification was achieved using fluorescence spectra recorded at $\Delta\lambda = 90$ nm. The synchronous fluorescence spectra showed different shapes. The width of synchronous spectra was larger for wine distillates than those for brandies. Wine distillates had higher fluorescence intensity regardless of wavelength but they were also more heterogeneous in this respect. Fig. 5d illustrates differences in the synchronous fluorescence spectra of brandy and wine distillates from various producers obtained at $\Delta\lambda = 90$ nm.

Fig. 6a shows that the plot of the first two PCs leads to a good discrimination of beverages according to origin. PC1 and PC2 describe 90.2% and 9.5%, respectively, of the total variance. The spectral pattern associated with the PC1 presented a broad positive peak between 300–400 nm and a negative peak at 520 nm (Fig. 6b). The spectral pattern associated with the PC2 exhibited a narrow positive peak at 230 nm, a broad positive peak around 350 nm and a negative peak at 460 nm.

Applying HCA to fluorescence spectra recorded at $\Delta\lambda = 90$ nm, the dendrogram shows that the wine distillates are well separated from brandies (Fig. 6c). The first main cluster contains wine distillate samples only, while the second one contains brandy samples. Wine distillates cluster consists of various small groups of very similar products. One small group is constituted of samples D_2 with 99% of similarity among them and by sample D_5 with 95% of similarity with the samples D_2 of this group. Another group is constituted of D_1, D_4 and D_6 with 96% of similarity among them and 85% in relation to the previous group. Brandy samples form three small subclusters of the second main cluster corresponding to three different producers. One subcluster is constituted of B_1 with 97% of similarity. Another subcluster is constituted of B_2 and B_3 with 95% of similarity among them and 82% in relation to B_1.

4. Conclusion

This preliminary study shows that brandies and wine distillates can be discriminated using differences in their front face fluorescence spectra. Although differentiation between samples was possible by visual inspection of the spectra, it was accomplished by multivariate data analysis methods more easy. Front face fluorescence spectroscopy offers a promising approach for the authentication of brandies as neither sample preparation nor special qualification of the personnel are required, and data acquisition and analysis are relatively simple.

Acknowledgements

This research was supported by the Scientific Grant Agency of the Ministry of Education of Slovak Republic and the Slovak Academy of Sciences VEGA No. 1/0318/08 and the Grant Agency of the Slovak Republic APVV-0319-07.

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Food Chemistry 117 (2009) 499–503

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Monitoring olive oil oxidation under thermal and UV stress through synchronous fluorescence spectroscopy and classical assays

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ARTICLE INFO

Article history:
Received 2 April 2008
Received in revised form 12 January 2009
Accepted 12 April 2009

Keywords:
Olive oil
UV
Thermal stability
Stress
Synchronous fluorescence spectroscopy

ABSTRACT

Synchronous fluorescence (SyF) spectra combined with principal component analysis (PCA) is proven to be a useful tool for monitoring olive oil deterioration under UV irradiation at 80 °C. Spectra acquired in the range 300–500 nm during 12 h accelerated oxidation stress using a surface per volume ratio of 0.95 cm^2 ml^{-1} reveal five different classes after PCA. Parallel monitoring of lipid oxidation parameters peroxide value (PV), anisidine value (AV) shows that after 12 h, TOTOX value increases 10-fold, 19-fold and almost 38-fold for extra virgin olive, olive and olive-pomace oil, respectively.

Linear correlations between parameter values versus exposure time were observed during extra virgin olive oil deterioration under UV at different temperatures: ambient (~20 °C), 40, 60 and 80 °C. The rate of increase was found to be 6.3, 18.7, 37 and 56 TOTOX h^{-1}, 2.8, 8.3, 16.5 and 25.6 meq peroxides kg^{-1} h^{-1} and 0.84, 2.0, 3.6 and 4.9 AV h^{-1}, respectively. The rates of parameter change increase linearly with temperature, the slopes being 0.84 ± 0.06 TOTOX h^{-1} °C^{-1}, 0.38 ± 0.03 meq peroxides kg^{-1} h^{-1} °C^{-1} and 0.07 ± 0.003 AV h^{-1} °C^{-1}.

1. Introduction

Oxidation results in deterioration of edible oils reducing shelf life and nutritional value, while at the same time toxic compounds are produced. Primarily oxidation products are hydroperoxides that subsequently decompose to secondary products such as aldehydes, alcohols, ketones and hydrocarbons. These compounds are responsible for the off-flavour characterising degraded edible oils. Oxidation is depended on light (Caponio, Bilancia, Pasqualone, Sikorska, & Gomes, 2005; Grigoriadou & Tsimidou, 2006; Nakajima & Hidaka, 1993) heat (Choe & Min, 2006; Naz, Siddiqi, Sheikh, & Sayeed, 2005) and endogenous metals, pigments, phospholipids and antioxidants (Baldioli, Servili, Perretti, & Montedoro, 1996; Hamilton, 1994; Velasco & Dobarganes, 2002). Moreover, the fatty acid composition i.e. saturated versus mono- and poly- unsaturated fatty acids, strongly affects the oxidative process.

Classical analytical methods are used for monitoring the oxidation state of edible oils: peroxide value (PV), anisidine value (AV), 2-thiobarbituric acid value, carbonyl value and total polar materials (Fritsch, 1981). Useful indices are also spectrophotometric absorption at 232 and 270 nm (Vieira & Regitano-d'Arce, 1999). Chromatographic methods that have been used to identify and quantify individual oxidation products are cumbersome and time consuming (Bilancia, Caponio, Sikorska, Pasqualone, & Summo, 2007). Recently, nuclear magnetic resonance (Guillen & Ruiz, 2006) and vibrational techniques (Muik, Lendl, Molina-Diaz, Valcarcel, & Ayora-Canada, 2007; Sinelli, Cosio, Gigliotti, & Casiraghi, 2007) which are rapid and easy to use have been used to monitor edible oil deterioration. Fluorescence spectroscopy has been recently used for monitoring deterioration of extra virgin olive oil during heating (Cheikhousman et al., 2005).

One of the modes of fluorescence spectroscopy is synchronous fluorescence (SyF). In SyF both the excitation and emission monochromators are scanned simultaneously in such a manner that a constant wavelength interval ($\Delta\lambda$) is kept between emission and excitation wavelengths. SyF enhances fluorescence spectroscopy in the analysis of complex multi-component samples such as olive oil, since it reduces spectral overlaps by narrowing spectral bands and simplifies spectra using a suitable wavelength interval (Patra & Mishra, 2002a; Poulli, Mousdis, & Georgiou, 2007). Total synchronous fluorescence (TSyF) spectra are obtained by plotting fluorescence intensity as a combined function of the excitation wavelength and the wavelength interval. In this way, spectra selectivity is increased and multi-component samples are better characterised (Patra & Mishra, 2002b).

This work presents an accelerated oxidation setup using a high surface per volume ratio, UV and thermal stress. SyF spectra are assessed as a tool for monitoring olive oil deterioration and differentiation of olive oils according to stress time by multi-dimensional chemometrics. Furthermore, the rate of PV, AV and TOTOX

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doi:10.1016/j.foodchem.2009.04.024

value changes for extra virgin olive oil during oxidation under UV irradiation at ambient temperature (~20 °C), 40, 60 and 80 °C is studied.

2. Materials and methods

2.1. Samples and reagents

Olive oils by Minerva S.A. (Athens, Greece) were purchased from a local shopping centre. Isooctane and glacial acetic acid of analytical reagent grade were purchased from Merck. Chloroform of analytical reagent grade was purchased from SDS. Potassium iodide 99.5%, sodium thiosulphate standard solution 0.1 M, starch and p-anisidine 99% were purchased from Ferak Berlin, Merck, Riedel-de Haën and Aldrich, respectively.

2.2. Analytical methods

PV of the samples was determined according to the official EC method (European Union Commission Regulation, 1991). AV was determined according to the International Union of Pure and Applied Chemistry standard method (IUPAC, 1979). Total oxidation value (TOTOX) is calculated as $2 \times PV + AV$ (Rossell, 1994).

2.3. Instruments

Fluorescence spectra were acquired by the Jobin Yvon fluorolog-3 spectrofluorometer which is a fully computer controlled instrument using a double-grating excitation and a single-grating emission monochromator. The spectrofluorometer uses a 950 W xenon lamp for excitation. Excitation and emission slit widths were set at 2 nm. A quartz cell from Hellma 104F-QS with an optical length of 4 mm was used. Right-angle geometry was used for spectral acquisition.

For SyF spectra, samples were used as received without any pretreatment to avoid noxious solvents reducing manipulation steps.

SyF spectra were collected in the excitation wavelength range 250–720 nm. TSyF spectra were obtained by measuring the excitation wavelength in the same spectral region and varying the wavelength interval from 20 to 120 nm in 20 nm intervals. The spectra were corrected for the excitation lamp, the photomultiplier detector spectral response and emission and excitation gratings.

A Perkin Elmer Lambda 19 UV/Vis/NIR spectrometer was used for p-anisidine value measurements. Quartz cells from Hellma 100-QX with 10 mm optical lengths were used.

Contour plots of TSyF spectra were plotted using the Origin Software Version 7.0 (OriginLab, USA, 2002).

2.4. Data analysis

Data analysis was performed using the Statistica Software Version 6.0 (StatSoft, USA, 2001). Principal component analysis (PCA) is an unsupervised classification procedure. The main aim of PCA is to reduce a large number of variables to a much smaller number of principal components (PCs) that capture the vast majority of variance in the data. This reduces the dimensionality of the original data considerably, enabling effective visualisation, regression and classification of multivariate data (Brereton, 1992).

2.5. Procedure

100 mL of olive oil were placed in a Petri dish of 11 cm diameter thermostated at 80 °C over a Heidolph hot plate. This setup provides a surface per volume ratio of 0.95 cm^2 ml^{-1}. A Hanovia UV lamp (200 Volt, 125 Watts) placed 21.5 cm above the dish was used for sample irradiation. Samples of approximately 5 g were withdrawn every 2 h. PV was measured immediately and the sample was stored at −40 °C for subsequent AV analysis and SyF spectra acquisition.

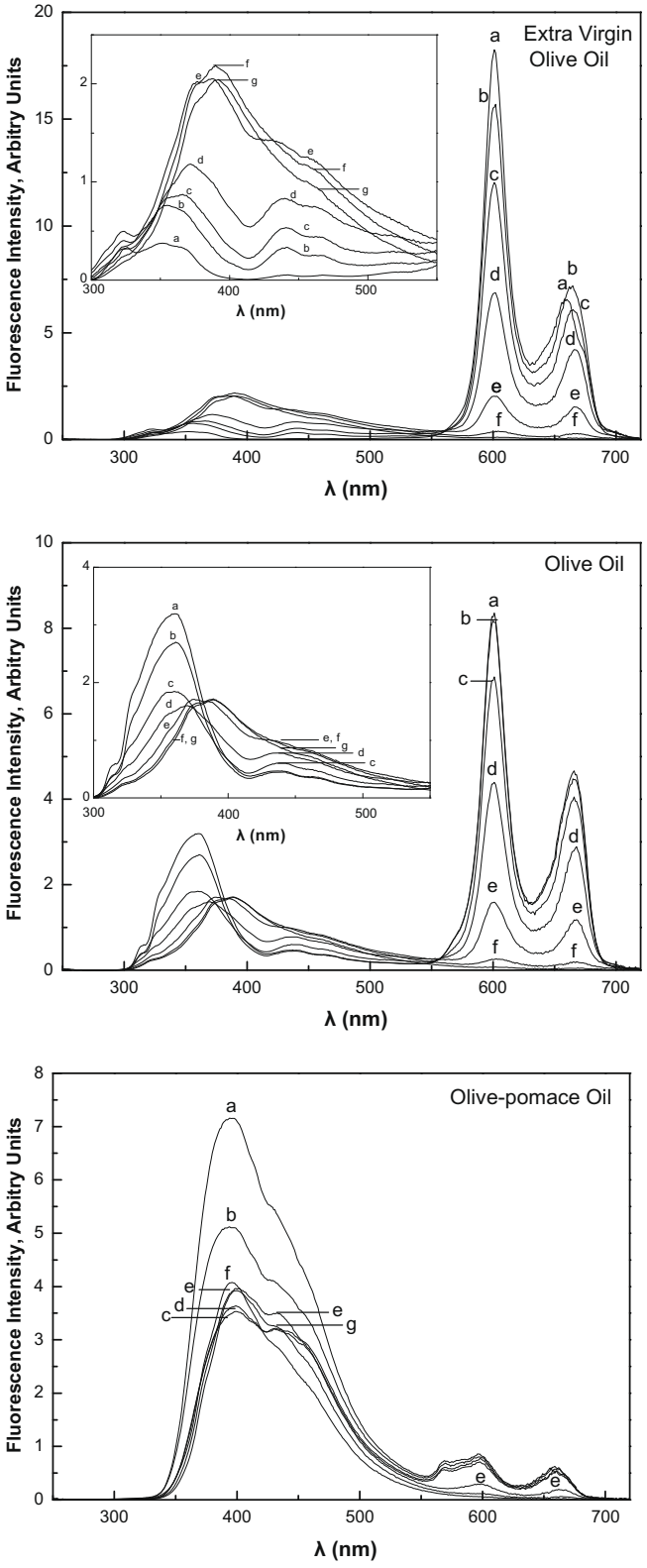

Fig. 1. Synchronous fluorescence spectra of extra virgin olive, olive and olive-pomace oil after 0 (a), 2 (b), 4 (c), 6 (d), 8 (e), 10 (f) and 12 h (g) under UV irradiation at 80 °C using 80 nm wavelength interval.

Extra virgin olive oil was used as a study case for a detailed investigation at different temperatures: ambient (~20 °C), 40, 60 and 80 °C. Samples, around 2 g, were withdrawn every few hours for PV, AV and TOTOX value analyses. The experiment was terminated when the peroxide value reached ~400 meq kg^{-1}.

For comparison, 100 ml extra virgin olive oil was placed in a Petri dish and stored in the dark for 6 months. A sample was withdrawn every 2 weeks for PV analysis.

3. Results and discussion

3.1. Synchronous fluorescence spectroscopy

Fig. 1 shows the evolution of SyF spectra of extra virgin olive, olive and olive-pomace oil during the time course of the experiment. All olive oils show fluorescence bands in the range of 550–700 nm which are attributed to chlorophyll pigments (Kyriakidis & Skarkalis, 2000). These bands are stronger for extra virgin olive oil and minimal for olive-pomace oil due to the different extraction procedure that involves organics solvents. During the time course of the experiment, these fluorescence bands decrease significantly due to deterioration of chlorophyll pigments that are involved in photo-oxidation (Choe & Min, 2006).

Extra virgin olive oil gives signals in the region 300–330 nm that decrease during oxidation and the region 350–550 nm that increase during the initial 8 h and then remain almost constant for up to 12 h.

Olive oil gives signals in the region 300–550 nm. The fluorescence bands in the region 300–370 nm decrease during oxidation whereas fluorescence bands in the region 370–550 nm increase during the initial 8 h and remain almost constant afterwards.

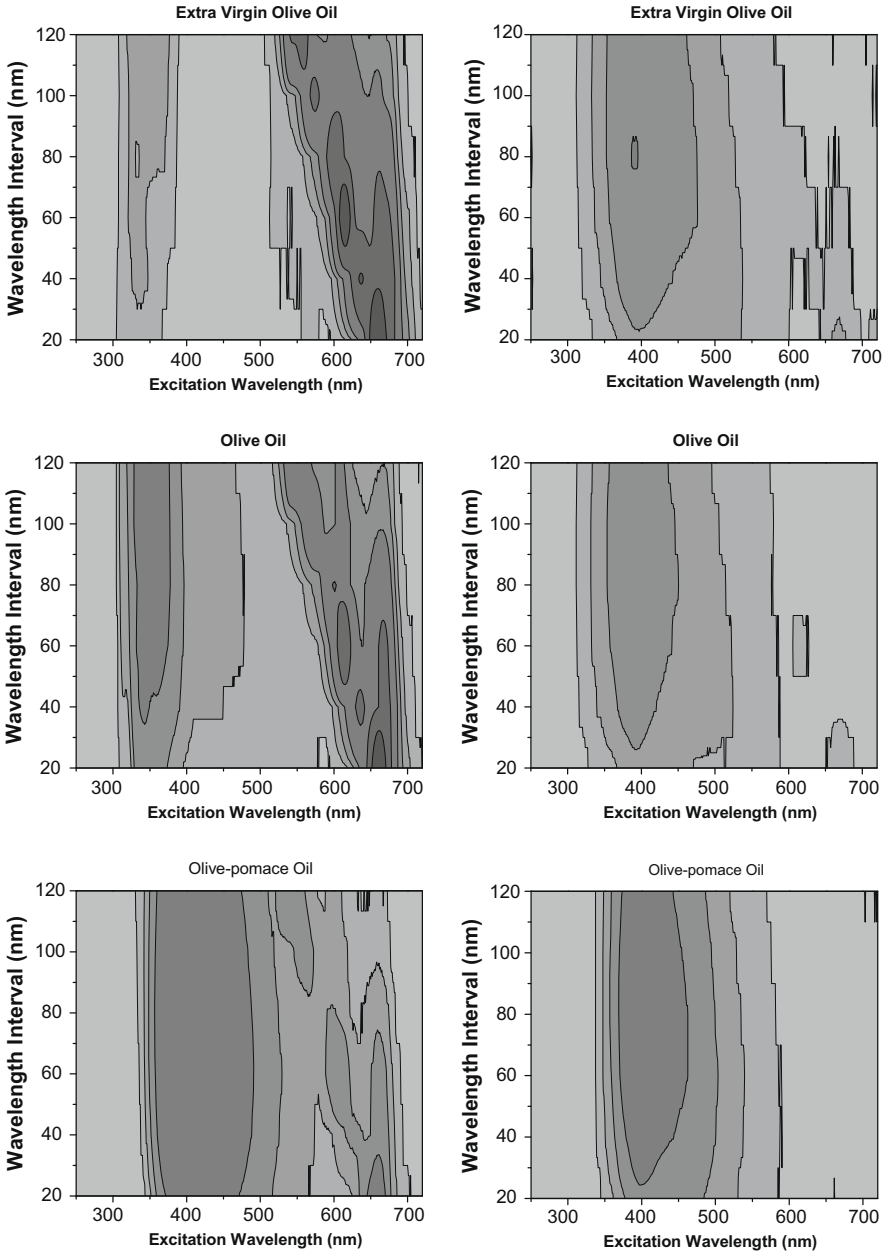

Fig. 2. Total synchronous fluorescence spectra contour plots of olive oils before (left) and after 12 h (right) exposure to UV light at 80 °C. Grayscale indicates fluorescence intensities.

Olive-pomace oil gives fluorescence bands in the region of 350–550 nm that decrease during the initial 6 h and then a small increase is observed.

As shown above, fluorescence spectra change even after just 2 h stress. This indicates that the combination of UV light and thermal stress using surface to volume ratio of 0.95 cm^2 ml^{-1} is sufficient for accelerated oxidation studies.

Initial TSyF contour plots change significantly after 12 h (Fig. 2). TSyF contour plot of extra virgin olive oil spreads in the excitation wavelength region 302–390 nm and 510–720 nm. After 12 h exposure to UV light at 80 °C, the fluorescence bands in the region 510–720 nm diminish and the region 302–320 nm disappear while fluorescence bands in the region 390–510 nm appear.

TSyF contour plot of olive oil spreads in the excitation wavelength region 305–480 nm and 515–720 nm. After 12 h under UV light at 80 °C, fluorescence in the wavelength region higher than 590 nm is almost suppressed whereas fluorescence bands in the wavelength region 480–515 nm appear.

Olive-pomace oil TSyF contour plot spreads in the excitation wavelength region 340–705 nm. After 12 h exposure to UV light at 80 °C the TSyF contour plot spreads in the excitation wavelength region 340–590 nm while the fluorescence bands in the region 590–705 nm disappear.

According to the results presented in the contour plots, the spectral characteristics of extra virgin olive, olive and olive-pomace oil change dramatically during the procedure. Fluorescence signals in the region 600–720 nm, attributed to chlorophyll, decrease significantly after 12 h while fluorescence bands in the low wavelength region expand up to 590 nm.

3.2. Principal component analysis

The spectra range selected was 300–500 nm using 80 nm wavelength interval after normalisation. The score for olive oil samples in the first two principal components that explain 95.1% of the data matrix variance is depicted in Fig. 3. Five different classes are well separated. Class A includes the initial olive-pomace oil spectrum and that acquired at 2 h stress. Olive-pomace oil spectra obtained after 2 h up to 12 h form class B. Extra virgin olive and olive oil spectra acquired after 4 h up to 12 h form class C. Classes D and E refer to extra virgin olive and olive oil, respectively. They include the initial spectrum and spectra at 2 and 4 h stress.

Extra virgin olive and olive oil (classes C, D and E) have positive scores in the first principal component (PC1) in contrast to olive-pomace oil (classes A and B). Furthermore, extra virgin olive (class D), in contrast to olive oil (class E) has positive scores in the second principal component (PC2). The results show that SyF spectra combined with PCA could be used for monitoring olive oils during stress under UV light at 80 °C if the surface to volume ratio is 0.95 cm^2 ml^{-1}.

3.3. Classical methods

As shown above, olive oil SyF spectra change substantially during oil oxidation. This could be attributed to evolution of primary and secondary oxidation products that are followed using PV, AV and TOTOX values.

Fig. 4 shows TOTOX value increase of extra virgin olive, olive and olive-pomace oil during the procedure. As shown, the TOTOX value increases 10-fold after 12 h for extra virgin olive oil while the same end point is reached after just 8 h for olive oil. Olive oil TOTOX value increases almost 19-fold after 12 h. The TOTOX value of olive-pomace oil increases 20-fold after 8 h and almost 38-fold after 12 h. It is clearly shown that extra virgin olive oil shows the lowest rate of deterioration while olive-pomace oil shows the highest.

Extra virgin olive oil was further used as a study case for a detailed investigation of olive oil deterioration.

Fig. 5 shows the change of extra virgin olive oil TOTOX value versus exposure time under UV light at different temperatures. TOTOX value increases linearly during oil deterioration. The increase of PV and AV with time is also linear. Rates of parameters increase are given in Table 1. In comparison to stress under UV light at ambient temperature (~20 °C), the rates for TOTOX and PV value are almost three times higher when the temperature increases to 40 °C, almost six times higher at 60 °C and nine times higher at 80 °C. The rate of AV change at 40 °C is almost two times higher than that at 20 °C, four times at 60 °C and six times at 80 °C. It is interesting to note the linear correlation between the rates and temperature, the slopes being 0.84 ± 0.06 TOTOX h^{-1} $°C^{-1}$, 0.38 ± 0.03 meq peroxides kg^{-1} h^{-1} $°C^{-1}$ and 0.07 ± 0.003 AV h^{-1} $°C^{-1}$ with correlation coefficients of 0.996, 0.995 and 0.998, respectively.

In contrast to the above results, the control sample that was stored in the dark for 6 months shows just a slight increase of PV from 12 to 23 meq kg^{-1}.

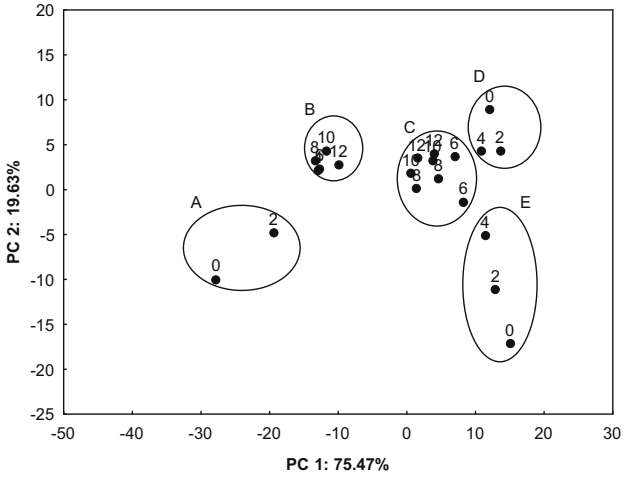

Fig. 3. PCA based on fluorescence spectra of extra virgin olive, olive and olive-pomace oil acquired in the range 300–500 nm during the time course of exposure to UV light at 80 °C: Classes A and B contain olive pomace oil, class C extra virgin olive and olive oil, class D extra virgin olive oil and class E olive oil. Numbers are the exposure time (h).

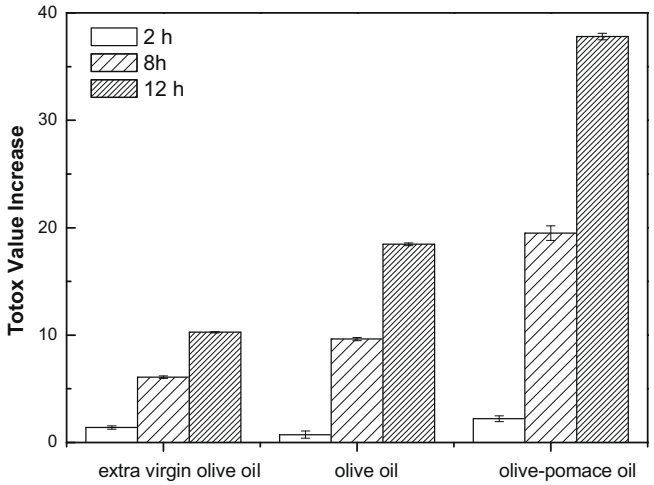

Fig. 4. TOTOX value increases during extra virgin olive, olive and olive-pomace oil deterioration under UV light at 80 °C. Values are means of three replicate measurements accompanied by standard deviations.

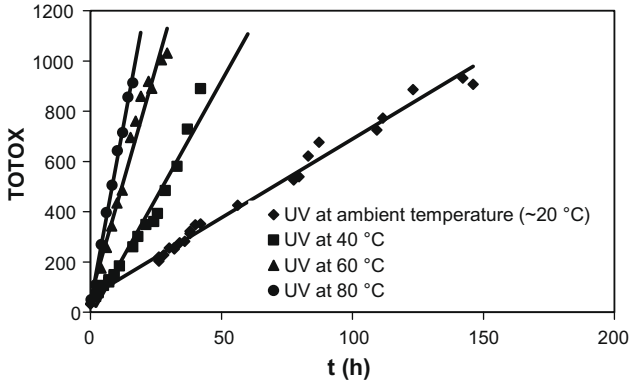

Fig. 5. Change of TOTOX value during extra virgin olive oil deterioration under different treatments.

Table 1
Rate of parameter changes during extra virgin olive oil oxidation under different treatments.

Parameter	Treatment	Correlation coefficient (r)	Rate ± SD
TOTOX			TOTOX × h^{-1}
	UV	0.994	6.3 ± 0.2
	UV at 40 °C	0.98	18.7 ± 0.9
	UV at 60 °C	0.99	37 ± 2
	UV at 80 °C	0.997	56 ± 2
PV			meq × Kg^{-1} × h^{-1}
	UV	0.992	2.8 ± 0.05
	UV at 40 °C	0.98	8.3 ± 0.4
	UV at 60 °C	0.98	16.5 ± 0.8
	UV at 80 °C	0.997	25.6 ± 0.6
AV			AV × h^{-1}
	UV	0.997	0.84 ± 0.01
	UV at 40 °C	0.97	2.0 ± 0.1
	UV at 60 °C	0.991	3.6 ± 0.1
	UV at 80 °C	0.997	4.9 ± 0.2

As shown above, extra virgin olive oil is very stable in the dark whereas it is susceptible to oxidation under UV light. Studying the combined effect of thermal stress and UV light is important for Mediterranean countries producing olive oil as the temperature could exceed 40 °C during summer rendering olive oil packed in transparent bottles prone to oxidation.

4. Conclusion

Synchronous fluorescence spectroscopy is a useful tool for monitoring olive oil deterioration under thermal and UV stress. Olive oils can be categorised according to stress time through synchronous fluorescence spectra combined with multi-dimensional chemometrics. Surface to volume ratio of 0.95 cm^2 ml^{-1} was found sufficient for accelerated oxidation tests. The experimental setup used proved to be appropriate for monitoring extra virgin olive oil deterioration under thermal and UV stress through classical methods such as peroxide, anisidine and TOTOX value. Linear correlations between parameters values versus exposure time as well as the rate of parameter changes versus temperature were observed.

Acknowledgments

This work was financially supported by the Greek General Secretariat for Research and Technology and Minerva S.A. (Athens, Greece) through a PENED 2003 grant.

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Food Chemistry 117 (2009) 504–508

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Supercritical fluid extraction of *Coriandrum sativum* and subsequent separation of isocoumarins by high-speed counter-current chromatography

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ARTICLE INFO

Article history:
Received 25 April 2008
Received in revised form 11 February 2009
Accepted 12 April 2009

Keywords:
Supercritical fluid extraction (SFE)
High-speed counter-current chromatography (HSCCC)
Coriander
Essential oil
Isocoumarins

ABSTRACT

A novel supercritical fluid extraction (SFE) method has been developed for extracting essential oil from the aerial part of *Coriandrum sativum*. Various experimental conditions were investigated to optimise the SFE. It suggested that the extraction of 35 °C, 8 MPa, 2 h might be the optimisation to get the maximum of coriander essential oil. Then, high-speed counter-current chromatography (HSCCC) was successfully used in one step for the isolation and purification of coriandrone B, coriandrin, dihydrocoriandrin and coriandrone A from the coriander oil with a two-phase system composed of *n*-hexane–ethyl acetate–methanol–water (3:7:5:5, v/v/v/v) for the first time. High-performance liquid chromatography (HPLC) was employed to analyse the HSCCC fractions and revealed that the purities of the isocoumarins were all above 90%, and the chemical structures were identified by spectroscopic analysis including ESIMS, ^{1}H NMR and UV.

1. Introduction

Coriandrum sativum L. (family Umbelliferae) is a culinary and medicinal plant originating from the Mediterranean region, and is nowadays grown widely all over the world. The leaves are variously referred to as coriander leaves in Britain; cilantro in the United States, and "yanshui" in China. It is featured in the cuisines of China, Southeast Asia, India, and South and Central America. As a medicinal plant, *C. sativum* has been credited with a long list of medicinal uses in traditional Chinese medicine to treat indigestion and abdominal distention. The effect of letting out skin eruptions and invigorating stomach by coriander was also recorded.

The essential oil and various extracts from coriander have also shown the activities of antibacterial (Kubo, Fujita, Kubo, Nihei, & Ogura, 2004), anticancerous and antimutagenic (Chithra & Leelamma, 2000), antioxidant (Madsen & Bertelsen, 1995; Ramadan, Kroh, & Morsel, 2003; Wangensteen, Samuelsen, & Malterud, 2004), antidiabetic (Gallagher, Flatt, Duffy, & Abdel-Wahab, 2003).

Research on the secondary metabolites has focused on the analysis of the essential oil from *C. sativum* from different origins and stages of maturity (Anitescu, Doneanu, & Radulescu, 1997; Bandoni, Mizrahi, & Juarez, 1998; Carrubba, la Torre, Di Prima, Saiano, & Alonzo, 2002; Msaada et al., 2007). However, none of studies has examined in detail on the preparative separation of coriander essential oil composition. As this plant exhibits an ample range of biological activity and is widely used in folk medicine and the human diet, it is important to extend the study to preparative scale.

The essential oil of aromatic herbs is traditionally obtained by hydrodistillation, steam-distillation or solvent extraction. As an alternative, supercritical fluid extraction (SFE) is a particularly suitable method. Carbon dioxide is an ideal solvent because it is non-toxic, non-explosive, readily available and easy to remove from extracted products. SFE has the ability to use low temperatures, leading to less deterioration of the thermally labile components in the extract with comparable or better recovery, and minimal alteration of the active ingredients; thus the curative properties can be preserved. So SFE is widely used to extract and separate active compounds in food, pharmaceutical and cosmetic industries (Mostafa, Yadollah, Fatemeh, & Naader, 2004).

High-speed counter-current chromatography (HSCCC) is a kind of support-free all-liquid partition chromatography that was first invented by Ito (1981). As no solid stationary phase is used, it eliminates irreversible adsorption of sample on the solid support, which is a common drawback in conventional chromatographic column. What is more, it can avoid the devitalisation of active components. As an advanced separation technique, HSCCC has been widely used for the separation of active components in traditional Chinese herbs and other natural products in recent years. Successful application of HSCCC has also been reported for the separation and purification of alkaloids (Liu, Chu, Sun, & Kong, 2005), quinines (Du, Li, & Ito, 2001), flavonoids (Chen, Song, Lan, Games, & Sutherland, 2005), coumarins (Wei, Zhang, & Ito, 2004) and other

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doi:10.1016/j.foodchem.2009.04.025

natural products (Yao, Li, & Kong, 2006; Yao, Liu, Huang, & Kong, 2007).

The preparative separation and purification of isocoumarins from the aerial parts of *C. sativum* by classical methods are tedious and usually require multiple chromatographic steps on silica gel and large amounts of organic environmentally unfriendly solvents. In the present work, the extraction condition was optimised first with a preparative-scale SFE system by an orthogonal test. The crude extract (contain essential oil and oleoresin) obtained was then purified by high-speed counter-current chromatography (HSCCC). There have no reports of using SFE to extract and HSCCC to isolate chemical compounds from coriander. The aim of this paper is to extract and separate the coriander essential oil rapidly, and to develop a new mode of the isolation of low polar compounds from essential oils.

2. Materials and methods

2.1. Plant material

Coriander herbs were collected from suburbs of Xuzhou, Jiangsu Province, China. Xuzhou was located in the southeast of North China Plain. The elevation was 30–50 m. Its coordinate of longitude and latitude was 34°16′N, 117°10′E. The average temperature during the year was 15.1 °C. On average it rains about 100 days of the year, and the mean annual rainfall is 881.1 mm.

For collection at the initial stage of maturity, only full green fruits were picked up in October, 2006. The aroma of the coriander was natural and pleasant.

The coriander roots were removed and the aerial parts were cleaned, cut into pieces and dried in a ventilated drying oven and stored in plastic bags at ambient temperature and protected from light.

2.2. Reagents

Carbon dioxide, 99.9% purity, was obtained from Topgrand Petrifaction Industry Gas Co., Jiangsu, China. All other solvents were of analytical grade.

2.3. Supercritical fluid extraction (SFE)

HA-130-50-05 supercritical extraction apparatus (Hua'an Supercritical Extraction Limited Company, Jiangsu, China) was used for all the extractions. The SFE system was equipped with 500 mL stainless steel vessel. In order to determine a suitable extraction condition in a wide range with a minimum number of trials, an orthogonal test design was employed where temperature, pressure and time were considered to be three major factors for effective extraction. Combinations of the two different levels of each factor are listed in Table 1. In each test, 500 g of the coriander was placed into an extraction vessel. Carbon dioxide with a purity of 99.9% was used as a solvent. After 10 min of static extraction (no liquid flow), the sample was then subjected to dynamic extraction by flowing gaseous carbon dioxide at a rate of 2 L/min. The coriander oil (contain essential oil and oleoresin) was trapped into a collection vessel and transferred into brown glass bottle and stored in a freezer until being subjected to HSCCC purification.

Table 1
Orthogonal test design in coriander SFE process.

No.	Temperature (°C)	Pressure (MPa)	Time (h)	Yield of coriander oil (% w/w)
1	25	5	1	0.189
2	25	8	2	0.306
3	35	5	2	0.332
4	35	8	1	0.237

All the experiments were done in three times and yields were averaged (n = 3).

2.4. Preparation of two-phase solvent and sample solution

Each component of the selected solvent system was added to a separatory funnel and thoroughly equilibrated at room temperature for 12 h. The upper phase and lower phase were separated and degassed by sonication for 30 min shortly before use. For HSCCC separation of coriander essential oil, the sample solution was prepared by dissolving 300 μL of essential oil in 5 mL of the upper phase and 5 mL of the lower phase of the solvent system for isolation, and then was filtered with micropore membrane filters (Φ = 0.45 μm).

2.5. HSCCC separation

The HSCCC separation was performed with a TBE-300 high-speed counter-current chromatography system manufactured by Tauto Biotech Co., Shanghai, China. The apparatus was equipped with three preparative coils (diameter of tube, 1.5 mm, total volume, 300 mL) and a 20 mL sample loop. The revolution radius was 5 cm, and the values β ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R is the revolution radius or the distance between the holder axis and central axis of the centrifuge) of the multilayer coil varied from 0.5 at internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. The system was also equipped with one S-1007 constant flow pump (Shenyitong Tech. and Exploitation, Beijing, China) and a Model 8823B-UV monitor (Bingdayingchuang Sci. and Tech., Beijing, China). The data were collected with the model N2000 chromatography workstation (Zhejiang University, Hangzhou, China).

In each separation, the coiled column was first entirely filled with the upper phase (stationary phase), then the apparatus was rotated at 850 rpm, while the lower phase (mobile phase) was pumped into the column at a flow rate of 1.2 mL/min. After the mobile phase front emerged from the tail outlet and the system established hydrodynamic equilibrium, the sample solution was injected into the column through the injection valve. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. The whole process of separation was carried out under room temperature. Each peak fraction was manually collected

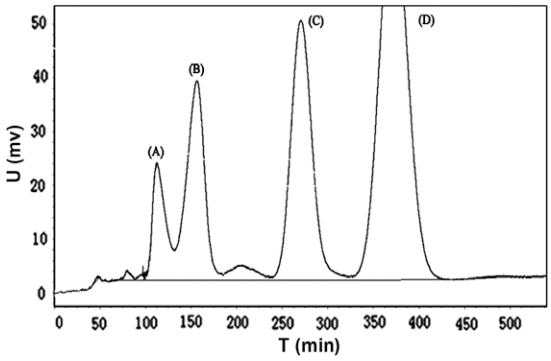

Fig. 1. HSCCC chromatogram of coriander essential oil from *Coriandrum sativum* L. *Conditions:* two-phase solvent system: *n*-hexane–ethyl acetate–ethanol–water 3:7:5:5 (v/v/v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 1.2 mL/min; revolution speed: 850 rpm; sample size: 300 μL of essential oil dissolved in 5 mL of the upper phase and 5 mL of lower phase; retention of the stationary phase: 59%.

according to the chromatogram (Fig. 1) and then evaporated under reduced pressure for later analysis. The retention of the stationary phase was calculated from the volume of the stationary phase collected from the column after the separation was completed.

2.6. HPLC analysis and identification of HSCCC fractions

The crude sample and peak fractions obtained by HSCCC were analysed by HPLC at room temperature. The HPLC equipment used was Agilent 1100 series system and Agilent HPLC workstation (Agilent, USA). The column used was a Shim-pack VP-ODS column (150 mm × 4.6 mm, 5 μm), with a gradient elution from 50% to 80% methanol in 40 min. The effluent was monitored at 254 nm and the flow rate was set at 1.0 mL/min constantly. Identification of HSCCC fractions was carried out by ESIMS (Agilent 1100 Series LC–MS Trap SL), ^{1}H NMR spectra (Bruker ACF-300), UV (Agilent 1100 with G1315B DAD).

3. Results and discussion

3.1. Optimisation of the SFE conditions

Since various parameters potentially affect the extraction process, the optimisation of the experimental conditions represents a critical step in the development of a SFE method. Pressures and temperatures of the fluid, and the extraction times are generally considered as the most important factors. In the present study, all selected factors were examined using an orthogonal test design. Reverchon (1997) suggested performing SFE of essential oils at 40–50 °C and pressure below 10 MPa would suppress co-extraction of higher molecular weight compounds. So the extractions were carried out at temperatures of 25 and 35 °C, pressures of 5 and 10 MPa, durations of 1 and 2 h.

For the experimental results of orthogonal test, the range of the extraction yields were 0.189–0.332%, the condition of 35 °C, 5 MPa, 2 h got the maximum extract yield. It suggested that the extracted time was the most important factor. It also meant that it could not achieve complete extraction at 2 h. However, the content of essential oil in the extract was higher. It suggested that the long extraction time, higher temperatures and higher pressure would get higher extract yield when the SFE performed at temperature below 40 °C and pressure below 10 MPa. The influence of different parameters on the extract yield of coriander SFE process was also confirmed by Illés in their study (Illés, Daood, Perneczki, Szokonya, & Then, 2000). Although the extract yield was high, the essential oil content of the extracts could possibly decrease and fatty acid composition would increase remarkably. The high temperature and low pressure could lead to the increase of the selectivity of the extraction. Although propane might increase the yield of extraction, considering the solvent residual, it was not chosen as the entrainer.

Reverchon (1997) suggested irrespective of pressure and temperature, the essential oil content of the extract rapidly decreased in accord with the increased quantity of the recovered oil. This means that essential oils are first solubilised by SC–CO_2. Therefore, in order to get as much amount of the coriander essential oil as possible and suppress co-extraction of higher molecular weight substances to simplify the procedure of purification, the optimal conditions for the extraction of coriander essential oil by SFE were at pressure of 5 MPa, temperature of 35 °C and extract time of 2 h.

3.2. Selection of two-phase solvent system

The selection of the two-phase solvent system for the target compounds is an important step in HSCCC. Optimal solvent system should satisfy two requirements. Firstly, the value of the partition coefficient (K) of the solvent system should be in the range of 0.5–2.0. The partition coefficient (K) reflects the solute distribution between the two mutually equilibrated solvent phases. Secondly, the higher retention of the stationary phase, the better peak resolution because the retention of the stationary phase is accomplished by a combination of coiled column configuration and the planetary motion of the column holder (Ito, 2005).

In the present study, the two-phase solvent systems composed of light petroleum (b.p. 60–90 °C)–methanol–water 5:4:1 (v/v/v), chloroform–methanol–water 5:4:1 (v/v/v), *n*-hexane–ethyl acetate–methanol–water 3:7:5:5 (v/v/v/v) and *n*-hexane–ethyl acetate–methanol–water 6:4:5:5 (v/v/v/v) were tested for the two parameters.

After the measuring of the K values and settling time of the above solvent systems, the target substances in the solvent system of *n*-hexane–ethyl acetate–methanol–water 3:7:5:5 (v/v/v/v) got suitable K values in the range of 0.5–1 and the settling time was 15 s. Therefore it was chosen for the HSCCC separation finally.

3.3. HSCCC purification and HPLC identification

The crude extract coriander oil (contain essential oil and oleoresin) (300 μL) was separated and purified by HSCCC. The retention of the stationary phase was 59%, and the separation time was about 600 min in each separation run. Separation of the coriander oil by the preparative HSCCC with *n*-hexane–ethyl acetate–methanol–water 3:7:5:5 (v/v/v/v) as a solvent system is shown in Fig. 1. Based on the HPLC analysis and the elution curve of the preparative HSCCC, all collected fractions were combined into different pooled fractions, yielding 3.3 mg of coriandrone B (fraction A, the purity is 90.21%), 12.0 mg of coriandrin (fraction B, 99.15%), 10.5 mg of dihydrocoriandrin (fraction C, 96.25%) and 5.7 mg of coriandrone A (fraction D, 90.98%) in only one-step separation. These results demonstrate the high resolving power of HSCCC. The chromatograms of HPLC and UV spectra of these compounds are shown in Fig. 2.

Fractions A–D were identified as coriandrone B, coriandrin, dihydrocoriandrin and coriandrone A, respectively (Fig. 3) based on the comparison of the ESI-MS and NMR spectroscopic data with the literature data (Ceska et al., 1988; Baba, Xiao, Taniguchi, Ohishi, & Kozawa, 1991).

HSCCC fraction A in Fig. 1: ESI-MS (m/z): 293 $[M+H]^+$; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.31, 1.44 (each 3H, s), 1.45 (d, J = 6.2 Hz), 2.65 (dd, J = 16.1, 4.5 Hz), 2.77 (2H, d, J = 5.1 Hz), 2.85 (dd, J = 16.1, 11.0 Hz), 3.80 (1H, t, J = 5.1 Hz), 3.87 (s), 4.51 (1H, m, J = 6.2, 11.0, 4.5 Hz), 6.25 (1H, s); UV λ_{max} (MeOH) nm: 223, 266, 300.

HSCCC fraction B in Fig. 1: ESI-MS (m/z): 230 $[M+H]^+$; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.25 (3H, d, J = 1.0 Hz), 4.23 (3H, s), 6.23 (lH, q, J = 1.0 Hz), 7.06 (lH, dd, J = 2.4, 1.0 Hz), 7.10 (lH, d, J = l.0 Hz), 7.61 (lH, d, J = 2.4 Hz); UV λ_{max} (MeOH) nm: 250, 285, 298, 340.

HSCCC fraction C in Fig. 1: ESI-MS (m/z): 487 $[2M+Na]^+$, 233 $[M+H]^+$; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.50 (3H, d, J = 6.4 Hz), 2.94 (1H, dd, J = 16.1, 5.0 Hz), 3.00 (1H, dd, J = 16.1, 9.2 Hz), 4.19 (3H, s), 4.57 (1H, m, J = 6.4, 9.2, 5.0 Hz), 7.00 (1H, dd, J = 2.3, 1.0 Hz), 7.04 (1H, d, J = 1.0 Hz), 7.57 (1H, d, J = 2.3 Hz); UV λ_{max} (MeOH) nm: 231, 259, 309.

HSCCC fraction D in Fig. 1: ESI-MS (m/z): 585 $[2M+H]^+$, 293 $[M+H]^+$; ^{1}H NMR(500 MHz, $CDCl_3$): δ 1.21 (3H, s), 1.37 (3H, s), 1.46 (3H, d, J = 6.3 Hz), 2.81 (1H, dd, J = 16.1, 4.8 Hz), 2.85 (1H, dd, J = 16.1, 10.6 Hz), 3.03 (2H, d, J = 9.2 Hz), 3.87 (3H, s), 4.54 (1H, m, J = 6.3, 10.6, 4.8 Hz), 4.79 (1H, t, J = 9.2 Hz), 6.22 (1H, s); UV λ_{max} (MeOH) nm: 227, 267, 306.

Of the four isocoumarins, coriadrin was found to be a potent inhibitor and inactivator of purified human P450 1A1. It could inactivate hepatic EROD activity and also possess anticarcinogenic

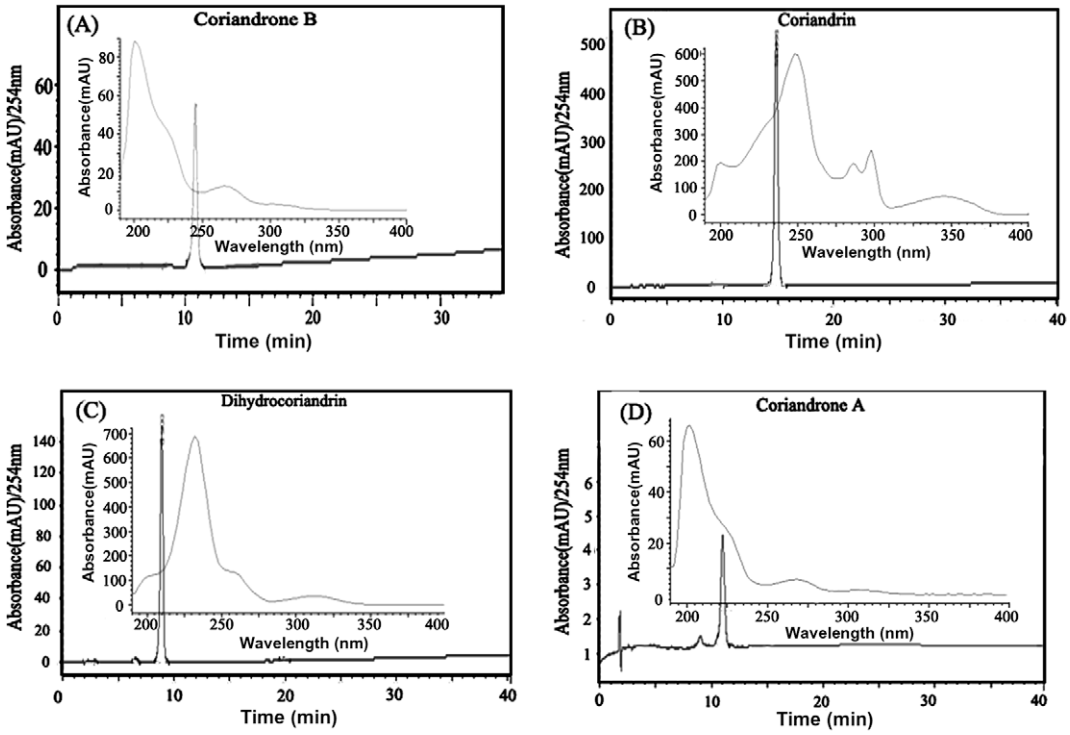

Fig. 2. HPLC chromatogram of the HSCCC peak fractions A–D. *Conditions:* column: Shim-pack VP-ODS column (150 mm × 4.6 mm, 5 μm). Mobile phase: a gradient elution from 50% to 80% methanol in 40 min. Detection wavelength: 254 nm.

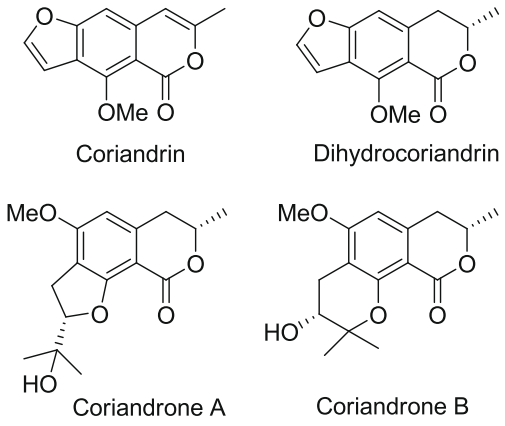

Fig. 3. The four isocoumarins isolated from the *Coriandrum sativum* L. by HSCCC.

effect towards chemical carcinogens such as polycyclic aromatic hydrocarbons by inhibition of DNA adduct formation (Cai, Baer-Dubowska, Ashwood-Smith, & DiGiovanni, 1997; Cai et al., 1996, 1997). Moreover, in contrast to other antiviral furocoumarins, such as 8-methoxypsoralen (MOP), the RNA-virus and the DNA-virus murine cytomegalovirus were substantially more sensitive to coriandrin. Human immunodeficiency virus, HIV-1, was also susceptible to coriandrin + UVA (Hudson, Graham, Harris, & Ashwood-Smith, 1993). Certainly additional work on other isocoumarins would be valuable. For example, the further study of all aspects, such as pharmacology and structural modification could be done for the other isocoumarins except coriandrin from coriander.

A few studies were carried out using GC–MS on the chemical compositions of the essential oil from *Coriandrum sativum* L. at different stage of maturity (Msaada et al., 2007). Isocoumarins have not been reported in these investigations. Therefore, the combinatorial application of SFE and HSCCC may be a good complemental method to the conventional GC–MS.

4. Conclusion

Four isocoumarins including coriandrone B, coriandrin, dihydrocoriandrin and coriandrone A were extracted, separated and purified by SFE and HSCCC from coriander. The SFE condition was optimised to be 35 °C, 8 MPa, 2 h. At last, high purities of isocoumarins were obtained by HSCCC in one step with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water at a volume ratio of 3:7:5:5 (v/v/v/v). The purity was assessed by analytical HPLC and the confirmation of chemical structures was performed by ESIMS, NMR and UV spectroscopy. The present method is suitable for preparing available quantities of pure isocoumarins and the quantification of coriandrone B, coriandrin, dihydrocoriandrin and coriandrone A in coriander.

Acknowledgement

The research work was supported by the Cultivation Fund of the Key Scientific and Technical Innovation Project, Ministry of Education of China (707033).

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Food Chemistry 117 (2009) 509–513

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analaytical Methods

A direct determination method for ethanol concentrations in alcoholic beverages employing a eukaryote double-mediator system

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ARTICLE INFO

Article history:
Received 24 February 2008
Received in revised form 30 March 2009
Accepted 12 April 2009

Keywords:
Ethanol determination
Alcoholic beverage
Small sample volume
Eukaryote double-mediator (EDM) system
Yeast
Food analysis

ABSTRACT

A fast, direct, small-sample measurement method of ethanol determination in alcoholic drinks was established by the application of attractive characteristics of a eukaryote double-mediator (EDM) system, as previously reported. The EDM system, which consists of yeast as well as both hydrophilic and hydrophobic mediators, demonstrates superior sensitivity to organic substances. For high performance, the EDM system was reoptimised for ethanol determination with a small sample volume of only 3 μL and a short incubation time of 5 min. Under optimal conditions, a practical calibration curve for ethanol determination in alcoholic beverages was obtained between 0.5 and 50 v/v% (r = 0.997, 9 points, n = 3, average of relative standard deviation, RSD_{av} = 2.75%), with a detection limit of 0.5 v/v%. To characterise our ethanol determination method, the effects of pH, organic substances, carbon dioxide gas, or sodium sulphite on EDM responses were studied in detail. No notable effects of pH on EDM responses were observed; however, glucose and ascorbic acid strongly increased the EDM response to ethanol. Real sample applications were mainly performed using distilled alcoholic drinks (shochu, brandy, vodka, gin, and whisky) and several kinds of beer and similar beverages (lager, draft, low-malt, non-malt, and sugar-free). When the results with sugar-free beverages were compared with those obtained by the available enzymatic-spectrophotometric (ES) method, an excellent correlation was obtained (r = 0.990, 7 points, n = 3, RSD_{av} of the EDM method = 7.90%), with an excellent slope of 1.05. Finally, a yeast suspension stored at 4 °C was stable for at least 7 days, with a response reduction of 88% (RSD_{av} for five testing days = 4.8%).

1. Introduction

In recent years, a need for ethanol determination has arisen in the fields of food, diagnostic, and environmental analyses. Of the various methods which have been developed, a refractive index method using an available refractometer is widely utilised for liquid samples; however, the influences of colour in the sample are not negligible. High-performance liquid chromatography (HPLC) methods are used to determine the ethanol concentration accurately but are expensive in terms of both equipment and measurement. In addition, enzymatic-spectrophotometric (ES) methods using alcohol dehydrogenase (ALDH), though widely used in experiments, involve costly measurements and are affected by the sample colour, gaseous ethanols such as breath ethanol, carbon dioxide gas, and so on. In addition, in the ES methods, the direct measurement of an alcohol beverage is not possible, and the samples must be diluted (~10,000 times).

For simple and rapid measurement, enzyme biosensors for ethanol have been developed using ALDH (Kitagawa et al., 1991; Niculescu, Mieliauskiene, Laurinavicius, & Csoregi, 2003) or alcohol oxidase (Azevedo, Prazeres, Cabral, & Fonseca, 2005; Huseyin & Levent, 2006; Lapa, Lima, & Pinto, 2003). However, both enzymes lack stability, and purification is expensive. In addition, these enzymatic methods require dilution of the sample, which requires precision and careful avoidance of contamination.

On the other hand, cost-effective microbial biosensing methods have been widely studied (Lei, Chen, & Mulchandani, 2006; Nakamura & Karube, 2003, 2005; Nakamura, Shimomura-Shimizu, & Karube, 2008). In recent years, we have actively developed many types of microbial biosensors for use in such environmental fields as soil diagnosis (Hashimoto, Nakamura, Asaga, & Karube, 2008), biological oxygen demand (BOD) measurements (Nakamura et al., 2007a, 2007b, 2007c, 2008a, 2008b; Yoshida, McNiven, Morita, Nakamura, & Karube, 2002; Yoshida et al., 2000; Yoshida et al., 2001a, 2001b), toxicity measurements (Ikebukuro, Nakamura, & Karube, 2000, chap. 19; Nakamura & Suzuki, 2007a, 2007b;

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doi:10.1016/j.foodchem.2009.04.026

Nakamura et al., 2007d), and composting monitoring (Yoshida et al., 2001c). Recently, microbial biosensors for use in food analyses have been developed. For example, to enhance koji quality control in the sake brewing process, Chiyo, Matsui, Murakami, Yokoyama, and Tamiya (2001) developed a microbial biosensor using a surface photovoltage (SPV) device. For ethanol measurement, *Gluconobacter oxydans* (Tkac, Vostiar, Gemeiner, & Sturdik, 2002; Tkac, Vostiar, Gorton, Gemeiner, & Sturdik, 2003) as a prokaryote or yeast strains (Rotariu, Bala, & Magearu, 2004) as eukaryotes have recently been used as bio-recognition elements.

Yeast is one of the most attractive microbes used for the fast, stable, low-cost determination of ethanol concentrations in alcoholic beverages. Thus, we have used yeast to investigate the possibility of a microbial biosensing method to ethanol determination utilising a eukaryote double-mediator (EDM) system which consists of yeast, potassium hexacyanoferrate(III) [HCF(III)] as a hydrophilic mediator, and menadione as a hydrophobic and lipophilic mediator (Nakamura et al., 2007a, 2008a). In the previous study, BOD measurements in real samples were performed by obtaining an electrical signal from a batch-type, carbon-paste electrode chip equipped with a micro-stir system. Characteristics of uniformly sustainable yeast in the incubation mixture of the sensor chip made it possible to obtain reproducible results. In this study, Baker's yeast was applied to the EDM system for ethanol determination as a readily available, safe, and easily handled microbe. The EDM system was optimised, and the measurement conditions for the direct measurement of ethanol in small samples of alcoholic beverages were characterised for real sample application. Finally, the stability test of a yeast storage suspension was performed for practical use.

2. Experimental

2.1. Reagents and solution

Potassium HCF(III), ethanol (99.5 v/v%; top grade according to Japanese Industrial Standards), and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemicals (Osaka, Japan). Menadione (2-methyl-1,4-naphthoquinone) was purchased from MP Biochemicals, LLC (Germany). Triton X-100™ (Triton™) was purchased from Sigma Chemicals (USA). The other chemicals used in this study were of reagent grade and were obtained from various distributors. Water was used after deionization.

A 100-mM sodium phosphate buffer (PB) solution was prepared (pH 7.0) and used after autoclaving. A 400-mM potassium HCF(III) solution was prepared using the PB solution. In addition, menadione was freshly prepared using DMSO to give a 20-mM solution, and a 0.05-v/v% Triton™ solution was prepared using sterilized water. A standard solution of ethanol was also prepared before use.

2.2. Preparation of yeast cells

Baker's yeast (Super Camellia) was purchased from Nisshin Foods, Inc. The procedures for the yeast cell preparation were performed according to the method described in the previous study ***(Nakamura et al., 2008a). Yeast cells were grown in a two-step process in a sterilized YPD medium (10 g yeast extract, 20 g glucose, and 20 g Polypeptone™ in 1 L; pH 6.5–6.6). The cells were grown at 180 rpm and 28 °C for 8 h in 2 mL of the YPD medium using a test tube for the pre-culture step. Of the pre-cultured medium, 0.5 mL was inoculated to 200 mL fresh YPD medium and incubated at 120 rpm for 15 h using a Sakaguchi flask.

After growth, the yeast cells were harvested by centrifugation (3 min and 3000 rpm) and washed three times with a 0.9% NaCl solution at room temperature. The yeast cells were resuspended in 35 mL of the NaCl solution in a centrifugation tube (50 mL) and starved by shaking at 120 rpm and 28 °C for 2 h. After starvation, the yeast cells were cooled in crushed ice for 10 min and washed three times using the Nacl solution (4 °C). For experimental use, the yeast cells were resuspended in the NaCl solution to an optical density (OD_{600}) of 20 and put on crushed ice. For storage, the yeast cell suspension was diluted 20 times using the NaCl solution and was stored at 4 °C.

2.3. Experimental procedure

The same measurement system of an electrochemical analyser (CHI-1202, BAS, USA) and a batch-wise carbon paste electrode chip (inner volume, 563 μL) equipped with the micro-stir system used in the previous study was employed (Nakamura et al., 2008a). Chronoamperometry was performed by poising the carbon-working electrode + 900 mV relative to the carbon reference/counter electrode for 3 s. The output obtained by the measurement system of the chronoamperometry was taken as the EDM response. All experiments were performed three times ($n = 3$) at room temperature and with a reaction volume of 400 μL.

The EDM sensor chip was preconditioned for each measurement using diluted bleach and stirring at ca. 300 rpm for 3 min. After adequate washing, the current response of the EDM sensor chip to 10 mM of a standard potassium HCF(II)-HCF(III) solution, which was prepared by dissolving equimolar amounts of both potassium HCF(II) and potassium HCF(III), was confirmed between 150 and 170 μA without stirring.

In the experiments using the EDM system, 40 μL of 400 mM HCF(III) solution, 4 μL of 20 mM menadione, 4 μL of 0.05% Triton™, 120 μL of a yeast suspension ($OD_{600} = 20$), 3 μL of the sample solution, and 229 μL of water were added to the sensor cuvette to give a final volume of 400 μL. The reaction in the sensor cuvette was started by the final addition of the yeast suspension under stirring at ca. 300 rpm. One electrochemical measurement was performed after 5-min incubation.

3. Results and discussion

3.1. Optimization

In Japan, beverages containing more than 0.5 v/v% ethanol are defined as alcoholic. In the present study, we investigated measurement conditions which did not require the dilution of alcoholic beverage samples containing between 0.5 and 50 v/v% of ethanol. As a result, the constituents of the incubation mixture were optimised to contain 10 mM PB solution (pH 7.0), 40 mM HCF(III), 0.2 mM menadione, 5 mg L^{-1} Triton™, and $OD_{600} = 6$ of yeast cells. A sample solution of 3 μL was added to the incubation mixture, and the final volume of the incubation mixture was fixed at 400 μL. The stirring condition was then adjusted to ca. 300 rpm, and the incubation time was defined as 5 min.

3.2. Calibration curves

In order to identify the fundamental condition of the EDM system, we made a calibration curve for HCF(II) using HCF(II)–HCF(III) mixtures prepared with a total of 40 mM HCF and containing 10 mM PB solution (pH 7.0). Under stirring at 300 rpm, the calibration curve was obtained at 0.03, 0.1, 0.3, 1.0, and 3.0 mM HCF(II) (sensitivity, 68.0 μA mM^{-1}, $r = 0.9997$, 5 points, $n = 3$, average of relative standard deviation, $RSD_{av} = 2.38\%$), with a detection limit of 30 μM HCF(II) and an upper limit of 20 mM HCF(II).

Next, a calibration curve for ethanol was investigated under optimum conditions. The results are shown in Fig. 1. The calibra-

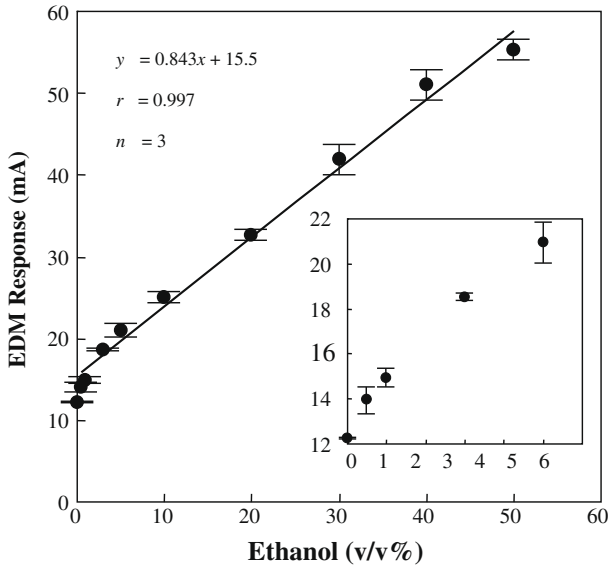

Fig. 1. Calibration curve for ethanol.

tion curve was obtained at 0.5, 1.0, 3.0, 5.0, 10, 20, 30, 40, and 50 v/v% ethanol ($r = 0.997$, 9 points, $n = 3$, $RSD_{av} = 2.75\%$) with a detection limit of 0.5 v/v% ethanol. Thus, the EDM system met the measurement conditions without dilution of the available alcoholic beverage samples.

3.3. Characterisation

The effects of pH on the EDM responses were investigated. Each pH value of a 100 mM PB solution was prepared, and 40 μL of the PB solution was added to the incubation mixture as a substitute for the 100 mM PB solution (pH 7.0). The results are shown in Fig. 2. The EDM responses to 25 v/v% ethanol were slightly increased by increasing the pH; however, as shown at pH 8.0, the relatively large error bar might be negligible due to the effects of pH. In addition, the RSD_{av} obtained between pH 5.0 and 9.0 was 3.98%. Because the sample volume was only 3 μL, the effects of pH on the EDM responses were ignored.

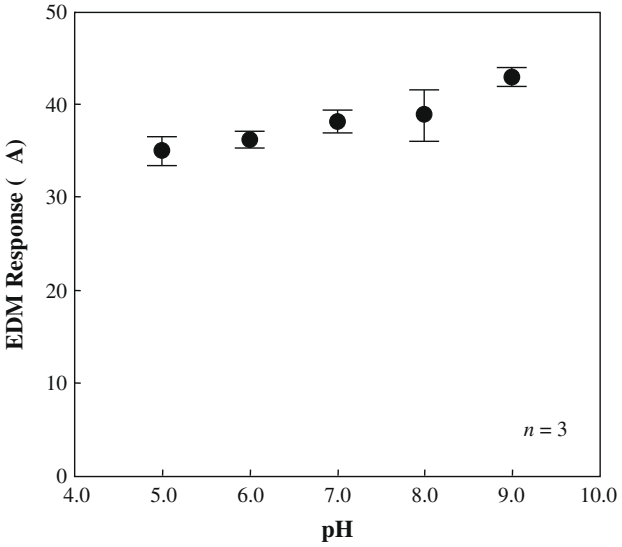

Fig. 2. Effects of pH.

Next, influences of organic substances in the EDM method were investigated. The EDM response to 5 M ethanol was measured as a control, and the EDM response to 5 M ethanol containing 50 mM of an organic substance was measured as a sample. In this examination, we chose glucose, fructose, sucrose, and maltose as sugars, ascorbic acid and citric acid as organic acids, glutamic acid as an amino acid, and methanol and 2-propanol (isopropyl alcohol) as other alcohols. The results are shown in Table 1. Except for the results obtained by maltose and 2-propanol, the EDM responses to organic substances were strongly increased (more than 110% as the relative value). The results obtained by the addition of glucose or ascorbic acid were remarkable, probably because yeast metabolic activity was strongly enhanced by two nutrients (ethanol or glucose). In addition, strong influences on the electrochemical signal were observed by the addition of ascorbic acid as a reducing substance. These results showed the necessity of some elimination methods of such organic substances or selective ethanol detection methods, for example, the use of membrane techniques (Rotariu & Bala, 2003; Tkac et al., 2002, 2003).

In addition, influences of inorganic substances in the EDM method were also examined using carbon dioxide gas or sodium sulphite. Sparkling water (SODA, 500 mL, Suntory Limited, Osaka, Japan) was used as the water containing carbon dioxide gas. Up to 350 mg L^{-1} sodium sulphite was used as the oxygen scavenger agent or antioxidant for alcoholic beverages. No notable influences were observed, although the EDM responses to 25 v/v% ethanol containing 350 mg L^{-1} sodium sulphite were slightly increased compared with the EDM responses to 25 v/v% ethanol (control) (Table 2).

Based on these results, we concluded that the present EDM method influenced coexisting substances in the alcohol beverages. Therefore, we tried to determine the ethanol concentrations in distilled alcohol beverages.

3.4. Real sample application and comparison with conventional method

As real samples, distilled alcoholic beverages and beers were used for the determination of ethanol concentrations utilising the present EDM method. In general, distilled beverages are defined as spirits that are low in sugars and contain at least 35 v/v% of ethanol by volume. The distilled beverages we chose were gin (375 mL, Gilbey's Special Dry Gin, W. & A. Gilbey Co., London, UK), vodka (375 mL, Gilbey's Imported Vodka), whisky made from malt and grain (180 mL, Finest Old Whisky, Suntory, Ltd.), brandy

Table 1
Influences of organic substances on the EDM response to 5 M ethanol*.

Organic substance (50 mM)	Relative value (%)	SD (±)	RSD (%)
Control	100.0		
Sugar			
Glucose	166.6	4.2	2.5
Fructose	125.1	7.9	6.3
Sucrose	118.2	5.1	4.3
Maltose	108.3	3.8	3.5
Organic acid			
Ascorbic acid	227.6	2.0	0.87
Citric acid	120.4	8.3	6.9
Amino acid			
Glutamic acid	129.6	12.3	9.5
Alcohol			
Methanol	128.0	9.6	7.5
Propanol	101.0	4.7	4.6

Undesirable values are underlined.
* Each control and sample was measured with three replications ($n = 3$).

Table 2
Influences of inorganic substances on the EDM response to 25 v/v% ethanol[a].

Inorganic substance	Relative value (%)	SD (±)	RSD (%)
Control	100.0		
Sparkling water[b]	104.3	5.6	5.3
Sodium sulphite[c]	109.2	5.2	4.8

[a] Each control and sample was measured with three replications ($n = 3$).
[b] The sample contained sparkling water and 50 v/v% ethanol with 1:1.
[c] The sample contained sodium sulphite at 350 mg L^{-1}.

made from grapes (640 mL, VO, Suntory, Ltd.), and shochu, a traditional Japanese alcoholic beverage (200 mL, Takara-Cup 35°, Takara Shuzo Co. Ltd., Kyoto, Japan). In Japan, beers are categorised into three groups: beer (normal), low-malt beer ("Happo-Shu"), and non-malt beer ("Daisan-no-Beer"). For normal beers, we chose lager (135 mL, Lager Beer, Kirin Brewery Co., Ltd., Tokyo, Japan), draft (135 mL, Super Dry, Asahi Breweries, Ltd., Tokyo, Japan), and black draft (350 mL, Yebis The Black, Sapporo Breweries, Ltd.). For low-malt beers, we used diet draft (350 mL, Diet 77 kcal, Suntory, Ltd.), carbohydrate-free (250 mL, Style Free, Asahi Breweries, Ltd.), and draft (135 mL, Tanrei, Kirin Brewery Co., Ltd.) beers. As the non-malt beer, a draft (350 mL, Draft One, Sapporo Breweries, Ltd.) was used.

In the EDM method, calibration was performed with 0 and 50 v/v% ethanol before the determination of ethanol. The sample volume was then fixed at 3 μL, and the sample was measured without any pretreatment such as dilution. For comparison, ethanol determination in alcoholic beverages was performed using an available enzymatic-spectrophotometric (ES) method (F-kit ethanol, Cat. No. 176290, J. K. International, Inc., Tokyo, Japan). In the ES method, an ADH reaction was employed, and the amount of NADH produced by the ADH reaction was measured. A spectrophotometer (Model DU-800, Beckman Coulter, Inc., Fullerton, CA, USA) was used, and absorbance at 340 nm was fixed and measured for the determination of ethanol. In the ES method, the ethanol content in the sample solution must be between 5 and 60 mg/L; therefore, all of the alcohol beverages used in this study were diluted several times.

Table 3 shows the results obtained by both methods. In our EDM method, the ethanol concentrations were relatively higher than those obtained through the ES method. One possible reason is that other nutrients contained in beers or spirits raised the

Table 3
Comparison of the ethanol determination results obtained by two methods using available alcohol beverages.

	Energy (kcal/ 100 mL)	Nutrition facts (g/100 mL)				Fig. 3		Lebeled alc. conc (v/v%)	EDM method			ES method		
		Protein	Fat	Carbo-hydrate	Fiber	a	b		Ethanol (v/v%)	SD (±)	RSD (%)	Ethanol (v/v%)	SD (±)	RSD (%)
Beer														
Lager	42	0.3	0	3.2	0–0.1	•		5	10.9	1.43	13.2	4.97	0.0725	1.46
Draft	42	0.2–0.4	0	3.0	0	•		5	12.4	1.46	11.7	5.41	0.0166	0.306
Draft (black)	45	2.1	0	12.8	0.7–1.1	•		5	15.4	1.50	9.71	5.36	0.0042	0.079
Low-malt beer														
Draft (diet)	**22**	–	–	–	–	•	•	3.5	3.34	1.01	30.2	3.38	0.0174	0.516
Carbo-hydrate free	**24**	0	0	**0**	0–0.1	•	•	4	6.06	0.14	2.31	3.86	0.0659	1.71
Draft	45	0.2–0.3	0	3.4	0–0.1	•		5.5	9.35	1.14	12.2	5.29	0.0249	0.470
Non-malt beer														
Draft	42	0–0.1	0	3.3	0–0.1	•		5	8.04	1.42	17.7	4.89	0.0706	1.44
Spirits														
Shochu	–	–	–	–	–	•	•	35	32.4	1.60	4.95	34.5	0.155	0.448
Brandy	–	–	–	–	–	•	•	37	37.2	3.74	4.95	36.3	0.444	1.22
Gin	–	–	–	–	–	•	•	37.5	40.4	1.65	4.07	35.5	0.268	0.754
Vodka	–	–	–	–	–	•	•	37.5	40.0	1.15	2.88	36.7	0.415	1.13
Whisky	–	–	–	–	–	•	•	40	47.2	2.82	5.96	42.3	0.0855	0.202

Remarkable values are displayed in **bold**, and undesirable values are underlined.

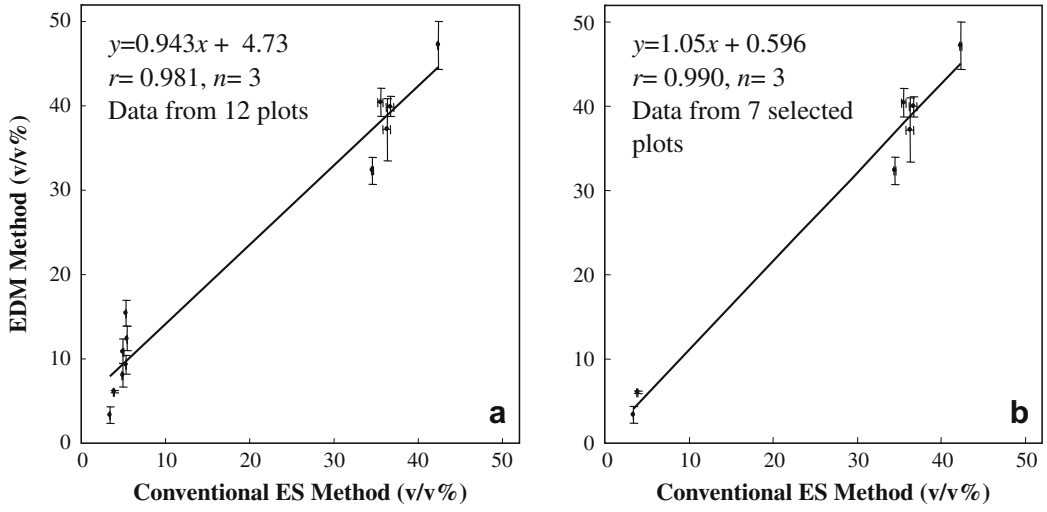

Fig. 3. Correlation of the ethanol concentration calculated from the response of the MDM method with the SE method for alcoholic beverage samples.

EDM values. The correlations between the two methods are shown in Fig. 3. All the data obtained by both methods are shown in Fig. 3a. In this figure, the slope is at a slightly low value of 0.943, which was induced by the relatively high values of beers evaluated by the EDM method. Thus, we next made a correlation between the two methods without the results from beers which contained other nutrients. As shown in Fig. 3b, the correlation was improved, with a slope of 1.05, and the correlation coefficient for these results was also improved from 0.981 to 0.990. The average RSD values (RSD_{av}) obtained at 7 points were around 7.90% for the EDM method and 0.855% for the ES method. However, in general, the reproducibility of microbial biosensor methods is around 10%; therefore, the RSD_{av} value obtained by our method can be considered a standard value. These results indicate that it is possible to determine ethanol concentrations by our EDM method, although several problems related to the influences of other nutrients need to be solved.

3.5. Storage stability

In the present study, the yeast cell suspension was diluted 20 times using a 0.9% NaCl solution after experimental use and stored at 4 °C. Before experimental use, the stored yeast cell suspension was centrifuged for 3 min at 3000 rpm, and the restrictive volume of the supernatant was removed. By adding a dilution procedure, we examined the effects of low-OD suspension and washing. The stability of the yeast suspension stored at 4 °C was examined for 25 days by estimation of the EDM response to 100 v/v% ethanol. The response reduction of 88% was observed during the first 7 days (RSD_{av} for five testing days, 4.8%), and the response reduction of 24% was observed during the first 25 days (RSD_{av} for seven testing days, 5.5%). Based on these results, we believe that the yeast suspension can be used for at least 7 days for ethanol measurements using the EDM system.

4. Conclusions

We have demonstrated the potential of the present EDM method as a direct ethanol determination method and have applied it to real samples of spirits and beers. In the present study, the problem of poor selectivity in the EDM method remained. Therefore, experiments to enhance the selectivity will be performed in the future.

Acknowledgements

The authors greatly acknowledge Professor Dr. Isao Karube for his participation in fruitful discussions and Mr. S. Okuma, Mr. A. Yoshimura, Mr. H. Hattori, and other laboratory members for their assistances with the experiments.

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Food Chemistry 117 (2009) 514–521

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Detection of genetically modified soya and maize: Impact of heat processing

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ARTICLE INFO

Article history:
Received 21 February 2008
Received in revised form 8 April 2009
Accepted 12 April 2009

Keywords:
Amplicon size
DNA degradation
Food processing
Genetically modified organisms
Polymerase chain reaction

ABSTRACT

The analysis of processed foods entails a number of complications, which negatively affect the performance of DNA based detection methods. Heat-processing methods viz. autoclaving and micro-waving, that mimic processing and manufacturing, as model unit operation systems were used to study their effect on the detection of genetically modified organisms (GMOs). This study confirms the premise that high temperature and/or pressure significantly reduce the level of detectable DNA. PCR methods were developed and adapted to target varying amplicon sizes of the trait, construct and event specific gene sequences that occur in MON-810 maize and Roundup Ready® soybean. Integrity of DNA, recovery and PCR amplicon size (<200 bp) are major factors that direct the successful detection of GMOs in processed foods. The model systems used provide a platform to devise better strategies in developing detection protocols, especially for processed foods containing GMOs.

1. Introduction

A tremendous and rapid increase in the global area dedicated to transgenic crops has occurred in recent years. Herbicide tolerant soybean is the principal genetically modified (GM) crop occupying 71% of global biotech area. USA, Argentina, Brazil, Canada and China currently share 98% of the worldwide area planted with GM crops whereas India accounts for 1% of the total global area (James, 2005). Since the approval of *Bt* cotton in 2002, to date 62 *Bt* cotton hybrids have been granted market authorisation in India. Consumer's awareness and concerns of the potential risks associated with GM crops on environment, biological diversity, human health and safety have burgeoned. A number of countries have therefore adopted, or are in the process of developing legislation related to the approval of GM-products. Regulatory provisions for labelling of products derived from genetically modified organisms (GMOs) have been introduced in the European Union, Japan, Korea and other countries with different threshold levels (EC 1829/2003; Notification No. 1775, 2000; Notification No. 2000-31, 2000). India too has a robust regulatory framework and an elaborate review process, which is backed by well-developed bio-safety guidelines (Warrier, 2006). Rule 37E and 48F of the recently enacted Food and Safety Standards Act (Food Safety and Standards Act, 2006) states that all imports of GMOs for the purpose of food, feed, industrial processing, research and development for environment release or commercialisation will be governed by and allowed only with the certification of the Genetic Engineering Approval Committee (GEAC). Moreover, imported consignments containing genetically modified products must carry a declaration stating that the product is genetically modified (Notification No. 2 RE-2006/2004–209, 2006). A process-based rather than a product-based labelling approach is under consideration so that any product derived from gene technology must be compulsorily labelled, even if the novel DNA and/or protein are absent (Draft notification on labelling of GM food, 2006).

Polymerase chain reaction (PCR) is a widely used technique considered as the method of first choice in GMO detection to comply with a robust labelling system. Several DNA based screening methods have been developed to detect GMOs in raw food materials (James, Schmidt, Wall, Green, & Marsi, 2003; Lau, Collins, Yiu, Xing, & Yu, 2004; Lipp et al., 2001). Food manufacturing entails a number of complex processing steps that are harsh and might negatively influence detection of GMOs. These include cooking, heating (dry as well as in presence of moisture), high pressure, pH treatments, physically shearing, extrusion at high temperatures and high torque settings. Each food and each food-processing step contribute to a unique environment in which, DNA undergoes deterioration and proteins are denatured. Hupfer, Hotzel, Sachse, and Engel (1998) reported that temperature and pH influenced degradation of a *cry*1A(b) sequence in *Bt*-maize during preparation of polenta. Thermal stress in combination with pH affects DNA integrity (Bauer, Weller, Hammes, & Hertel, 2003). Murray, Butler, Hardacre, and Vaughan (2007) demonstrate a substantial degradation of endogenous maize DNA at high temperature and torque settings. The degree of DNA degradation adversely affects the limit of detection by qualitative PCR, which is dependent on the effective concentrations of amplifiable DNA sequences (Terry, Harris, & Parkes, 2002).

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doi:10.1016/j.foodchem.2009.04.028

Heat transfer, an operation that occurs repeatedly in food manufacture namely cooking, baking, drying, sterilizing or freezing is part of the processing of almost every food. It is therefore important to understand the effect of heat transfer on the target molecules viz. DNA and protein. The investigations on how various food-processing parameters affect the level and quality of DNA are limited.

MON-810 maize and Roundup Ready® soybean (RR-soy) are currently the major GM crops. The qualitative detection of these two transgenic events are based on PCR amplification using primers that recognise regulatory regions of the promoter derived from Cauliflower mosaic virus (CaMV35S) and *Agrobacterium tumefaciens* nopaline synthase (TNOS) terminator, both of which have a significant risk of obtaining false positives. These two elements are natural DNA sequences occurring in the growing environment (Brodmann et al., 1997). Furthermore, the trait specific transgenic gene, the enoyl pyruvyl shikimate phosphate synthase (EPSPS) or *cry*1A(b) can be incorporated in various independent transformation events of various species. The detection of the transgene cannot distinguish between an authorised and unauthorised GMO. In the present study, PCR methods were developed to detect the event: the junction between the newly introduced genes and the integration into the host genome, referred to as event specific methods (Burns, Shanahan, Valdivia, & Harris, 2003). In addition, PCR methods to amplify the cross border sequence of the transgenic construct were also developed. The specific genetic elements of RR-soy and MON-810 maize were targeted and their capability to direct PCR in unit heat operations (a) microwave treatment (MWO) at 540 W and 900 W, (b) autoclaving, (c) baking (180 °C) and (d) freezing (−80 °C) were evaluated. Microwave treatment simulates dry heating, a common method of processing used in post-baking, drying and moisture control of biscuits, crackers, bread and other bakery products. Autoclaving simulates moist heating under pressure, used to manufacture soy drink, textured vegetable protein, soybean meal, maize instant tortilla, etc. The key questions to be answered were: (1) can the DNA be detected when GMO containing grain/seed is subjected to heat processing and (2) what are the implications on PCR based detection of such GMOs? The PCR detection methods described are based on amplifying much smaller segments (200 bp) of the target DNA in processed foods.

2. Materials and methods

2.1. Materials

Standard flours (Certified reference materials, IRMM-410S 5% Roundup Ready® soy and IRMM-413 5% MON-810) containing defined percentages of GMO material prepared and certified by the Institute of Reference Materials and Measurements (Geel, Belgium) were purchased from Fluka, (Riedel-deHaën, Germany). Crushed seed powder of Roundup Ready® (RR) soybeans and Yieldgard® (MON-810) were a gift from Monsanto Co. (St Louis, MO, USA) obtained with the approval of the Research Committee for Genetic Manipulation, Department of Biotechnology, Government of India (India). For simplicity sake, the term RR-soy and MON-810 will be used hereafter to refer Roundup Ready® soy and MON-810 maize, respectively. A conventional non-GM-soy and maize were procured from the pilot plant, Department of Protein Chemistry and Technology, Central Food Technological Research Institute (Mysore, Karnataka, India). Wizard™ magnetic DNA purification kits for food were purchased from Promega Corporation (Madison, USA). *Taq* PCR core kit was obtained from Qiagen Gmbh (Hilden, Germany). Agarose was from Amresco (Solon, Ohio, USA). Ethidium bromide was purchased from Sigma–Aldrich Chemicals Private Limited (Bangalore, India). Mass loading dye (6×) and 100 bp ladder size marker were obtained from Fermentas Inc., (Maryland, USA).

2.2. Oligonucleotide primers

Oligonucleotide primers EPSPS-101F (5′-AAGTCGATCTCCCACCGGTC-3′), EPSPS-101R (5′-TTGCCCGTATTGATGACGTC-3′), EPSPS-210F (5′-ATCGAACTCTCCGATACGAAG-3′), NOS-210R (5′-CCCATCTCATAAATAACGTCAT-3′), GTS-181F (5′-GCATGCTTTAATTTGTTTCTAT-3′), GTS-181R (5′-ATCTTGAACGATAGCCTTTCCT-3′), GTS-121F (5′-TAGCGCGCAAACTAGGATAAA-3′), GTS-121R (5′-CGGTGATGCGCGTTTCA-3′), hsp-95F (5′-AGCACCTCGACCTCAGGGTT-3′) and cry1A(b)-95R (5′-TTGTAATGCAGATACCAAGCG-3′) employed in this study were designed using Primer Express™ 2.0 software (Applied Biosystems, Foster City, CA, USA), to amplify distinctly different sized products of the host genome-junction and cross border junction DNA sequences unique to either RR-soy or MON-810. The primers chosen from literature, target gene, primer sequence and expected amplicon lengths are listed in Table S1 (Supplementary data). The single-copy lectin gene (*Le*1) of soy and zein (*Ze*1) of maize were selected as internal controls. The primers synthesised by Sigma–Genosys (Sigma–Aldrich Chemical Private Limited, Bangalore, India) were diluted to a final concentration of 1 nmol μL^{-1} with nuclease free water and stored at −20 °C.

2.3. Preparation of raw material

The RR-soy and MON-810 crushed seed powders were further ground to a fine powder and sieved (Aperture size of 450 μm). One% RR-soy and MON-810 flour were prepared in-house by spiking the defatted conventional soy or maize flour with an appropriate quantity of RR-soy or MON-810 flour, respectively. Adequate care was taken to avoid contamination during spiking and the subsequent sieving step. The proportion of GM-ingredient in the spiked samples was deliberately set at 1% (w/w) to assess the degree of the expected effects at a level that would be of practical interest for threshold labelling.

2.4. Heat processing operations

All of these experiments were bench top experiments. Utmost care was taken to avoid the intermixing of transgenic samples during heat processing. All the processing steps were carried out individually and in triplicate.

2.4.1. Autoclaving

One gram of flour was directly autoclaved using an automated high-pressure steam steriliser (HA-240MIV/300 mV, Hirayama Manufacturing Corporation, Japan) at 121 °C and 15 lbs pressure for 20 min. The time did not include raising and lowering of the temperature.

2.4.2. Microwave treatment

One gram of flour was spread uniformly and heated in a microwave oven [(MWO) LG, India] at 540 and 900 W for 2 min. The MWO exposure was restricted to 2 min as charring occurred when subjected to 3 min.

2.4.3. Baking

Biscuits containing 0.67 wt.% level of RR-soy and MON-810 were prepared by conventional baking. The dough containing GMOs was sheeted to about 3.5 mm thickness and cut into biscuit shapes and placed on a tray. Biscuits were baked at 180 °C for 15 min and cooled to room temperature.

2.4.4. Freezing

One gram of the flour in an air tight container was kept at −80 °C for 48 h.

2.5. Extraction of genomic DNA

DNA was extracted from finely ground individual powders (0.2–0.5 g) using Wizard™ Magnetic DNA purification kits following the manufacturers protocol, with some modifications. The RNase (10 mg ml^{-1}) added to the autoclaved and MWO flours suspended in lysis buffer was increased from 10 to 25 μL and incubated at 25 ± 2 °C for 10 min. The suspensions were centrifuged at 13,000*g* for 10 min. Fifty microlitres of paramagnetic particle (PMP) solution were added to the supernatant, washed thoroughly with 70% ethanol and air dried. Bound DNA was eluted in 100 μL of nuclease free water and PMPs removed by magnetic separation. The DNA concentration was checked by spectrophotometry (UV-601, Shimadzu, Japan) after dilution. The DNA concentration and impurity factor (A_{260}/A_{280} ratio) were recorded. DNA integrity was evaluated by 1% agarose gel electrophoresis in 0.5× TBE (5× TBE: 450 mM Tris–Borate, 10 mM EDTA) at 100 V. The Gel Doc 2000™ documentation system with Quantity One software (Bio-Rad Laboratories Inc., CA, USA) was used to visualise the ethidium bromide stained DNA.

2.6. Qualitative PCR for detection of RR-soy and MON-810 DNA fragments

The PCR reactions performed in a total volume of 50 μL contained 1× PCR buffer containing 1.5 mM $MgCl_2$, 20 pmol of each primer, 200 μM dNTP and 1.5 U of *Taq* polymerase. The concentration of template DNA was varied depending on the percentage of GMO and the degree of heat processing (unprocessed: 80 ng; autoclaved: 1000 ng; MWO: 500 ng and baking: 850 ng). The PCR thermal profile included an initial 10 min denaturation step at 95 °C, followed by 40 cycles of denaturation at 94 °C (20 s), annealing at either 60 °C (lectin inner, lectin outer, CaMV35S-CTP, EPSPS-TNOS) or 61 °C (TNOS-soy genome, EPSPS, *hsp*70 intron1-*cry*1A(b)-95) or 59 °C (soy genome-CaMV35S) (60 s) and extension at 72 °C (30 s). A final extension step was performed at 72 °C for 3 min. The thermal cycling program used for Zein (*Ze*1) (Hohne, Santisi, & Meyer, 2002), CaMV35S promoter-*hsp*70 intron1 and *hsp*70 exon1 (Zimmermann, Hemmer, Liniger, Luthy, & Pauli, 1998), maize genome-CaMV35S promoter (ISO/FDIS 21569: 2005) and *hsp*70–*cry*1A(b) (Yamaguchi, Sasaki, Umetsu, & Kamada, 2003) were as reported. The PCR was carried out using a Gene Amp® PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The amplified DNA fragments were separated by electrophoresis in a 2% agarose gel (supplemented with 0.5 μg ml^{-1} ethidium bromide) in 0.5× TBE buffer at 150 V and visualised using The Gel Doc 2000™ documentation system (Bio-Rad Laboratories Inc., CA, USA). Samples were analysed in triplicate.

2.7. Direct sequencing analysis of PCR products

The PCR products were purified using a PCR product purification kit (Promega, Madison, USA) following the manufacturer's instructions and subjected to direct dideoxy sequencing on an automated DNA sequencer (ABI 310, Applied Biosystems, Foster City, CA, USA).

3. Results

3.1. Influence of processing on DNA quantity and integrity

The DNA extracted from 200 mg of MWO and baked products were quantifiable. In contrast, the extracted DNA from autoclaved flours was not quantifiable. Increasing the initial sample size to 500 mg yielded sufficient DNA (30 ng/mg flour). The quantity of extractable DNA is therefore affected by heat-processing (Fig. 1). Yoshimura et al. (2005a) reported that increasing the initial weight of cornstarch from 2 to 5 g resulted in the extraction of sufficient DNA. Statistical analyses performed for DNA extracted reveals a significant impact of the processing method applied (Fig. 1). The quantity of extracted DNA varied with the severity of the processing method used. The highest yields were obtained with unprocessed flours, followed by baking, MWO and autoclaved. Within the MWO model system, flours microwaved at 900 W yielded less DNA than the 540 W. The most notable effects occurred during autoclaving, wherein the yield of DNA was <70% of unprocessed

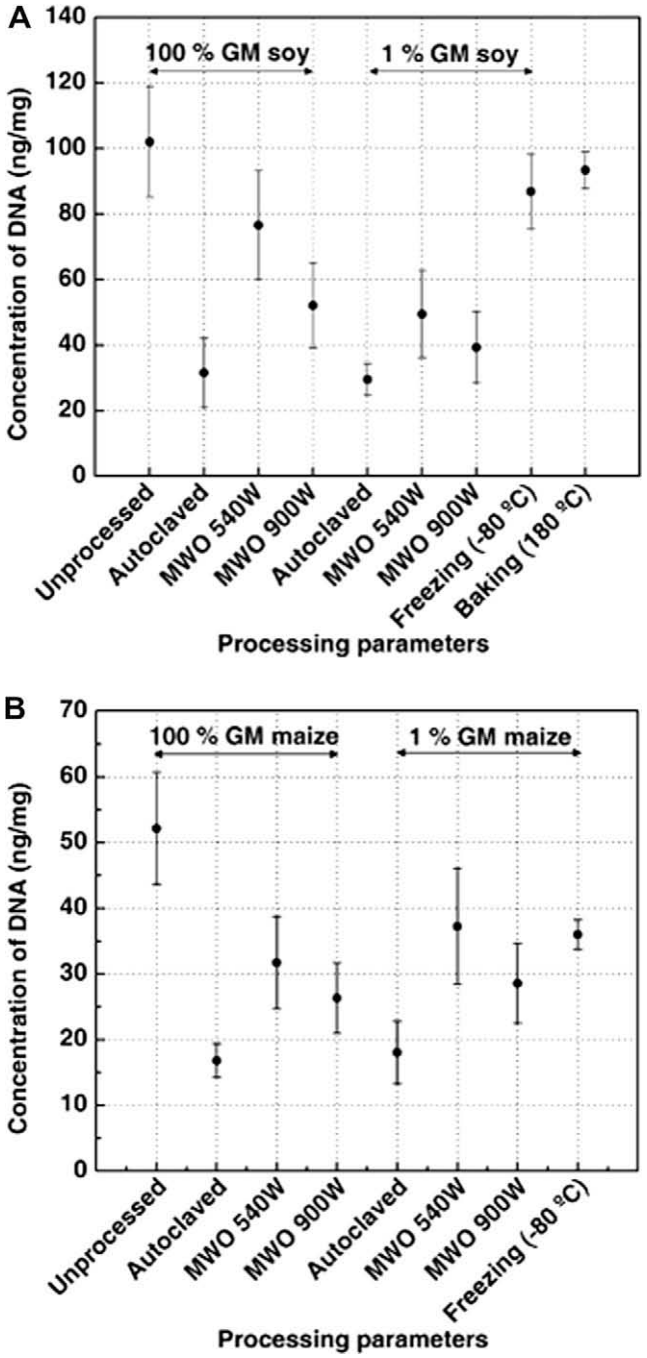

Fig. 1. Concentration of DNA extracted from model heat processed samples. (A) RR-soy and (B) MON-810. Vertical bars denote 95% confidence intervals. The mean values are derived from five extractions for each model system.

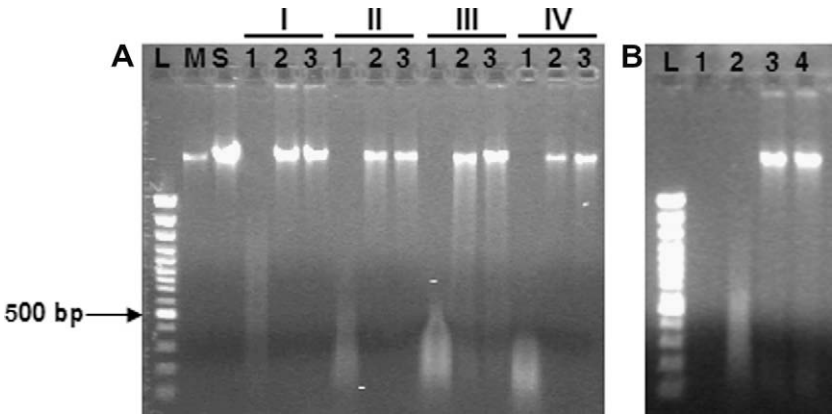

Fig. 2. Degradation of DNA induced by (A) autoclaving and microwave treatment. I: 100% RR-soy; II: 1% RR-soy; III: 100% MON-810; IV: 1% MON-810. Lane L, 100 bp ladder size marker; Lane M, unprocessed MON-810; Lane S, unprocessed RR-soy; Lane 1, autoclaved; Lane 2, MWO 540 W; Lane 3, MWO 900 W, (B) baking and freezing. Lane 1, control biscuit; Lane 2, 0.67 wt.% GM-biscuit; Lane 3, 1% MON-810 and Lane 4, 1% RR-soy. DNA loaded was 250 ng for all samples except autoclaved and baking where 1000 ng was loaded.

flours. Freezing at −80 °C also yielded less DNA. In addition, it is also evident that the DNA yield from soy was higher when compared to maize, either unprocessed or heat processed (Fig. 1). This lower yield in maize can be ascribed to the albuminous nature of the grain. Differences in the DNA size, a parameter of DNA integrity is dependent on the degree of processing the sample has been subjected to. DNA extracted from unprocessed RR-soy and MON-810 appear as clear distinct bands of high intensity >3000 bp (the highest band of the DNA marker 3000 bp) (Fig. 2). The DNA from autoclaved flours shows the complete absence of the longer DNA with the concomitant appearance of a smear smaller in size indicating DNA deterioration (Fig. 2). In contrast, a major portion of the DNA from the MWO treated is still intact with minimal smearing (<500 bp) when compared with DNA ladder accounting for some sheared DNA. These results reckon that the shearing of DNA during MWO though not severe, yet occurs. The fragmentation of DNA was most extensive in the autoclaved flours with the long DNA fragment being totally absent (Fig. 2, Lane 1). This is probably because of the intensity of the heat and the presence of moisture during autoclaving. Exposure of DNA to high temperature and pressure is known to cause fragmentation of DNA and breaks in strands thus reducing the average fragment size. Nevertheless DNA fragments <500 bp are present reckoning that amplification of DNA through PCR is still possible. The quantity of DNA loaded for detection was approximately five times greater for the autoclaved in comparison to the MWO. These results reckon that heat processing not only affects the yield of DNA but also has a significant impact on DNA deterioration. Low temperatures (−80 °C) had no effect on the DNA. The impurity factor defined by the A_{260}/A_{280} ratio showed values ranging from 1.6 to 1.8, which indicate that the DNA extracts are sufficiently pure for PCR amplification.

3.2. Specificity of the oligonucleotide primers

On the basis of published recombinant DNA sequences of RR-soy and MON-810 maize and data obtained by sequencing, primer pairs were designed to distinctly amplify specific DNA sequences for RR-soy and MON-810 (Table S1 (Supplementary data)). DNA degradation and amplicon length are crucial factors in the success of a PCR designed to identify GMOs in highly processed foods (Van Hoef, Kok, Bouw, Kuiper, & Keijer, 1998). The primers used in this study were designed to generate shorter amplicons, ~200 bp, targeting the trait (EPSPS gene), construct (EPSPS-NOS) and event (integration site: soy genome-CaMV35S promoter and NOS-soy genome) specific gene sequences of RR-soy. The primer pair *hsp*–95F/*cry*1A(b)-95R amplifies a 95 bp fragment unique to MON-810, which incidentally has a high GC content. Specificities of the primers designed were individually assessed and validated using DNA extracts of IRMM-410S (5% RR-soy) and IRMM-413 (5% MON-810). Compact and distinct bands of the expected length are visible (Fig. 3). These amplicons are visibly absent in the non-GM-soy and -maize (Fig. 3A and B). Specificity of the PCR, was

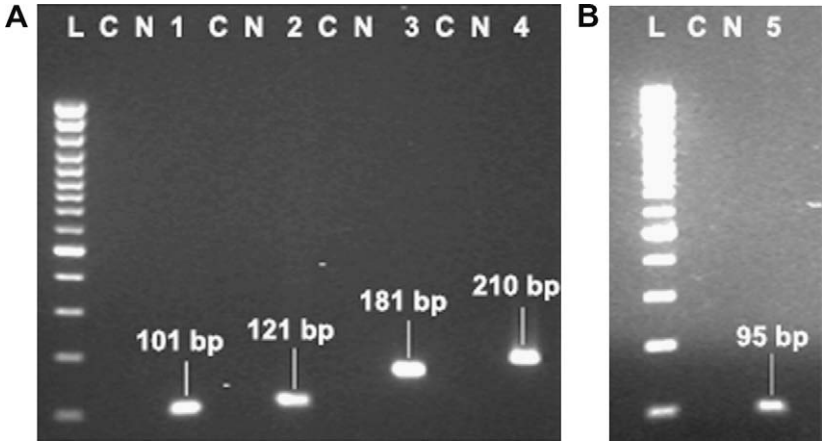

Fig. 3. Agarose gel electrophoresis of PCR products amplified from genomic DNA of (A) RR-soy (IRMM-410S). Lane L, 100 bp ladder size marker; Lane C, premise control; Lane N, non-GM-soy; Lane 1, EPSPS (101 bp); Lane 2, TNOS-soy genome (121 bp); Lane 3, Soy genome-CaMV35S promoter (181 bp); Lane 4, EPSPS-TNOS (210 bp) and (B) MON-810 (IRMM-413). Lane N, non-GM maize; Lane 5, *hsp*70 intron1–*cry*1A(b) (95 bp).

further evidenced when DNA isolated from rice (*Oryza sativa*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) showed no amplification when used as template (data not shown).

3.3. Effect of microwave treatment and autoclaving on detection

The detection of a species-specific single copy gene is often used to assess the amplifiability of DNA from soybean-derived products. The primer pairs GMO1/GMO2 and GMO3/GMO4 directed to amplify a 413 bp and an internal 118 bp fragment, respectively, specific for soy lectin *Le*1 gene (Meyer & Jaccaud, 1997) were used. The 413 bp amplicon was discernable in the MWO but absent in the autoclaved flours (Fig. 4A). This result is not unexpected considering the highly fragmented DNA of <500 bp in the autoclaved samples (Fig. 2). In contrast, a smaller internal fragment (118 bp) was amplifiable in all the heat processed samples (Fig. 4B). This result emphasises the suitability of primers that generate shorter PCR products when analysing processed foods. This observation was the premise for designing the other primers listed in Table S1 (Supplementary data).

The primer pair EPSPS-101F/EPSPS-101R was designed to amplify a 101 bp region of EPSPS gene. The amplicon size was minimised to obtain the highest possible sensitivity without the interferences of primer–dimer formation. A 101 bp fragment was observed in both the autoclaved and MWO processed RR-soy (Fig. 4C). The EPSPS gene of *A. tumefaciens* has been used in developing several herbicide tolerant GMOs, therefore the specificity is restricted to this trait and not the GM-crop. Construct specific primer pair EPSPS-210F/NOS-210R attaches to the EPSPS sequence and the adjacent nopaline synthase gene which is used to end the expression of EPSPS in RR-soy. Positive amplification of the 210 bp fragment was observed in both the MWO samples (Fig. 4D). The 210 bp fragment was amplified in the autoclaved 100% but not in 1% RR-soy (Fig. 4D). The primer pair 35SF-2/petuR-1 amplifies a 172 bp junction sequence between the CaMV35S promoter and the petunia hybrid chloroplast transit signal sequence (ISO/FDIS 21569: 2005). The 172 bp product is observed in both the autoclaved and MWO processed flours (Fig. 4E). It is feasible that this construct is present in several GMOs; therefore two independent GMO events cannot be distinguished by this method. Ultimate specificity for GMO detection is achieved by the use of an event- or line-primers that identifies a DNA sequence that spans the junction between the newly introduced genes and the host plant DNA. This site of integration in the plant genome will be unique to a GMO and therefore will specifically identify only the GMO in question. For RR-soy event specific detection, primer pairs GTS-181F/GTS-181R and GTS-121F/GTS-121R that encompass the sequence of the integration site of the transgene and soy plant genome were designed to amplify 181 and 121 bp, respectively. The pair GTS-181F/GTS-181R amplifies a 181 bp region between the soy plant genome and CaMV35S promoter whereas GTS-121F/GTS-121R pair detects the NOS terminator and soy plant genome integration site at the other end. A 181 bp product was visible in both autoclaved and MWO samples (Fig. 4F). A 121 bp fragment was amplified in the autoclaved and MWO RR-soy flours (Fig. 4G). The results reckon that these intergenic DNA sequences were stable to the processing deterioration. The lower limit of detection for the amplification of these RR-soy line-specific genes under this simulated model study was 1% (Fig. 4F and G).

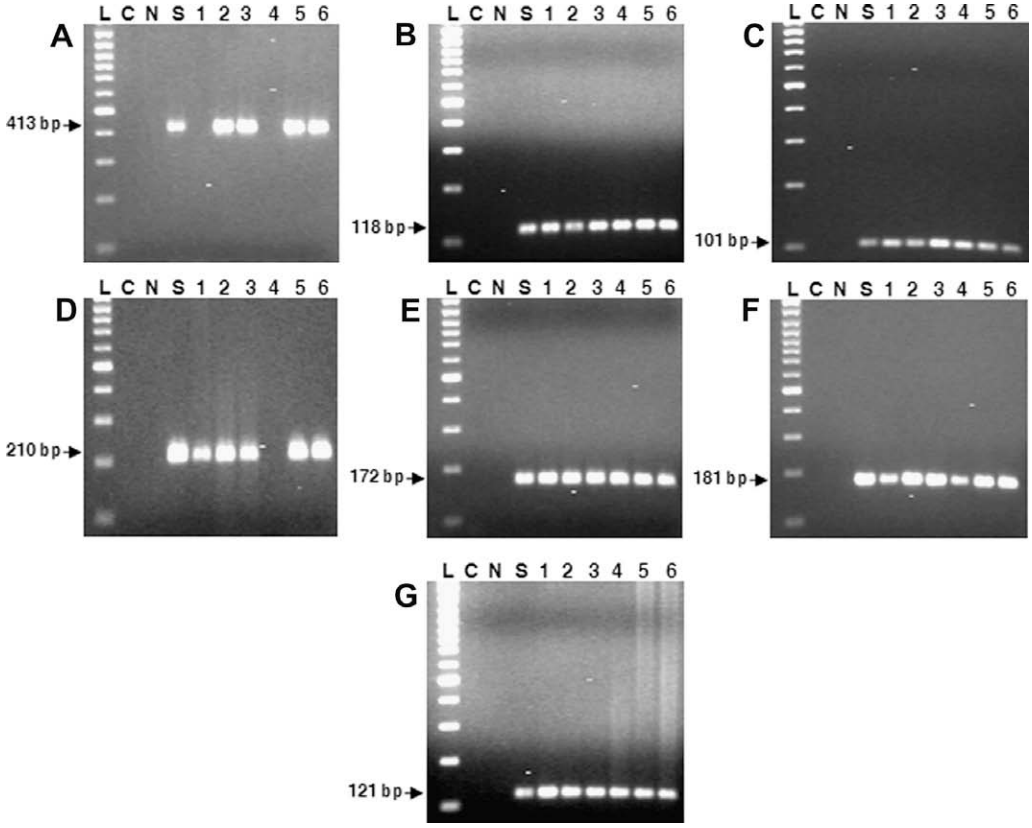

Fig. 4. Agarose gel electrophoresis of PCR products amplified from genomic DNA of heat processed RR-soy. The arrows indicate the expected PCR amplification products. Primer pairs (Table S1 in the Supplementary data section) were used for the detection of lectin outer (A), lectin inner (B), EPSPS (C), EPSPS-TNOS cross border (D), CaMV35S promoter-Petunia-CTP cross border (E), soy genome-CaMV35S promoter junction region (F) and TNOS-soy genome junction region (G). Lane L, 100 bp ladder size marker; Lane C, premise control; Lane N, non-GM-maize in (A) and (B) non-GM-soy in (C)–(G); Lane S, unprocessed 100% RR-soy; Lane 1, autoclaved (100% RR-soy); Lane 2, MWO 540 W (100% RR-soy); Lane 3, MWO 900 W (100% RR-soy); Lane 4, autoclaved (1% RR-soy); Lane 5, MWO 540 W (1% RR-soy) and Lane 6, MWO 900 W (1% RR-soy).

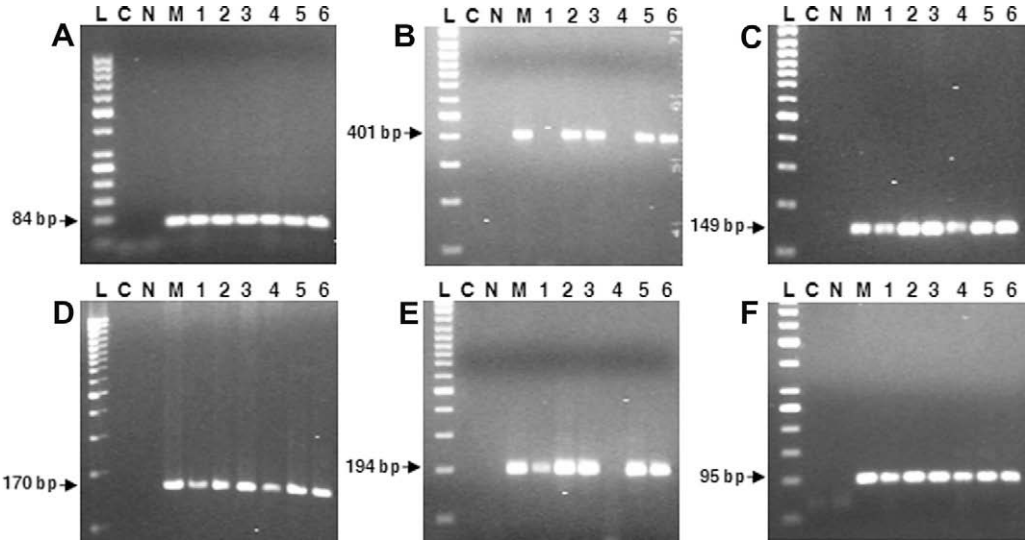

Fig. 5. Agarose gel electrophoresis of PCR products amplified from genomic DNA of heat processed MON-810. The arrows indicate the expected PCR amplification products. Primer pairs (Table S1 in the Supplementary data section) were used for the detection of Zein gene (A), CaMV35S promoter–*hsp*70 intron1 cross border (B), CaMV35S promoter-*hsp*70 exon1 cross border (C), maize genome-CaMV35S promoter junction region (D), *hsp*70 intron1–*cry*1A(b) cross border (E) and *hsp*70 intron1–*cry*1A(b) smaller fragment (F). Lane L, 100 bp ladder size marker; Lane C, premise control; Lane N, non-GM-soy in (A) non-GM-maize in (B)–(F); Lane M, Unprocessed 100% MON-810; Lane 1, Autoclaved (100% GM); Lane 2, MWO 540 W (100% GM); Lane 3, MWO 900 W (100% GM); Lane 4, Autoclaved (1% GM); Lane 5, MWO 540 W (1% GM) and Lane 6, MWO 900 W (1% GM). A 50 bp ladder size marker was used for A and F.

The amplifiability of DNA extracted from the processed GM-maize (MON-810) was confirmed using a primer pair ZeinF/ZeinR for the endogenous reference gene Zein (*Ze*1) (Hohne et al., 2002). An 84 bp amplicon was detected in both the MWO and autoclaved MON-810 flours (Fig. 5A). The gene stretch of CaMV35S promoter, followed *hsp*70 intron1 and the *hsp*70 exon1 is unique and specific to only MON-810. The primer pairs mg1/mg2 and mg3/mg4, which amplify parts of the CaMV35S promoter, *hsp*70 intron1 and *hsp*70 exon1 have been previously used for the specific detection of MON-810 (Zimmermann et al., 1998). The outer primers (mg1 and mg2) yield an amplicon of 401 bp spanning the sequence between the CaMV35S promoter and *hsp*70 intron1 whereas the inner primers (mg3/mg4) amplify a 149 bp fragment comprising the CaMV35S promoter and *hsp*70 exon1. When the primer pair mg1/mg2 was used positive amplification was observed only with the MWO processed samples. The signal for 401 bp was not discernable in the autoclaved flours (Fig. 5B). In contrast, the smaller 149 bp inner fragment was observed in both MWO and autoclaved maize samples (Fig. 5C), clearly demonstrating that primers targeting shorter fragments are more effective in detection of GMOs in processed foods.

The primer pair VW01F/VW03R (ISO/FDIS 21569: 2005) is MON-810 specific detecting the integration site between the maize genome and CaMV35S promoter. Using this primer pair a 170 bp fragment was observed for both autoclaved and MWO samples. The fluorescence intensity for autoclaved samples was visibly lower than that observed for the MWO (Fig. 5D). These PCR results are concurrent with the observed extensive fragmentation of the extracted DNA following autoclaving (Fig. 2). The primer pair MonF/MonR has often been used to amplify a 194 bp fragment, by hybridizing with the cross border region between *hsp*70 and *cry*1A(b) of MON-810 maize (Yamaguchi et al., 2003). Though the signal for the 194 bp amplicon was visible in the autoclaved 100% MON-810, it was not discernable in the 1% MON-810 (Fig. 5E). This can be explained by the fact that the copy number of the inserted sequence is much lower. Moreover, with increased processing, the size of the genomic DNA fragments available for detection also decreases. In consequence a primer pair hsp-95F/

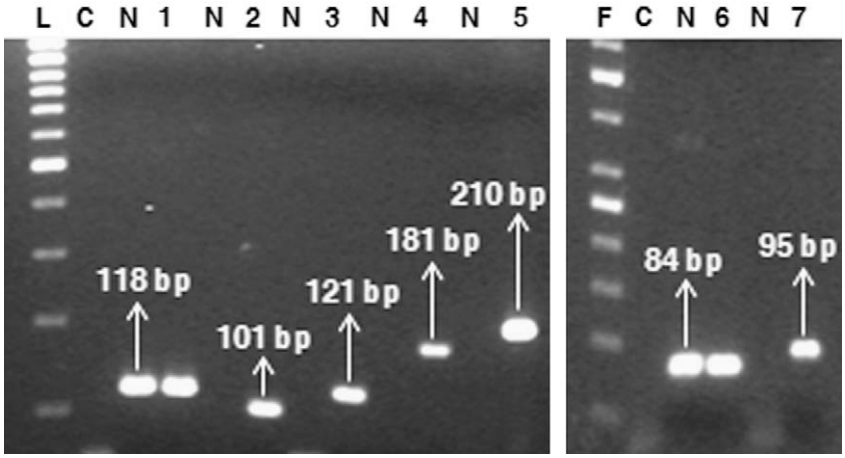

Fig. 6. Agarose gel electrophoresis of PCR products amplified from genomic DNA of baked product. Lane L, 100 bp ladder size marker; Lane C, premise control; Lane N, control biscuit; Lane 1, lectin inner (118 bp); Lane 2, EPSPS (101 bp); Lane 3, TNOS-soy genome (121 bp); Lane 4, Soy genome-CaMV35S promoter (181 bp); Lane 5, EPSPS-TNOS (210 bp); Lane F, 50 bp ladder size marker; Lane 6, Zein (84 bp) and Lane 7, *hsp*70 intron1–*cry*1A(b) (95 bp).

cry1A(b)-95 R was designed to amplify a much smaller inner fragment of the 194 bp sequence. A 95 bp fragment was amplified in both the autoclaved and MWO treated flours (Fig. 5F). These results further advocate that amplification of smaller fragments facilitate detection of GMOs in highly processed foods. The sequences of all the PCR products shown in Figs. 3–5 were verified by direct sequencing.

In the present study, the concentration of template DNA was varied (80–1000 ng) depending on the percentage of GMO and the degree of heat processing. The DNA required for amplification of the transgenes from MWO heated 100% MON-810 and RR-soy was ~4–5 folds greater than that of the unprocessed flours. MON-810 and RR-soy flours subjected to autoclaving required more template DNA as compared to MWO treated. A 9–13 fold increase of the autoclaved processed GMO-DNA was required for positive amplification over that of the raw material. It is clearly evident that the PCR assays of heat processed samples require a higher concentration of template DNA. It is reckoned that as the defined percentage of GMO decreases, a higher amount of template DNA is required.

In all the above PCR assays, appropriate controls were used. PCR set up without template DNA served as premise control labelled as Lane 'C' (Figs. 3–5). PCR set up with template DNA from the conventional non-GM-soy or non-GM-maize served as a negative control (Figs. 3–6, Lane N). PCR set up with unprocessed 100% RR-soy and MON-810, served as positive controls (Lanes S and M, respectively). The absence of any amplification product in the premise control indicates the absence of contaminating DNA from the environment, buffers or reagents used.

3.4. Detection of RR-soy and MON-810 under practical conditions: effect of baking

Baking was chosen as a practical food processing method for monitoring DNA degradation and detection of GMOs. Biscuits containing 0.67 wt.% each of RR-soy and MON-810 were prepared. Control biscuits contained exactly the same amount of conventional soya and maize flours. The DNA of longer length (>3000 bp) was visibly absent in the baked products (Fig. 2B). The extracted DNA showed a smear of short DNA fragments <500 bp with a total loss of the long DNA fragment as observed in the processed flours (Fig. 2B). These results are in close agreement with the DNA smearing reported for bread and cookies (Gryson, Dewettinck, & Messens, 2007; Straub, Hertel, & Hammes, 1999). Although the extracted DNA was fragmented this difference was not observed in the impurity factor (A_{260}/A_{280} = 1.7). The quantity of DNA extracted was greater than that extracted from the same amount of autoclaved or MWO processed flours. Although the extracted DNA was <500 bp in length, the sequences required for the construct and line specific event detection were intact as evidenced by the positive PCR amplification of these sequences (Fig. 6). These results further affirm that the newly designed PCR systems dovetailed to detect RR-soya and MON-810 specific sequences exhibit sufficient specificity for qualitative PCR analyses of a practical unit operation of food processing. The near to identical signal intensity for both the plant specific (lectin/zein) and line-specific sequences of RR-soy/MON-810 (Fig. 6A and B) suggest the usefulness of these assays in the quantitation of GMOs in processed foods.

4. Discussion

It is generally considered difficult to accurately determine the GMO content of processed foods. The suitability of isolated DNA as an analyte for PCR based detection or characterisation technique depends on the concentration, purity, and integrity, each of which, may be influenced by the sample matrix and extraction technique (Terry et al., 2002). Although several factors are important in achieving successful amplification, the template DNA concentration that is often overlooked was also found to be a critical factor in the present study. Despite the genomic DNA of autoclaved RR-soy and MON-810, not being visible by agarose gel electrophoresis (Fig. 2), the PCR detection of smaller fragments suggests that the extracted DNA was capable of directing amplification in the subsequent PCR (Figs. 4 and 5). The copy number of the transgene decreases with considerable physical damage and fragmentation as a result of high temperature and pressure associated with food processing. It can be raised above the detection limit by increasing the amount of template DNA used in PCR (Yoshimura et al. 2005a, 2005b). The results of this study reconfirm that the amount of the DNA present in autoclaved GM-foods is several orders of magnitude lower than the DNA found in the corresponding unprocessed samples (Fig. 1). The difficulty in recovering amplifiable DNA target sequences increases proportionately to the severity of processing. The higher the temperature and/or pressure, and longer the processing time, greater the damage to DNA (Fig. 2). Degradation of genomic DNA is primarily linked to processes carried out at low pH and increases dramatically with thermal stress. DNA fragmentation has been observed as a result of enzymatic hydrolysis (Klein, Altenbuchner, & Mattes, 1998) and to a certain extent on milling and grinding owing to the impact of both shear forces and mechanical stress (Moreano, Busch, & Engel, 2005). The quantity of DNA and DNA degradation in complex matrices, differences in DNA recovery and presence of PCR inhibitors are factors that direct a successful amplification. Baking served not only as a practical food processing condition, but also the effect of the food matrix could be evaluated. In the baking process although a significant deterioration of intact DNA was visible, amplifiable sequences still existed. These results indicate that the complex food matrix is not a protective factor. A pH-dependent fragmentation of DNA has also been reported during baking (Straub et al., 1999). Degradation of both transgene and endogenous reference gene sequences during the processing of soybean to soy powder, tofu and soymilk have been reported by Chen, Wang, Ge, and Xu (2005). Murray et al. (2007) using maize nuclear sequences encoding chloroplast glyceraldehyde-3-phosphate dehydrogenase and cell wall invertase show that maize DNA was degraded by a number of heat processing procedures including extrusion at high temperatures and/or high torque settings.

Sufficient amounts of DNA may be extractable following heat processing, yet it is the extent of degradation, which is a critical factor in determining whether a specific sequence can be amplified or not. Our results also demonstrate that amplification of <200 bp fragments are most suited for the detection of GMOs in highly processed foods. Our results are in agreement with those of Lin, Wei, Lin, and Shih (2006) who showed that the effect of DNA degradation from heating could be reduced by targeting shorter genes. Hupfer et al. (1998) showed that DNA fragments of 1914 bp were no longer detectable after boiling *Bt*-176 maize flour for 5 min at pH 2 whereas boiling for 60 min at pH 8.5–9.5 a shorter 211 bp fragments remained detectable. Therefore, it was recommended that the primers used in PCR detection of GMOs in processed foods, yield amplification products of 150–300 bp (Hupfer, Hotzel, Sachse, & Engel, 1999). This is further validated from this study as all the primers designed by us amplify fragments of ~200 bp. It is also likely that the degradability of the PCR target sequences also depends on the GC content. DNA regions containing high GC content are generally considered to be more heat stable. As observed all the target sequences with 40% GC content or higher were amplifiable (Table S1 (Supplementary data)). Yoshimura et al. (2005b) demonstrated that the construct specific region of MON-

810 and CaMV35S was more strongly degraded than the SSIIb1–4 as the GC content was lower.

Yoshimura et al. (2005) based on their heat processing studies conclude that the amplification regions used to quantitate recombinant and taxon-specific DNA should be closely similar in terms of size. To this end the primers designed in this study to amplify 101 bp (EPSPS-101F/EPSPS-101R) and 121 bp (GTS-121F/GTS-121R) meet this criterion and therefore would be very useful to quantify RR-soy in processed foods owing to their closeness in size to the 118 bp *Le*1 taxon specific gene of soya. In addition, the primer pair hsp-95F/cry1A(b)-95R amplifies a 95 bp fragment of MON-810, which once again in similar in size to the 84 bp product of *Ze*1 the maize taxon specific product.

5. Conclusion

The models of simulated food processes revealed a strong correlation between the degree of processing and the recovery of target DNA. Such simulation studies can be used to predict the availability of target DNA sequences for GMO detection. We believe that they provide an appropriate and a reliable basis that can be extrapolated to commonly used food technological processes such as retorting and rendering, which combine heat and high pressure to sterilise, and cook food and animal feed, respectively. When analysing products that have undergone processing, one can expect the DNA to be highly fragmented and hence can choose an appropriate method to meet the testing requirement, preferably one which targets smaller fragments. The effects of processing steps on DNA degradation must be clearly understood for each processed food on a case to case basis before any DNA based method can be applied to detect GMOs. The present study therefore contributes to the accumulation of basic data necessary to consider the impact food processing has on DNA based detection.

Acknowledgements

The authors thank Dr. A.G. Appu Rao, Head, Department of Protein Chemistry and Technology and Dr. K.N. Gurudutt, Head, Food Safety and Analytical Quality Control, CFTRI, Mysore, India for their keen interest in this work. This research work was supported by Grant-in-Aid projects from the Department of Biotechnology, New Delhi, India and the Council of Scientific and Industrial Research, New Delhi, India (CSIR Networking Project COR 0017).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2009.04.028.

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Food Chemistry 117 (2009) 522–528

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Furan in the baby-food samples purchased from the Finnish markets – Determination with SPME–GC–MS

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ARTICLE INFO

Article history:
Received 24 September 2008
Received in revised form 20 February 2009
Accepted 12 April 2009

Keywords:
Furan
Baby-food
Solid-phase microextraction
GC–MS

ABSTRACT

A method applying solid-phase microextraction followed by gas chromatography–mass spectrometric determination was in-house validated and used to study furan concentrations in baby-food samples purchased from the Finnish markets. The validation parameters showed that the method was well applicable for the reliable analysis of furan. Furan was analysed in 21 different baby-food samples as three independent replicates. The mean levels of furan varied between 4.7 and 90.3 μg kg^{-1} being well in accordance with the levels reported in other studies. The mean concentrations of similar product formulas based on their ingredients were 9.2, 37.0 and 49.6 μg kg^{-1} for fruit-, vegetables- and meat-containing baby-foods, respectively. According to the statistical analyses, fruit-based baby-food samples had significantly lower concentrations of furan as compared to other formulas. Based on our exercise, it seems that a low margin of safety exists between the extreme worst case infant exposures and the deduced NOAEL of furan on experimental animals, particularly for a clear rodent carcinogen.

1. Introduction

Chemical food safety is a topic with an increasing concern within the respective community, although due to the present risk management procedures the acute threat posed by harmful chemicals is very uncommon. Instead, more interest has been focused on the minute concentrations and consequent potential chronic symptoms. It has been postulated, that chemical contaminants found in foods (and feeds) might be aetiological factors behind several diseases (e.g. cancer), the causes of which are still greatly unknown. In addition to the environmental and agrochemical residues as well as natural toxins, contaminants produced during the food processing or storage, such as acrylamide, polycyclic aromatic hydrocarbons (PAH compounds), benzene and furan, are becoming an issue.

Furan is a lipophilic contaminant, which is formed during heating process used for the manufacture of foods. Furan is an aromatic heterocyclic compound (Fig. 1) which is highly volatile with a boiling point of 31.36 °C (The Merck Index, 2006). Furan has been detected in hot-air dried, baked, fried and roasted food items, such as cereal products and coffee as well as canned or jarred prepared foods (EFSA, 2005; US FDA, 2004a; Zoller, Sager, & Reinhard, 2007). Using experimental models it has been proposed that there exist multiple precursors and alternative routes for the formation of furan in foods rather that a single mechanism. Non-enzymatic browning involving reducing sugars only (Strecker degradation) or together with amino acids (Maillard reactions) has shown to produce furan and furan derivatives, the latter being important aroma compounds (Beliz, Grosch, & Schieberle, 2004). Ascorbic acid has proved to form furan through the same pyrolytic pathway than sugars even in less harsh conditions (Becalski & Seaman 2005; Limacher, Kerler, Conde-Petit, & Blank, 2007). Secondly, amino acids serine and cysteine can rearrange to form both molecular moieties needed for aldol condensation and subsequent furan formation (Perez Locas & Yaylayan, 2004). The third alternative, especially potent in prepared foods, is the formation of furan from oxidised polyunsaturated lipids, either produced via radical attack or lipoxygenase activity (Becalski & Seaman, 2005; Perez Locas & Yaylayan, 2004). However, most studies have been conducted using simple mixtures, and only few recent papers try to resolve which reactions prevail in more complicated or food systems (Limacher, Kerler, Davidek, Schmalzried, & Blank, 2008; Märk, Pollien, Lindinger, Blank, & Märk, 2006).

Acute exposure effects of furan are poorly studied but can be considered of low priority as far as the food contamination is concerned. Instead, the chronic low level exposure seems to be more relevant with cancer as an endpoint. In a two-year bioassay conducted by the National Toxicology Program, furan administered by gavage to rats induced for example hepatic cholangiocarcinoma

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doi:10.1016/j.foodchem.2009.04.029

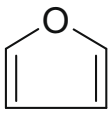

Fig. 1. The chemical structure of furan.

and hepatocellular adenoma and carcinoma (NTP, 1993). A preliminary report from a second two-year bioassay in female mice found increased incidence and multiplicity of hepatic tumours at exposure level of 4 mg/kg bw/day exposure level (Goldsworthy et al., 2001). Furan is regarded as a possible human carcinogen (Group IIB) by the International Agency for Research on Cancer (IARC, 1995). The mode of action in furan induced carcinogenesis has two schools of thoughts: a genotoxic mode of action is supported for example by the data that metabolic activation by cytochrome P450 enzyme (CYP2E1) generates *cis*-2-butene-1,4-dial which has been found to irreversibly bind to proteins (Burka, Washburn, & Irwin, 1991) and nucleosides (Byrns, Predecke, & Peterson, 2002). However, genotoxic mechanism is not supported by a recently conducted in vitro and *in vivo* micronucleus assays (Durling, Svensson, & Abramsson-Zetterberg, 2007). Alternative hypothesises are metabolite induced cell proliferation and uncoupling of mitochondrial oxidative phosphorylation (Kedderis & Ploch, 1999; NTP, 2002).

The determination of furan is very challenging due to its highly volatile nature. The most used analytical technique used to date is (automated) head-space extraction combined with gas chromatography–mass spectrometric (GC–MS) determination (e.g. Becalski et al., 2005; Hasnip, Crews, & Castle, 2006; US FDA, 2004b). Later, however, sample extraction based on solid-phase microextraction (SPME) followed by GC–MS has been developed (e.g. Bianchi, Careri, Mangia, & Musci, 2006; Goldmann, Périsset, Scanlan, & Stadler, 2005). Both approaches are very convenient sample preparation techniques for head-space analyses of volatiles, as they are very simple and demand no expensive equipment for sample extraction/concentration. Both techniques also give satisfactory results, if applied correctly (Wenzl, 2008). Instead, attention should be paid to the sample storage and preparation, careful standard preparation, calibration using internal standard as well as proper quality control procedures (Wenzl, 2008). In 2007 the European Commission put out the recommendation for the monitoring of furan in different foods that have undergone heat treatment during processing. To provide competent analytical data for risk assessment, a reliable determination method is, certainly needed.

The aims of this study were (i) to develop and validate an in-house SPME–GC–MS – method for the determination of furan in baby-foods, (ii) to apply the developed method to study the concentration levels of furan in different kind of baby-food samples (fruit purées, vegetable purées and meat-containing foods) purchased from the Finnish markets, and (iii) to estimate the exposure of Finnish infants to furan based on the levels detected.

2. Materials and methods

2.1. Standards

Furan (0.94 g/ml, ⩾99%) was purchased from Fluka Chemie (Buchs, Switzerland) and deuterated furan d_4 (0.99 g/ml, internal standard) was purchased from Isotec (Miamisburg, Ohio, USA). Standard solutions of 0.94 mg/ml, 0.94 and 0.094 μg/ml for furan and 1.19 mg/ml and 1.55 μg/ml for deuterated furan were prepared in methanol and stored at +4 °C.

2.2. Chemicals

Methanol was of HPLC-grade and purchased from J.T. Baker (Deventer, The Netherlands). De-ionised water was purified with a Millipore Milli-Q Plus system (Millipore, Espoo, Finland). Sodium chloride (NaCl) was of p.a (proanalysis) – grade and purchased from Merck (Darmstadt, Germany).

2.3. Samples

Twenty-one different baby-food samples were purchased from the local markets in February–March 2008. The samples represented all the brands available on the markets. All brands were represented by three different product categories (if available): (i) a fruit-based, (ii) a vegetable-based and (iii) a meat–vegetable based purée packed in a glass-jar. Unfortunately, exactly corresponding samples were not available for all of the brands. The main ingredients of the baby-food samples analysed are presented in Table 1. All samples were analysed as three independent replicates originating from the same batch. The sample jars were stored unopened at +4 °C before the analysis.

2.4. Sample preparation

The method used was a modification of the method of Goldmann et al. (2005). In brief, the sample was homogenised in an ice-bath, after which 2.5 g of homogenous sample was weighed into a 40 ml headspace vial (Supelco, Bellefonte, PA, USA) with a PTFE/silicone-septum. 2.5 g of ice-cold 20% NaCl was added followed by the blending of the mixture with a Polytron 1200 rod-homogenisator (Kinematica Ag, Lucerne, Switzerland). About 250 μl of MeOH and 50 μl of internal standard (deuterated furan, 1.55 μg/ml in MeOH) were added. Furan was statically extracted from the sample using SPME (CAR/PDMS-fibre, 75 μm, Supelco) at +45 °C using a magnetic stirring for 20 min.

2.5. GC–MS analysis

Furan was analysed using an Agilent 6890 GC and an Agilent 5973N MS (Agilent Technologies, Palo Alto, CA, USA). The capillary column used was a 30 m × 0.32 mm × 20 μm HP-PLOT/Q (J&W Scientific, Folsom, CA, USA). The injection port temperature was 290 °C with injection in the splitless mode. The hold time of the injector was 1 min. Helium was used as a carrier gas with a flow rate of 2.0 ml/min. The initial GC temperature was 40 °C with a hold time of 8 min, after which the temperature was increased to 220 °C at 40 °C/min and then held for 1 min. Selected ion monitoring (SIM) was used for the detection of furan and internal standard. The ions monitored were *m/z* 68 and 39 and *m/z* 72 and 42 for furan and deuterated furan, respectively. After the extraction, the holder was removed from the head-space of the vial and the fibre immediately inserted into the injector of the GC–MS-system. The retention time of furan and deuterated furan was approximately 12.28 min. The extracted ion chromatograms for a spiked carrot-purée and a baby-food sample are presented in Fig. 2.

Furan was quantified using the internal standard method. The calibration points were prepared as follows: 2.5 g of MQ-water and 2.5 g of 20% NaCl was weighed into a headspace vial. Different volumes (50–250 μl) of furan working standard solutions and a constant volume (50 μl) of internal standard working solution were added. Finally, MeOH was added to ensure that corresponding volume of solvent was added to all vials. The calibration curve covered the concentration range of 1.9–93.6 μg kg^{-1} furan in baby-food.

Table 1
The different baby-food samples analysed with the ingredients and nutritional values reported by the manufacturers. All samples were analysed as three independent replicates (see text).

Sample number	Manufacturer	Product	Ingredients	Protein (g/100 g)	Carbohydrates (g/100 g)/sugars (g/100 g)	Fat (g/100 g)
1	A	Carrot and potato purée	Carrot, water, potato	0.7	5.4/3.4	0.1
2	A	Banana and peach purée	Banana, peach, water, rice flours, citron juice, vitamin C	1.1	15.2/12.1	0.01
3	A	Vegetables and bovine meat	Carrot, potato, water, bovine meat, maize oil	2.2	6.3/2.4	2.3
4	B	Apricot purée	Water, apricot, fructose, tapioca starch, vitamin C	<0.5	15/11	<0.5
5	B	Carrot and potato purée	Carrot, water, potato, rice starch, rape seed oil	0.5	7.5/2.5	1.0
6	B	Vegetables and meat	Water, potato, pork meat, Apple purée, rice starch, bovine meat, onion, peas, tomato purée, wheat flours, salt	3.0	9.0/1.0	3.5
7	C	Fruit purée	Apricot, apple juice, water, mango, pineapple, tapioca starch, vitamin C	0.5	12/7	<0.5
8	C	Vegetable purée	Carrot, water, potato, maize, rice starch, rapeseed oil	1	10/2	1.5
9	C	Meat casserole	Water, potato, pork meat, carrot, peas, maize starch, onion, rapeseed oil, salt, bay, allspice	3	8.5/1	3.3
10	D	Apple and apricot purée	Apple juice, water, apple, apricot, rice starch, vitamin C, iron	0.2	12/6.5	0.1
11	D	Carrot purée	Carrot, water, rapeseed oil	0.5	7	2.4
12	D	Vegetables and pork meat	Water, potato, ham, carrot, peas, orange juice, rice starch, skimmed milk powder, rapeseed oil, white pepper, rosemary	4	10	3.5
13	E	Fruit purée	Apple purée, peach purée, apple juice, rice flours, vitamin C	0.6	14/9.8	0.04
14	E	Carrot purée	Carrot, water, rice flours	0.8	5.6	0.2
15	E	Vegetables, rice and pork meat	Zucchini, apple juice, rice, tomato, carrot, pork meat, wheat starch, maize oil, parsley, yeast extract	3.2	10.9	1.7
16	F	Organic carrot and veal meat	Water, maize, potato, carrot, veal meat, vegetable oil	2.5	9.5/1.5	2.5
17	G	Organic peach purée	Peach, apple	0.7	11.1/8.5	0
18	G	Organic carrot purée	Carrot, maize	1.1	6.2/2.2	0.7
19	G	Organic vegetables and meat stew	Potato, peas, carrot, onion, water, low-fat ham, parsley	3.9	7.1/1.2	0.5
20	H	Apple and banana purée	Apple, banana, water, rice flours	1.0	18.6/13.1	0.2
21	H	Vegetables purée	Carrot, water, potato, spinach, parsnip, leek	0.6	5.1/2.0	0.3

2.6. Validation

The following validation parameters were determined for the method used: specificity, recovery percent, repeatability, reproducibility, linearity, limit of detection (LOD) and limit of quantification (LOQ).

2.7. Quality control procedures

The quality control practices included the daily determination of furan concentration in a positive control sample (commercial baby-food sample) and the recovery of furan from spiked samples (self-made carrot purée). The ion ratio (*m/z* 68/39) of the samples was compared to that of standards and used for further confirmation of a positive identification of furan (data not shown).

2.8. Measurement uncertainty

The measurement uncertainty of the method was determined according to EURACHEM/CITAC Guidelines (2000).

2.9. Statistical analyses

The results from furan analyses were subjected to statistical analyses (two-sided *t*-test and one-way analysis of variance (ANOVA)) using Statistix for Windows, version 7.0 (Analytical Software, Tallahassee, FL, USA). The *p*-values <0.05 were considered statistically significant.

3. Results

3.1. Method validation

The specificity of the method was determined by analysing 12 blank samples (self-made carrot purée). The analyses showed, that a signal (both at *m/z* 68 and 39) was recorded at the retention time of furan. Hence, the method can not be concerned as absolutely specific with respect to the common validation guidelines. The signal of blank samples was, however, taken into consideration with the calculation of LOD and LOQ (see later). According to the observations it was concluded that the method is specific for the determination of furan only when all identification parameters (detection of both ions, ion ratio corresponding to standard, correct retention time) are compatible.

The repeatability was tested by analysing six replicates of spiked blank samples at three different spiking levels (Table 2). The experiment was repeated to test the in-house reproducibility. The repeatability (as well as the reproducibility) of the method was good, as the relative standard deviations (RSD) for the determinations varied between 6.3–14.3% depending on the spiking level (Table 2). The same samples were also used to determine the recovery of furan. The mean recovery was excellent, varying between 97.5% and 115.8% (depending on the spiking level). It must be noted, however, that the signal observed in the blank matrix was not taken into consideration in the calculations of recovery, which caused the mean recovery of >100% at the lowest spiking level (4.7 μg kg^{-1}).

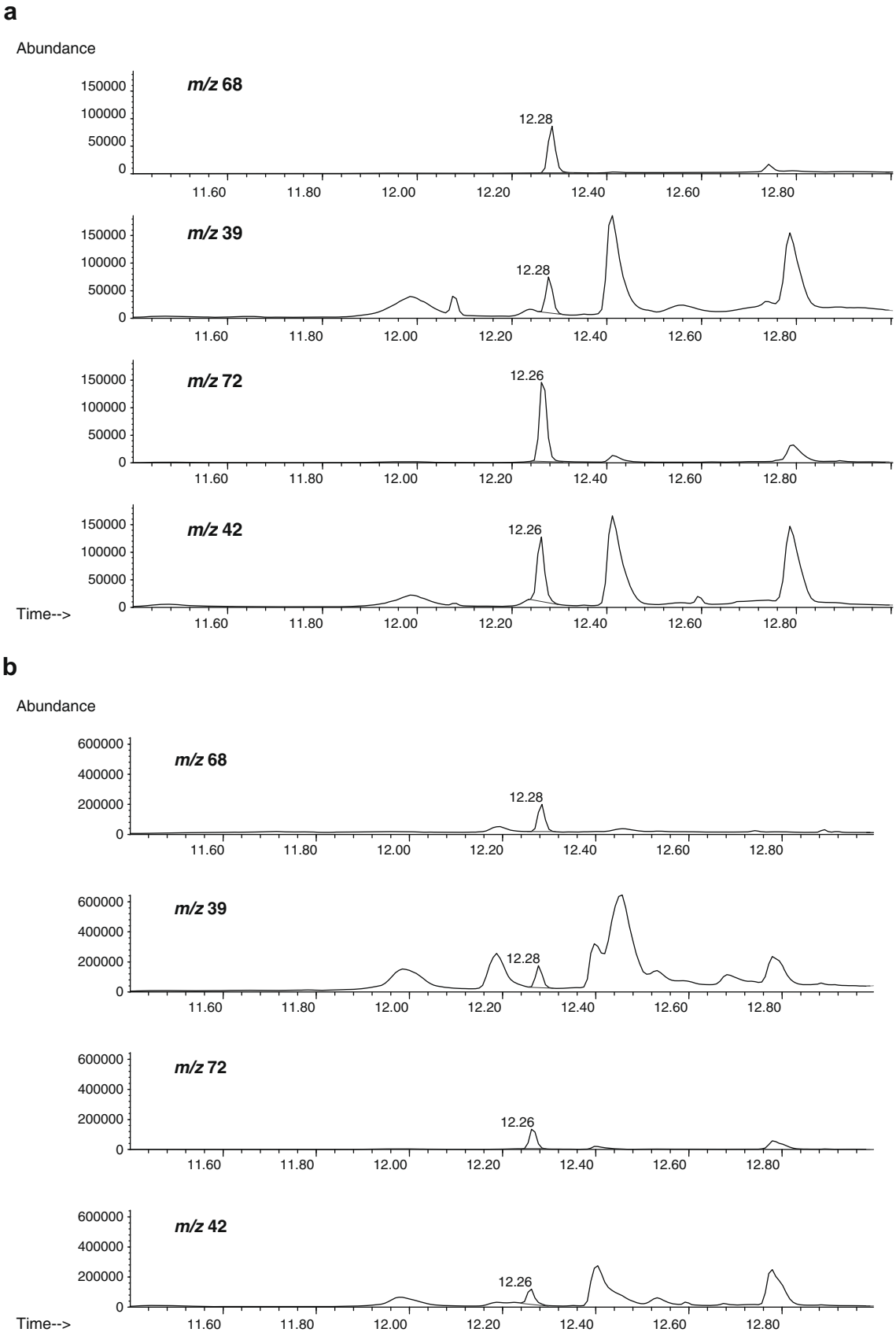

Fig. 2. The extracted ion chromatograms for (a) a spiked carrot purée sample (28.1 μg kg^{-1}) and (b) a baby-food sample (58.5 μg kg^{-1}).

The acceptable linearity of each point of the calibration curves was tested with the method of van Trijp and Roos (1991). A tolerance of 100 ± 10% was accepted for the separate calibration points for good linearity. On that basis, the method can be considered as

Table 2
The mean recoveries and relative standard deviations of furan determinations at three different spiking levels used. Each spiking level is represented by 12 replicates.

	Spiking level 4.7 μg kg^{-1}	Spiking level 28.1 μg kg^{-1}	Spiking level 74.9 μg kg^{-1}
Mean recovery (%)	115.8	97.5	104.7
RSD (%)	13.2	14.3	6.3

being linear for the determination of furan in the range tested (1.9–93.6 μg kg^{-1}) (data not shown).

LOD and LOQ for furan were calculated from the responses of the SIM signal for the "blank" matrix (n = 12). The calculated LOD (blank matrix mean response +3 × standard deviation) was 1.9 μg kg^{-1} and the calculated LOQ (blank matrix mean response +10 × standard deviation) was 4.0 μg kg^{-1}.

3.2. Measurement uncertainty

The measurement uncertainty calculated based on the systematic error (spiked samples) and random error (positive control samples) of the method is 29% (at the level of 5 μg kg^{-1}), 23% (at the level of 25 μg kg^{-1}) and 20% (at the level of 75 μg kg^{-1}).

3.3. Furan in the baby-food samples

All samples analysed had quantifiable amounts of furan (Table 3). The highest mean concentration (90.3 μg kg^{-1}) was determined in sample 15 (vegetables, rice and pork meat), whereas the lowest mean concentration (4.7 μg kg^{-1}) was observed in sample 2 (banana and peach purée). The three replicates gave very comparable results, the relative standard deviations (RSD) of the three independent measurements ranging between 1.7–31.4%. In previous studies, it has been claimed that reproducibility of the quantitative analysis of food volatiles is greatly affected by the changes in individual CAR–PDMS fibres (Mestres, Sala, Mart, Busto, & Guasch, 1999; Natera Marín, Castro Mejians, Garcia Moreno, Garcia Rowe, & Garcia Barroso, 2002), but based on our data this does not apply. The statistical analyses (two-sided t-test) showed that the concentration levels measured from a positive control sample (n = 20) did not differ significantly (t-test), although two different fibres were used in parallel. However, variation may arise when using fibres originating from different batches. This kind of problems will, however, be overcome by careful quality assurance procedures used in the analytical laboratory. The use of two fibres was practical, as this increased the laboratory efficiency remarkably, when manual injections were applied.

The mean concentrations of similar product formulas based on their ingredients were 9.2, 37.0 and 49.6 μg kg^{-1} for fruit-, vegetables- and meat-containing baby-foods, respectively.

Table 3
The concentration of furan in the baby-food samples analysed.

Sample	Jar 1 (μg kg^{-1})	Jar 2 (μg kg^{-1})	Jar 3 (μg kg^{-1})	Mean concentration (μg kg^{-1})	RSD (%)
1	29.3	30.0	30.3	29.9	1.65
2	4.4	4.6	5.1	4.7	7.6
3	52.2	53.1	58.5	54.6	6.2
4	13.4	10.0	18.8	14.1	31.4
5	19.2	25.6	23.1	22.6	14.2
6	28.1	32.4	30.3	30.3	7.2
7	12.9	14.4	14.9	14.1	7.5
8	39.8	37.9	40.4	39.4	3.3
9	13.7	12.3	12.3	12.8	6.2
10	8.2	8.6	7.6	8.1	6.2
11	18.1	26.0	24.6	22.9	18.4
12	41.2	37.3	37.3	38.6	5.8
13	7.7	9.9	8.1	8.6	13.6
14	36.2	30.2	34.0	33.5	9.1
15	91.8	83.8	93.8	90.3	4.8
16	81.0	71.4	72.1	74.8	7.2
17	5.3	5.9	5.4	5.5	5.7
18	35.6	41.0	34.5	37.0	9.4
19	49.9	45.9	41.0	45.6	9.8
20	9.0	8.9	10.3	9.4	8.5
21	82.8	67.8	69.7	73.4	11.2

4. Discussion

The method performance parameters determined during the in-house validation showed that the analytical method is well applicable for the reliable determination of furan in baby-foods with limit of detection and limit of quantification suitable for the purpose. The sample analyses showed RSD of less than 20% for all other samples except sample 4, although three independent replicates were analysed. This further demonstrates that the method is repeatable for the determination of furan in baby-foods, although two different fibres from the same batch were used in parallel. In addition, the low RSD values emphasise, that only minor difference in the furan concentrations occurs in the jars prepared in the same batch.

We used calibration curve prepared in water in contrary to the recommendations of Wenzl (2008). This was because the standard addition method was not found suitable as the determination was accomplished manually and therefore the suggested method would have restricted the laboratory efficiency dramatically. In addition, as the recovery of furan from the spiked samples was around 100% at all spiking levels, it is unlikely, that the matrix would affect the quantitative result. Crews, Hasnip, Roberts, and Castle (2007) compared these two different quantification methods (standard addition and calibrants prepared in solvent) and found that they both provide nearly identical results and suggested that the relative partitioning of furan and deuterated furan is not significantly affected by the nature of the sample matrix. Additionally, Limacher et al. (2007) used calibrants diluted to water to perform a reliable quantitative analysis of furan in vegetable purée.

The furan concentrations in baby-food samples from the Finnish markets of our study are well in accordance with the levels determined in other studies (Becalski et al., 2005; Bianchi et al., 2006; Morehouse, Nyman, McNeal, DiNovi, & Perfetti, 2008; US FDA, 2004a; Zoller et al., 2007). Lowest concentrations have typically been found in fruit-based purées and juices, and highest in meat-containing baby-foods. However, Zoller et al. (2007) reported low concentrations of furan (3–6 μg kg^{-1}) in the meat-containing baby-foods, but it is noteworthy that these samples did not contain vegetables. US FDA (2004a) internet database is rather extensive, and data from years 2004–05 shows furan concentrations in fruit-based baby-foods below 8 μg kg^{-1}, vegetables and mixed vegetables up to 112 μg kg^{-1} and meat containing mixed baby and toddler foods up to 90 μg kg^{-1}. It must be noted, however, that all samples in our study represent baby-foods prepared by autoclaving in glass jars, which has been suggested to favour furan formation over other technologies used for commercial baby-food preparation and domestic practises (Roberts, Crews, Grundy, Mills, & Matthews, 2008; US FDA, 2004a; Zoller et al., 2007).

Statistical analyses (ANOVA) showed that furan concentrations in baby-food samples were not significantly affected by the agricultural practise (organic/conventional) or the manufacturer whereas a significant difference was found between the recipes: the fruit-based products had significantly lower concentrations of furan as compared to vegetable- or meat-based products. As dis-

cussed above, same tendency has been obtained in other studies. It must be noticed that the fruit-based products with added ascorbic acid contain also organic acids. It has been reported that the formation of furan from sugars or ascorbic acid is less likely at low pH (Becalski & Seaman, 2005; Limacher et al. 2008), although Fan, Huang, and Sokorai (2008) have recently reported completely opposite findings. So it seems that the effect of pH on the formation of furan is much more complex than what has been previously suggested. Apparently preparation of autoclaved baby-food does not include process conditions of low water activity at high temperatures typical for non-enzymatic browning reactions to occur, thus more likely furan is formed through other mechanisms than Strecker degradation or Maillard reaction. Although not directly confirmed in our study, it is possible to speculate, that most potential precursors for furan in such baby-foods are polyunsaturated lipids such as polyunsaturated fatty acids and carotenoids (Becalski & Seaman, 2005; Perez Locas & Yaylayan, 2004). This is also supported by the findings of Limacher et al. (2008) and Fan et al. (2008).

Based on the EFSA database on the occurrence of furan in baby-foods the concentration levels ranged from nondetectable to 112 μg kg^{-1} (EFSA, 2005). In our exercise the levels are well in agreement with those figures ranging from 4.7 to 90.3 μg kg^{-1}. A consumption of 195 g/day of baby-foods (ready-to-eat or -drink) in Germany is reported (Kersting, Alexy, Sichhert-Hellert, Manz, & Schöch, 1998) which is somewhat higher than the corresponding Finnish value of 172 g/day for industrial baby-foods (Tapanainen & Virtanen, unpublished data). By using the EFSA database the German consumption level result to the exposure which range from less than 0.03 μg kg^{-1} bw/day to 2.9 μg kg^{-1} bw/day (EFSA, 2005) assuming a body weight of 7.5 kg for a 6 months old baby (Scientific Committee for Food, 1993). If we apply the mean Finnish consumption figure (172 g/day) to our data, the furan intakes in Finnish infants range from 0.8 to 15.5 μg/day resulting to exposures of 0.1 μg kg^{-1} bw/day and 2.1 μg kg^{-1} bw/day, respectively. Two extreme worst case scenarios can, however, be deduced from our data: (i) the maximum value in consumption of canned baby-foods (883 g/day) (Tapanainen & Virtanen, unpublished data) and the maximum concentration of furan detected (90.3 μg kg^{-1}) and (ii) 95 percentile consumption of canned baby-foods (384 g/day) (Tapanainen & Virtanen, unpublished data) and the maximum concentration of furan detected (90.3 μg kg^{-1}). These two extreme worst case scenarios gave intakes of 79.7 and 34.7 μg, respectively. National Research Council deduced as a compromise value for no observable adverse effect level (NOAEL) of furan 0.08 mg kg^{-1} bw/day from the lowest observed adverse effect level (LOAEL) in gavaged rats of 2 mg kg^{-1} bw/day by applying the benchmark dose rate as well as safety-factor approaches (NRC, 2000). If the margin of safety (MOS) is calculated by using the deduced NOAEL of 0.08 mg kg^{-1} bw/day (600 μg/day for 7.5 kg baby) and the extreme worst case exposure scenarios of Finnish infants, the MOS values of 7.5 and 17.3 can be derived. In the case of low exposure scenario (mean consumption of 172 g/day and furan concentration of 4.7 μg kg^{-1}) the MOS value is 750, respectively. Based on our exercise, it seems that a low margin of safety exists between the extreme worst case infant exposures and the deduced NOAEL on experimental animals, particularly for a clear rodent carcinogen. That conclusion is in line with the conclusion EFSA (2005) derived. However, a significant reduction to the exposure scenarios is provided by the fact that at least in Finland the use of fruit-based baby-foods with low furan content (as observed in our study) is comprising a substantial proportion (mean consumption 78 g/day, maximum consumption 488 g/day and 95 percentile consumption 197 g/day) (Tapanainen & Virtanen, unpublished data) of the consumption of total industrial baby-foods. Additionally, although a rising cancer trend among children in various organs/tissues in 15 European countries is reported due to the changes in lifestyle and exposure to a variety of cancer causing agents (Kaatsch et al., 2006), the authors did not observe any changes in the incidence of hepatic tumours, the clear target organ (cholangiocarcinoma, hepatocellular adenoma and carcinoma) of furan caused cancers in experimental animals (NTP, 1993). If additional safety precautions are hoped to avoid furan exposure, heating in an open can and applying occasional stirring is reported to considerably lower furan concentration in baby-food (Bianchi et al., 2006; Roberts et al., 2008; Zoller et al., 2007).

Acknowledgements

The authors want to thank Milla Rantala, Sanna Ylikarjula and Goran Šarić for their excellent technical assistance during the method development and Tiina Ritvanen for her assistance with the statistical analyses.

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Food Chemistry 117 (2009) 529–537

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Evolution of tebuconazole residues through the winemaking process of Mencía grapes

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ARTICLE INFO

Article history:
Received 28 July 2008
Received in revised form 4 April 2009
Accepted 13 April 2009

Keywords:
Fungicides
Winemaking process
Yeast
Bacteria
Alcoholic fermentation
Malolactic fermentation

ABSTRACT

The removal of tebuconazole residues during the winemaking process, applied initially to red must elaborated from Mencía variety grapes from A.O.C. Valdeorrras (Ourense, N.W. Spain), was studied. Analytical determination of tebuconazole residues in grapes, musts and wines were perfomed by gas chromatography equipped with an ion trap mass spectrometry detector (GC–ITMS). Tebuconazole is retained on the solid matter (cakes and lees) and clarification agent. The eliminated percentage of tebuconazole in the final wine is 86%. The influence of this fungicide on the fermentative activity of *Saccharomyces cerevisiae* yeast and the *Oenococcus oeni* was also studied through *in vitro* assays. Liquid chromatography equipped with triple quadrupole mass spectrometer (LC–MS/MS) was used to determine tebuconazole residues in synthetic must and wine used for *in vitro* assays. No effect on the alcoholic or malolactic fermentation was observed; and no degradation of tebuconazole originated by these microorganisms was registered.

1. Introduction

Grey mold (*Botrytis cinerea*), powdery mildew (*Uncicula necator*) and downy mildew (*Plasmopara viticola*) are the most common fungi encountered in vineyards control (Sala et al., 1996). Fungicides are widely used in the treatment of diseases of grapes for vinification. Tebuconazole is a fungicide widely used in Galicia (N.W. Spain), an important vineyard area which produces white and red wines protected by five Appellation d'Origine (A.O.C) – Rías Baixas, Ribeiro, Valdeorras, Monterrei and Ribeira Sacra.

Although the correct use of fungicides does not cause problems of public concern in health and environmental areas, if inappropriate abusive treatments are applied without respecting safety recommendations, undesirable residues can remain on grapes after harvest and they can be transferred to the wine. Luckily, the winemaking process and the oenological steps carried out contribute to their reduction (Cabras & Angioni, 2000; Cabras et al., 1997, 1999; De Melo et al., 2006; Jiménez, Bernal, del Nozal, Bernal, & Toribio, 2007; Navarro, Barba, Oliva, Navarro, & Pardo, 1999; Navarro et al., 2000; Oliva, Payá, Cámara, & Barba, 2007; Sala et al., 1996). However, several studies are focused on the connection between fungicide residues and stuck and sluggish alcoholic and malolactic fermentations (Cabras & Angioni, 2000; Cabras et al., 1999, 2000; Ruediger, Pardon, Sas, Godden, & Pollnitz, 2005) and therefore the negative effect on the aromatic composition of wines (García et al., 2004; Oliva, Navarro, Barba, Navarro, & Salinas, 1999). As a conclusion, to decrease the risk to the consumer's health and to increase the quality of wine is necessary to know the evolution of fungicide residues and their degradation products during the winemaking process.

No studies have reported the fate of fungicides in Galicia. The first aim of this study is to report the tebuconazole residues through the winemaking process considering red grapes, *Vitis vinifera* var. Mencía, produced in A.O.C Valdeorras (Ourense, N.W. Spain). The second aim is to evaluate, through *in vitro* experiments, the effect of tebuconazole on the behaviour of alcoholic fermentation by *Saccharomyces cerevisiae* yeast and malolactic fermentation by *Oenococcus oeni* lactic bacteria, as well as to determine if yeast and/or bacteria could produce a decrease on tebuconazole pesticide residues in wine.

2. Materials and methods

2.1. Vinification experiments

Red grapes, *Vitis vinifera* var. Mencía, produced in A.O.C. Valdeorras were harvested in September 2007. Two vinification experiments (A and B), by duplicate, were performed at the experimental cellar belonging to Consejo Regulador from A.O.C. Valdeorras. Experiment A, used as a vinification control, consisted of performing the vinification processing with uncontaminated

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doi:10.1016/j.foodchem.2009.04.030

crushed and destemmed Mencía grapes. For experiment B, crushed and destemmed grapes were spiked with a pure standard of tebuconazole at level of 3.3 mg/kg. The winemaking process with maceration was identical for all experiments. The selected contamination level is corresponding to around 1.5 maximum residue limit (MRL, 2.0 mg/kg) from tebuconazole established by European legislation to control pesticide levels in grapes from vinification (Commission Regulation, 2008).

2.1.1. Winemaking process

Intact bunches of freshly harvested grapes were weighed into four replicates of 40 kg. Winemaking process, itemised in Fig. 1, was identical for all vinification experiments, as it was commented above. Briefly, each grape sample were crushed and destemmed, introduced into independent metallic vessels of fermentation and supplied with SO_2 (40 mg/L) and the fungicide (only for experiment B). After 24 h, commercial yeast (*S. cerevisiae*) was added. During alcoholic fermentation-maceration (conducted at temperatures below 18–20 °C during 10–11 days), the mixture was re-pressed two times a day, and the temperature and density values were measured in all containers. At the end of the process the wine was strained off, grapes were pressed and the wine-must mixture transferred again to metallic vessels for the malolactic fermentation where commercial lactic bacteria (*O. oeni*) was added). At the end of malolactic fermentation, the wine was racked, supplied with SO_2 and clarified with fresh egg albumin. Finally, wine was bottled.

2.1.2. Sampling

For studying the effect of the different winemaking steps in elimination of tebuconazole, the following samplings (summarized in Fig. 1) were carried out for each vessel such it follows: (1) (crashed grape samples before to start alcoholic fermentation); (2) (wine and pomace samples collected at the end of alcoholic fermentation); (3) (wine samples at the end of malolactic fermentation after racking); and finally, (4) (bottled wine samples after clarification process).

2.2. Chemicals

2.2.1. Tebuconazole extraction and analysis

Tebuconazole [(RS)-1-p-chlorophenyl-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol], of certified purity > 98%, was obtained from Riedel-de-Haën (Seelze, Germany). Tebuconazole D6 (100 mg/L, in acetone) from Dr. Ehrenstorfer, was used as internal standard (IS) to correct for variability in chromatographic injection and mass spectrometric detection response. The 3-ethoxy-1,2-propanediol (98%), D-sorbitol (>99%) and L-gulonic acid γ-lactone (>98%), used as analyte protectants, were obtained from Aldrich (Steinheim, Germany). Solvents (residue analysis grade) were acetone, acetonitrile, formic acid (85%), ultra-pure water (Panreac, Barcelona, Spain), dichloromethane (Merck, Darmstadt, Germany), methanol and toluene (Scharlau, Barcelona, Spain). Sodium chloride, anhydrous sodium sulfate and ammonium formiate were purchased from Panreac. Standard solutions of fungicides and analyte protectants were prepared according to a previous work (González-Rodríguez, Rial-Otero, Cancho-Grande, & Simal-Gándara, 2008a).

2.2.2. Assays of in vitro fermentations

Ingredients and culture media (peptone, yeast extract, agar and MRS broth) were obtained from Cultimed (Barcelona, Spain). Glucose PA–ACS, ethanol 96% PA–ACS, glycerol PA–ACS–ISO, malic acid PRS–CODEX, NaCl PRS–CODEX, $CaCl_2$ PA–ACS, H_2SO_4 95–98% PRS–CODEX, NaOH PA–ACS–ISO, 3,5-dinitrosalicylic acid PB and Na–K-tartrate PA–ACS–ISO were obtained from Panreac (Barcelona, Spain).

2.3. Materials and small apparatus

The sorbent material used for solid-phase extraction was Supelclean Envi-Carb II/PSA, 6 mL size (Supelco Corp., Bellefonte, PA, USA). For solid–liquid and liquid–liquid extractions, samples were placed in 50 mL polypropylene screw-capped centrifuge tubes (Sterilin, Staffordshire, UK). Samples initially were vigorously homogenised in an ultrasounds bath (Ultrasons-H, J.P. Selecta, Barcelona, Spain). Centrifugation was performed in a Rotina 35R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). Organic extracts were evaporated under a stream of nitrogen in Turbo Vap LV evaporator (Caliper Life Sciences, Hopkinton, MA, USA). *In vitro* fermentation assays without agitation were performed in a refrigerated incubator FOC 225E (Velp Scientifica, Milano, Italy). Agitated incubations were done in a thermostatized orbital shaker (Unitron incubator, Infors, Bottmingen, Switzerland). For absorbance measurements a 6505 UV/VIS spectrophotometer (Jenway, Essex, England) was used.

2.4. Extraction procedure

For the extraction of tebuconazole residues in grapes, musts, pomaces and wines, a fungicide multiresidue method, previously developed in our laboratory, was used (Rial-Otero, Cancho-Grande, & Simal-Gándara, 2003); some modifications were performed to improve the purification process and to reduce the matrix contribution (González-Rodríguez et al., 2008a). Briefly, crushed grapes (5 g), must and wine (5 mL) samples were weighed inside a 50 mL polypropylene screw-capped centrifuge tubes. A volume (25 mL) of dichloromethane–acetone (75:25, v/v) was added and the container was vigorously homogenised in an ultrasounds bath for 10 min. Sodium chloride (3 g) and anhydrous sodium sulphate (12 g) were added followed by vigorous shaking for 5 min. After phase partitioning (10 min, 4000 rpm) in a centrifuge, an aliquot of 15 mL of the organic layer was transferred to a 40 mL glass vial and evaporated to dryness under a stream of nitrogen in Turbo Vap LV evaporator and the residue was redissolved in acetonitrile (2 mL). For clean-up, a multi-layer Supelclean Envi Carb-II/PSA SPE cartridge was conditioned with 5 ml of acetonitrile:toluene (3:1, v/v). Acetonitrile extract was loaded and the retained pesticide was eluted weakly, in a 40 mL glass vial, with a volume of 20 ml of acetonitrile:toluene (3:1, v/v). The eluate was evaporated till dryness under a stream of nitrogen and filled up to a final volume of 2 mL with acetone containing the three analyte protectants (3-ethoxy-1,2-propanediol at 10 g/L; and D-sorbitol and L-gulonic acid γ-lactone at 1 g/L; respectively). Use of analyte protectants mixture provided the best results in terms of effective compensation for matrix-induced enhancement effect. Tebuconazole D6 (0.1 mg/L) as internal standard was also added and the acetone extract was finally homogenised by vortex shaking. An aliquot of the final acetone extract was placed via 350 μL glass inserts into 2 mL vials prior to chromatographic analysis.

2.5. Tebuconazole analysis

Residues of this fungicide were determined by gas chromatography-ion trap mass spectrometry (GC–ITMS) in grapes, musts and wines. Liquid chromatography equipped with triple quadrupole mass spectrometer (LC–MS/MS) was instead used to determine tebuconazole residues in synthetic must and wine used for *in vitro* assays.

2.5.1. GC–ITMS analysis

GC analyses were carried out on a Trace GC Thermo Finnigan gas chromatograph (Rodano, Italy) equipped with a PolarisQ ion trap mass selective detector (ITMS), interfaced to a PC computer running the software Xcalibur 1.4, from Thermo Electron Corpora-

tion (Italy). Chromatographic separations were done by using a SPB-5 fused-silica capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness) from Supelco. PTV was used for the 2 μL injection volume into a silcosteel liner (120 × 2 mm id). The temperature programming of the PTV was 85 °C for 0.3 min; 600 °C/min to 270 °C and hold for 2 min; 840 °C/min to 300 °C for 5 min. The carrier gas, helium, operates at 100 kPa in constant pressure mode. Splitless mode was selected for 45 s. The oven temperature was programmed as follows: 80 °C for 3 min; 15 °C/min ramp to 270 °C for 5 min. The transfer line temperature was 270 °C, and the ion-trap manifold temperature was 250 °C. The ion energy for electron impact (EI) was always 70 eV. Mass detection was performed in full scan mode to identify possible degradation products of tebuconazole and in single ion monitoring (SIM) mode to measured tebuconazole residues in samples. The selected ions (m/z) were 163 and 250 for tebuconazole, and 256 for internal standard (tebuconazole D6).

2.5.2. LC–MS/MS analysis

LC analyses were performed with a Thermo Separation Products system equipped with a vacuum membrane degasser, a Spectra Series P200 quaternary pump, an AS1000 autosampler, a column heater and a SN4000 system controller. The LC system was coupled on-line to a TSQ Quantum Access triple quadrupole mass spectrometer (TQMS) (Thermo Fisher Scientific) equipped with an electrospray ionisation (ESI) source and operated in the positive ion mode. The analytical column was a Luna C_8 (150 × 2 mm ID, 5 μm particle size), coupled with a C_8 Security Guard cartridge system (4 × 2 mm ID, 5 μm particle size), both from Phenomenex (Torrance, CA, USA). An aliquot (5 μL) was injected into the column and eluted at 45 °C, with a constant flow-rate of 0.25 mL/min, using as solvents 5 mM ammonium formiate in water (solvent A), 5 mM ammonium formiate in methanol (solvent B) and 0.5% formic acid (solvent C). Elution was performed with a gradient of 35% B (0–2 min), 35–95% B (2–4 min), 95% B (4–9 min), 95–35% B (9–9.1 min) and 35% B (9.1–22 min), using a constant 5% C along all gradient program. A divert valve was placed between the analytical column outlet and the mass spectrometer inlet, and the flow was diverted to waste during the first 5.9 min and the last 8.5 min of the chromatographic run. The MS conditions were as follows: spray voltage 4000 V; capillary temperature 350 °C; sheath gas pressure (N_2) 20 units; auxiliary gas pressure (N_2) 5 units; collision gas (Ar) 1.5 mTorr; and scan time 0.5 s. The mass spectrometer was operated in full scan mode to identify possible degradation products of tebuconazole and in selected reaction monitoring (SRM) mode to quantify tebuconazole residues. The parent mass, the product mass and the collision energy selected were 308 and 314, 70 and 72, and 22 and 30, for tebuconazole and for internal standard, respectively. For instrument control, data acquisition and processing, Xcalibur software version 2.1.0 (Thermo Electron Corporation) was used.

2.6. In vitro assays. Inoculation and fermentation

2.6.1. Microorganisms

2.6.1.1. (A) Yeast inoculation. The commercial dry yeast used was Fermol Arôme Plus (*S. cerevisiae* var. *cerevisiae*) from AEB Group (Brescia, Italy). Before using it as inoculum, the yeast was purified to eliminate unknown coadyuvants that could make difficult the analysis of possible tebuconazole degradation products. It was done by rehydrating the commercial powder as described by the manufacturer, and inoculating it on YPD-agar plates, which were incubated without agitation at 30 °C for 24 h and kept at 5 °C until use. Inoculum was prepared by transferring cells from a sole colony in the plates to YPD liquid broth placed in an Erlenmeyer flask, and incubating it in an orbital shaker (150 rpm, 30 °C). The exponentially growing cells were separated from the supernatant by centrifugation (5,000 rpm/15 min), and washed twice and suspended in an enough volume of sterile water to reach a cell concentration of 2.5 g/L (measured as turbidity in a spectrophotometer at 750 nm).

2.6.1.2. (B) Bacteria inoculation. The commercial freeze dried lactic bacteria used was Uvaferm Alpha (*O. oeni*) from Lallemand S.A. (Saint Simon, France). The bacterium was also purified in the same way as the yeast substituting YPD by MRS (Liofilchem, Roseto degli Abruzzi, Italy) media. Precultures were prepared inoculating MRS liquid broth with a sole colony form the MRS-agar plates, and incubating it without agitation (30 °C) in consecutive batches with increasing ethanol concentrations (0, 10, 40 and 80 g/L) to adapt the microorganism to the alcohol. Cells from exponential cultures with 80 g/L ethanol were then washed twice and suspended in 0.8% of NaCl. The determination of biomass concentration in concentrated inoculum for *in vitro* malolactic fermentation experiments was carried out by optical density measurement at 600 nm ($1x10^9$ cells/mL) (Guilloux-Benatier, Remize, Gal, Guzzo, & Alexandre, 2006).

2.6.2. Alcoholic fermentation assays

Three series of experiments were carried out: in series A′ *S. cerevisiae* was cultured in presence of tebuconazole, in series B′ the yeast grew without the pesticide, and series C′ was a control of the tebuconazole stability in the culture medium. A simple culture medium reproducing the main must characteristics was elaborated with 200 g/L glucose, 7 g/L peptone and 3.5 g/L yeast extract, and sterilized by filtration through 0.22 μm membrane filters (Millipore, Bedford, MA, USA). Part of the culture medium (experiments A′ and C′) was supplemented with a tebuconazole concentrated standard solution in ethanol (400 ppm) to give a final concentration of 2 mg/L in the medium, and the rest (for experiment B′) was supplemented with an equivalent volume of ethanol (which corresponded to a 0.5% of the total volume). These liquid culture media were distributed into 49 mL replications in 100 mL erlenmeyer flasks (12 replications for each experiment A′, B′ and C′). Erlenmeyer flasks of experiments A′ and B′ were inoculated with 1 mL of the yeast suspension, and 1 mL of sterile water was added to the flasks of experiment C′. All flasks were incubated for 13 days in an orbital shaker at 20 °C and 75 rpm. Two samplings of each experiment consisting in the whole culture of a flask were carried out at 0, 3, 4, 6, 9 and 13 days after inoculation. The following analyses were made: biomass, reducing sugars, fermentation metabolites (ethanol and glycerol) and tebuconazole (free and adsorbed to yeast cells). A 15 mL aliquot of the culture medium (in experiments A′ and B′) was centrifuged at 4000 rpm for 20 min. The supernatants were stored at -20 °C for reducing sugars and fermentation metabolites analysis, and the biomass was washed twice to measure the biomass concentration by dry weight. Reducing sugars were assayed by the 3,5-dinitrosalicylic acid (DNS) method (Bernfeld, 1951) with glucose as standard, and ethanol and glycerol were measured by a high-performance liquid chromatograph equipped with a RI-150 Spectra System refractive index detector (Thermo Separation Products) and a column model ION-300 (Transgenomic, San Jose, CA) eluted with 0.0085 M H_2SO_4 at 35 °C and a flow rate of 0.4 mL/min during 40 min. On the other hand, a 30 mL aliquot of the culture medium (experiments A′ and C′) was centrifuged to quantify free tebuconazole and tebuconazole degradation products in the supernatant, while the biomass was washed twice with water, re-suspended in 0.5 M $CaCl_2$, incubated in an orbital shaker for 60 min at 200 rpm to extract the fungicide residues adsorbed on yeast cells, and centrifuged to obtain the supernatant for quantifying the adsorbed fungicide, according to Zadra and co-workers (Zadra, Cardinali, Corte, Fatichenti, &

Marucchini, 2006). Free and adsorbed tebuconazole was directly analysed in LC–TQMS following the instrumental conditions described before. Quantification was performed by external calibration using standards made in their correspondent matrix ($CaCl_2$ and YPD broth medium).

2.6.3. Malolactic fermentation assays

Three series (A″, B″ and C″) were again performed as for *Saccharomyces* fermentation assays, just substituting the yeast by the bacterium in series A″ and B″, reducing the concentration of tebuconazole from 2 to 0.4 mg/L in series A″ and C″, and changing the medium, which consisted on a synthetic wine (pH = 4.9) containing 1 g/L glucose, 7 g/L peptone, 3.5 g/L yeast extract, 5 g/L malic acid and 80 g/L ethanol, sterilized by filtration through 0.22 μm membrane filters and supplemented with tebuconazole as described. These liquid culture media were distributed into 245 mL replications in 500 mL glass bottles (2 replications for each experiment A″ and B″, and 1 replication for experiment C″). Bottles of experiments A″ and B″ were inoculated with 5 mL of the bacteria inoculum, and 5 mL of sterile 0.8% NaCl was added to the bottle of experiment C″. All bottles were closed and incubated without agitation at 25 °C under anaerobic conditions for 31 days. An aliquot of 5 mL from each bottle was taken each 2–3 days. Firstly, biomass was measured by absorbance readings at 600 nm (using the synthetic wine as a blank). The samples were then centrifuged at 4000 rpm for 20 min, and the supernatants were stored at −20 °C for fermentation metabolites (malic and lactic acids) and free tebuconazole and tebuconazole degradation products analysis. Malic and lactic acids were measured by HPLC-RI following the same analytical conditions of ethanol and glycerol analysis described above, and free tebuconazole was directly analysed in LC–TQMS (synthetic wine was used as matrix to make the calibration standards).

3. Results and discussion

3.1. Analytical performance

Determination of pesticide residues in food matrices may be adversely affected by a phenomenon commonly known as the "matrix-induced chromatographic response enhancement effect", contracted to matrix-induced response enhancement, which was first described by Erney and co-workers (Erney, Guillespie, Gilvydis, & Poole, 1993). When pesticides are in absence of matrix during the injection step, poor peaks with low response resulted for analytes susceptible to be degraded and/or absorbed on the GC injector; however, peak intensity and shape of affected compounds improved when they are injected in the presence of a complex matrix (carbohydrates, proteins and/or lipids). This effect is presumably the most discussed matrix effect negatively impacting quantitation accuracy of certain analytes in GC (Hajšlová & Zrostlíková, 2003). The use of analyte protectants, which are compounds that interact with active sites in the GC system, decreases degradation and/or adsorption of co-injected analytes (Anastassiades, Maštovská, & Lehotay, 2003; Maštovská, Lehotay, & Anastassiades, 2005). The mixture of these three analyte protectants (3-ethoxy-1,2-propanediol at 10 g/L; D-sorbitol and L-gulonic acid γ-lactone at 1 g/L; respectively) was previously adopted in the determination of pesticides in leafy vegetables (González-Rodríguez et al., 2008a; González-Rodríguez, Rial-Otero, Cancho-Grande, & Simal-Gándara, 2008b) and the use of fortified blank samples as calibration standards was avoided. In this case, the use of this mixture was also adopted to evaluate the compensation of this effect in the determination of tebuconazole residues in grapes, musts and wines samples.

The performance of the SLE/SPE/GC–ITMS and SLE/SPE/LC–TQMS methods for determining tebuconazole in grapes, musts and wines was assessed by evaluating quality parameters (such as linearity range, precision, limits of detection and quantitation), which experimental values are summarized in Table 1, such it was described as follows. Uncontaminated Mencía crushed grapes, musts and red wine previously fortified for tebuconazole at different levels (ranging from 0.1 to 1.0 mg/kg or mg/L for GC–ITMS/ranging from 0.01 to 0.1 mg/kg or mg/L for LC–TQMS) were treated following the experimental conditions described above. Linearity of each method was established by plotting the relative area of tebuconazole to the tebuconazole D6 (A/A_{is}), versus the concentration of the fungicide (C_i). Acceptable linearity was obtained for each matrix evaluated with a correlation coefficient > 0.99 (Table 1). Absolute recovery value for the whole calibration line was obtained from the ratio between the slope of the calibration line corresponding to the spiked sample and the slope of the calibration line corresponding to the fungicide standards injected directly onto the analytical column (standards ranging from 0.15 to 1.5 mg/L for GC–ITMS and ranging from 0.015 to 0.15 mg/L for LC–TQMS, in acetone, containing the same concentration of internal standard and analyte protectants). Absolute recovery values ranged from 77 to 92%. One-way analysis of variance between mean recovery values obtained with both chromatographic techniques (GC–ITMS and LC–TQMS) for each type of sample (grapes, musts and wines) were performed. The p-values of the F-test (95% probability) in the ANOVA was greater than 0.05 for each type of sample evaluated; then, there is not a statistically significant difference between the mean recoveries for each type of sample obtained with both techniques at the 95.0% confidence level. Moreover, the use of analyte protectants for both chromatographic techniques eliminated practically the differences between calibrations obtained in different samples versus matrix-free solutions. Another aspect to comment is that recoveries in must and wine samples were higher than values obtained in grapes; this fact could be due to the presence of solid particles in grapes (as skins or nuggets), where tebuconazole could be adsorbed and then, the extraction efficiency reduced. Precision of the SLE-SPE procedure, were calculated by analysing, on the same day, five replicates of each type of sample (grape, must and wine) fortified at concentration of 0.4 mg/kg or mg/L for GC–ITMS and 0.05 mg/kg or mg/L for LC–TQMS; precision was expressed as relative standard deviation (RSD%), which was lower than 7% for the three samples evaluated and injected with both chromatographic techniques. Limits of detection (LOD) and quantitation (LOQ) were evaluated following the recommendations of the American Chemical Society (American Chemical Society, 1980); they were calculated from the signal-to-noise ratios obtained by analysing unspiked samples ($n = 7$); thus LOD and LOQ were taken to be the concentrations of tebuconazole resulting in a signal-to-noise ratio of three and ten, respectively. Under these conditions, LOD and LOQ obtained by LC–TQMS were eight times lower than LOD and LOQ obtained by GC–ITMS. For both cases, the SLE/SPE procedure followed of the chromatographic techniques allowed to determine residue levels 40 (for GC–ITMS) and 250 times (for LC–TQMS) than those established in European legislation (Commission Regulation, 2008).

3.2. Decrease of tebuconazole residues during Mencía winemaking process

Red grapes, *Vitis vinifera* var. Mencía, were previously analysed to guarantee the absence of tebuconazole before beginning the vinification experiments. When red grapes were crushed and destemmed, a pure standard of tebuconazole was added at 3.3 mg/kg only in vessels corresponding to experiments B; this contamination level is 1.6 times higher than European maximum residue level (2.0 mg/kg) established to control pesticide levels in grapes for

Table 1
Performance of the optimised method for the determination of tebuconazole in grape, must and wine.

Sample	GC–ITMS					LC–TQMS				
	Linearity[a] (r^2)	Recovery (%)	RSD[b] (%)	LOD[c]	LOQ[c]	Linearity[d] (r^2)	Recovery (%)	RSD[b] (%)	LOD[c]	LOQ[c]
Grape	0.991	83	5	0.028	0.050	0.993	77	2	0.004	0.008
Must	0.991	88	7	0.023	0.041	0.990	83	6	0.003	0.006
Wine	0.991	87	6	0.024	0.042	0.994	92	7	0.003	0.006

[a] Linear range: 0.1–1.0 mg/kg or mg/L ($n = 7$).
[b] Precision, expressed as relative standard deviation (RSD), $n = 5$.
[c] LOD and LOQ, expressed as mg/kg for grape and as mg/L for must and wine, $n = 7$.
[d] Linear range: 0.01–0.1 mg/kg or mg/L ($n = 7$).

vinification (Commission Regulation, 2008). We opted for this contamination level to identify possible new compounds originated by degradation of the initial tebuconazole; moreover, we opted for addition of tebuconazole as pure standard instead of a commercial formulation to eliminate interferences associated to other substances such as additives and/or excipients.

One day after the addition of tebuconazole standard, necessary to provide the liquid–solid partitioning in experiment B, the winemaking process started. Sampling followed in experiments A and B are described in Fig. 1. Residual concentrations of tebuconazole found in the different control stages of vinification experiment B, by duplicate, are shown in Table 2. No tebuconazole residues were detected in the duplicates of vinification experiment A.

Comparing the alcoholic fermentation course in experiments A and B, followed by the evolution of must density, it can be concluded that both fermentation had a regular course (10–15 days). This seems to indicate that this yeast is not negatively affected by tebuconazole in the vinification conditions here assayed, as also reported by other authors with this microorganism and other fungicides like iprodione and fludioxonil (Ochiai et al., 2002) and fenhexamid (Cabras et al., 2001). On the contrary, the maceration-alcoholic fermentation stage was the most influential one in the

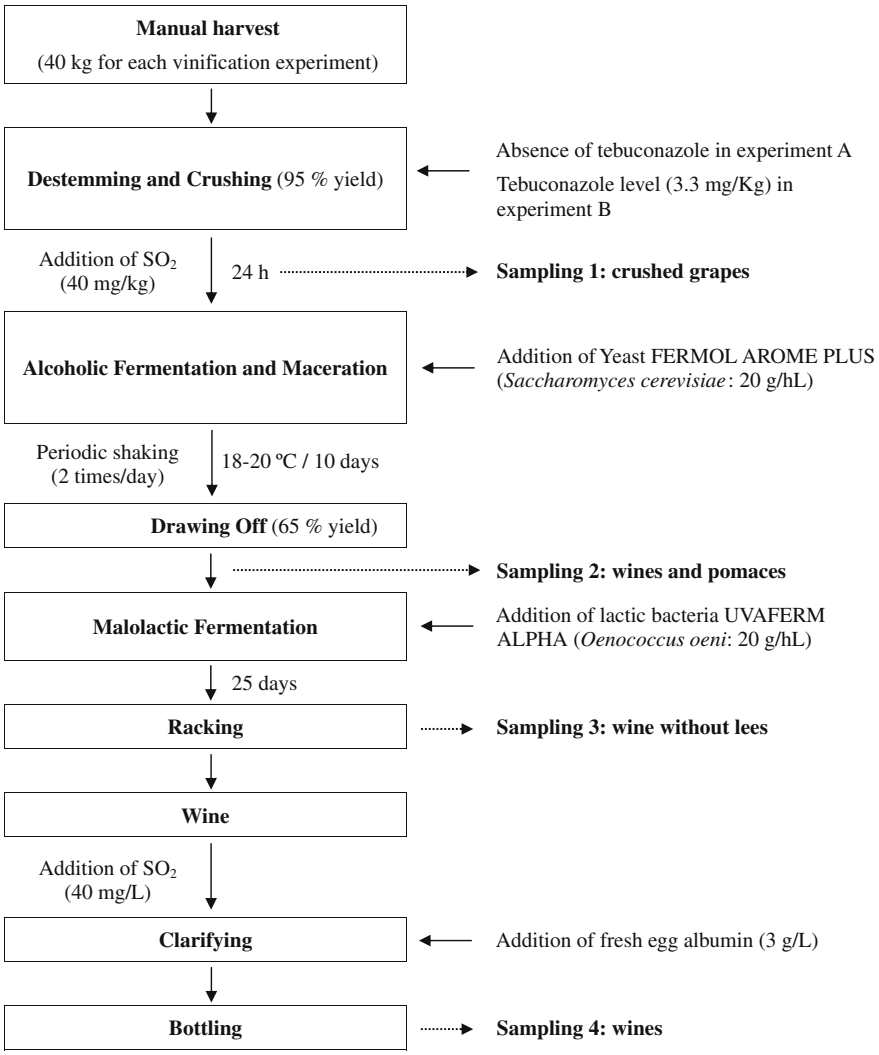

Fig. 1. Flowsheet and sampling for Mencía wine vinification.

Table 2
Average (experiment A) of residual concentrations of tebuconazole ($n = 2$) found in the different control stages of vinification (mg/kg or mg/L ± SD) by GC–ITMS.

Sample 1	Sample 2		Sample 3	Sample 4
Crashed grapes	Must (65%)	Pomace (35%)	Racked wine	Clarified wine
3.49 ± 0.57	0.64 ± 0.15	6.12 ± 0.49	0.59 ± 0.03	0.45 ± 0.01

levels of tebuconazole present in the wine and the pomace. From the initial enrichment of the must to the end of the alcoholic fermentation-maceration, the fungicide was distributed between the fermenting must (the liquid phase) and the pomace (the solid phase). Tebuconazole did not remained completely dissolved in the liquid phase due to its low water solubility (0.032 g/L), unlike other fungicides studied by other authors like metalaxyl (7 g/L) which 64% remains dissolved (Navarro et al., 2000). The remaining amount in the wine was only around 12% of the total amount in comparison to the 88% present in pomace samples (see Table 2); it shows the high affinity of this compound to be adsorbed on the suspended solid matter. For other fungicides, such as quinoxyfen, myclobutanil and tetraconazole (another fungicide of the triazole family), no determinable residues were found at the end of the alcoholic fermentation (Cabras & Angioni, 2000; Cabras et al., 2000).

Malolactic fermentation, followed by thin layer chromatography, had a regular course in all vessels too, also showing the resistance of the bacteria used to the tebuconazole concentration remaining in the wine after the first stage of alcoholic fermentation (0.6 mg/L). Although studies on lactic acidic bacteria inhibition by pesticides (Bordons, Masqué, & Vidal, 1998; Cabras et al., 1994; Haag, Kreiger, & Hammes, 1988) report minimum inhibitory concentrations for various pesticides ranging from as low as 1 to > 30 mg/L, in most cases pesticide residues were found to have little or no effect on malolactic fermentation. Recently, Ruediger and co-workers (Ruediger et al., 2005) evaluated the effect of red wine malolactic fermentation on the fate of seven fungicides (carbendazim, chlorothalonil, fenarimol, metalaxyl, oxadixyl, procymidone and triadimenol); only dicofol has an inhibitory effect on the catabolism of malic acid. On the other hand, the malolactic fermentation stage resulted in no significant reduction on tebuconazole levels (around 0.6 mg/L). Published studies showed that a minimum number of pesticides were degraded or adsorbed by the bacteria during this process (Cabras et al., 1999, 2000; Navarro et al., 2000; Ruediger et al., 2005).

Clarification was performed with fresh egg albumin; it is a clarifying agent used in high prestige red wines. It removes a large number of phenols and mellows wines with a high content in astringent tannins. Compared to other clarifying agents, it offers the advantage of not modifying the sensory qualities (Oliva et al., 2007). Clarification as well as filtration allows achieving the clearness demanded of wines but they also lead to remove other exogenous substances such as pesticide residues. In this case, tebuconazole residues decreased around 25% in this stage with regard to racked wine due to this fungicide was also adsorbed in lees formed along the clarification (see Table 2). Similar results were obtained by Oliva and co-workers (Fernández, Oliva, Barba, & Cámara, 2005; Oliva et al., 2007) who studied the effects of clarification and filtration processes on the removal of other fungicides in the winemaking process of red wine elaborated from Monastrell variety grapes. Fresh egg albumin presented the highest elimination, in the clarified wine, for famoxadone, flunquiconazole and trifloxystrobin (50% for the three target fungicides) (Oliva et al., 2007); and for the blood albumin was observed highest elimination for cyprodinil, pyrimethanil (70%) and fludioxonil, quinoxyfen (30–40%).

At the end of the winemaking process, the residue remaining in the final wine was lower than in grapes, presenting a mean transfer factor from grape to wine of 0.14. These results are in accordance with those published for other fungicides of the triazole family in red grapes winemaking process. As example, penconazole and propiconazole decreased by as much as 90% of the initial value in red wines (Navarro et al., 1999); levels of tetraconazole fell by 100% of the initial value found in grapes (Cabras et al., 1998) and the loss of tebuconazole in the final red wine ranged from 50% to 73% (Cabras et al., 1997; Jiménez, Bernal, del Nozal, Toribio, & Bernal, 2004). For white and rosé wines, the reduction of the contamination levels for tebuconazole is lower as a consequence of the absence of the malolactic fermentation and the minor presence of the matter in suspension that can adsorb the tebuconazole; Cabras et al. (1997) established that tebuconazole residues in wine were ca. 50% that levels found in grapes.

Some fungicides, which are unstable in an acid environment, started to degrade after pressing, and at the end of fermentation new related compounds were present in wines. The results here presented also appear to show the absence of tebuconazole degradation processes in this system since no degradation products could be detected. Jiménez and co-workers (Jiménez et al., 2004) determined the degradation products of bromopropylate, trichlorphon, parathion-methyl and tebuconazole, formed during the winemaking process; degradation products were only identified for the three initial fungicides meanwhile tebuconazole, remained intact. It is necessary to make *in vitro* assays to confirm if tebuconazole is degraded and/or their degradation products were also adsorbed on solid matter.

As a conclusion, tebuconazole showed a continuous decrease through the red winemaking process. Nowadays any European legislation to control maximum residue levels of pesticides in wine exists. In future, according these results, MRL for tebuconazole in red wines could be established as a eight times lower (0.25 mg/L) than MRL established in wine grapes (2 mg/kg).

3.3. Evaluation of in vitro assays

Although the previous winemaking experiments allowed to discard negative effects of tebuconazole on the alcoholic and malolactic fermentation at the concentration assayed, the presence in the must of the solids coming from the grapes during the maceration–alcoholic fermentation stage makes difficult to analyse the real effect of the fungicide on yeast grown and fermenting activity since solids reduce the concentration of soluble tebuconazole by adsorption and interfere in yeast biomass determination. On the other hand, the complex composition of must and wine makes also difficult to detect, identify and quantify possible tebuconazole degradation products produced by the yeast and the bacteria.

Consequently, *in vitro* alcoholic and malolactic fermentation assays were carried out employing liquid synthetic media with a simpler composition than must and wine, but reproducing their main characteristics (sugars, ethanol and malic acid concentration, and pH) and enriched in tebuconazole to evaluate the effect of the fungicide on the main fermentation parameters.

3.3.1. Effect of tebuconazole on alcoholic fermentation

To evaluate whether the presence of tebuconazole can negatively affect yeast growth and alcoholic fermentation of the must by *S. cerevisiae*, experiments in the synthetic medium described in Materials and Methods were carried out using the maximum residue limit of tebuconazole in grapes for vinification (MRL, 2.0 mg/kg). Evolution of sugars consumption, yeast growth and ethanol production, with and without presence of tebuconazole residues, is shown in Fig. 2.

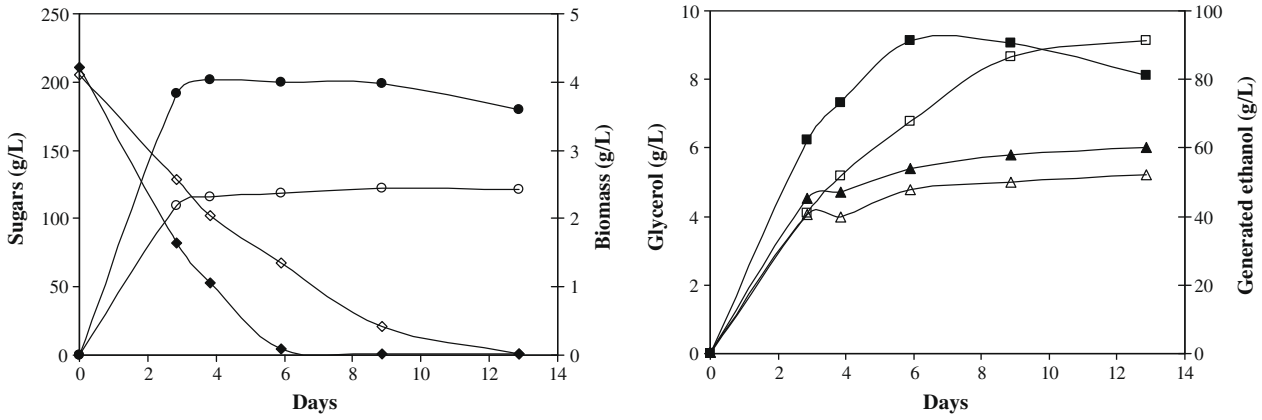

Fig. 2. Time course of sugars consumption (◇, ♦) and biomass (○, ●), glycerol (△, ▲), and ethanol (□, ■) production during alcoholic fermentation of a synthetic medium with *Saccharomyces cerevisiae*, in presence and absence of tebuconazole residues, respectively.

The presence of tebuconazole in the culture medium at a concentration of 2 mg/L caused a decrease of the sugar consumption rate and, consequently, a slower growth and ethanol production rate. Moreover, the maximum biomass reached was clearly lower in the series incubated with tebuconazole, all of that showing the toxic effect of this fungicide for the yeast. These results are in accordance with the general inhibitory effect of azole compounds on the transformation of lanosterol into ergosterol in the cell membranes of the yeasts (Ji et al., 2000). Glycerol production was not affected, while ethanol production was slower than in the control but reached a maximum concentration 4–5 days later that was comparable to the series without the fungicide. Although the final ethanol concentration was lower in the control, this can be explained by the earlier sugars depletion in this series, what causes the stop of the ethanol production and, consequently, a more evident effect of ethanol evaporation as well as the beginning of ethanol consumption as a new substrate.

According to these results the presence of tebuconazole at the concentration assayed has a toxic effect for *S. cerevisiae*, reducing the biomass and slowing the fermentation. Nevertheless, and for practical purposes, it is expected that during red winemaking the levels of soluble tebuconazole in the must are reduced by adsorption on grape solids to a concentration low enough to make these effects irrelevant, as it happened in this work.

There are some works showing the ability of *S. cerevisiae* cells to adsorb inorganic and organic contaminants as fungicides (Aksu & Dönmez, 2003; Gomes, Fragoso, Riger, Panek, & Eleutherio, 2002; Goyal, Jain, & Banerjee, 2003: Martín-Esteban, Fernández, & Cámara, 1997; Razmkhab et al., 2002), which is attributed to the cell wall polysaccharides (Ballou, 1988). To investigate if the tebuconazole concentration in the must during red winemaking is reduced not only by adsorption on grape solids but also by the yeast, pesticide residues were determined both in the fermentation medium (free tebuconazole) and in the yeasts (adsorbed tebuconazole) during the fermentative process (Table 3). In this case the levels of soluble tebuconazole were not modified during the fermentation and the amount of tebuconazole extracted from the cells was not significant. Moreover, degradation products of tebuconazole were not identified. These results indicate that yeast activity during fermentation did not induce any degradation of the pesticide during must fermentation.

3.3.2. Effect on malolactic fermentation

Several studies have directly or indirectly investigated the effect of pesticide residues on the rate of malolactic fermentation. In most cases, pesticide residues were found to have little or no effect on malolactic fermentation, and very few pesticides were degraded or adsorbed by the bacteria during this process (Bordons et al., 1998; Haag et al., 1988). In other cases, the fermentation activity of *O. oeni* was affected by the presence of certain pesticides, azoxystrobin, cyprodinil, fludioxonil, pyrimethanil, (Cabras et al., 1994; Oliva et al., 2007) and dicofol (Ruediger et al., 2005) with a lower degradation of malic acid; however, the amount of these pesticides was not affected significantly during MLF, except in the case of dicofol. For quinoxyfen (Cabras et al., 2000), mepanipyrim (Cabras et al., 1999), carbaryl, carbendazim, chlorothalonil, fenarimol, metalaxyl, oxadixyl, procymidone and triadimenol (Ruediger et al., 2005) no effect on the MLF was observed, and, as before, bacteria do not have any degradative effect on them.

To evaluate whether tebuconazole can negatively affect the malolactic fermentation of wine by *O. oeni* and be adsorbed or degraded by the microorganism, *in vitro* experiments with the wine synthetic medium described in Materials and Methods were carried out using a concentration of 0.6 mg/L of tebuconazole, 80% lower than the maximum residue limit in grapes according to the tebuconazole reduction after alcoholic fermentation in our field experiments. The evolution of malic and lactic acid, as well as tebuconazole residues were analysed during the incubation.

The results in Fig. 3 show that this concentration of tebuconazole did not affect significantly the conversion rate of malic to lactic acid, as well as the final concentration of both acids at the end of the fermentation with respect to the control. As reported by other

Table 3
Tebuconazole residues (mg/L ± SD) during alcoholic fermentation of *Saccharomyces cerevisiae* yeast determined by LC–MS/MS.

Sample	Residues after inoculation					
	0 days	3 days	4 days	6 days	9 days	13 days
Control	2.14 ± 0.04	1.89 ± 0.02	2.01 ± 0.10	1.92 ± 0.04	2.10 ± 0.07	2.01 ± 0.08
Fermentation medium	1.97 ± 0.10	1.97 ± 0.01	1.82 ± 0.13	1.71 ± 0.21	1.92 ± 0.08	1.90 ± 0.05
Yeast cells	n.d.	0.04 ± 0.00	0.05 ± 0.01	0.04 ± 0.00	0.07 ± 0.00	0.07 ± 0.00

n.d.: not detected.

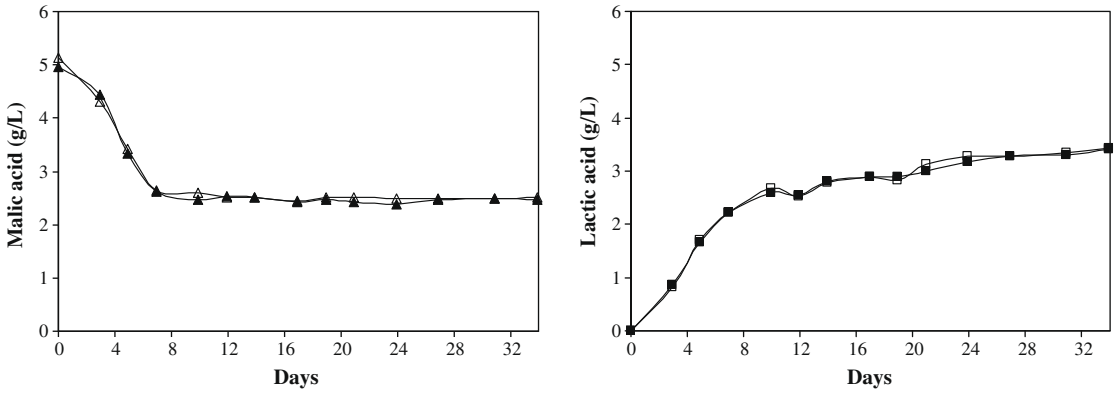

Fig. 3. Time course of malic acid (△, ▲) and generated lactic acid (□, ■) during malolactic fermentation of *Oenococcus oeni* bacteria, in presence and no presence of tebuconazole residues, respectively.

authors (Cabras et al., 1999), there is a remnant malic acid concentration that was not degraded, what is probably due to the lost of cell viability after a long period of exposure to ethanol and lactic acid. Finally, the levels of tebuconazole remained constant during the MLF and no degradation products were detected, what allows concluding that this bacterium does not have any adsorptive or degradative effect on it at the fungicide concentration assayed.

4. Conclusions

The winemaking process followed in A.O.C. Valdeorras for producing red wines allowed to decrease the tebuconazole residues of the initial musts. Tebuconazole presented high affinity to be adsorbed on the suspended solid matter. The eliminated percentage of tebuconazole in the final wine is 86%. In future, according these results, MRL for tebuconazole in red wines could be established as a eight times lower (0.25 mg/L) than MRL established in wine grapes (2 mg/kg). Results of *in vitro* assays showed that the presence of tebuconazole did not affect alcoholic nor malolactic fermentations. At the same time, these two fermentative processes did not affect the amount of tebuconazole either by degradation or by adsorption.

Acknowledgements

This work was funded by the AGL2007–62075 (Spanish Ministry of Education and Science) and PGIDIT07TAL016383PR (Galician Council of Innovation and Industry) contracts. R.M. González-Rodríguez acknowledges Xunta de Galicia for her contracts sponsorship through the Maria Barbeito program. A. Torrado Agrasar also acknowledges Xunta de Galicia for her contracts sponsorship through a Parga Pondal research program.

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Food Chemistry 117 (2009) 538–544

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Phytochemicals and antioxidant properties of solvent extracts from *Japonica* rice bran

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ARTICLE INFO

Article history:
Received 6 August 2008
Received in revised form 20 March 2009
Accepted 12 April 2009

Keywords:
Rice bran
Phytochemicals
Antioxidant property
Methanol
Ethyl acetate
Hexane

ABSTRACT

The yield, major phytochemicals (oryzanols, tocopherols (T), and tocotrienols (T3)) and antioxidant properties of *Japonica* rice bran extracts were investigated, for illustrating the major effects from solvent property. Generally, the extract yield varied with the solvent used in the order of methanol (MeOH) > ethyl acetate (EtOAc) > hexane. In contrast to hexane extracts, both MeOH and EtOAc extracts exhibited a higher total content in phenolic compounds (~2.5 g gallic acid equivalent/kg bran), oryzanols (1.6–1.8 g/kg bran), or tocols (126–130 mg/kg bran), and a higher T3% in tocols (24–26%). The MeOH extract (at 1 mg/ml) showed the greatest capability in inhibiting linoleic acid peroxidation (57%), scavenging DPPH radicals (93%), reducing power (78%), and Fe^{2+} chelating activity (~1300 μg EDTA equivalent/g) than the other two extracts, partly attributed to its high antioxidant contents. It is newly found that the yield, total content in phenolic compounds, oryzanols, and tocols, and T3% in tocols of the extracts increased with increasing Synder's polarity value in a quite good linear manner (R^2 = 0.923–1.000), associated with an increased solvent viscosity. This clearly suggests the potential of using Synder's polarity value as an index in isolation of desired rice bran phytochemical extracts.

1. Introduction

Rice bran contains a significant amount of natural phytochemicals such as oryzanols, tocopherols and tocotrienols that have been reported as the strongest antioxidants in rice bran (Godber & Juliano, 2004; Godber & Wells, 1994; Orthoefer & Eastman, 2004). The tocopherols and tocotrienols can be used as radical scavengers, terminating the propagation of radical chain reactions by reacting with peroxyl radicals and generating unreactive phenoxyl radicals as well as hydroperoxide products (Kitts, 1997). Another research discovered that rice bran contains a phenolic content (2.51–3.59 mg/g) higher than wheat bran (Iqbal, Bhanger, & Anwar, 2005). The phenolic compounds show higher antioxidant activities, in contrast to tocopherols (Chen & Ho, 1997; Ohnishi et al., 1994). In addition to lipophilic antioxidant activity, tocotrienols have shown peculiar physiological potential including antitumor properties toward mammary cancer (Guthrie, Gapor, Chambers, & Carroll, 1997; Nasaretnam, 2005), reducing serum cholesterol effects (Qureshi, Mo, Packer, & Peterson, 2000), and anti-inflammation (Akihisa et al., 2000).

Recent studies on the phytochemicals and antioxidant properties of rice bran involve using various solvents such as hexane (Chen & Bergman, 2005; Hu, Wells, Shin, & Godber, 1996; Kim, 2005; Lloyd, Siebenmorgen, & Beers, 2000; Rohrer & Siebenmorgen, 2004), methanol (Aguilar-Garcia, Gavino, Baragaño-Mosqueda, Hevia, & Gavino, 2007; Chotimarkorn, Benjakul, & Silalai, 2008; Renuka Devi & Arumughan, 2007), acidic 80% methanol (Iqbal et al., 2005; Shen, Jin, Xiao, Lu, & Bao, 2009), isopropanol (Hu et al., 1996), and ethyl acetate (Renuka Devi & Arumughan, 2007). However, there have been few investigations clarifying the dependence of rice bran antioxidant compositions on solvent property during extraction. Accordingly, this study was to elucidate the influence of solvent property on the extract yield, total phenolic content, oryzanol content and tocol compositions of the extracts with methanol, ethyl acetate and hexane. The antioxidant activity of each extract was also analyzed, in relation to the phytochemical compositions.

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doi:10.1016/j.foodchem.2009.04.031

2. Materials and methods

2.1. Rice material

Rice bran of TaiKung 9 (TK9), a *Japonica* rice cultivar hybrid from Japanese Koshihikari cultivar and popular as cooked rice in Taiwan, was collected during milling brown rice to polished rice with a degree of milling of 88% in Hung-Chang Rice Mill Factory, Tainan, Taiwan.

2.2. Stabilisation of rice bran

Rice bran stabilisation was carried out in a microwave oven, following the method of Malekian et al. (2000). Rice bran (100 g) packed in a polyethylene microwave-safe bag was heated in a preheated (120 °C, 3 min) microwave oven (2450 MHz, output power 550 W), followed by cooling at room temperature overnight. The heating–cooling process was repeated twice to ensure complete inactivation of endogenous lipase. The free fatty acid content of the stabilised rice bran was low (3.1–3.6% on dry bran basis), in contrast to the unstabilised bran (3.8% increasing to 18.4%), during 6-week storage at room temperature, as detected with the *m*-cresol purple titration method (Hoffpauir, Petty, & Guthrie, 1947). The stabilised bran was immediately used for solvent extraction.

2.3. Extraction of rice bran phytocompounds

Ten grams of stabilised rice bran were extracted with 100 ml of methanol (MeOH), ethyl acetate (EtOAc) or *n*-hexane at room temperature for 3 h under gentle stirring, followed by filtering through Whatman No.1 filter paper. The residue was re-extracted twice. The filtrates were then collected, vacuum dried in a rotary evaporator (50 °C), and weighed immediately. The extraction procedure was done in three replications for each of the extracts. The extraction yield was expressed as the weight percentage on dry rice bran basis. All extracts were stored in a sealed container at 4 °C for further analysis.

2.4. Total phenolic content measurement

The total phenolic content of the extract was measured with the Folin–Ciocalteau method as described by Taga, Miller, and Pratt (1984), using gallic acid as a standard. A 0.1 ml of the extract solution (1.0 mg/ml DMSO) was sampled into 2 ml of 2% Na_2CO_3 and mixed for 3 min. After adding 0.1 ml of 50% Folin–Ciocalteau reagent, the final mixture was left for 30 min before reading the absorbance at 750 nm (Hitachi U-2000, Tokyo, Japan). All measurements were conducted in triplicate and the data were expressed as g gallic acid equivalent (GAE) per kg of the extract, based on the calibration curve of gallic acid.

2.5. Oryzanol content determination

The content of oryzanols was detected on a reverse-phase HPLC system consisting of a Hitachi L-7100 pump, Hitachi L-7200 autosampler, Hitachi L-7420 UV detector, Hitachi D-7500 integrator (Tokyo, Japan), and C18 column (Nova Pak C18, 150 × 3.9 mm, Waters, Alliance Milford, MA). The mobile phase was the mixture of methanol, acetonitrile, dichloromethane and acetic acid (50:44:3:3 by volume) and eluted at a flow rate of 1.4 ml/min at ambient temperature (McBride & Evans, 1973). The detector wavelength was set at 330 nm. The injection volume for the sample was 50 μl. The data were produced in three replicates and in reference to the peak area-concentration calibration curve of oryzanol standards (Wako Pure Chemical Industries Ltd., Osaka, Japan).

2.6. Tocopherol and tocotrienol quantification

The reverse-phase HPLC conditions for quantifying tocopherols and tocotrienols were similar to those for the oryzanol content mentioned above, except that a Hitachi L-7480 fluorescence detector (Ex = 298 nm; Em = 328 nm), gradient mobile phase, and flow rate of 0.8 ml/min were applied instead. According to Rogers et al. (1993), the mobile phase acetonitrile–methanol–isopropanol–water mixture was initiated at 45:45:5:5 (by volume), programmed linearly to 50:45:5:0 for the first 10 min, and then maintained for a further 15 min before being returning to the initial condition. Total tocopherol or tocotrienol content was determined as the summation of their isomers (α, β, γ and δ) quantified from the peak area-concentration calibration curves of corresponding tocopherol or tocotrienol standards (Sigma Chemical Co., St Louis, MO and ChromaDex , Santa Ana, CA, USA, respectively), where β and γ isomers coeluted on a C18 column.

2.7. Inhibition on linoleic acid peroxidation

Following the suggestions of Mitsuda, Yasumoto, and Iwami (1966), rice bran extract (0.5 mg) dissolved in 0.5 ml of DMSO was mixed with linoleic acid (LA) emulsion (2.5 ml, 0.02 M, pH 7.0) and phosphate buffer (2.0 ml, 0.2 M, pH 7.0) in a test tube. The mixed solution was then incubated at 37 °C for 72 h for completing colour development rising from $FeCl_2$–thiocyanate interaction. The absorbance at 500 nm ($A_{500\ nm}$), an index of the peroxide value, of the resultant solution was then checked (Hitachi U-2000, Tokyo, Japan). Butylated hydroxyl anisole (BHA) was examined for reference. All data were means of triplicate analyses. The percentage of LA peroxidation inhibition was calculated as Eq. (1):

$$\text{Inhibition on LA peroxidation } (\%) = \left[1 - \left(A_{500\ nm,sample}/A_{500\ nm,control}\right)\right] \times 100 \quad (1)$$

2.8. Scavenging effect on DPPH radical

DPPH radical scavenging activity was determined using the method originally developed by Blois (1958). A portion (0.1 ml) of the extract solution (1.0 mg/ml DMSO) in a test tube was well mixed with 3.9 ml of methanol and 1.0 ml of α,α-diphenyl-β-picrylhydrazyl (DPPH) solution (1.0 mM in methanol). The mixture was kept at ambient temperature for 30 min prior to measurement of the absorbance at 517 nm ($A_{517\ nm}$). BHA was used as the reference. All measurements were done in triplicate. The scavenging effect was derived following Eq. (2):

$$\text{DPPH scavenging effect } (\%) = \left[1 - \left(A_{517\ nm,sample}/A_{517\ nm,control}\right)\right] \times 100 \quad (2)$$

2.9. Reducing power determination

According to the method of Oyaizu (1986), 5.0 ml of the extract solution (1.0 mg/ml DMSO) was mixed with 5.0 ml of phosphate buffer (0.2 M, pH 6.6) and 5.0 ml of 1.0% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min, followed by adding 5.0 ml 10% trichloroacetic acid and centrifugation. A portion of the supernatant (5.0 ml) was mixed with 5.0 ml water and 1.0 ml 1% ferric chloride. The absorbance at 700 nm ($A_{700\ nm}$), indicative of the reducing power, was recorded on the final mixture. All data were run in triplicate and compared with BHA reference.

2.10. Metal chelating activity

The method of Decker and Welch (1990) was applied with modifications in preparation of the sample solution. A portion of the extract solution (1 mg/ml DMSO, 0.2 ml) was added to a solution of 2 mM $FeCl_2$ (0.1 ml), followed by adding 5 mM ferrozine (0.2 ml). The mixture was stirred vigorously and left standing at room temperature for 10 min for equilibrium, and finally measured its absorbance at 562 nm ($A_{562\ nm}$) indicating the level of ferrozine–Fe^{2+} complex. All tests were run in triplicate. The metal chelating activity was calculated according to Eq. (3), using disodium ethylenediaminetetracetate (Na_2EDTA) as standard and expressing as EDTA equivalent (EDTAE, ug/g).

$$\text{Metal chelating activity } (\%) = \left[1 - \left(A_{562\ nm,sample}/A_{562\ nm,control}\right)\right] \times 100 \quad (3)$$

2.11. Statistical analyses

Analysis of variance (ANOVA) and Duncan's multiple range tests among data were carried out using the Statistical Analysis System software (v 8.2, SAS Institute Inc., Cary, NC).

3. Results and discussion

3.1. Yield and phytochemical profile of rice bran extracts

Table 1 indicates that the yield of the rice bran extracts significantly varied with the solvents applied, in the order of MeOH (16.3%) > EtOAc (12.9%) > hexane (7.85%). For the extract compositions, the total phenolic content was significantly greater for the EtOAc extract (~20 g GAE/kg extract) than for the MeOH and hexane (~15 g GAE/kg extract). The oryzanol content was similar for both EtOAc and hexane extracts (13–14 g/kg extract), higher than the MeOH (9.8 g/kg extract). Both total tocopherol (T) and tocotrienol (T3) contents in the extracts were EtOAc > MeOH >> hexane and predominated with β + γ-isomers rather than α- and δ-isomers, regardless of the solvents used. However, if the extraction efficiency on a rice bran weight basis (data in parenthesis) was concerned, both MeOH and EtOAc exhibited comparably high extraction efficiencies in total phenolic substances (~2.5 g GAE/kg bran), oryzanols (1.6–1.8 g/kg bran), and tocols (126–130 mg/kg bran; 93–99 and 31–33 mg/kg bran for T and T3, respectively), in contrast to hexane.

Generally, the yield of MeOH extract in this study was similar to the reported values (~16%) for the MeOH extract at RT for 3 days (Akihisa et al., 2000) and Drew rice bran hexane extract using an accelerated solvent extractor (10.3 MPa, 50 °C, 30 min) (Rohrer & Siebenmorgen, 2004). It was 2-folds of the hexane extract at ambient temperature (this study) but is lower than those (17–21%) of hexane or isopropanol extracts at an elevated temperature (40–60 °C) (Hu et al., 1996; Kim, 2005; Shin & Godber, 1996). These results are higher by 12–17% than those for defatted rice bran (Renuka Devi & Arumughan, 2007).

The total phenolic contents for TK9 rice bran extracts (1.2–2.5 g GAE/kg bran) are close to that reported for Thailand long-grain (LG) rice bran with MeOH at RT for 12 h (2.2–3.2 g GAE/kg bran) (Chotimarkorn et al., 2008) and the rice flour extracts from 481 China rice cultivars with acidic MeOH at RT for 24 h (1.1–2.5 g GAE/kg flour) (Shen et al., 2009). They are in the range between those reported for Pakistan rice bran extract with acidic 80% MeOH at RT for 3 h (2.5–3.6 g GAE/kg bran) (Iqbal et al., 2005) and Venezuelan rice bran extract with MeOH at RT for 3 min (1.1–1.2 g GAE/kg bran) (Aguilar-Garcia et al., 2007). These results are certainly lower than the pigment rice bran extracts with MeOH at RT for 17 h (8–45 g GAE/kg bran) (Goffman & Bergman, 2004).

3.2. Antioxidant activity of rice bran extracts

Fig. 1 demonstrates that, at the concentration examined (1.0 mg/ml), a general tendency of MeOH extract > EtOAc extract > hexane extract was found for inhibition on linoleic acid (LA) peroxidation (Fig. 1A), DPPH scavenging activity (Fig. 1B), reducing power (Fig. 1C), and metal (Fe^{2+}) chelating activity (Fig. 1D), where the MeOH and EtOAc extracts displayed statistically similar inhibition capability on LA peroxidation. The antioxidant activity of the MeOH extract was the most promising among the extracts examined, accounting for ~57%, 93%, and 78% of BHA in inhibition on LA peroxidation, DPPH scavenging activity, and reducing power, respectively.

The reason for that MeOH extract had superior antioxidant activities to the EtOAc, which exhibited a comparatively greater amount of the antioxidant compositions, may be explained by the possibility of more polar phenolic compounds and lipids in the MeOH extract than in the EtOAc. Chlorogenic acid (Ohnishi et al., 1994), caffeic acid, hydroxycinnamic acid (Chen & Ho, 1997), and anthocyanes (Iqbal et al., 2005) may also contribute to the total polyphenol content and antioxidant activities. Besides, α-tocopherol has been found to show a DPPH radical scavenging

Table 1
Yields and antioxidant compositions of the extracts from TK9 rice bran with various solvents.

Composition[A]	Type (unit)	MeOH	EtOAc	Hexane
Extract yield	(%, w/w)	$16.27 \pm 1.55^{a,B}$	12.91 ± 1.33^{b}	7.85 ± 0.88^{c}
Total phenolic content	(g GAE/kg)	15.7 ± 0.6^{b} (2.55 ± 0.10^{a})[C]	19.7 ± 0.8^{a} (2.54 ± 0.10^{a})	14.7 ± 1.2^{b} (1.15 ± 0.09^{b})
Oryzanol	(g/kg)	9.8 ± 0.4^{b} (1.60 ± 0.07^{a})	13.8 ± 0.9^{a} (1.78 ± 0.11^{a})	13.1 ± 0.5^{a} (1.03 ± 0.04^{b})
Tocopherol (T)	α (mg/kg)	46 ± 9^{a}	48 ± 4^{a}	18 ± 6^{b}
	β + γ (mg/kg)	509 ± 10^{b}	662 ± 24^{a}	115 ± 23^{c}
	δ (mg/kg)	18 ± 0^{b}	60 ± 2^{a}	63 ± 5^{a}
	Total T (mg/kg)[D]	573 ± 13^{b} (93 ± 9^{a})	770 ± 24^{a} (99 ± 10^{a})	196 ± 24^{c} (15 ± 2^{b})
Tocotrienol (T3)	α (mg/kg)	26 ± 4^{b}	83 ± 4^{a}	ND[E]
	β + γ (mg/kg)	151 ± 3^{a}	136 ± 5^{b}	16 ± 1^{c}
	δ (mg/kg)	25 ± 1^{a}	18 ± 1^{b}	1 ± 0^{c}
	Total T3 (mg/kg)[D]	202 ± 5^{b} (33 ± 3^{a})	237 ± 6^{a} (31 ± 3^{a})	17 ± 1^{c} (1 ± 0^{b})
Total tocols (T + T3)	(mg/kg)	775 ± 14^{b} (126 ± 9^{a})	1007 ± 25^{a} (130 ± 10^{a})	213 ± 24^{c} (16 ± 2^{b})

[A] Extract yields are on a dry weight basis of rice bran; all chemical compositions except in parenthesis are on a dry weight basis of the extract.
[B] Means ± standard deviations ($n = 3$); means followed by different letters in the same row differ significantly ($P < 0.05$).
[C] Data in parentheses are on a dry weight basis of rice bran.
[D] Sum of α, β, γ, and δ-isomer contents.
[E] Not detectable.

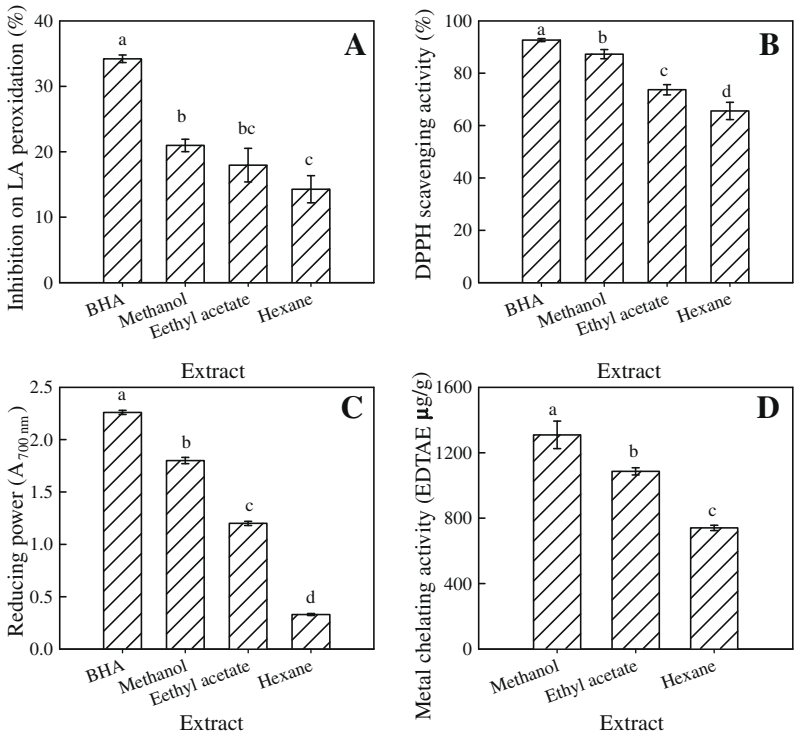

Fig. 1. Antioxidant activities of rice bran extracts (A: inhibition on linoleic acid peroxidation; B: DPPH radical scavenging activity; C: reducing power; D: metal chelating activity). Values are expressed as means of three replicates ± standard deviations; means indicated with different letters differ significantly.

capability high up to ~7-folds of those does γ-oryzanol (Juliano, Cossu, Alamanni, & Piu, 2005). However, a great antioxidant activity for oryzanol as 10-folds of tocopherols in different biological systems is also reported (Abdel-Aal & Hucl, 1999). Comparatively, the DPPH radical scavenging ability of the MeOH in this study is higher than the MeOH extract from oat groat (Sun, Xu, Godber, & Prinyawiwatkul, 2006). The Fe^{2+} chelating abilities (0.06–0.21 EDTAE mg/g bran) of all extracts in this study are less than those of Pakistan rice bran extract with 80% MeOH (0.61–0.72 EDTAE mg/g bran) (Iqbal et al., 2005).

Table 2
Antioxidant compositions of rice bran extracts with respect to the rice source and extraction conditions reported.

No	Rice bran source[a]	Extractioncondition	Oryzanol	Tocol composition				Reference
			Content (g/kg bran)	Total (mg/kg bran)	T%	T isomer α:(β+γ):δ	T3 isomer α:(β+γ):δ	
1	Taiwan *Japonica* rice (*n* = 1)	MeOH, RT, 3 h	1.6	126	74	8:89:3	13:75:12	This study
2	Venezuelan rice (*n* = 3)	MeOH, RT, 3 min	1.6–2.7	196–219	21–28	56:44:0–0:100:0(γ)[b]	0:89:11–15:81:4 (γ)[b]	Aguilar-Garcia et al. (2007)
3	Pakistan MG rice (*n* = 5)	80% MeOH (+0.15% HCl), RT, 3 h	0.5–0.8	756–990	51–55			Iqbal et al. (2005)
4	US rice (*n* = 4, MG & LG)	MeOH, RT, 3 min	4–5	329–392	33–38	68:32:0 – 78:22:0	24:76:0–30:70:0	Chen and Bergman (2005)
5	Thailand LG rice (*n* = 5)	MeOH, RT, 12 h	0.6–1.1	760–1230	46–73	34:57:9–49:48:3(β)[c]	35:65:0–51:49:0 (β)[c]	Chotimarkorn et al. (2008)
6	Taiwan *Japonica* rice (*n* = 1)	Hexane, RT, 3 h	1.0	17	92	9:59:32	0:94:6	This study
7	US rice (*n* = 2, LG & MG)	Hexane, 60 °C, 30 min	2.6–6.4	69–117	74–83			Lloyd et al. (2000)
8	Southern US rice (*n* = 7, LG & MG)	Hexane, 60 °C, 30 min	2.5–6.9	179–389	28[d]	78:22:0(γ)[bd]	54:46:0 (γ)[bd]	Bergman and Xu (2003)
9	US LG rice (*n* = 2)	Hexane, 10.3 MPa, 50 °C, 30 min	10–12[e]	1001–1017[e]	25–45[e]			Rohrer and Siebenmorgen (2004)
10	South Korea rice (*n* = 1)	Hexane, RT, several min x3[f]		287	28	63:39:0(γ)[b]	44:55:1 (γ)[b]	Kim (2005)
11	Rice (*n* = 1)	Hexane		271	39	59:39:2(γ)[c]	23:73:4 (γ)[b]	Shin and Godber (1996)

[a] *n* = sampling number; LG = long-grain, MG = medium grain.
[b] Only γ- (no β-) isomer present is indicated in parenthesis.
[c] Major isomers in β + γ mixture are indicated in parenthesis.
[d] Average data of the seven samples because no individual data are listed in the report.
[e] HPLC examination on the supernatant soluble in acetonitrile:methanol (75:25) mixture directly after methanol extraction.
[f] Composition analysis on the partially refined oil sample after saponification.

3.3. Factors influencing the phytochemical compositions of rice bran extracts

For the oryzanol and tocol compositions of rice bran, the data in this study and that reported for rice bran extracts are compared in Table 2, in order to understand the variations with rice source, extraction condition, and other factors to the extent as great as possible. Briefly, the MeOH extracts from rice cultivars of Taiwan (#1), Venezuelan (#2), Pakistan (#3) and Thailand (#5) showed a lower oryzanol content in rice bran than those of US rice (#4). This implies that the cultivation environment is important for the oryzanol content, as reported for southern US rice (Bergman & Xu, 2003), since many diverse rice cultivars (including MG and LG rice) and a wide extraction period (3 min–12 h) have been studied in these reports. Besides, the MeOH extracts possessed a diverse tocol content in the order of Pakistan MG rice (#3) and Thailand LG rice (#5) (756–1230 mg/kg bran) > US LG and MG rice (#4) > Venezuelan rice (#2) > Taiwan rice (#1) and T% in tocols varying from 21% to 28% (#2) to >70% (#1 and parts of #5).

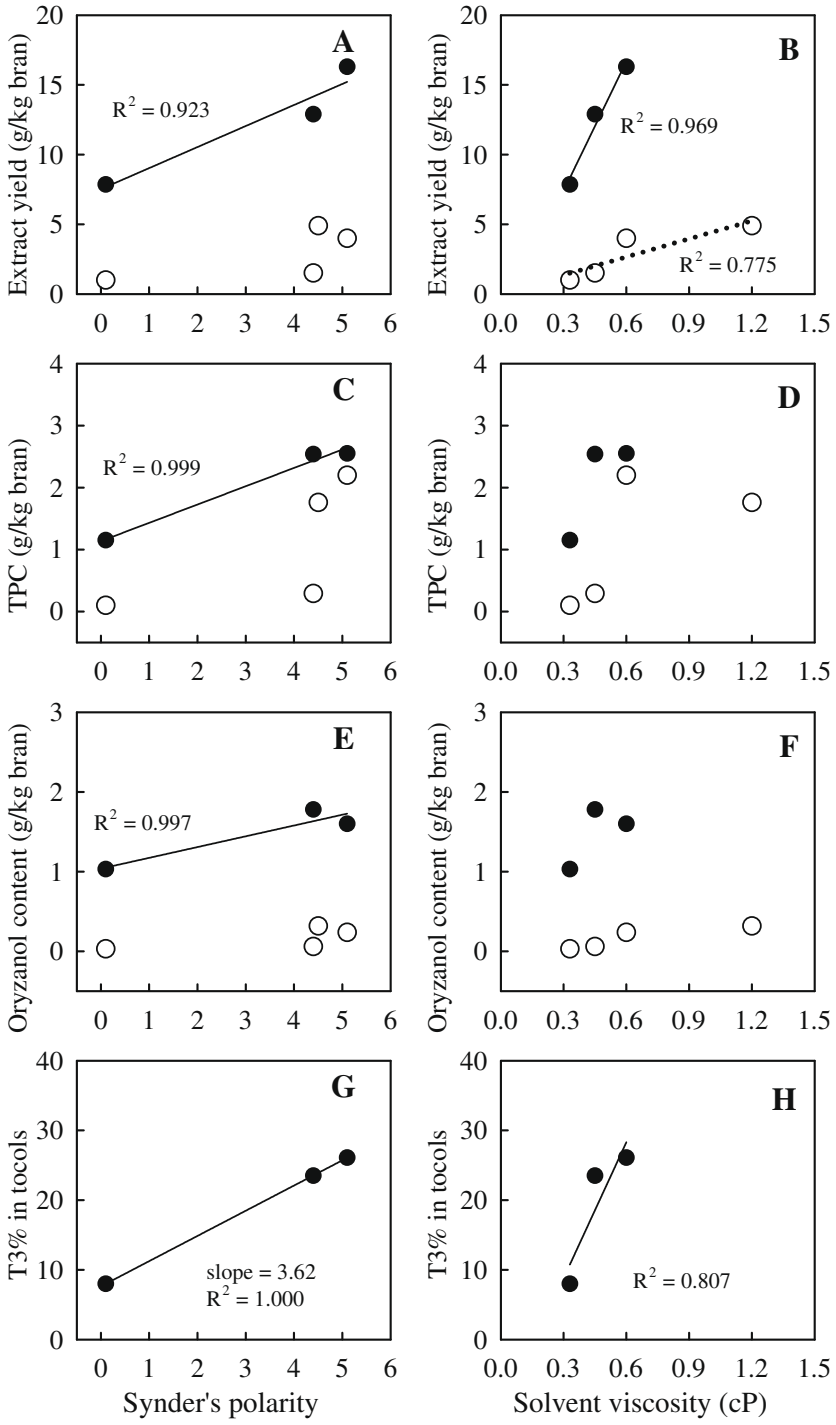

Fig. 2. Effects of solvent polarity and viscosity on the yield, total phenolic content (TPC), oryzanol content and tocotrienol (T3) percentage in tocols of TK9 rice bran extracts (filled circle), in comparison to defatted rice bran extracts (Renuka Devi and Arumughan, 2007) (open circle). R^2 values are the regression coefficients by the least-squared fits.

In contrast to the MeOH extract at RT (#1) (Table 2), applying hexane instead (#6) resulted in a noticeably reduced content in oryzanol and total tocols, lower (β + γ)-T% in T, but greater T% in total tocols and (β + γ)-T3 isomer% in T3. These differences between MeOH and hexane are hardly perceivable for the US rice (#7, #8 vs. #4) including the same rice cultivars (e.g. Cypress, Bengal, and La-Grue) since warm hexane (60 °C) was employed. Nonetheless, comparison of #9 with #8 and #4 (based on the same rice cultivar Cypress) reveals that pressurised extraction (#9) promotes the yield of oryzanols and total tocols by about 2–4 and 3–5-folds, respectively, of the traditional hexane extraction (#4 and #8).

Regarding the T and T3 isomer distribution, it is conclusive from Table 2 that those extracts that predominated with α-T isomer in T composition are those from US rice with either MeOH (#4) or hexane (#8), the partial refined oil of South Korea rice bran (#10), and that reported by Shin and Godber (1996) (#11). The extracts from Taiwan *Japonica* rice with MeOH (#1) or hexane (#6), most of Thailand LG rice cultivars (#5), and parts of Venezuelan rice (#2) are mainly with γ- or β-T isomers in T composition. As to T3 isomer distribution, γ-, or β-T3 isomers are generally the majority for most of the extracts studied, except some of Thailand LG rice (#5) and US rice (#8).

For taking insight into the effects of solvent property on the yields and phytochemical compositions of rice bran extracts, three kinds of solvent polarity scales defined by Synder, Hildebrand and Reichardt, dielectric constants (Berthod, 1994), and solvent viscosity (Seidel, 2006) were evaluated. Synder's polarity values resemble those reported by Seidel (2006) and are developed based on both the solubility parameter and the eluting power of the solvent in thin-layer chromatography (Berthod, 1994). It is encouraging to find that the yield and major phytocompound compositions of rice bran extracts related very closely with Synder's polarity value, partly associated with solvent viscosity (Fig. 2), where hexane, EtOAc, MeOH, and ethanol possess a Synder's polarity value of 0.1, 4.4, 5.1, and 4.5 (Berthod, 1994) and viscosity of 0.33, 0.45, 0.60, and 1.2 cP (Seidel, 2006), respectively. Ethanol was compared herein for the reported defatted rice bran extracts using four solvents including ethanol (Renuka Devi & Arumughan, 2007). When on a rice bran basis, the extract yield (Fig. 2A), total phenolic content (TPC) (Fig. 2C), oryzanol content (Fig. 2E), and T3% in tocols (Fig. 2G) of the rice bran extracts in this study increased with increasing Synder's polarity value in a very good linearity (R^2 = 0.923–1.000), accompanying with a positive dependence on solvent viscosity especially for the yield and T3% in tocols (R^2 = 0.969 and 0.807, respectively). The variations for total tocol content (data not shown) were similar to those of oryzanol. The yield, TPC and oryzanol content for the extracts from defatted rice bran (Renuka Devi & Arumughan, 2007) also tended to increase with increasing Synder's polarity value and solvents viscosity, even that rice bran has been previously defatted and subject to Soxhlet extraction at an elevated temperature.

Generally, hexane is traditionally preferred for rice bran oil extraction for cost consideration (Orthoefer & Eastman, 2004). However, it is a worse solvent than MeOH, EtOAc, ethanol (in this study; Renuka Devi & Arumughan, 2007) and isopropanol (Chen & Bergman, 2005; Hu et al., 1996) for extracting antioxidative phytocompounds from rice bran. This can be attributed to the amphipathic characteristics of tocopherols, tocotrienols, or oryzanols, involving the methyl groups on a chroman ring and the hydroxyl groups on an aromatic ring of vitamin E homologs, or the carboxylic group on a ferulated moiety, long side chain, and methyl groups on a hydropyrano moiety and sterol of oryzanols (Chen & Bergman, 2005; Godber & Juliano, 2004). In addition to the extraction solvent, the other factors influencing the product composition and antioxidant activity may include stabilisation temperature for raw rice bran (Shin & Godber, 1996), oil refining treatment (Orthoefer & Eastman, 2004; Rogers et al., 1993), rice cultivars and milling time (Chen & Bergman, 2005; Godber & Juliano, 2004; Lloyd et al., 2000), and cultivated environment for rice (Bergman & Xu, 2003).

4. Conclusions

Extraction of antioxidant phytocompounds from non-defatted rice bran with MeOH and EtOAc produced the extracts of a significantly greater yield and total content in phenolic compounds, oryzanols, or tocols (either tocopherols or tocotrienols) than did those with hexane. The composition variations in the extracts could partly account for the high antioxidant activities of both MeOH and EtOAc extracts in inhibiting linoleic acid peroxidation, DPPH radical scavenging ability, reducing power, and metal chelating activity. Comparison with the data of rice bran antioxidant extracts from TK9 and the reported diverse rice cultivars illustrated some phytochemical differences between rice sources or cultivation environments, in addition to the effects of extraction conditions concerned. It is newly discovered that either extraction yield or the antioxidant compositions examined were basically interpretable by a linear function of Synder's polarity value of the solvents used. Further studies on the synergism of phenolic compounds or oryzanol and tocols are needed in the future, for developing rice bran phytocompounds with preferred antioxidant and physiological potential.

Acknowledgments

This work was financially supported by the National Science Council of Taiwan [NSC 94-2214-E-273-002]. The authors thank Hung-Chang Rice Mill Factory for providing rice bran samples.

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Food Chemistry 117 (2009) 545–552

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Multiresidue determination of tetracyclines, sulphonamides and chloramphenicol in bovine milk using HPLC-DAD

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ARTICLE INFO

Article history:
Received 27 August 2008
Received in revised form 27 January 2009
Accepted 12 April 2009

Keywords:
Antimicrobials
Tetracyclines
Sulphonamides
Chloramphenicol
HPLC
Milk

ABSTRACT

A high-performance liquid chromatographic method for the simultaneous determination of tetracycline, oxytetracycline, chlortetracycline, sulfamethazine, sulfaquinoxaline, sulfamethoxazole and chloramphenicol in milk has been developed. The determination of these antimicrobials was carried out using HPLC-DAD with a C_{18} hybrid column and gradient elution with a mobile phase composed of an aqueous phase of 0.075 mol L^{-1} sodium acetate, 0.035 mol L^{-1} calcium chloride and 0.025 mol L^{-1}sodium EDTA, pH 7 and an organic phase of methanol:acetonitrile, 75:25 v/v. Sample preparation involved protein precipitation followed by solid-phase extraction using a polymeric cartridge. The method was validated and applied for the analysis of pasteurised milk samples commercialised in Brazil. The limits of quantitation for all antimicrobials, with the exception of choramphenicol, were below the maximum residue limit, which indicates that the method is appropriate for the determination of these antimicrobials in milk.

1. Introduction

Antimicrobial agents are widely used in food of animal origin for therapy to treat diseases, to control and prevent infection and for growth promotion and production efficiency.

The presence of residues of antimicrobial agents in food of animal origin in excess of the established maximum residue limits (MRLs) indicates that good veterinary practices were not respected and may contribute to the generation of resistance to bacteria in humans; nevertheless, current evidence suggests that the risk is low. In milk, however, their presence may cause allergic reactions in sensitive individuals and may interfere with starter cultures for cheese and other dairy products (Schenk & Callery, 1998).

The sulphonamides, among them, sulfamethazine (SMZ), sulfaquinoxaline (SQX) and sulfamethoxazole (SMX) play an important role as effective chemotherapeutics of bacterial and protozoal diseases in veterinary medicine. The Committee for Veterinary Medicinal Products considers that a tissue MRL of 100 μg kg^{-1} of the original drug substance should be applied to all substances of the sulphonamide group. In addition, the combined residues of all sulphonamides in bovine milk should not exceed 100 μg kg^{-1} (EMEA, 2007).

The Joint FAO/WHO Expert Committee of Food Additives and Contaminants (JECFA), at its 50th Meeting in 1998, established a group acceptable daily intake (ADI) of 0–0.03 mg kg^{-1} body weight for the tetracyclines (oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC)), alone or in combination. The committee also recommended MRLs in milk of 100 μg L^{-1}. Tetracyclines, in particular chlortetracycline, have been routinely employed to prevent and treat mastitis in lactating dairy cows (JECFA, 1998).

Chloramphenicol (CLP) is a broad-spectrum antibiotic that is an effective therapeutic agent for the treatment of mastitis in cattle. Historically, epidemiological data suggested that the treatment of humans with chloramphenicol was associated with induction of blood dyscrasias. Due to the potential risk to human health posed by its residues in food the use of CLP is prohibited in food-producing animals destined for the human consumption in many countries, among them, the USA, Australia, Europe and Brazil. Due to the lack of toxicological data available, no ADI was established for CLP and, in consequence, no MRL could be attributed (JECFA, 2004).

In many regions of the world antimicrobial drug residues are known to be present in food (Ekuttan, Kang'ethe, Kimani, & Randolph, 2007; Helena, Zdenka, Ksenija, Andrej, & Vesna, 2007; Husam et al., 2007). Lack of a control can be attributed to the costs of analyses. In many instances this is beyond the reach of most low income countries. Helena et al. (2007), reported the presence of CLP in some samples of raw milk taken in the autumn of 2002 in Slovenia. Studies conducted in Kenya showed samples of milk to contain tetracyclines at levels exceeding the established MRL (Ekuttan, Kang'ethe, Kimani, & Randolph, 2007).

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.04.032

The governmental institution responsible for public health in Brazil (ANVISA, National Agency of Sanitary Surveillance) reported for the period 2004/2005 that 7% of the milk samples analyzed (312) showed to be positive in the screening test for the presence of tetracyclines, however none of them was confirmed by a chromatographic method. In addition, 7% of 306 milk samples analyzed indicated the presence of CLP, however the confirmatory test was not implemented at this time (ANVISA, 2006).

Thus, there is a need for countries to establish surveillance systems that allow for obtaining reliable data on veterinary antimicrobial usage. Therefore, it is necessary to establish simple, sensible and reliable analytical methods for the determination of multiresidues of different classes of antimicrobials in food and, in particular in milk.

The structures of the sulphonamides, tetracyclines and chloramphenicol used in veterinary medicine and evaluated in this work are shown in Fig. 1.

While rapid screening methods (immunological or microbial inhibition assays) are commonly used to detect the presence of antimicrobials in food, more accurate chromatographic methods are required by the governmental regulatory agencies to identify and confirm the presence of these compounds. Milk is consumed all over the world and its quality control is important to guarantee food safety.

Chromatographic methods require elaborate sample preparation procedures before quantitation in order to eliminate interferents from the food matrix and concentrate the analyte. The extent of sample preparation depends on the detection device of the chromatographic system. Conventional sample treatment protocols involve protein precipitation, centrifugation and analyte extraction, followed by clean-up of the extract over solid-phase cartridges and eluate concentration under a nitrogen flow. Alternatively, liquid–liquid extraction, combined or not with solid-phase extraction and matrix solid-phase extraction has been employed (Andersen et al., 2005; Bogialli et al., 2005; Furusawa, 2003; Schenk & Callery, 1998).

Many liquid chromatographic methods have been published for the determination of tetracyclines (Oka, Ito, & Matsumoto, 2000; Andersen et al., 2005), sulphonamides (Furusawa, 2000; Van Rhijn, Lasaroms, Berendsen, & Brinkman, 2002) and chloramphenicol (Long et al., 1999; Perez et al., 2002) in milk. However, almost all of them aim at the determination of antimicrobials of a single group. Simultaneous determination of multiresidues of antimicrobials of different groups has been carried out using liquid chromatography coupled to tandem mass spectrometry (Koesukwiwat, Jayanta, & Leepipatpiboon, 2007). Samanidou and Nisyriou (2008) presented a review of the state of the art in the analytical strategies concerning the multiresidue analysis of antibiotics in milk.

This paper focuses on the development and validation of a simple HPLC-DAD method for the simultaneous multiresidue determination of TC, CTC, OTC and SMZ, SQX, SMX and CLP in milk which could be applied to quality control in the routine analysis.

2. Materials and methods

2.1. Equipment

The HPLC-DAD measurements were carried out using a binary gradient chromatographic system from Waters (USA), model 1525, coupled to a Waters photodiode array detector (PDA) model 2996 and a Rheodyne injector, model 7725 (sample loop of 50 μL). Data acquisitions were performed by the Millenium^{32} 4.0 software.

For the solid-phase extraction procedures a 12 port vacuum manifold from Alltech (USA) was employed.

Measurements of pH were made with a DM-20 pH-meter from Digimed (Brazil), using a combined glass electrode.

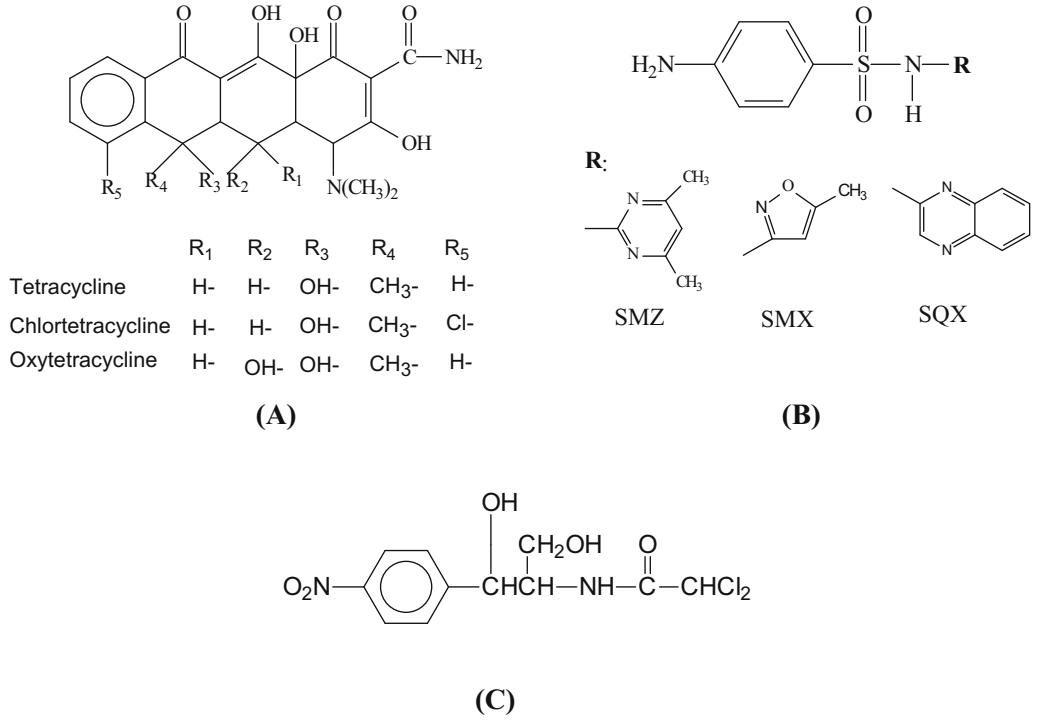

Fig. 1. Structures of (A) tetracyclines, (B) sulphonamides and (C) chloramphenicol.

2.2. Chromatographic columns and solid-phase extraction cartridges

The separation of the antimicrobials by HPLC was evaluated on the following chromatographic columns: (i) LiChrocart®, 125 x 4.6 mm, containing 5 μm LiChrospher® from Merck (Germany) with a guard column (6.0 x 4.0 mm, 5 μm) of the same stationary phase; (ii) X-Terra® C_{18} hybrid silica column (250 mm × 4.6 mm, 5 μm) from Waters (USA) with a guard column (4.0 x 4.6 mm, 5 μm) of the same stationary phase.

The solid-phase extraction procedures were carried out using endcapped octadecyl silica cartridges (500 mg, 3 mL, Waters, EUA) and polymeric cartridges (200 mg, 6 mL, OASIS HLB, Waters, EUA).

2.3. Standards and reagents

Tetracycline hydrochloride (92.6%) was purchased from Sigma (USA), while oxytetracycline dihydrate and chlortetracycline hydrochloride (99.8%) were purchased from ICN Biomedical Inc. (USA). Sulfamethazine (99%), sulfaquinoxaline (95%) and sulfamethoxazole (99.7%) were purchased from Sigma (USA) and chloramphenicol (99%) was purchased from ICN Biomedical Inc. (USA). Enrofloxacin (99.7%) was purchased from Fluka (Switzerland).

Analytical grade disodium hydrogenphosphate (sodium $HPO_4{\cdot}7H_2O$), sodium hydroxide, calcium chloride, sodium ethylenediaminetetraacetate (sodium EDTA), hydrochloride acid and phosphoric acid were purchased from Merck (Germany). Methanol and dimethylformamide, HPLC-grade solvents, were purchased from Tedia (USA). Citric acid was purchased from Nuclear (Brazil). Trichloroacetic acid (TCA) and sodium acetate were purchased from Sigma (USA) and J.T. Baker (USA), respectively.

Throughout the study, water was obtained from a Milli-Q system from Millipore (USA). Before the HPLC analysis, all the solutions were filtered through 0.45 μm membrane filters from Millipore (Brazil).

The mobile phase was composed of an aqueous phase (AP) (0.075 mol L^{-1} sodium acetate, 0.035 mol L^{-1} calcium chloride and 0.025 mol L^{-1} sodium EDTA, pH 7) and an organic phase (methanol:acetonitrile, 75:25 v/v), with gradient elution. The pH of the aqueous phase was adjusted with 3 mol L^{-1} ammonium hydroxide or 3 mol L^{-1} hydrochloric acid.

2.4. Standard solutions

Standard stock solutions of TC, OTC, CTC, SMZ. SMX, SQX and CLP were prepared by dilution of appropriate volumes of the standards in methanol to a final concentration of 1.0 mg mL^{-1}. These solutions were stored under refrigeration (−18 °C) until use. Working solutions in the concentration range of 50 μg mL^{-1} were prepared daily by dilution of aliquots of the standard stock solution in water. The working solution was used directly for sample fortification to produce calibration standards of 50, 100, 200, 300 and 500 ng mL^{-1} concentrations. The sodium EDTA-McIlvaine buffer was prepared as previously described (Pena, Lino, & Silveira, 1999) and stored at 4 °C. All the stock solutions were stable for a period of at least 90 days.

2.5. Milk samples

Sixteen samples of pasteurised whole bovine milk types A, B and C were purchased at retail markets in Campinas, São Paulo State, Brazil. All samples were acquired in 2006.

2.6. Sample preparation

A volume of 5 mL of the milk sample (blank sample or fortified sample) was apportioned into 50 mL polypropylene centrifuge tubes. The sample was added to 2.5 mL 30% TCA in methanol, sonicated for about 10 min and centrifuged for 10 min at 1000*g* at room temperature. The upper phase was transferred to another sample tube and the remaining solid was re-extracted with a second portion of 10 mL McIlvaine buffer, pH 4. Sample was again sonicated for 10 min and centrifuged at 1000*g* for 20 min. The extracts were combined and cleaned up over hydrophobic–lipophilic-balanced SPE cartridges (OASIS HLB, Waters USA) using a 12 port manifold under atmospheric pressure. The SPE cartridges were previously conditioned with 5 mL methanol, 10 mL acetonitrile and 5 mL McIlvaine buffer, pH 4. The sample extracts were applied and the cartridges were washed with 10 mL of a mixture of methanol, acetonitrile and McIlvaine buffer (6:7.5:86.5, v/v/v). The retained antimicrobials were eluted with 5 mL of methanol. The milk extracts obtained were concentrated to dryness at 35 °C under a nitrogen flow and the residue was dissolved in 500 μL of the aqueous phase of the chromatographic mobile phase which composed of 0.075 mol L^{-1} sodium acetate, 0.035 mol L^{-1} calcium chloride and 0.025 mol L^{-1} sodium EDTA, pH 7. After filtration through a 0.45 μm membrane filter (Millipore, Brazil) a volume of 50 μL was injected into the HPLC system.

2.7. HPLC analysis

Chromatographic separation of the antimicrobials on the octadecyl X Terra® column was achieved using a mobile phase containing an aqueous phase (AP) of 0.075 mol L^{-1} $CaCl_2$, 0.035 sodium acetate and sodium EDTA, pH 7.0 and an organic phase (OP) of methanol:acetonitrile (75:25, v/v). Gradient elution of 90:10 AP:OP v/v to 50:50 AP:OP v/v (0–30 min) then to 90:10 AP:OP v/v (30–37 min) was used. The flow rate was 0.7 mL min^{-1}.

Quantitation was performed through internal standardization at 265 nm (SMZ, SMX, SQX), 311 nm (ENRO and CLP) and 385 nm (TC, CTC, OTC). For this purpose blank milk samples were fortified at five concentration levels (50, 100, 200, 300 and 500 ng mL^{-1}), using 300 ng mL^{-1} ENRO as an internal standard.

2.8. Method validation

The method was validated using the following performance criteria: linearity and linear range, sensitivity, selectivity, intra-assay and inter-assay precision, accuracy, limit of detection (LOD) and limit of quantitation (LOQ). The linearity, linear range, sensitivity and LOD were established through the calibration curve obtained by triplicate analysis of SMZ, SMX, SQX, TC, CTC, OTC and CLP at five concentration levels (50, 100, 200, 300 and 500 ng mL^{-1}) in the milk matrix. The sensitivity is the slope of the calibration graph.

The selectivity of the method was evaluated by exposing SMZ, SMX, SQX, TC, CTC, OTC and CLP, at a concentration level of 1000 ng mL^{-1}, to the following stress conditions for 1 h: 0.1 mol L^{-1} HCl, 0.1 mol L^{-1} NaOH, 3% v/v H_2O_2 and temperature (55 °C). The solutions were analyzed considering the resolution between the analyte and other substances formed during the experiment and the analytical signal before and after the exposure of the analyte to the stress conditions, expressed as recovery.

The intra-assay precision of the method, expressed as the relative standard deviation (RSD) of peak area measurements ($n = 5$), was evaluated through the results obtained with the method operating over 1 day under the same conditions, using a blank milk sample fortified at a single concentration level of 200 ng mL^{-1}. The inter-assay precision was determined at two concentration levels, 100 and 200 ng mL^{-1}, and the analyses were performed for 5 days. According to the European Commission, Regulation 2002/657/EC, the RSD should be lower than 15%.

The accuracy of the method was determined through the recovery at three fortification levels (100, 200 and 300 ng mL^{-1}) in triplicate analyses. Enrofloxacin at a concentration of 300 ng $mL^{-1}$was used as internal standard. The European Communities guidelines recommend a recovery in the range of 80–110% of the target concentration (European Commission, Regulation 2002/657/EC).

The limit of detection (LOD) and limit of quantitation (LOQ) were determined at the signal-to-noise ratios of 3 and 10, respectively, measured at the approximate retention time of the corresponding analyte peak.

3. Results and discussion

3.1. HPLC-DAD method development

Initially, a silica-based octadecyl column (LiChrocart®) was employed for optimisation method and the mobile phase was prepared according to Houglum and Larson (1997), for the quantitation of chlortetracycline in animal feeds by HPLC with fluorescence detection.

The main aim was the separation of the seven antimicrobials from three different classes in a single run, using a photodiode array detector and measuring each analyte in its best detection condition, i.e. maximum absorption wavelength. The optimum wavelengths are: 260 nm (SMZ, SQX and SMX), 311 nm (CLP) and 385 nm (OTC, TC and CTC). Minocycline (MC) was initially evaluated as internal standard, however enrofloxacin (ENRO) was later employed for this purpose due to the fact that this antimicrobial did not elute at the same time as other components did from the milk matrix and it is not recommended for application to dairy animals.

When the silica-based octadecyl stationary phase column was employed with a mobile phase containing an aqueous phase (AP): 0.075 mol L^{-1} sodium acetate, 0.035 mol L^{-1} $CaCl_2$, 0.025 mol L^{-1} sodium EDTA, pH 7 and an organic phase (OP): MeOH:ACN 65:35 v/v, it was possible to separate SMZ, SQX, SMX, TC, CTC and CLP with adequate resolution. However, the tetracyclines, especially OTC, showed broad peaks with inadequate asymmetry factors. The poor peak shape of the tetracyclines could be explained due to the residual acidic silanols in the stationary phase of the silica-based octadecyl column.

To overcome this limitation a hybrid octadecyl column (X Terra®) and different organic phases (MeOH:ACN 65:35, 75:25 and 80:20 v/v) were evaluated. The aqueous phase was the same as described before and the separation was conducted using gradient elution. The use of the hybrid octadecyl column and 75:25 v/v MeOH:ACN in the organic phase of the mobile phase resulted in adequate efficiency and selectivity for the separation of the antimicrobials under study (Fig. 2).

Co-elution of SMZ with TC was observed using 65:35 and 80:20 v/v MeOH:ACN as organic phase. At pH 7 the sulphonamides ($pK_a \approx 5.5$) are in their anionic form, which is the reason for their lower retention times due to their lesser interaction with the apolar stationary phase in comparison with tetracyclines, which will be mainly in their zwitterionic form. CLP is the last compound eluted from the chromatographic column. This is attributed to its non-ionic form at pH 7 (pK_a 11) and to their lipophilic property. The system suitability parameters are in the acceptable range: retention factor ($1 < k$), plates ($N > 2000$) and resolution ($Rs > 2.0$) (EC, 2002).

3.2. Extraction of the antibiotics from milk

Due to the different chemical structures and physicochemical properties of the antimicrobials under study the establishment of a unique sample preparation procedure is a difficult task and therefore all steps must be carefully evaluated. The main experimental steps evaluated were: protein precipitation, extraction, clean-up using solid-phase cartridges and concentration of the eluate.

For protein precipitation different solutions have been recommended in the literature for the determination of tetracyclines in milk: succinic acid (Andersen et al., 2005), ACN (Fletouris, Psomas, & Botsoglou, 1990), 1 mol L^{-1} hydrochloric acid (Moats & Harik-Kahn, 1995; Zhao, Zhang, & Gan, 2004) and McIlvaine buffer/EDTA (Abete, Genta, & Squadrone, 1997; Brandsteterova et al., 1997) and for sulphonamides: ACN (Van Rhijn et al., 2002) and 20% TCA (Furusawa, 1999). The procedures have not been harmonised for the same antimicrobial group and considering that compounds from three different groups would be evaluated in this work, protein precipitation conditions were studied. The solutions evaluated were: McIlvaine buffer/EDTA, 1 mol L^{-1} hydrochloric acid, ACN and 20% and 30% TCA in MeOH. For these studies fortified blank milk samples (1000 ng mL^{-1}) were employed. Before HPLC quantitation, the extracts obtained after protein precipitation were cleaned up using octadecyl silica-based cartridges. As wash solution 10 mL of 97:3 v/v McIlvaine buffer, pH 4:MeOH was employed. The analytes were eluted with 5 mL MeOH and concentrated under N_2 flow. The residues were reconstituted in 1.0 mL of mobile phase before injecting into the HPLC system.

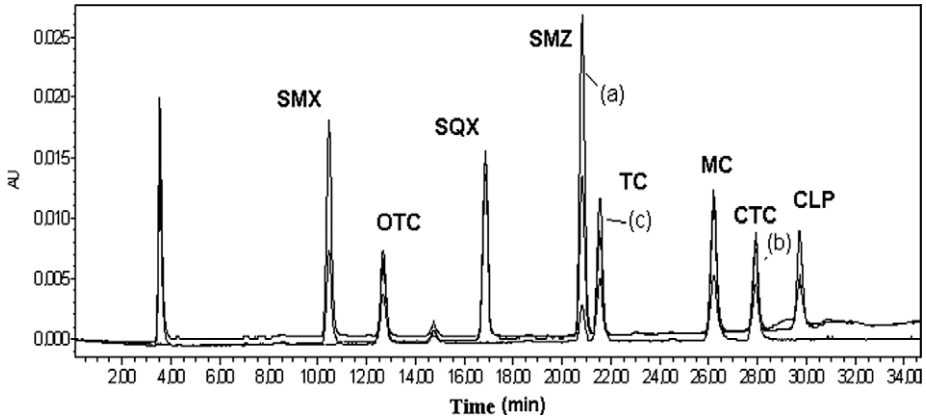

Fig. 2. Chromatogram of SMX = sulfametoxazole, OTC = oxytetracycline, SQX = sulfaquinoxaline, SMZ = sulfamethazine, TC = tetracycline, MC = minocycline, CLP = chloramphenicol and CTC = chlortetracycline (1000 ng mL^{-1}) on a hybrid-polymeric column. Mobile phase, aqueous phase (AP): sodium acetate 0.075 mol L^{-1}, $CaCl_2$ 0.035 mol L^{-1}, Na_2EDTA 0.025 mol L^{-1}, pH 7 and organic phase (OP): MeOH:ACN 75:25 v/v. Gradient elution: AP:OP 90:10 v/v – 50:50 AP:OP v/v (0–30 min); AP:OP 90:10 v/v (30–37 min). Flow rate: 0.7 mL min^{-1}. λ: 250 nm (a), 278 nm (b) and 385 nm (c).

All solutions, with exception of the TCA, did not result in complete protein precipitation and the chromatograms obtained with the fortified blank milk samples showed a large number of interferents and/or low recoveries of the analytes. Using McIlvaine buffer/EDTA a suspension was obtained even after centrifugation indicating an incomplete protein precipitation. Among all protein precipitation solutions evaluated the best results for all compounds under study were obtained with TCA in MeOH. Even then, 20% TCA in MeOH gave low recoveries of the antimicrobials. The use of 30% TCA in MeOH provides adequate protein precipitation with better recoveries than those obtained with 20% TCA. However, some interferents were not eliminated and some compounds were co-eluted with SMZ and CLP. To overcome these problems, different SPE-cartridges (octadecyl silica-based, endcapped octadecyl silica and copolymer OASIS HLB) were evaluated for clean-up. In addition, a liquid–liquid extraction (LLE) with n-hexane after protein precipitation and before SPE clean-up with octadecyl silica-based cartridges, in order to eliminate some interferents, was also evaluated. The results are summarised in Table 1. The LLE with hexane, before clean-up on the silica-based octadecyl sorbent, showed an increase in the extraction efficiency for all antimicrobials, with the exception of OTC, which was not recovered. It is well known that basic compounds (e.g. OTC) interact with the acidic residual silanols from silica-based octadecyl sorbents. Due to this fact, octadecyl endcapped and copolymer OASIS HLB were evaluated and the results are also presented at Table 1. It was verified that the OASIS HLB allowed higher recovery values and lower interferences near the retention time of each analyte.

Table 1
Average ($n = 2$) extraction efficiency (%) using different SPE sorbents.

Compound	Extraction efficiency[a] (%)			
	C_{18}	LLE[b]+C_{18}	C_{18} endcapped	OASIS HLB
SMX	67	64	52	59
SQX	54	52	10	60
SMZ	45	47	26	21
OTC	–	–	56	72
TC	39	51	46	63
CTC	33	53	34	50
CLP	70	72	66	80

[a] The extraction efficiency was calculated from the recovery of the analyte in the fortified blank milk sample (100 ng mL^{-1}) in relation to the same concentration of the standard in the solvent (analytical curve with internal standard).
[b] LLE with n-hexane.

Table 2
Average ($n = 2$) extraction efficiency (%) using OASIS HLB cartridges and different washing solutions.

Antimicrobial	Extraction efficiency[a] (%)	
	MeOH:ACN:buffer[b]	ACN:buffer
	3:7:90 v/v/v	8:92 v/v
SMX	64	22
SQX	48	52
SMZ	19	25
OTC	80	63
TC	64	62
CTC	61	58
ENRO	90	88
CLP	89	85

[a] The extraction efficiency was calculated from the recovery of the analyte in the fortified blank milk sample (100 ng mL^{-1}) in relation to the same concentration of the standard in the solvent (analytical curve with internal standard).
[b] McIlvaine buffer, pH 4.0.

In order to increase the recovery of the antimicrobial residues and to diminish the interference the washing step of the cartridge was evaluated. SMZ showed to be the compound most susceptible to the effect of the organic solvent in the washing step (Table 2).

The use of the ACN in the elution step decreases the recoveries of the sulphonamides, because this solvent is less polar than methanol. A mixture of MeOH and ACN in the proportion MeOH:ACN:-buffer 6:7.5:86.5 v/v/v, was adequate for antimicrobial recoveries.

The results indicated that it was not possible to establish a single condition that allowed high recoveries for all the antimicrobials, using one SPE cartridge for extraction in the clean-up step. Thus, the best compromise between recovery and time of analysis was reached using the procedure described in Section 2.6.

3.3. Method validation

The HPLC-DAD method was *in-house* validated for the determination of the tetracyclines, sulphonamides and chloramphenicol by evaluating the following parameters: selectivity, linear range, linearity, sensitivity, intra- and inter-assay precision, accuracy, limit of detection and limit of quantitation. The results are summarised in Table 3.

Selectivity indicates the ability of the method to accurately measure the analyte response in the presence of potentially interfering sample components or degradation products. In this study selectivity was evaluated by exposing the analytes to stress conditions, such as 0.1 mol L^{-1} HCl, 0.1 mol L^{-1} NaOH and 3% v/v H_2O_2. The solutions were analyzed considering the resolution between the analyte and other substances formed during the experiment and the analytical signal before and after the exposure of the analyte to the stress conditions. Co-migration of degradation products

Table 3
Validation parameters for the HPLC-DAD method developed for the determination of SMX, SQX, SMZ, CLP, OTC, TC and CTC in milk.

Parameter	SMX	SQX	SMZ	CLP	OTC	TC	CTC
Linear range (ng mL^{-1})	60–500	60–500	60–500	60–500	60–500	60–500	60–500
Sensitivity (ua mL ng^{-1})	0.0058	0.0072	0.0020	0.0012	0.0027	0.0034	0.0019
Linearity (r)	0.998	0.996	0.995	0.998	0.996	0.995	0.992
Linear coefficient	−0.019	−0.174	−0.067	−0.014	0.090	0.053	0.022
Intra-assay precision ($n = 5$) (RSD%) 200 ng mL^{-1}	6.5	3.9	3.1	2.6	3.9	4.9	2.7
Inter-assay precision ($n = 5$) (RSD%)							
100 ng mL^{-1}	8.1	5.4	13.2	7.4	15.8	6.4	6.0
200 ng mL^{-1}	4.8	9.0	7.1	7.3	14.6	10.0	7.1
300 ng mL^{-1}	12.5	7.6	15.6	8.3	5.4	10.0	12.7
LOD (ng mL^{-1})	20	20	20	20	20	20	20
LOQ (ng mL^{-1})	60	60	60	60	60	60	60
Accuracy, ($n = 5$) recovery/s (%)							
100 ng mL^{-1}	108/8	104/4	103/10	109/7	92/14	112/6	103/6
200 ng mL^{-1}	99/4	110/8	101/7	102/10	100/2	106/7	107/6
300 ng mL^{-1}	104/11	106/7	107/14	101/7	93/5	83/8	100/12

RSD: relative standard deviation, LOD: limit of detection, LOQ: limit of quantitation, s: estimated standard deviation.

was not observed due to the fact that peak purity for each analyte was confirmed by the overlay of the spectra before and after degradation process. It was verified that the stability of the tetracyclines depends on their chemical structure. Under the three stressing conditions, CTC and CLP were the least stable compounds to alkaline treatment and OTC was the least stable to acid and oxi-

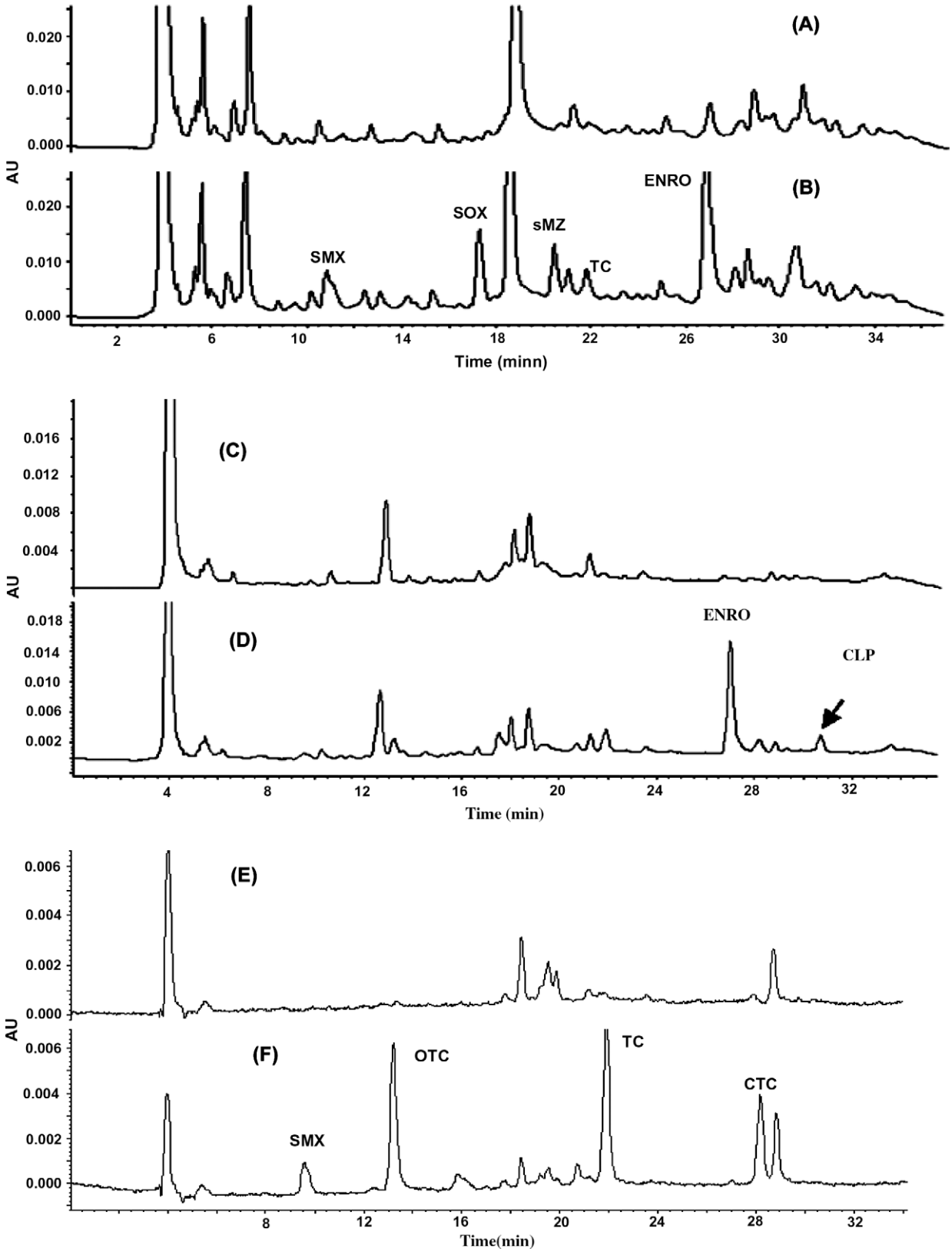

Fig. 3. Chromatograms: (A) blank milk sample, detection λ: 265 nm; (B) blank milk sample fortified, detection λ: 265 nm; (C) blank milk sample, detection λ: 311 nm; (D) blank milk sample fortified, detection λ: 311 nm; (E) blank milk sample, detection λ: 385 nm; (F) blank milk sample fortified, detection λ: 385 nm. Fortification level: 100 ng mL^{-1} of SMX, SQX, SMZ, CLP, OTC, TC, CTC and 300 ng mL^{-1} of ENRO (internal standard).

dant media. The sulphonamides were more stable under the evaluated stress conditions. Even though some degradation products under the evaluated stress conditions are formed, they present retention times different from the analytes and, in consequence, they do not affect the selectivity method.

Endogenous interferences were also appraised. For this purpose five blank milk samples were analyzed. Characteristic chromatograms from blank milk samples registered at the three different wavelengths (265 nm, 311 nm and 385 nm) are presented in Fig. 3A–F. The chromatogram shown in Fig. 3A, obtained at 265 nm, shows the presence of peaks near to the retention times of SMX (t_R 10.8 min), SQX (t_R 17.3 min) and SMZ (t_R 20.5 min). However, none of them have any effect on the accuracy of the method, since the recoveries were in the range of 96–108%.

At 311 nm, no potential interference was observed for the signal of ENRO (internal standard) and CLP, since all the compounds from the milk matrix eluted at lower retention times (Fig. 3C and D). At 385 nm, even though some compounds eluted in the range of 18–20 min and 29 min, these compounds did not interfere in the tetracycline determinations (Fig. 3E and F).

Due to the fact that no potential interference was detected in the blank samples at the retention time of each analyte, it could be concluded that the method developed presents adequate selectivity for the determination of the antimicrobials.

The linearity, linear range and sensitivity were obtained from calibration graphs using an internal standard (ENRO, 300 ng mL^{-1}) and fortifying blank milk samples at five concentration levels (60, 100, 200, 300 and 500 ng mL^{-1}) for each analyte under study, with triplicate analyses. The linearity was tested using a pure error lack of fit test with simple regression, which was not significant at the 5% level.

The precision of the method was evaluated using the results obtained over 1 day of operation under the same conditions (intra-assay) and during 5 days (inter-assay precision). The intra-assay precision was evaluated by means of analysis in one single day with five blank milk samples fortified at 200 ng mL^{-1}. The RSD for all the antimicrobials studied (TC, CTC, OTC, SMZ, SQX, SMX and CLP) was in the range of 2.6–6.5%. The inter-assay precision was evaluated through the analysis, during five different days, of fortified blank milk samples at three concentration levels (100, 200 and 300 ng mL^{-1}). The inter-assay precision (RSD) was in the range of 4.8–15.8% (Table 3). Considering that the European Commission Decision 2002/657/EC, 2002 (European Commission, 2002) recommends that the inter-assay precision should be lower than 23%, the values obtained by the proposed HPLC method are adequate.

The accuracy of the method was assessed by recovery tests, analyzing fortified blank milk samples at three concentration levels (100, 200 and 300 ng mL^{-1}) over 5 days. The results are presented at Table 3. The recovery varied from 83% to 112% and is in accordance with the EU guidelines, which established a range of 80–120% for these concentration levels (EC, 2002).

The limit of detection (LOD) and limit of quantitation (LOQ) were determined at a signal-to-noise ratio of 3 and 10, respectively, measured at the approximate retention times of the corresponding analyte peaks. These values were validated by the analyses of fortified samples with these values. The LOD for all antimicrobials was similar, i.e. 20 ng mL^{-1}. The quantification limit (LOQ) is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational condition of the method. The LOQ was established at 60 ng mL^{-1} for all the antimicrobials and fulfils the requirement that this value must be lower than the MRL, with the exception of CLP. In the case of CLP residues in milk, as well as meat, eggs, aquaculture products and honey, the European Commission established a minimum required performance limit (MRPL) of 0.30 ng g^{-1} to ensure the same level of consumer protection throughout the community (EC, 2002). Considering this MRPL, the CLP LOQ value of the developed method (60 ng mL^{-1}) does not fulfil adequate detectability for the quality control. However, the required EC detectability might be possible using a LC–MS–MS system.

3.4. Analysis of commercial samples

In Brazil, fluid pasteurised bovine milk that is available to the consumers at the retail market is commercialised in three different categories (A, B and C). These categories differ in terms of total quality of the product (control of the milk origin, veterinary control of the cows and chemical, physical–chemical and microbiological quality). The pasteurised bovine milk types A and C belong to the highest and lowest categories of quality, respectively.

To evaluate the antimicrobial presence in milk the three types of pasteurised (A, B and C type) milk were considered. Analyses of the selected milk samples show that all the antimicrobials were below the LOD of the method (20 ng mL^{-1}) and therefore below the established MRL values (EC, 2002). It is worth emphasising that the aim of this work is not to conduct a quality control program, but to verify the performance of the method in the different milk matrices.

4. Conclusions

The method developed using high-performance liquid chromatography associated with photodiode array detection was shown to be adequate for the simultaneous determination of multiresidues of SMX, SQX, SMZ, OTC, TC and CTC in milk. Considering the maximum residue limits established for those antimicrobials (100 ng mL^{-1}), the LOQ value, as well as the other parameters of validation of the method, is adequate for the monitoring of their residues in milk. In the case of CLP, which has a LMPR of 0.3 ng mL^{-1}, the proposed method does not have adequate detectability for the quality control of this antimicrobial in milk.

Acknowledgements

The authors gratefully acknowledge the financial support from CNPq and FAPESP (01/14072-6, 04/07312-9) and thank Professor C.H. Collins for language assistance.

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Food Chemistry 117 (2009) 553–560

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Analytical Methods

Heavy metal uptake in the enological food chain

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ARTICLE INFO

Article history:
Received 27 June 2008
Received in revised form 26 March 2009
Accepted 11 April 2009

Keywords:
Heavy metals
Enological food chain
Bioaccumulation

ABSTRACT

The research presented here describes the assessment of a number of physiological and xenobiotic metal concentrations occurring throughout the enological production processes of two native wines from Irpinia, *Aglianico* and *Fiano of Avellino*. In order to evaluate the wines' metal content throughout the entire wine-making process, samples of grapes and musts from both wines were taken at time intervals during the fermentation and maturation phases. Copper, chromium, nickel, iron, zinc, lead and cadmium levels were analysed by atomic absorption spectrophotometry. The concentration of metals obtained were compared to the limits fixed by the Organization Internationale de la Vigne et du Vin (O.I.V.) and in the Italian ad European legislation.

1. Introduction

Wine contains hundreds of different substances (more than 600 known substances), the organoleptic properties (Maarse, Slump, Tas, & Schaefer, 1987) of which are not only influenced by its major components, such as water and ethanol, but also by traces of minor components like the 2,4,6-trichloroanisole which gives to certain wines an unpleasant cork taste.

Due to the wide range of wine-making techniques, it is difficult to define the average composition of wine. However the following components are always present: water; alcohols: ethanol, methanol, super alcohols, glycerin; organic acid: tartaric, malic, citric, lactic, acetic (Maarse et al., 1987); sugars: glucose, fructose; rubber and pectin; polyphenols (Singleton, 1988): anthocyanins (Mazza & Miniati, 1993; Ribèreau-Gayon, 1982), tannins; mineral substances: anions and cations; volatile substances (Maarse et al., 1987; Moret, Scarponi, & Cescon, 1984; Noble, Flath, & Forrey, 1980; Rapp, Guenter, & Ullmeyer, 1985; Rapp & Gunter, 1985; Rapp, Suckrau, & Versini, 1993; Rapp, Volkmann, & Niebergall, 1993; Schreier, Drawert, Junker, & Reiner, 1976): volatile acids, esters, aldehydes, terpenes; vitamins; dissolved gases: carbon dioxide, sulphur dioxide, oxygen.

The organoleptic characteristics of this beverage are a function of the interactions which occur between these elements and the chemical balance during wine-making and ageing.

As for many other foods, wine analysis may be broken down into a number of measurements: normative, nutritional and qualitative.

The normative aspects are linked to the analytical parameters that each enological product must have in order to be marketed, including alcoholic grade, total acidity, partial and fixed dry extract, etc., although the production techniques are not as restrictive from this point of view.

The nutritional aspect of wine relate to the beneficial properties (within certain limits of consumption) that it may bring to one's health, in particular, through the intake of antioxidant substances such as polyphenolic compounds (Minussi et al., 2003).

Finally, qualitative assessment of a wine refers to its aroma and bouquet, and therefore to the hundreds of volatile substances contained within a wine, many of which are yet to be identified and/or quantified.

At the European Community level, EC Regulation No. 2676/90 dictates the methods of analysis which must be used by wineries or wine companies. This law describes all of the important parameters relating to the nutritional safety of wine. Subsequent updates resulting from the developments within our understanding of wine chemistry have allowed significant new improvements to be made brought through the introduction of new methodologies.

Like those in other European countries, Italian wine-making laws relating to analytical methodologies refer to those set out by the EU.

With regards to the upper limit of the concentration of undesired substances in wine, there are still no laws set out by the EU. Mean-

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doi:10.1016/j.foodchem.2009.04.033

while, industrial waste, gas emissions and the use of pesticides have all contributed to a significant increase in the concentrations of toxic metals which occur in the environment – unlike natural organic and synthetic substances, once absorbed into the soil, these seldom degrade due to their more extensive latency period. Metal toxicity varies according to chemical form, as well as concentration and modality of exposure. Pollution caused by non-physiological metals is one of the most important fields of study in the epidemiological investigations relating to the health of living systems. The analytical determination of metals in wines is still used frequently not only to assess the wine's origin, but also to certify its quality and to show that the tolerable limits established by law have been respected throughout the production process (Azenha & Vasconcelos, 2000; Gennaro, Mentasti, Sarzanini, & Pesticcio, 1986; Jaganathan, Reisig, & Dugar, 1997; Lapa, Lima, & Santos, 1996; Maarse et al., 1987; Moret et al., 1984; Thiel, Bauer, Danzer, & Eschnauer, 1998).

Moreover, it is important to consider the different factors that determine the content of elements in wine such as: the soil on which the vines grow; the capacity of the grapes to absorb mineral substances from the soil, preliminary treatment of the grape harvest, the fermentation reaction, the addition of composts possessing different functions, preservation and bottling.

The final content is therefore the result of a number of different variables, such as the chemical and physical characteristics of the soil in which the vines have grown, as well as other factors that cannot be controlled or are non-discriminating.

The so-called 'natural' elements are those of which the concentration in wine is not influenced by the productive cycle, but is instead determined by the degree to which the mineral is present within the soil and the capacity of the vine to assimilate it. These elements include mineral substances such as aluminum, boron, barium, lithium, magnesium, molybdenum, silicon, strontium, and titanium. Other elements are Lanthanides and metals of the second and third transition series which are present in trace quantities.

Furthermore there are elements that occur both naturally and from 'artificial' sources, the concentration of which is determined by a number of different factors; these include elements such as calcium and magnesium; also copper and zinc are also taken up naturally from the soil and are also derived from fungicidal treatment (Bordeaux mixture, zinc thiocarbamates) or from wine-making equipment.

The concentration of iron is due in part to natural (e.g. presence of residual earth particles in the must), and in part to artificial sources (wine-making equipment, steel containers); potassium is the predominant cation in grapes, but it is also added as metabisulphite or carbonate during wine making. Sodium comes from the soil or is added illicitly like chloride, when salting; phosphorous is naturally present as both organic and non-organic phosphites, but it is also added to wine as a calcium or ammonium salt. There are also elements of which the concentration is almost exclusively influenced by artificial sources.

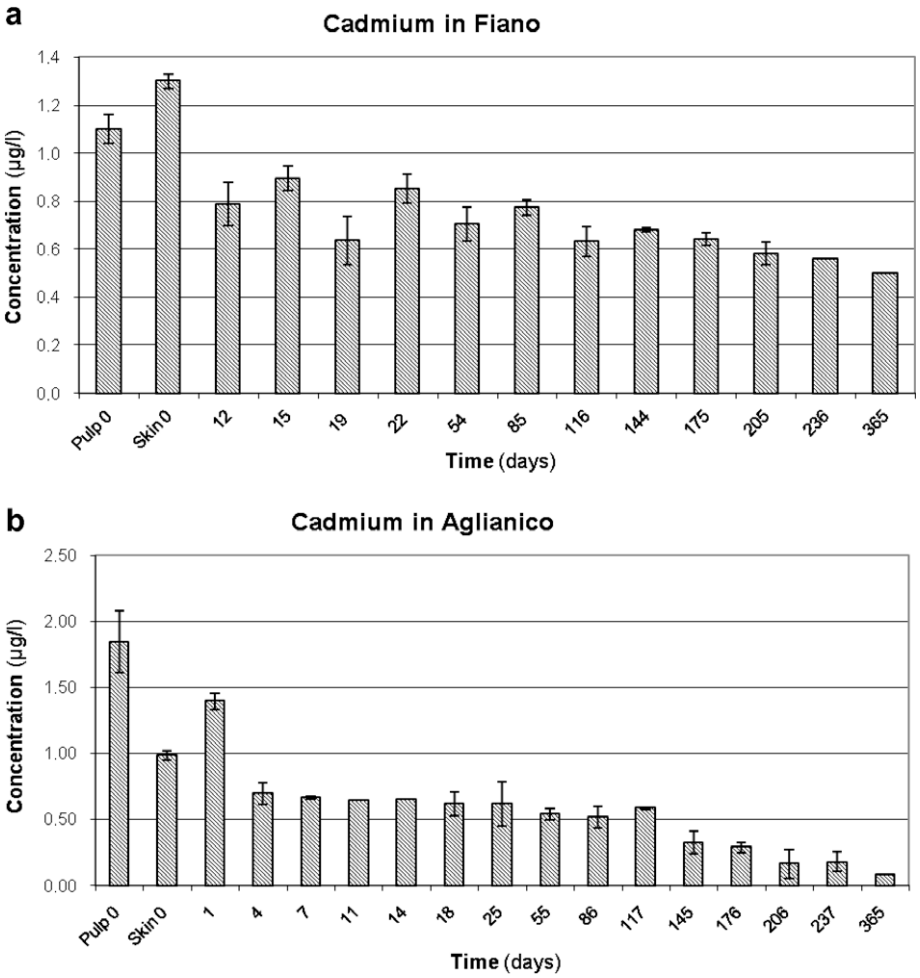

Fig. 1. Cadmium concentration, expressed in µg/l, along the production process of *Fiano of Avellino* (a) and *Aglianico* (b) wines.

Lead comes predominately from fungicides treatment, sealed containers or pollution of the atmosphere (for example, vines located near roads subjected to heavy traffic) and in small quantities from the soil.

Cobalt, chromium, nickel and vanadium, normally present in wine at low concentrations, are most probably derived from the interaction of the must and wine with the metal containers, rather than from natural sources. However, it is thought that cadmium originates exclusively from atmospheric pollution and it has the remarkable capacity to accumulate in the ground.

Heavy metals, in particular cadmium, mercury and lead are those that are the most toxic to man. The accumulation of these elements in the organism leads to a number of diverse and deleterious effects, both in the long and the short term, and varies according to the type of metal. They can cause damage to the kidneys, to the nervous and immune systems, and in some cases they can have cancerous effects. The classic symptom of heavy metal intoxication include irritability, mood changes, depression, headaches, tremor, loss of memory and reduced capacity of sight (Järup, 2003).

In contrast, metals such as copper, zinc and iron are essential for man. For example copper is important in respiration because it is necessary for synthesis of hemoglobin, the protein that transports oxygen in the blood stream. Moreover, it participates in the synthesis of collagen and the neurotransmitter noradrenalin, as well as being one of the most important blood antioxidants and helping to prevent breakdown of polyunsaturated fatty acids which help to keep the cell membrane healthy. Iron is also necessary for the synthesis of collagen and is present in the brain as a co-factor in the synthesis of the serotonin, dopamine and noradrenalin neurotransmitters. Its principle function is through its interaction with proteins and copper in the formation of hemoglobin. Zinc performs various functions: it plays a part in the normal absorption, it interacts with vitamins, pertaining in particular to the B complex, and is present as a component of over 200 enzymes required for digestion and metabolism (e.g. carbonic anhydrase necessary for tissue oxygenation) (Vallee & Falchuk, 1993).

Furthermore, when zinc is present at good levels, it competes for uptake with cadmium, reducing absorption of the latter and minimising its detrimental effects.

Numerous studies have proven that moderate consumption of wine, especially red wine, is considered to be beneficial to health when combined with a balanced diet (Doll, Peto, Holl, Wheatlag, & Gray, 1994; Gronbaek et al., 1995; Klatsky, Armostrong, & Friedman, 1992). It does, in fact, contribute significantly to the demands for essential elements including potassium, calcium, magnesium, chromium, cobalt, iron, fluorine, iodine, copper, manganese, molybdenum, nickel, selenium and zinc (Eschnauer & Neeb, 1988).

On the other hand many metals like lead, cadmium and arsenic, are known for being potentially toxic, like copper (Scheinberg, 1991), aluminum, iron and zinc when present in high concentration (Gennaro et al., 1986). Many elements, amongst them copper, iron, aluminum, zinc and nickel, also contribute to the formation of opacity and sometimes to the colour, aroma and taste of the wine.

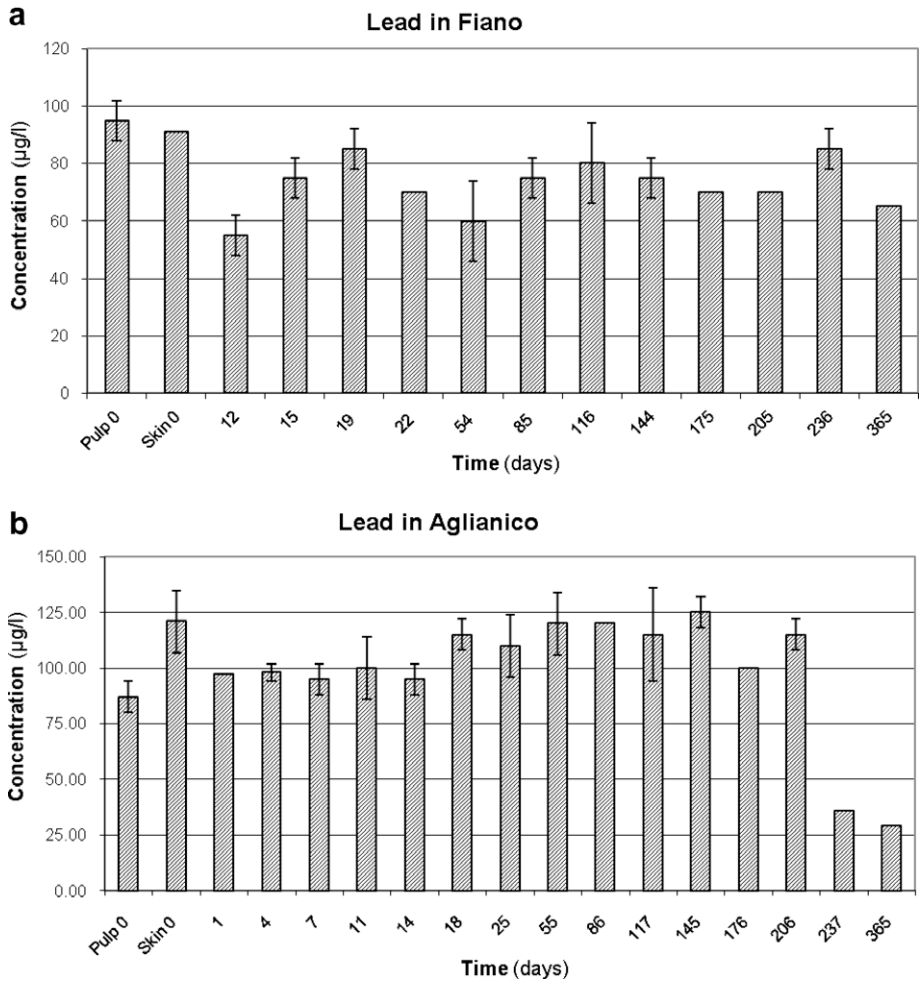

Fig. 2. Lead concentration, expressed in µg/l, along the production process of *Fiano of Avellino* (a) and *Aglianico* (b) wines.

Analysis of the specific metal content of wines can be useful in the identification of its geographic area of origin (Almeida, Bastos, Cardoso, Ferreira, & Lima, 1992; Almeida, Cardoso, & Lima, 1994). The determination of lead in grapes has previously been investigated by Teissedre, Cabanis, Champagnol, and Cabanis (1994) who showed that this metal is present at significant but varying levels in several parts of a grape: seed concentration was found to be 352 μg/kg, in the skin 90 μg/kg and in the pulp it was only 3 μg/kg. Other researchers have analysed the concentration of iron and cadmium in local wines and musts (Lazos & Alexakis, 1989).

On this basis, it is necessary to determine a wine's metal content not only to verify its authenticity, but also in order to confirm that levels are within legal limits, and to obtain knowledge pertaining to the wine's components to set the conditions necessary to proceed, prior to packaging, to the corrective treatments suggested by modern enological techniques. It is important to stress that although existing laws (both EU and national rules) describe the minimum or maximum limits for some parameters, in most cases, accurate estimation of an authenticity can only be derived from final examination of the specific analytical data.

The main objective of this work will be to monitor the concentration (μg and mg/l) levels, through the use of Atomic Absorption Spectrophotometry, of iron, copper, nickel, chromium, zinc, lead and cadmium which occur throughout the production process of two typical Irpinia vines, *Aglianico* (red) and *Fiano of Avellino* (white) from grape-harvesting up to wine bottling.

The concentration of physiological (iron, copper, zinc, chromium, nickel) and toxic metals (lead and cadmium) will be compared to the limits described by the Organization International de la Vigne et du Vin (O.I.V.), bearing in mind the recent decision ENO 13/2006 which fixed a new maximum permitted lead concentration of 0.15 mg/l. The analytical methodology used has been according to CEE Regulation No. 2676/90, the European Community's Official Journal L. 272 dated 03/10/1990. The only Italian law on the subject, specific for the elements copper, iron, manganese and zinc, is the D.M. 29/09/1976.

2. Materials and methods

2.1. Samples collection

Grape samples of *Aglianico* and *Fiano of Avellino* were collected from the Institute of Agrarian Technology of Avellino vines in correspondence to their harvesting dates and stored at −20 °C. The same thing was done with samples of wine withdrawn at several time intervals.

2.2. Sample treatment

For each type of grape, pulp and skin were manually separated, samples were then coarsely crushed in a mortar with rough and pestled walls. Samples were processed using a simple strainer to

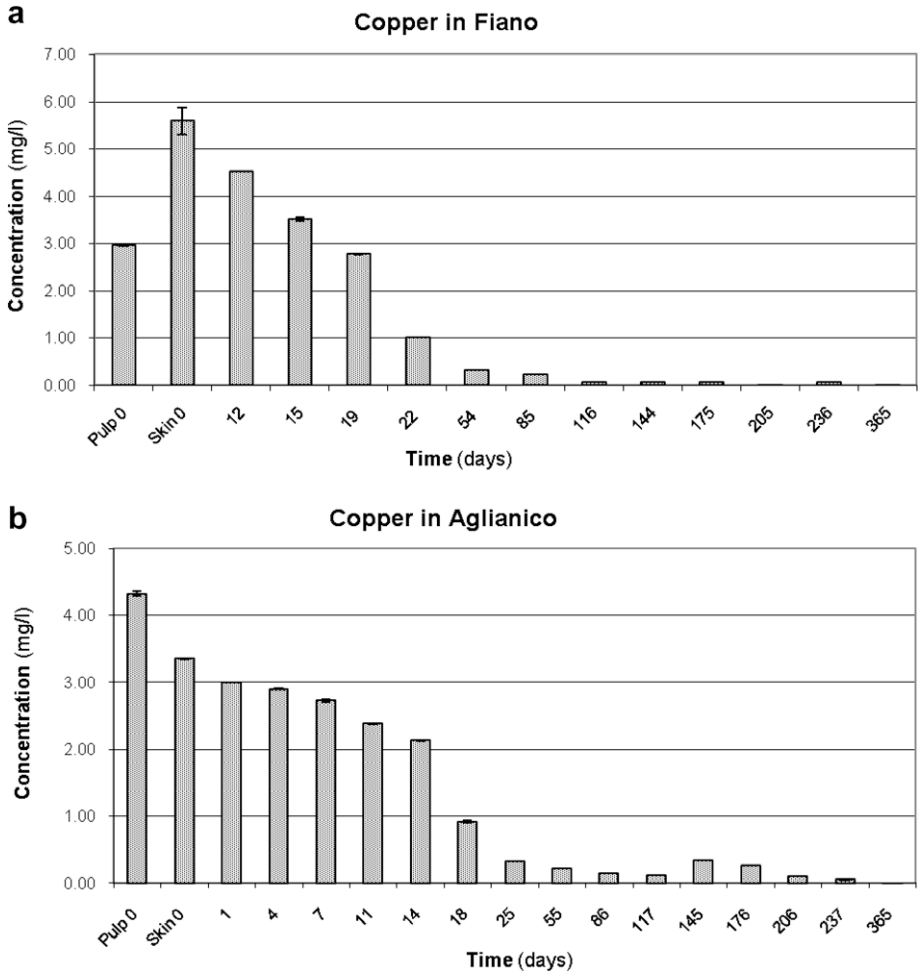

Fig. 3. Copper concentration, expressed in mg/l, along the production process of *Fiano of Avellino* (a) and *Aglianico* (b) wines.

roughly filter the contents in order to remove the suspended deposits, followed by a finer progressive filtration on a sterile Millex-HA (Millipore, 0.45 μm filters).

The samples of must and wine, withdrawn at regular intervals, were centrifuged directly at 4.500 rpm for 15 s; the floating supernatant was gathered and then placed in a RotoVapor at 30 °C, in order to remove the alcoholic component. The watery solution obtained was acidified with 1% HNO_3 in order to resuspend the metals, and the concentration subsequently optimised for atomic absorption spectrophotometry analysis.

2.3. Atomic absorption spectrophotometry analysis

In the present work, we have evaluated the concentration of five physiological metals; copper, chromium, nickel, iron and zinc, and two xenobiotic metals, lead and cadmium. To determine the metal concentrations, a Varian-SpectrAA 200 spectrophotometer (Mulgrave – Victoria, Australia) equipped with an optical bench with a double beam and a monochromator (Czerny-Turner type) was used. Two methods were used for analysis: flame (for quantitation of cadmium and lead) and graphite furnace (mod. GTA 100) (for Co, Cr, Ni, Fe and Zn), completely controlled by the SpectrAA computer. The oven's temperature was set between 40 °C and 3000 °C, with a maximum ramp of 2000 °C/s.

Real temperature controls was achieved through sensors that indicating the resistance to the electrode heads and the temperature of the cooling water. The gas flow used for the flame analysis was programmed at 3 l/min. The standard solutions used for the calibration lines, useful for the determination of each metal (1000 ppm concentration), were as follows.

Cadmium and lead standard solutions diluted in HNO_3 ($Cd(NO_3)_2{\cdot}4H_2O$ and $Pb(NO_3)_2$, respectively); nickel, copper and zinc standard solutions diluted in HCl ($NiCl_2{\cdot}6H_2O$, $CuCl_2{\cdot}2H_2O$ and $ZnCl_2$, respectively); all "Carlo Erba" reagents.

The iron standard consisted of a solution of Fe in 3% HNO_3 (Solutions *Plus Inc.*), and the chromium (III) standard from $Cr(NO_3)_3$ in 3% HCl (Spectro Pure).

The wavelengths used to determine the absorbance of the metals were: lead – 217.0 nm, chromium – 425.4 nm, nickel – 232.2 nm, zinc – 213.9 nm, copper – 324.8 nm, iron – 248.3 nm and cadmium – 228.8 nm.

The measurement of absorbance detected alongside the concentrations, expressed in mg/l and in μg/l, were characterised by standard deviations of less than 1%, for measurements taken with a flame, and less than 5%, for measurements taken with a graphite furnace. For each measured standard and for each sample of grapes, must and wine analysed, the readings were performed in duplicate and each result was the average value of three subsequent replicates (Lara, Cerutti, Salonia, Olsina, & Martinez, 2005).

3. Results and discussion

Out of the metals tested, the results for chromium are not reported, as this was not detected to be present at any point throughout the wine production procedure, Figs. 1–6 show the concentration of the following elements: cadmium and lead (expressed in μg/l), copper, iron, zinc and nickel (expressed in mg/l) occurring throughout the production of the *Fiano of Avellino* (a) and *Aglianico* (b) wines.

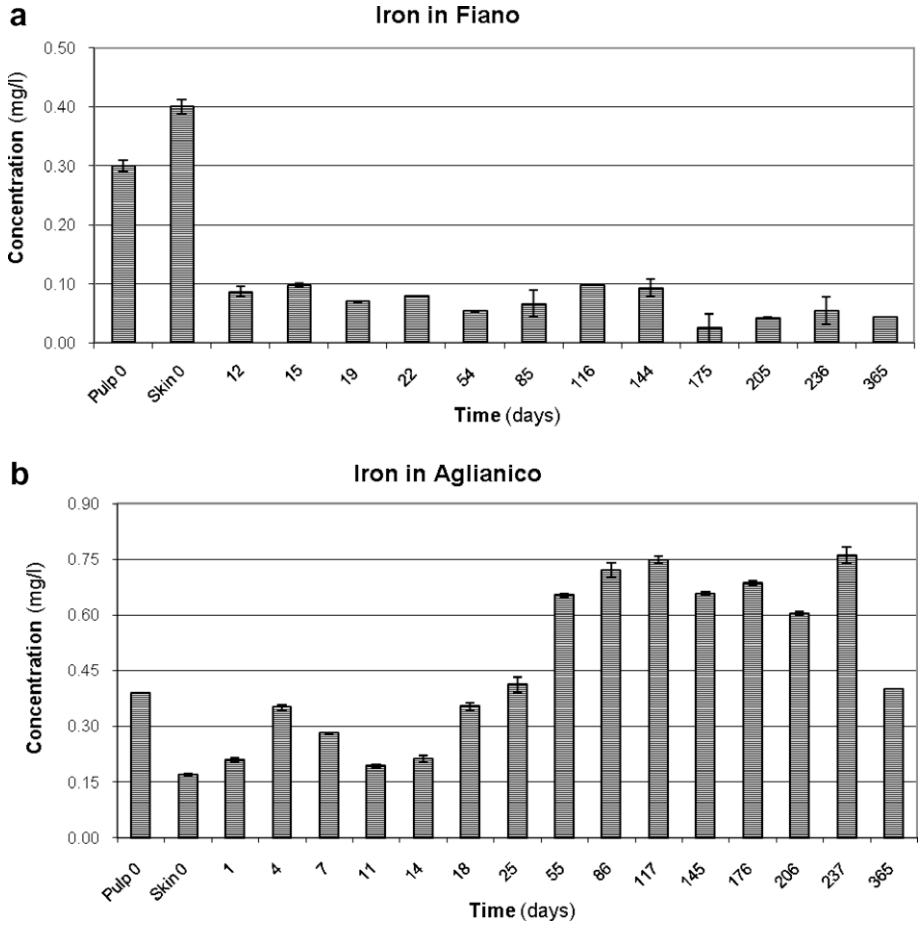

Fig. 4. Iron concentration, expressed in mg/l, along the production process of *Fiano of Avellino* (a) and *Aglianico* (b) wines.

3.1. Cadmium

The maximum limit set by the O.I.V. for the concentration of cadmium in wines is 10 μg/l. Relative to *Fiano* wine, this element was found to decrease both constantly and progressively; in fact the pulp and skin cadmium concentration was found to be more than 1.0 μg/l, and was found to gradually decrease from 12 days to one year (0.5 μg/l) (see Fig. 1a).

The pulp of the *Aglianico* showed higher values compared to the *Fiano* wine, whereas an opposite trend was observed in the skin. After four days, a gradual decrease in metal concentration was observed (see Fig. 1b). Whilst both red and white wines showed a similar trend, i.e. a decrease in cadmium concentration, after one year, cadmium concentration in *Aglianico* was lower than that in *Fiano* wine.

3.2. Lead

The O.I.V. states that 150 μg/l is the maximum lead concentration permitted in wine. In both the *Fiano* and *Aglianico* wines, the monitored values were always under the limit and each showed a regular trend (see Fig. 2a and b). Whilst, after one year, there appeared to be little change in the values for Fiano wine, with the values remaining almost constant, the *Aglianico* wine showed a significant decrease in lead concentration.

3.3. Copper

The copper concentration limit set by the O.I.V. and the Italian norm D.M. 29/09/1976 in wines equal to 1 mg/l; Fig. 3a and b show the copper concentrations for *Fiano* and *Aglianico* wines respectively, revealing that pulp and skin of both wines possess a concentration value higher than the O.I.V. limit allowed. This is most probably due to the cupric treatment that both wines are subjected to during the production process. In fact copper sulphate in particular is used to prevent parasitic infection during growth of the grapes. However, throughout the whole fermentation process, there is a constant decrease in copper levels, down to extremely low values and, after one year, the copper concentrations for both wines reach a level which is well below the legally permitted limit.

3.4. Iron

O.I.V. rules define no limit stated relating to the concentration of iron in wines, while the old Italian law establishes the standard limits at 2.5 mg/l (in the case of soils rich in iron the concentration can reach 10–12 mg/l).

As shown in Fig. 4a, the pulp and skin of *Fiano* grapes contain iron concentrations ranging from 0.3 to 0.4 mg/l, significantly higher than the level seen from the 12th day onwards (around 0.08 mg/l). In red wine however, the iron content remains uniform (ranging from 0.4 to 0.8 mg/l) throughout the production process (Fig. 4b).

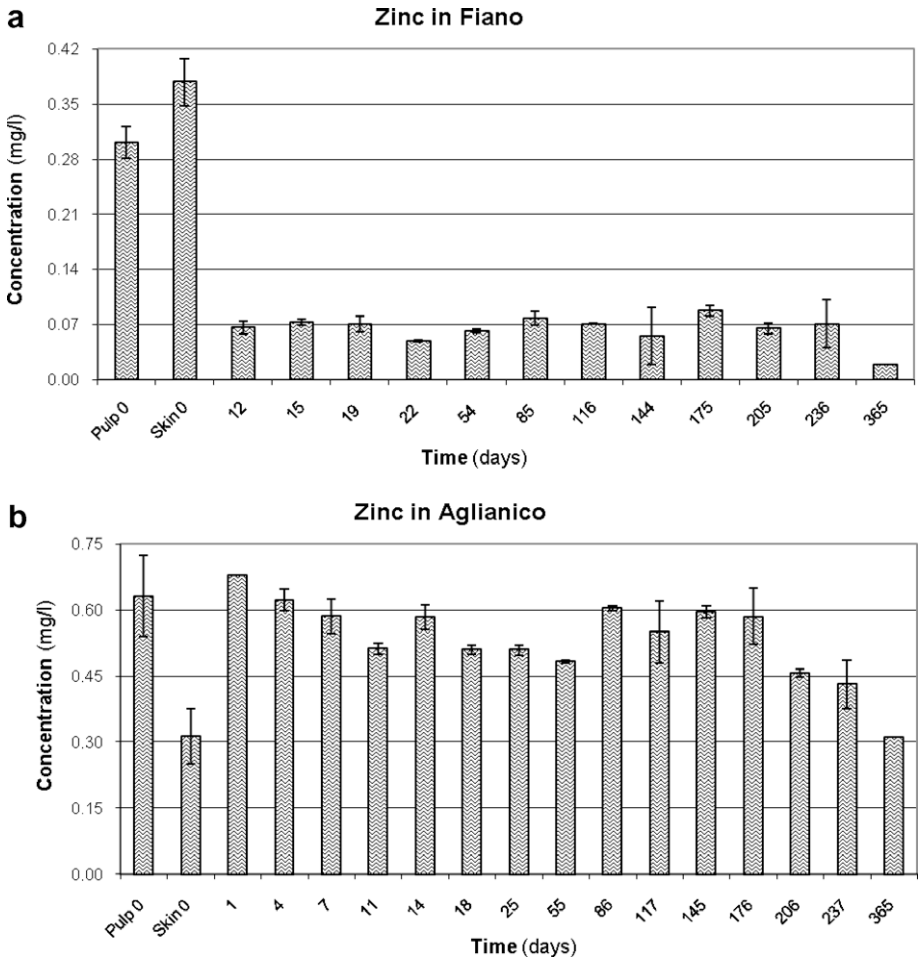

Fig. 5. Zinc concentration, expressed in mg/l, along the production process of *Fiano of Avellino* (a) and *Aglianico* (b) wines.

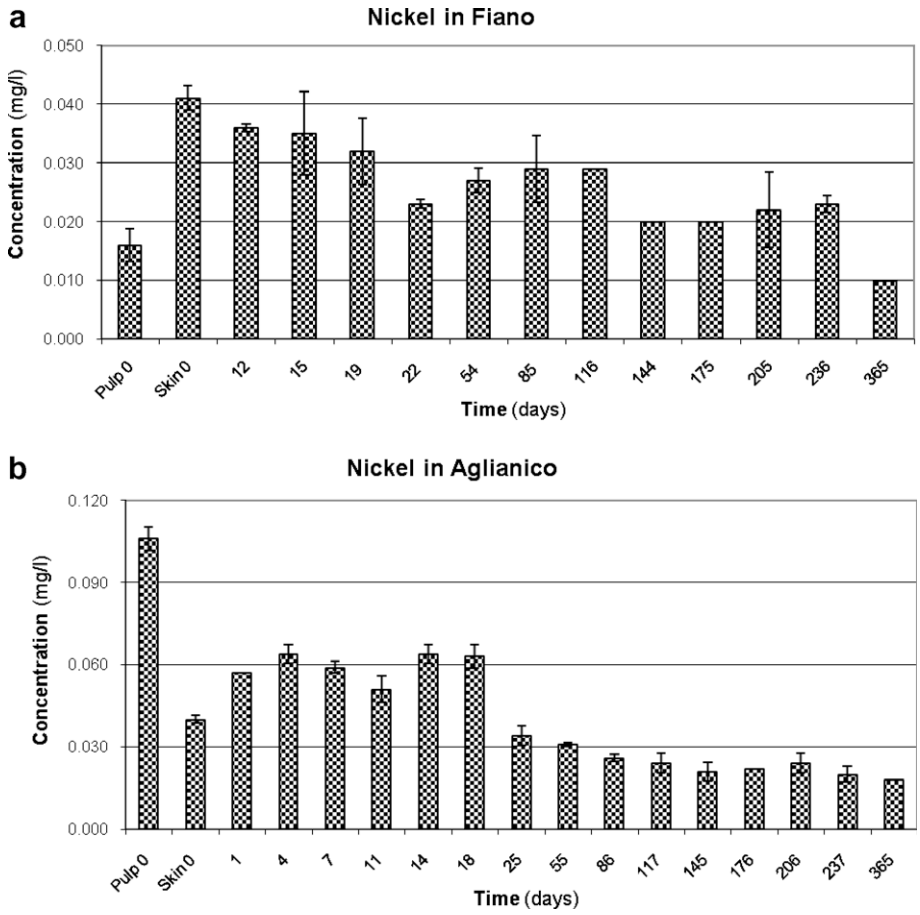

Fig. 6. Nickel concentration, expressed in mg/l, along the production process of *Fiano of Avellino* (a) and *Aglianico* (b) wines.

In both cases, this may be due to the soil being particularly poor in iron.

3.5. Zinc

The presence of this type of metal in wines is usually linked to the plan's capacity of absorbing it from the soil. With regards to its concentration, both the O.I.V. and the Italian law agree on an upper concentration limit of 5 mg/l in the enological production chain.

For zinc, the white wine shows a trend similar to that seen for iron (see Fig. 5a): in fact, pulp and skin values are about 3–4 times higher than wine. In general, red wine contains zinc values are higher in red wine than in white, the trend being in the range of 0.40–0.60 mg/l (Fig. 5b).

3.6. Nickel

Enological containers are made of stainless steel; generally referred to as 18/10, they contain 18% chromium plus 10% Nickel and are internationally classified, according to American abbreviation as AISI 304 or, according to European abbreviation, as X5CrNi1810 (or W. 1.4301) from the norm EN 10088/3 (in Italy as UNI EN 10088/3). Italian and European law do not describe limits relating to nickel concentration in wine, whereas the O.I.V. sets the limit at 0.01 mg/kg. The average nickel content in plants varies between 0.05 and 8.00 μg/g dry weight. Whilst this concentration very rarely exceeds 5 μg/g in normal growth conditions, it may reach up to 50 μg/g (100 μg/g) in ultrabasic substrates. Furthermore, the pH regulates mobility and therefore nickel can be assimilated from the ground to the plant. The effects of pH on nickel's chemical mechanisms have been demonstrated by way of various studies relating to the application of mud purifiers to soil, on plant nutrition, on the re-utilisation of serpentinitic soil and a study on ionic retention in soil. The studies collectively show that the quantity of nickel held by soil increases concomitantly with an increase in pH. In areas that receive mud depurates containing a high load of toxic minerals, or in which soils have a pH higher than 6.5, nickel and other metals should not cause toxicity to crops or damage the food chain. The critical concentration of nickel is much lower in acidic soils compared to calcareous or alkaline soils.

The data presented here show that throughout the production process of both *Aglianico* and the *Fiano* nickel levels are extremely low, and show a more or less constant level over time, with mg/l values around the second decimal place (see Fig. 6a and b); in some cases, particularly for *Aglianico*, the value is significant on the third decimal place.

4. Conclusions

Few statistical studies exist pertaining to the metal content of Italian wines. Recently Marengo and Aceto (2003) characterised the metal content of five classes of wines derived from the *Nebbiolo* grapes of northern Italy. Comparison of the data reported here with that of Marengo and Aceto highlights some interesting differences which exist between northern Italian wines and the *Fiano* and *Aglianico* wines of Southern Italy.

In particular, the initial copper concentration of both the *Aglianico* and *Fiano* is higher than that reported for *Nebbiolo* wines, though the values observed at the end of production process are

very low. Lead, cadmium and zinc concentrations of *Aglianico* and *Fiano* are in the same values range of *Nebbiolo* wines, while nickel is significantly inferior. Moreover, due to the scarcity of iron in the soils in which the Irpinia vines are grown, the iron concentration interval determined in our study is somewhat inferior to that observed for the *Nebbiolo* wines.

With regards to white wine (*Fiano*), and with the exception of lead and cadmium, the concentration of physiological and xenobiotic metals is almost always lower than in red wine; this is primarily due to the production process of the former, in which fermentation proceeds without grapes skin, the latter containing higher metal concentrations than pulp.

In conclusion, by way of an extremely rigorous control procedure characterised by ongoing monitoring of physiological and xenobiotic metal concentrations, the data presented confirms that both the wine production processes described here are well within the toxicological safety limits. With the exception of copper and zinc (whose concentration is unusually high for both wines due to antiparasitic treatments and plant zinc uptake, respectively), the concentration of the metals monitored throughout the production process of both wines fall well within the limits stated by both the Italian and O.I.V norms. The acceptable concentration limit of nickel is yet to be legally defined, and therefore remains difficult to compare to a standard reference.

During wine storage, it is very important to use a container that guarantees non-contamination with the liquid that it holds: being unassailable by wines or musts, 18/10 stainless steel described above certainly represents the best currently available option for this purpose. It is important to remember that for wine makers of coastal zones, i.e. those with vineyards in proximity to the sea and therefore in an area with a salty atmosphere, not only is the use of austenitic stainless steel advisable, but also those containing chromium and nickel with 2% of molybdenum, the latter being particularly effective in preventing corrosion caused by both the mixtures and reactions occurring during the productive cycle and wine maturation and that caused by the saline nature of the external atmosphere. Apart form this, an infinitesimal quantity of nickel will normally be present throughout a wine's enological production process, the cause of which is difficult to assign. Finally, treatments should be sought which will increase the concentration of iron that is particularly low in both wines.

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Food Chemistry 117 (2009) 561–567

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Validation of a headspace solid-phase microextraction procedure with gas chromatography-electron capture detection of pesticide residues in fruits and vegetables

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ARTICLE INFO

Article history:
Received 24 July 2008
Received in revised form 17 March 2009
Accepted 11 April 2009

Keywords:
HS-SPME
Organochlorine
Organophosphorous
GC-ECD

ABSTRACT

Headspace solid-phase microextraction (HS-SPME) was evaluated for the determination of pesticide residues in fruits and vegetables by gas chromatography with an electron capture detector (GC-ECD). The fibre used was coated with polydimethylsiloxane (100 μm thickness) and the analytical conditions employed have been developed and optimised in a previous work [Chai, M. K., Tan, G. H., & Asha, L. (2008). Optimisation of headspace solid-phase microextraction for the determination of pesticide residues in vegetables and fruits. *Analytical Sciences, 24* (2), 273–276]. The results show that the HS-SPME procedure gave a better linear range, accuracy, precision, detection and quantification limits and is adequate for analysing pesticide residues in fruits and vegetables. The average recoveries obtained for each pesticide ranged between 71% and 98% at three fortification levels with the relative standard deviation of less than 5%. Repeatability (0.3–3.7%) and intermediate precision (0.8–2.5%) were shown to be satisfactory. The limits of detection (0.01–1 μg L^{-1}) and the limits of quantification (0.05–5 μg L^{-1}) of these pesticides were much lower than the maximum residue levels (MRL), allowed for fruits and vegetables in Malaysia.

1. Introduction

The public awareness pertaining to the health hazards posed by pesticide residues in fruits and vegetables have led to the development of many analytical methods (Stajnbaher & Zupancic-Kralj, 2003) for measuring these residues. The main focus is on simplification, miniaturisation, and improvement of sample extraction and cleanup methods with universal microextraction procedures, namely supercritical fluid extraction (SFE) (Lehotay & Garcia, 1997; Nerin, Batlle, & Cacho, 1998; Stefani, Buzzi, & Grazzi, 1997), matrix solid-phase dispersion (MSPD) (Blasco, Font, & Pico, 2004; Bogialli, Curini, Corcia, Nazzari, & Tamburro, 2004; Kristenson, Haverkate, Slooten, & Ramos, 2001), solid-phase extraction (SPE) (Abhilash, Jamil, & Singh, 2007; Juan-Garcia, Pico, & Font, 2007; Sharif, Man, Hamid, & Chin, 2006; Stajnbaher & Zupancic-Kralj, 2003) on cartridges to replace liquid–liquid extraction (LLE), enzyme-linked immunosorbent assay (ELISA) (Bushway, Savage, & Ferguson, 1990; Watanabe, Yoshimura, Yuasa, & Nakazawa, 2001) and solid-phase microextraction (SPME) (Beltran, Peruga, Pitarch, Lopez, & Hernandez, 2003; Cai, Gong, Chen, & Wu, 2006; Chen, Su, & Jen, 2002; Zambonin, Cilenti, & Palmisano, 2002). Among these extraction and cleanup methods, SPME has become a popular technique in recent years. It is an inexpensive, environment-friendly and solvent-free technique with reliable and excellent sensitivity as well as good selectivity.

SPME was developed by Pawliszyn and co-workers in 1990 in an attempt to redress the limitations inherent in the SPE and LLE techniques (Kataoka, Lord, & Pawliszyn, 2000). It is a sample preparation technique using a fused-silica fibre which is coated on the outside with an appropriate stationary phase and is then employed to extract the analytes from a variety of matrices, which are subsequently transferred into the injector of a GC system for analysis. This sample preparation prior to the GC analysis can be carried out by direct immersion of the fibre into the sample (DI-SPME) or via the exposure of the fibre in the headspace above a liquid or solid sample (HS-SPME).

In a previous paper (Chai, Tan, & Asha, 2008), the optimisation of a HS-SPME extraction and thermal desorption procedure coupled to gas chromatography with electron capture detection for the determination of 8 pesticide residues in fruits and vegetables was carried out. A 100 μm polydimethylsiloxane (PDMS) coated fibre was found to be the most efficient in extracting the investigated pesticide residues. Parameters such as the effects of extraction time

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0308-8146/$ - see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2009.04.034

and temperature, salting-out effect, stirring speed, pH, desorption time and temperature, the effects of dilution and types of organic solvent were developed and optimised.

The purpose of the work reported here is to perform the validation of the optimised HS-SPME analytical method for the analysis of eight organochlorine (OC) and organophosphorous (OP) pesticide residues. The method, after validation, has been applied to various types of samples of fruits and vegetables found in Malaysia.

2. Materials and methods

2.1. Chemicals and standard solutions

All the solvents used were HPLC grade. Acetone and methanol were purchased from Fisher Scientific, Longhborough, UK. Eight pesticides standards >95% pure (diazinon, chlorothalonil, malathion, chlorpyrifos, quinalphos, profenofos, α-endosulfan and β-endosulfan) which are commonly used by local farmers in fruit and vegetable cultivation (Suzuki, 2003) were purchased from AccuStandard Inc., New Haven CT, USA. A range of standard mixture stock solutions containing 0.5–50 mg L^{-1} were prepared in methanol and stored at 4 °C. Preparation of different concentration levels of the stock solutions is carried out to correspond to the sensitivity of the ECD detector towards different compounds. Working standard solutions of a mixture of pesticides were freshly prepared daily by volume dilution in distilled water. 1-Chloro-4-fluorobenzene (98.0%), purchased from AccuStandard Inc. was used as the internal standard to compensate for sample and injection volume changes and was added to the vial prior to the GC-ECD analysis.

2.2. Sample preparation

In the multiclass and multiresidue analysis of pesticides in fruits and vegetables, three types of fruits namely strawberry (*Fragaria ananassa*), star fruit (*Averrhoa carambola*) and guava (*Psidium guajava*) and three types of vegetables namely cucumber (*Cucumis sativus*), tomato (*Lycopersicon esculentum*) and pakchoi (*Brassica parachinensis*) were obtained from a pesticide-free farm in the Malaysian Agricultural Research and Development Institute (MARDI), Selangor, Malaysia. For the HS-SPME analysis, 100 g of the individual fruit or vegetable sample was weighed and finely chopped. A 30 g subsample was accurately weighed and placed in a 150 mL beaker. Three concentration levels – low, medium and high, were spiked into the sample to provide the spiked control sample. After being kept at room temperature for 1 h, 30 g of distilled water was added to the spiked sample which was then blended and homogenised in a food processor. The sample was then placed in separate vials.

2.3. HS-SPME analysis

A homogenised spiked sample was added with 2% (vol/weight) of methanol/acetone (1:1) and optimum dilution was made with distilled water containing 10% NaCl until the total sample in the vial was equal to 5.00 g. Then, the internal standard was added. The sample was extracted via the headspace SPME method using a 100 μm PDMS coated fibre mounted in a manual syringe holder (obtained from Supelco, Bellefonte, PA, USA) at 60 °C for 30 min; with sample agitation at 800 rpm without any pH adjustment. Desorption was done at 240 °C for 10 min.

2.4. Gas chromatography-electron capture detector (GC-ECD)

A Shimadzu GC 17A version 2.21 gas chromatograph coupled with an electron capture detector (ECD) which was purchased form Kyoto, Japan was used. A SGE BPX5, 30 m × 0.32 mm i.d. capillary column with a 0.25 μm film was used in combination with the following oven temperature program: initial temperature 120 °C, then heated at 7 °C min^{-1} to a final temperature of 250 °C, and then held for 4.5 min. The total run time was 23.07 min. A silanised narrow-bore injector liner (0.75 mm i.d.) for the SPME injections was installed and the fibre was inserted into this injector using the splitless mode. The injector temperature was held at 240 °C and the detector temperature was maintained at 300 °C. Nitrogen (99.999%) was used as the carrier gas with a gas flow at 24.4 cm s^{-1} linear velocity and the pressure maintained at 94 kPa.

2.5. Validation studies

The calibration graph of each pesticide was constructed using samples spiked with six different concentrations of the standard mixture solutions. The calibration standard mixture solutions over the concentration range of interest were prepared by serial dilution of the mixed standard stock solution with methanol and then spiked to the fruit and vegetable samples. The analyte peaks obtained were integrated and plotted as functions of the concentration. The standard mixture solutions were analysed in triplicate by GC-ECD at each concentration level.

Samples spiked at three different concentrations and three replicates for each concentration were analysed on three different occasions together with a calibration curve were performed to establish the repeatability (intra-day precision), intermediate precision (inter-day precision) and accuracy of the method. The accuracy was determined as the mean of the measured value relative to the theoretical spiked values and is reported in percentage (%). The precision is represented by the intra- and inter-day relative standard deviation (RSD).

The selectivity of the method was assessed by comparing the chromatograms obtained with and without the analytes in the blank samples. Each analyte was injected separately to ensure that no interfering peaks with the same retention times were present.

The limits of detection (LOD) and the limits of quantification (LOQ) were evaluated as the signal-to-noise ratios of 3:1 and 10:1, respectively. The LOD and LOQ in distilled water were evaluated for each pesticide as follows:

(a) Retention times were determined by running the chromatogram of a standard solution.
(b) The average noise levels were measured by running the chromatogram of a blank sample.
(c) The concentration that led to a signal of three or 10 times the noise level was evaluated using the average of the peak areas of the spiked samples in triplicate and taking into account the values of the noise level.

Recovery tests were based on the addition of known amounts of pesticides to the fruit and vegetable samples. Since the SPME technique is a non-exhaustive extraction procedure, the relative recovery, which is defined as the ratio of the concentration found in the samples and working solution, spiked with the same amount of analytes was used instead of the absolute recovery associated with an exhaustive extraction procedure. The recoveries and linearity of the method was examined on pesticide-free fruit and vegetable samples. All recoveries were determined in triplicates at three concentration levels. The different spiking levels were carried out to reflect the sensitivity of the ECD detector towards different compounds.

3. Results and discussion

The development of the HS-SPME technique for trace analysis of multiresidue pesticides in fruits and vegetables without any pre-

treatment of the samples has been demonstrated in the previous paper (Chai et al., 2008). The developed HS-SPME method has resulted in a drastic reduction of the analysis time and organic solvent consumption. The use of the 100 μm PDMS fibre is most suitable for the analytes in this study which consist of non-polar to semi-polar compounds. The extraction time of 30 min was selected after taking into consideration factors such as sample preparation time, loss of analyte during extraction and the sensitivity of the method. The extraction temperature of 60 °C was found to be the optimum temperature. This is due to the fact that the extraction efficiency decreases when the extraction temperature exceeds 60 °C as a result of the degradation of the pesticides with increasing water vapour pressure in a gas tight vial and the formation of air bubbles. The use of 10% NaCl to increase the extraction efficiency is due to the salting-out effect. The extraction efficiency decreases when the percentage of NaCl exceeds 10% because of the predominant salt interaction with the analyte in the solution. The addition of water and 2% (vol/weight) of methanol/acetone (1:1) is necessary to enhance the release of the analytes from complex matrices such as fruits and vegetables.

When a method has been developed, it is important to validate it to confirm that it is suitable for its intended purpose. In this study, the analytical performance characteristics of the optimised HS-SPME method were validated.

3.1. Calibration curve (linearity)

The linearity of an analytical method is its ability to produce test results that correspond directly to the concentration of the analyte in the samples within a given range. For the linearity studies, a minimum of five different concentrations are recommended. It is also recommended that a specific range, normally from 80% to 120% of the expected concentration range (Shah, 2001) be employed.

Usually, spiked solutions are made with a known amount of a mixture of the analytes and the calibration curves are plotted by relating the peak areas obtained when desorption occurs at the concentrations used for spiking the samples. However, in real samples, the number of analytes and their concentrations are unknown, and some matrix effects which exist can affect the calibration curves. In order to ensure that no competition occurs between the analytes during the partition process, the calibration curves of the analytical method in this study were determined under three conditions: (a) with only one pesticide spiked in the sam-

Table 1
Comparison of the linearity range, r^2 and RSD (%) values of investigated pesticides in distilled water and in cucumber sample.

Pesticide	In distilled water			In cucumber sample		
	Range (μg L^{-1})	r^2	RSD (%)	Range (μg L^{-1})	r^2	RSD (%)
Diazinon	0.3–2000	0.9991	4.09	10–1000	0.9982	4.71
Chlorothalonil	0.3–2000	0.9974	3.20	10–1000	0.9971	6.50
Malathion	10–10000	0.9990	3.68	50–5000	0.9974	6.09
Chlorpyrifos	0.02–100	0.9988	4.36	0.5–50	0.9977	5.80
Quinalphos	10–10000	0.9980	3.59	50–5000	0.9973	4.16
α-Endosulfan	0.01–50	0.9980	3.03	0.1–20	0.9953	3.98
Profenofos	0.05–350	0.9993	2.11	1–100	0.9990	6.96
β-Endosulfan	0.05–350	0.9985	3.09	1–100	0.9960	3.58

Table 2
Repeatability of the optimised HS-SPME method in spiked cucumber and strawberry samples at three concentration levels.

Pesticide	Spiking levels (μg L^{-1})	Cucumber ($n = 3$)		Strawberry ($n = 3$)	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Diazinon	40	79.1	2.5	74.7	0.9
	160	81.1	1.9	79.9	0.9
	480	80.7	1.5	81.9	0.8
Chlorothalonil	20	78.9	2.0	70.5	1.2
	80	80.8	1.5	74.4	1.5
	240	81.7	1.6	71.1	1.2
Malathion	50	77.7	1.5	75.1	2.5
	250	70.6	2.0	83.5	2.2
	600	73.8	1.8	75.1	1.1
Chlorpyrifos	1	79.6	0.4	70.0	1.0
	4	71.4	3.7	70.3	0.7
	12	77.6	2.0	74.8	0.8
Quinalphos	50	76.6	1.7	71.5	0.9
	250	74.8	2.6	80.2	1.2
	600	73.9	2.0	81.3	1.4
α-Endosulfan	0.5	81.4	1.5	72.8	0.7
	2	77.4	1.5	71.0	1.0
	6	80.3	1.7	81.8	0.8
Profenofos	5	78.9	1.9	78.3	1.4
	20	78.0	0.8	80.9	0.9
	60	80.8	0.4	81.6	2.0
β-Endosulfan	1	81.9	1.8	73.4	0.3
	4	80.7	2.5	82.8	0.8
	12	73.0	2.8	78.7	0.8
Range	0.5–480	71.4–81.9	0.4–3.7	70.0–83.5	0.3–2.5

ples (cucumber), (b) a mixture of eight pesticides spiked in the samples (cucumber), and (c) a mixture of eight pesticides spiked in distilled water.

The method of internal standard quantification was carried out at six levels of concentrations using three different conditions in triplicate. The peak area ratio (peak area of analytes/peak area of internal standard) was used for each compound.

The results show that the three calibration curves are almost similar with the r^2 value >0.9950 for all the calibration curves. These results are significant and imply that, the partition process is reproducible using these conditions, thus indicating that the method is quantitative for these pesticides.

Table 1 shows the comparison of the linearity range, with the r^2 and RSD values of investigated pesticides in distilled water and in the cucumber sample. The linearity range, from the r^2 and RSD values are slightly better in distilled water when compared to the cucumber sample, showing that the vegetable sample has a small matrix effect in the analysis of the investigated pesticides. Overall, the linearity using these two conditions is acceptable and the r^2 values are better than 0.9950 in all cases with the RSD value of less than 7% for all the investigated pesticides.

3.2. Precision

The precision of an analytical method is the closeness of a series of individual measurements of an analyte concentration when the analytical procedure is applied repeatedly to multiple samplings of a homogeneous sample. The precision is usually expressed as the relative standard deviation (RSD). The measured RSD can be subdivided into three categories: repeatability (intra-day precision), intermediate precision (inter-day precision) and reproducibility (inter-laboratory precision as in a collaborative study). In this study, we have measured the repeatability and intermediate precision.

The accuracy of an analytical method is a measure of the degree of closeness between the true value of the analytes in the sample and the value as determined by the method and is sometimes called "*trueness*" (Shah, 2001). Accuracy can be measured by analysing samples with known concentrations and comparing the measured values with the true values.

(a) Repeatability

The repeatability of an analytical method refers to the use of the procedure within a laboratory over a short period of time, carried out by the same analyst with the same equipment. According to the International Conference on Harmonisation (ICH), it is recommended

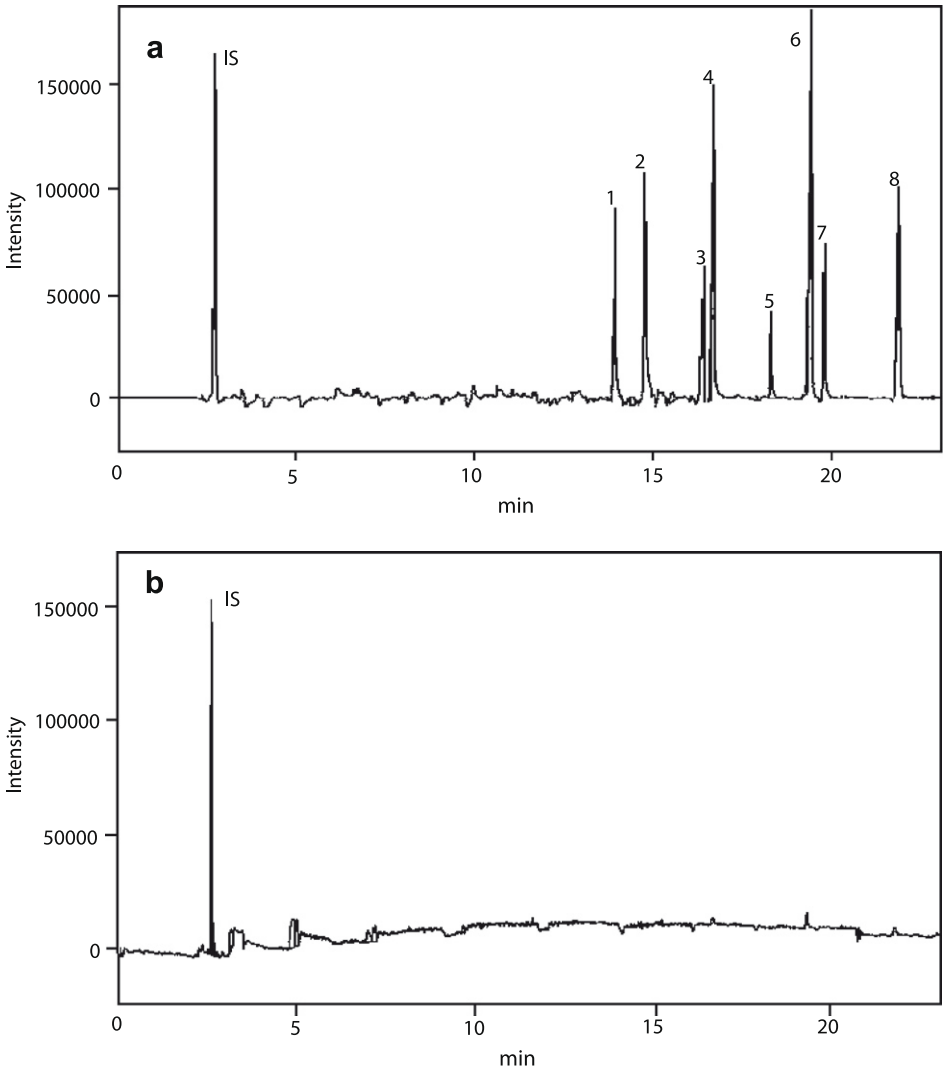

Fig. 1. Selectivity chromatograms (a) spiked cucumber sample (b) blank cucumber sample. IS (internal standard), 2.68 min; 1. Diazinon, 13.58 min; 2. Chlorothalonil, 14.74 min; 3. Malathion, 16.42 min; 4. Chlorpyrifos, 16.65 min; 5. Quinalphos, 18.30 min; 6. α-Endosulfan, 19.37 min; 7. Profenofos, 19.76 min; 8. β-endosulfan, 21.83 min.

that repeatability be assessed using a minimum of nine determinations covering the specified range (i.e., three concentrations and three replicates for each concentration) or a minimum of six determinations of 100% of the test concentration (ICH-Topic Q2b, 1996).

The intra-day accuracy and repeatability was assessed, at three concentration levels and with three replicates for each concentration on the same day. Table 2 shows the repeatability of the method for the investigated compounds in the spiked cucumber and strawberry samples.

The results show that the intra-day accuracies ranged between 71.4% and 81.9% with the RSD values between 0.4% and 3.7% for the cucumber samples. For the strawberry samples, the intra-day accuracies ranged from 70.0% to 83.5% with the RSD values from 0.3% to 2.5%. For the analysis of pesticide residues at the ppb or ppm levels, accuracy and recovery of 70% to 120% are considered as acceptable (Herdman, Pahrham, & Shen, 1988). Hence, the results obtained above can be considered to be acceptable for the concentration levels being investigated.

(b) Intermediate precision

The intermediate precision in this study is based on the mean repeatability values of a set of spiked samples at three concentration levels and analysed daily for a period of 4 days. The inter-day accuracies ranged from 70.7% to 83.9% with the RSD values from 0.8% to 2.5% for both samples indicating that the proposed HS-SPME method produces acceptable intermediate precision.

With the use of the internal standard and the relative recovery method, the RSD values of less than 4% are obtained. The internal standard and relative recovery method can overcome the common problems of the SPME technique such as inconsistency of the fibre quality and poor repeatability.

3.3. Selectivity/specificity

According to the ICH classification, specificity is defined as the ability to assess unequivocally the analyte in the presence of other components, which may be expected to be present such as impurities, degradation products and matrix components. Other authoritative international organisations such as IUPAC and AOAC have preferred the term selectivity, reserving specificity for those procedures that are completely selective (Soh & Abdullah, 2005).

The selectivity of the analytical method in this study was determined by comparing the chromatograms of a blank matrix solution with another spiked matrix solution. Fig. 1 shows the chromatograms of the spiked cucumber sample and the blank cucumber sample by GC-ECD. The analytes of interest were well-separated from the other components present in the samples. SPME is an equilibrium method which is more selective when compared to other exhaustive methods as it takes full advantage of the differences in the extracting phase/matrix distribution constants to separate the mixture of pesticides from the interferences. This SPME technique has demonstrated its selectivity since it does not require an additional cleanup step to remove any interference prior to GC analysis.

3.4. Limits of detection (LOD) and limits of quantification (LOQ)

The LOD is the lowest concentration of the analyte in a sample which can be detected but not necessarily quantified. The LOQ is the lowest concentration of the analyte in a sample which can be quantified with an acceptable degree of accuracy and precision. The LOQ should have an accuracy of 80–120% and the RSD value not exceeding 20% for its precision (Shah, 2001).

The LOD values obtained (Table 3) are below the first calibration level. These values are lower than the Maximum Residue Levels as stipulated in the Codex Alimentarius (EU, 2004) for fruits and vegetables. α-Endosulfan showed the lowest LOD values for all the samples because it is the most sensitive analyte measured by the ECD detector compared to the other analytes in this study.

Table 3
Limit of detection (LOD), limit of quantification (LOQ) and Codex Alimentarius maximum residue limits (MRL) of the investigated pesticide using the optimised HS-SPME method.

Pesticide	Pesticide level ($\mu g\ L^{-1}$)	Pesticide Level ($\mu g\ L^{-1}$)					
		Cucumber	Tomato	Pakchoi	Guava	Starfruit	Straw berry
Diazinon	LOD	0.2	0.2	0.2	0.2	0.2	0.2
	LOQ	1	1	1	1	1	1
	MRL	20	50	20	20	20	20
Chlorothalonil	LOD	0.2	0.2	0.2	0.2	0.2	0.2
	LOQ	1	1	1	1	1	1
	MRL	1000	2000	5000	3000	3000	3000
Malathion	LOD	1	1	1	1	1	1
	LOQ	5	5	5	5	5	5
	MRL	3000	3000	3000	500	500	500
Chlorpyrifos	LOD	0.02	0.02	0.02	0.02	0.02	0.02
	LOQ	0.1	0.1	0.1	0.1	0.1	0.1
	MRL	50	50	50	200	200	200
Quinalphos	LOD	1	1	1	1	1	1
	LOQ	5	5	5	5	5	5
	MRL	50	50	50	50	50	50
α-Endosulfan	LOD	0.01	0.01	0.01	0.01	0.01	0.01
	LOQ	0.05	0.05	0.05	0.05	0.05	0.05
	MRL	50	500	50	50	50	50
Profenofos	LOD	0.1	0.1	0.1	0.1	0.1	0.1
	LOQ	0.5	0.5	0.5	0.5	0.5	0.5
	MRL	50	50	50	50	50	50
β-Endosulfan	LOD	0.1	0.1	0.1	0.1	0.1	0.1
	LOQ	0.5	0.5	0.5	0.5	0.5	0.5
	MRL	50	500	50	50	50	50
Range	LOD	0.01–1					
	LOQ	0.05–5					

3.5. Recovery

The mean recovery data and its RSD values obtained in the analysis of fortified fruit and vegetable samples are as listed in Table 4. Acceptable relative recoveries were obtained, ranging between 71% and 97% for the vegetable samples with the RSD values ranging from 0.1% to 4.7%, and the relative recoveries of 76–98% for the fruit samples with the RSD values ranging from 0.5–4.7%. The percentage relative recoveries and the RSD values obtained for the fruit samples were slightly better than those obtained for the vegetable samples. This is probably due to the presence of higher total suspended solids in the vegetable samples. When all the fruit and vegetable samples are compared, the relative recoveries obtained in "*pakchoi*" are lower than those obtained from the other samples. This could be due to the water content of the "*pakchoi*" being the lowest among all the samples. From the results, it can be seen that the matrix has little effect on the developed HS-SPME method.

3.6. Real sample analysis

The developed HS-SPME method has been applied to the analysis of 10 fruits and vegetables purchased from a local market. Table 5 shows the pesticide levels detected in the selected fruit and vegetable samples. The residue levels of the pesticides determined were found to be lower than the MRLs specified by the EU (2004).

To ensure the validity of the results when the proposed method is applied for routine analysis, the following internal quality control criteria were applied in order to check if the system is under control:

A blank extract was carried out daily to eliminate any false positive via contamination in the extraction process, instrument or reagents used.

A blank extract spiked at the intermediate concentration level was run prior to the analysis of the real sample in order to assess the extraction efficiency. Recoveries between 70% and 120% are considered as acceptable in any method development.

Calibration curves are prepared daily to check for both, sensitivity and linearity in the working concentration range in order to avoid errors in quantitation caused by possible matrix effects or instrumental fluctuation ($r^2 > 0.99$ are required).

4. Conclusions

The developed HS-SPME method offers significant advantages and can overcome the common problems of SPME. The use of the

Table 4
Spiked concentration levels and relative recoveries in fortified fruits and vegetables by using GC-ECD.

Pesticide	Spiking levels (μg L^{-1})	Recovery (%) (RSD %, $n = 3$)					
		Cucumber	Tomato	Pakchoi	Guava	Starfruit	Straw berry
Diazinon	40	84 (1.9)	94 (1.5)	88 (0.1)	76 (3.1)	84 (1.1)	82 (0.6)
	160	88 (2.3)	88 (1.5)	94 (2.5)	88 (1.4)	84 (4.3)	83 (2.5)
	480	94 (2.2)	92 (1.5)	90 (0.4)	83 (3.7)	89 (1.5)	93 (1.1)
Chlorothalonil	20	88 (3.1)	88 (0.6)	86 (1.0)	82 (4.7)	78 (3.1)	76 (0.8)
	80	88 (0.4)	89 (4.4)	94 (4.7)	85 (1.0)	79 (1.1)	78 (2.9)
	240	97 (0.6)	92 (1.2)	85 (2.0)	84 (4.2)	74 (4.6)	78 (1.2)
Malathion	50	82 (3.2)	97 (2.8)	97 (3.7)	94 (1.0)	90 (1.1)	84 (3.2)
	250	80 (3.9)	91 (1.4)	96 (1.8)	95 (2.9)	90 (1.2)	90 (0.9)
	600	86 (3.0)	97 (4.2)	95 (1.7)	95 (1.3)	87 (2.0)	84 (2.6)
Chlorpyrifos	1	83 (1.0)	86 (2.1)	77 (1.1)	94 (1.0)	78 (3.1)	76 (0.8)
	4	88 (2.1)	80 (0.2)	84 (0.5)	95 (1.1)	82 (1.1)	80 (0.6)
	12	91 (2.6)	82 (0.8)	74 (1.7)	94 (1.0)	81 (1.7)	81 (1.8)
Quinalphos	50	80 (3.2)	91 (3.0)	93 (2.7)	95 (1.0)	90 (3.4)	79 (2.7)
	250	84 (1.6)	94 (1.1)	89 (1.7)	93 (3.4)	94 (2.7)	97 (2.0)
	600	86 (4.0)	95 (0.9)	96 (1.7)	89 (1.6)	90 (2.2)	91 (2.8)
α-Endosulfan	0.5	88 (3.0)	94 (2.0)	71 (2.8)	95 (1.0)	78 (2.3)	80 (4.2)
	2	89 (4.4)	92 (1.2)	78 (1.6)	92 (1.0)	77 (1.9)	78 (3.8)
	6	95 (4.5)	93 (1.3)	76 (3.1)	92 (1.1)	79 (2.6)	86 (4.4)
Profenofos	5	85 (1.5)	86 (1.2)	88 (1.1)	93 (3.3)	88 (1.2)	87 (1.9)
	20	84 (1.6)	93 (1.3)	91 (3.7)	93 (3.1)	93 (1.0)	88 (0.6)
	60	81 (3.6)	90 (1.0)	81 (1.7)	97 (1.3)	88 (2.9)	95 (1.0)
β-Endosulfan	1	89 (0.8)	81 (2.7)	72 (1.0)	97 (3.0)	89 (1.2)	83 (0.5)
	4	94 (1.5)	82 (1.1)	78 (1.6)	96 (1.0)	90 (1.2)	91 (2.0)
	12	97 (1.5)	82 (1.4)	71 (3.2)	98 (2.2)	88 (1.5)	85 (1.1)
Range	0.5–600	Vegetables: 71–97 (0.1–4.7)			Fruits: 76–98 (0.5–4.7)		

Table 5
Pesticide level detected in selected fruits and vegetables.

Pesticide	Pesticide level, μg L^{-1} (RSD, %, $n = 3$)									
	Cucumber	Tomato	Pakchoi	Chili	Spinach	Guava	Starfruit	Strawberry	Mango	Papaya
Diazinon	n.d	9.4 (1.5)	n.d	n.d	n.d	7.0 (0.6)	n.d	n.d	n.d	n.d
Chlorothalonil	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Malathion	n.d	n.d	56.8 (0.7)	n.d	53.5(1.0)	n.d	n.d	n.d	n.d	n.d
Chlorpyrifos	6.2 (1.4)	n.d	n.d	n.d	n.d	n.d	4.9 (0.5)	n.d	6.0 (0.6)	n.d
Quinalphos	n.d	21.4 (2.0)	n.d	35.3 (0.6)	n.d	20.5 (2.2)	n.d	41.3 (2.2)	n.d	n.d
α-Endosulfan	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Profenofos	n.d	n.d	17.4 (3.6)	n.d	19.7 (0.7)	n.d	n.d	15.0 (0.7)	n.d	n.d
β-Endosulfan	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d

n.d – not detected.

internal standard and relative recovery test can compensate for any inconsistency in the quality of the fibre to give high precision (<4%) and good recoveries (71–98%). The use of the headspace method can prolong the lifetime of the fibre until 120 extractions. By using optimum dilution and 2% organic solvents, the recoveries can be further improved. The validation results for the developed HS-SPME method in this study are comparable or even better when compared with the results reported by Berrada, Font, and Molto (2004), Zambonin, Quinto, Vietra, and Palmisano (2004), Dong, Zeng, and Li (2005), Cai et al. (2006) and Fytianos, Raikos, Theodoridis, Velinova, and Tsoukali (2006) for various food matrices.

The developed HS-SPME method is fast, cheap and environment-friendly because it employs a very simple sample preparation procedure with very minimal organic solvent consumption.

Acknowledgements

The authors would like to acknowledge the support given by Universiti Tenaga Nasional and University of Malaya. In addition, the authors would also like to thank the Malaysia Toray Science Foundation for the financial support.

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Food Chemistry 117 (2009) 568–575

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Acidification, crushing and thermal treatments can influence the profile and stability of folate poly-γ-glutamates in broccoli (*Brassica oleracea* L. *var. italica*)

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ARTICLE INFO

Article history:
Received 14 July 2008
Received in revised form 6 April 2009
Accepted 13 April 2009

Keywords:
Acidification
Blanching
Broccoli
Folates
Crushing
Poly-γ-glutamates

ABSTRACT

The influence of different treatments, i.e., crushing, high temperature short time (90 °C/4 min) (HTST) and low temperature long time (60 °C/40 min) (LTLT) blanching, acidification (pH 4.3), and sequences of these treatments on the folate poly-γ-glutamate profile and stability were investigated. In this study, broccoli was used as a case study. Regarding the folate poly-γ-glutamate profile, endogenous folate poly-γ-glutamates in broccoli florets were found predominantly as hepta- and hexa-γ-glutamates. Crushing raw broccoli, acidification and LTLT blanching enhanced folate deconjugation resulting in monoglutamate, di- and tri-γ-glutamates. Compared to other treatments, HTST blanching preformed prior to crushing resulted in the highest concentration of long chain poly-γ-glutamates. Regarding folate poly-γ-glutamates stability, acidification combined with LTLT blanching decreased folate stability whereas HTST blanching combined with different sequences of blanching and crushing did not affect folate poly-γ-glutamates stability. It was concluded that crushing (prior to heating), acidification and blanching could be strategically applied to increase the folate monoglutamate content of broccoli.

1. Introduction

Folates have been attracting a lot of attention due to their association with reduction in the occurrence of neural tube defects, certain types of cancer and diseases, e.g., cardiovascular disease by lowering homocysteine levels (Lucock, 2002; McKillop et al., 2002; Stranger, 2002). Man cannot synthesize folates and therefore has to obtain them from the diet. Dietary sources rich in folates include yeast extracts, liver, eggs, kidney, green leafy vegetables, legumes and citrus fruits (Scott, Rébeillé, & Fletcher, 2000).

The bioavailability of folates from plant based foods has been reported to vary from 50% to 98% in comparison with that of synthetic folic acid (Brouwer et al., 1999; Hannon-Fletcher et al., 2004; Sauberlich, Kretsch, Skala, Johnson, & Taylor, 1987; Seyoum & Selhub, 1998). Low folate bioavailability could be attributed to various factors such as folate instability and existence of dietary folates as poly-γ-glutamates (Seyoum & Selhub, 1998). Due to their state of reduction at the 5–8 positions of the pteridine moiety, dietary folates are quite unstable during processing, and can easily undergo oxidation (McKillop et al., 2002; Murphy, Boyle, Weir, & Scott, 1976; Murphy, Keating, Boyle, Weir, & Scott, 1978). The stability of folates during processing is influenced by temperature, pressure, pH, oxygen, light, metal ions, antioxidants and duration of heating (Gregory, 1989; Hawkes & Villota, 1989; Indrawati et al., 2004; McKillop et al., 2002; Verlinde, Oey, Hendrickx, & Van Loey, 2008).

In fruits and vegetables, folates mainly occur as poly-γ-glutamates with typically 2–11 γ-glutamic acid residues (Eitenmiller & Landen, 1999; Gregory, 1989; Lucock, 2002; Melse-Boonstra et al., 2002; Scott et al., 2000; Seyoum & Selhub, 1998). Before absorption in the intestines, folate poly-γ-glutamates must first be deconjugated to monoglutamate by the intestinal γ-glutamyl hydrolase (GGH, EC 3.4.19.9). However, the activity of intestinal GGH is said to be susceptible to inhibition by constituents found in some foods (Bhandari & Gregory, 1990), and such inhibition may further jeopardize the bioavailability of dietary folates. Therefore, increasing folate monoglutamate concentration in foods prior to ingestion could increase the bioavailability of dietary folates. Plant GGH, the enzyme that catalyses the hydrolysis (deconjugation) of folate poly-γ-glutamates to shorter chain poly-γ-glutamates and monoglutamates (Orsomando et al., 2005), has been reported to occur in the cytosol, extracellularly and in the vacuole (Huangpu, Pak, Graham, Rickle, & Graham, 1996; Lin, Rogiers, & Cossins, 1993; Orsomando et al., 2005). Therefore, matrix disruption (e.g., cutting, crushing, processing) facilitate the GGH catalyzed hydrolysis of native folate poly-γ-glutamates to shorter poly-γ-glutamates and monoglutamate (Leichter, Landymore, & Krumdieck, 1979; Melse-Boonstra et al., 2002).

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.04.036

Despite the potential of exploiting processing techniques to increase the bioavailability of dietary folates (by enhancing poly-γ-glutamate deconjugation), only limited data on the folate poly-γ-glutamate profile and the influence of processing on the poly-γ-glutamate profile of fruits and vegetables is available (Ndaw, Bergaentzlé, Aoudé-Werner, Lahély, & Hasselmann, 2001; Verlinde et al., 2008; Zheng, Lin, Lin, & Cossins, 1992). Hereto, the objectives of this research were to evaluate the native profile of folate poly-γ-glutamates; the effects of different treatments namely blanching, crushing and acidification on stability and deconjugation of folate poly-γ-glutamates and the effect of different sequences of performing these treatments on the stability and deconjugation of folate poly-γ-glutamates. In this study, broccoli was used as a case study since it is rich in folate poly-γ-glutamates.

2. Materials and methods

2.1. Reagents and folate standards

All chemicals used were of analytical or HPLC grade. Unless otherwise stated, all reagents were prepared using double demineralized water (18 MΩ cm at 25 °C) produced with a water purification system (Simplicity 185, Millipore Massachusetts, USA). The following folate poly-γ-glutamate standards were obtained from Schircks Laboratories (Switzerland); (6R,S)-5-methyl-5,6,7,8-tetrahydrofolic acid poly-γ-glutamates (5-CH_3–H_4PteGlu$_{2-5}$) trihydrochloride salts, pteroylhexa-γ-L-glutamic acid (PteGluγ$_6$) and pteroylhepta-γ-L-glutamic acid (PteGluγ$_7$). 5-CH_3–H_4PteGluγ$_6$ and 5-CH_3–H_4PteGluγ$_7$ were obtained by chemical conversion of PteGluγ$_6$ and PteGluγ$_7$ as outlined by Ndaw et al. (2001). (6S)-5-Methyl-5,6,7,8-tetrahydrofolic acid calcium salt (5-CH_3–H_4PteGlu$_1$) was obtained as a gift from Merck Eprova AG (Schaffhausen, Switzerland).

2.2. Sample preparation

To determine the native folate poly-γ-glutamate profile, one batch of each of two broccoli varieties (Belstar (BS) from Belgium, and unknown variety from Holland (HK)) was purchased from a local greengrocer. The broccoli was stored at 4 °C for maximum five days before use. For each of the two varieties the florets (including 1 cm of stalk) were cut from the broccoli heads, mixed to obtain a homogenous sample and then divided into two portions. One portion of intact florets was vacuum-packed in plastic bags (50 g per bag) and given a heat shock treatment by boiling in water for 10 min to inactivate endogenous GGH and other enzymes. The heat shocked florets were then cooled in ice water, frozen in liquid nitrogen and stored at −80 °C until folate extraction. The other portion of intact florets was vacuum-packed and directly frozen in liquid nitrogen without heat shock treatment (no inactivation of GGH and other enzymes) and stored at −80 °C until extraction. Prior to folate extraction, the frozen samples were ground (Grindomix, GM 200, Germany) to a frozen powder.

To determine the influence of different treatments on the stability and profile of folate poly-γ-glutamates, one batch of broccoli (20 kg, variety Grammel, Spain) was purchased from a local supplier. During the study, the broccoli was stored at 4 °C for a period not longer than 5 days. Both the florets and the stalks were used for the treatments. Broccoli heads were obtained by cutting the main stalk at the point slightly below the emergence of the smaller stalks. The florets, together with about 1 cm of stalk, were cut off from the rest of the stalk and these were used as broccoli floret samples. The rest of the stalk was cut into pieces of about 1 cm and used as broccoli stalk samples. The bottom main stalk was discarded.

2.3. Treatments

Two different crushing-blanching sequences were carried out as illustrated in Fig. 1. Mechanical (i.e., crushing) and thermal (blanching) treatments were conducted at the natural pH of broccoli around 6.5 and at acidic pH of 4.3. To obtain pH 6.5 and 4.3, broccoli florets or stalks were crushed (Buchi mixer, B-400, Switzerland) in the presence of distilled water or acetate buffer (0.2 M, pH 3.7) respectively, at a ratio of 1:1 (w/v). A crushing time of 20 s was standardized.

For thermal treatments, the samples were vacuum-packed (Multivac A300/16, Wolfertschwenden, Germany) in plastic bags (50 g per bag) and heated in a water bath (Memmert, WBU 45, Germany) either before or after crushing. High temperature short time (HTST) blanching was carried out at 90 °C for 4 min and low temperature long time (LTLT) blanching at 60 °C for 40 min. For the samples crushed before blanching, a 30 min period (including the time for vacuum-packing) after crushing and before blanching was standardized. For the samples crushed after the thermal treatment, a 15 min period after crushing (including the time for vacuum-packing) was standardized. After the various treatments, samples were cooled in ice water, frozen in liquid nitrogen and then freeze dried. The samples were then packed in opaque plastic containers and stored in a desiccator containing phosphorous pentoxide (P_2O_5) at room temperature for maximum 21 days.

2.4. Folate analysis

Folate analysis including extraction, chemical conversion of broccoli folates to 5-CH_3–H_4PteGlu$_n$, purification, and quantification was performed according to the method of Ndaw et al. (2001) optimized for broccoli (Verlinde et al., 2008) with a few modifications. Regarding folate extraction, 20 g heat shocked or 20 g non-heat shocked samples was respectively mixed with 50 ml cold or 50 ml boiling extraction buffer (phosphate buffer (0.1 M, pH 7, containing ascorbic acid)). Afterwards, the mixtures were homogenized (Ultra Turrax T25, IKA Labortechnik, Staufen, Germany) for 30 s. The non-heat shocked samples were hot extracted by immersion of the sample-containing tubes in boiling water for 10 min and subsequently cooled in ice water. The heat shocked samples did not receive a hot extraction to avoid folate degradation due to excessive heating and so that both categories of samples received approximately the same total heating intensity.

To extract folates from treated samples, 2 g freeze-dried broccoli was mixed with 50 ml boiling extraction buffer (phosphate buffer (0.1 M, pH 7, containing ascorbic acid) and 17.8 ml of double distilled water. The subsequent extraction steps were performed as described by Verlinde et al. (2008). The heat shocked and non-heat shocked samples were analyzed in triplicate while the treated samples were analyzed in duplicate. The dry matter content of broccoli folate and stalks was determined by oven drying broccoli at 105 °C for 16 h.

Quantification of the folates from broccoli was done using an external calibration with folate standards (5-CH_3–H_4PteGlu$_{1-7}$) and was based on peak height. The detection limit for the various poly-γ-glutamates and monoglutamates ranged from 0.015 to 0.100 pmol on column. The recovery of different folate derivatives as determined by spiking broccoli with standard (5-CH_3–H_4PteGlu$_{1-7}$) was taken into account in the quantification.

2.5. Method validation and recovery determination

The efficiency of the chemical conversion used in the current study had been previously demonstrated to be high (Verlinde et al., 2008). However, since the quality of the folate binding proteins (in the affinity chromatography columns) degrade with

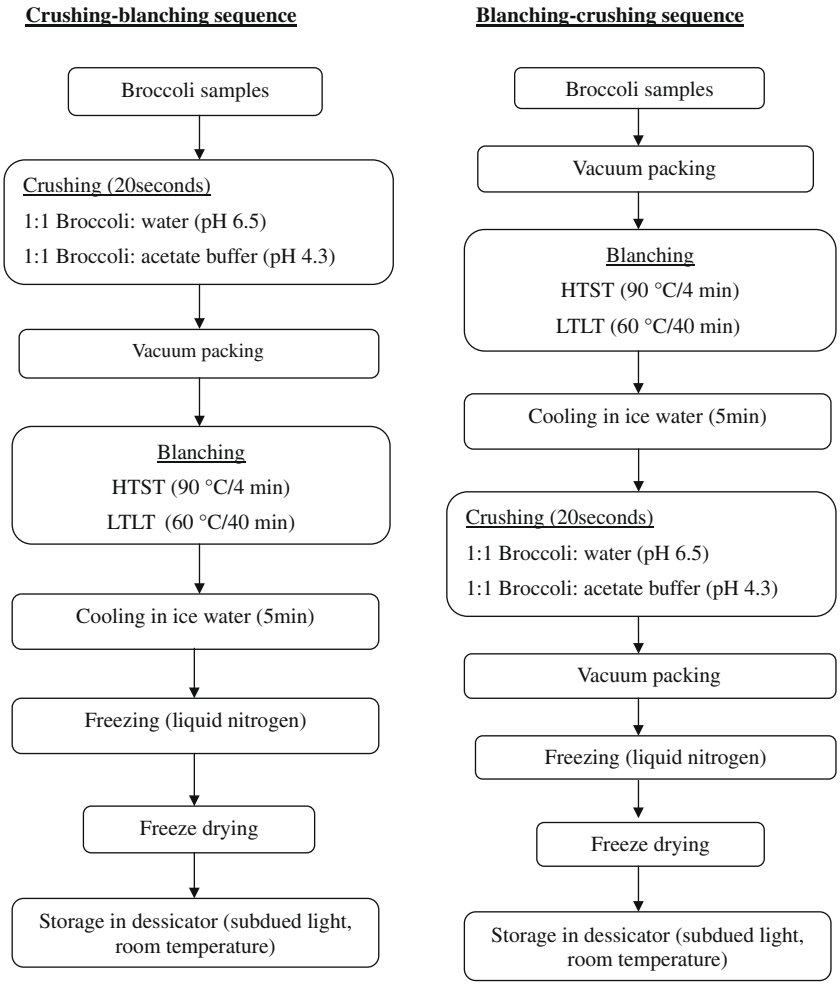

Fig. 1. Schematic representation of the different treatments applied to broccoli florets and stalks.

time, freshly made affinity chromatography columns were used in the current study. To ensure maximum folate recovery from samples, the maximum binding capacity of the affinity chromatography columns was checked using an excess of 5-CH_3–H_4PteGlu$_1$ (4.02 nmol). During folate purification, only 5 ml of chemically converted samples (with folate content less than 25% of the maximum binding) was applied on the columns.

The entire method of folate analysis was validated by analyzing a certified reference material consisting of lyophilized mixed vegetables (CRM 485, BCR, Geel-Belgium) and by spiking heat shocked broccoli with folate standards. Six grams of the CRM 485 was analyzed in triplicate. The dry matter content of the reference material was determined by oven drying the samples for 2 h at 105 °C.

Heat shocked broccoli was used during the spiking experiment to avoid enzymatic interconversion of folate poly-γ-glutamates which might interfere with the quantification of the different folates. Ten grams of the heat shocked broccoli was spiked with a mixture of 5-CH_3–H_4PteGlu$_{1-7}$ (total 29.4 nmol) in the beginning of the extraction procedure. The 5 ml chemically converted (spiked) sample applied on the affinity columns contained 0.32 nmol folates (approximately 10% of the maximum binding capacity). The folate content in the spiked broccoli ($n = 3$) was compared with the folate content in non spiked broccoli ($n = 3$).

2.6. Data analysis

Significance of differences for the mean of total folate contents and percent distribution of various folate derivatives in differently treated samples were analyzed by one way ANOVA using Tukey method (S-Plus 6.1.2, Release 1 for Microsoft Windows: 2002). The mean of total folate contents and percent distribution of various derivatives in all treated samples were compared with that of unblanched crushed (pH 6.5) samples.

3. Results and discussion

3.1. Method validation and recovery of folates

The recovery of folates during the chemical conversion step had been previously demonstrated to be close to 100% (Verlinde et al., 2008). The maximum binding capacity of the affinity columns as determined with 5-CH_3–H_4PteGlu$_1$ ranged from 2.24 to 3.15 nmol for the five columns used in the current study. The entire method of folate analysis was validated by analyzing a certified reference material (CRM 485). Using the current method, 2.87 ± 0.22 μg PteGlu1/g DM (6.50 ± 0.50 nmol/g DM) was found in CRM 485. This value was based on a dry matter content of 98.5% for CRM 485 and was in the range of 3.15 ± 0.28 μg PteGlu/g DM (7.15 ± 0.63 nmol/g DM) stated in the certification for this material. The predominant folate derivative in CRM 485 was monoglutamate (2.19 ± 0.23 μg PteGlu/g DM).

The average recovery of the folates spiked into broccoli was 91%. The recovery for individual folates is summarized in Table 1. These recoveries were taken into account in quantifying the folate content in broccoli samples.

Table 1
Individual folate derivative recovery from heat shocked broccoli spiked with 5-CH_3–$H_4PteGlu_{1-7}$ standards.

Folate derivative	Percent recovery
Hepta-γ-glutamate	90 ± 12[a]
Hexa-γ-glutamate	90 ± 15
Penta-γ-glutamate	96 ± 16
Tetra-γ-glutamate	88 ± 18
Tri-γ-glutamate	99 ± 6
Di-γ-glutamate	89 ± 11
Monoglutamate	91 ± 12

[a] Mean ± SD (n = 3).

3.2. Native folate poly-γ-glutamates profile of broccoli

To determine the native profile of broccoli folates, intact broccoli florets were first subjected to a heat shock treatment in order to inactivate endogenous broccoli GGH. The folate profile of the heat shocked broccoli was compared with that of non heat shocked (raw) broccoli. Although differently substituted folate derivatives (10-formyldihydrofolate (10-HCO–$H_2PteGlu_{1)}$, 5-formyltetrahydrofolate (5-HCO–$H_4PteGlu_1$), tetrahydrofolate ($H_4PteGlu_1$)) have been isolated from broccoli, results from several authors show that broccoli folates are mainly 5-CH_3 substituted (Konings et al., 2001; Stea, Johansson, Jägerstad, & Frølich, 2006; Zheng et al., 1992).

Table 2 shows the poly-γ-glutamate profile of both heat shocked and raw broccoli florets. Folates with 1–7 glutamate residues were found in broccoli. The folates in heat shocked broccoli predominantly occurred as hexa- and hepta-γ-glutamates. However, in comparison to the heat shocked broccoli, raw broccoli contained lower concentration of hexa- and hepta-γ-glutamates and higher concentration of tri-, di-γ-glutamate and monoglutamate. Results from the current study indicate that the native profile of broccoli folates consist mainly of hepta- (>50%) and hexa-γ-glutamates (>20%) and very little of folate monoglutamates. However, upon matrix disruption in the presence of active endogenous GGH the hepta- and hexa-γ-glutamates are deconjugated to shorter chain folate poly-γ-glutamates and monoglutamates. From these observations it was concluded that endogenous broccoli GGH was active in the raw samples and was responsible for the deconjugation of hepta- and hexa-γ-glutamates to shorter folate poly-γ-glutamates and folate monoglutamates. Since the raw broccoli was frozen with liquid nitrogen in the intact form and stored at −80 °C before extraction, not much enzymatic reaction due to GGH activity occurred in the frozen state. However, it is likely that most of the deconjugation took place during thawing and during the early stages of the hot extraction before the broccoli samples reached temperatures sufficient to inactivate GGH.

The native profile of broccoli folate poly-γ-glutamates observed in the current study is different from that reported in literature. Verlinde et al. (2008) reported that two broccoli varieties predominantly contained tri-γ-glutamates (>50%) while Zheng et al. (1992) reported that broccoli consisted of 80% di-γ-glutamates. Zheng et al. (1992) sliced the raw vegetables prior to hot extraction while Verlinde et al. (2008) extracted folates from frozen but raw broccoli. The high percentage of tri-γ-glutamate (Verlinde et al., 2008) and di-γ-glutamates (Zheng et al., 1992) could be the result of deconjugation of longer chain poly-γ-glutamates to the shorter chain poly-γ-glutamates during extraction of raw samples containing active endogenous GGH.

Regarding the poly-γ-glutamate profile of other vegetables, Zheng et al. (1992) reported folates with 2–8 γ-glutamyl residues for cauliflower, lettuce, cabbage pea leaves and carrot while Ndaw et al. (2001) found folates with 1–6 γ-glutamyl residues in spinach and peas. The latter authors reported relatively high percentage of monoglutamate (24%) in peas and spinach, an indication of GGH activity during sample handling and/or extraction.

Based on the findings of the current study, it was concluded that endogenous GGH activity can influence the poly-γ-glutamate profile of vegetables. In order to accurately determine the native folate poly-γ-glutamate profile of a vegetable, it is important to first inactivate the endogenous GGH prior to matrix disruption.

3.3. Effect of treatments on the native folate poly-γ-glutamate profile of broccoli

The different treatments investigated in the current study influenced the profile of broccoli poly-γ-glutamates in different ways. The percentage distribution of the various folate poly-γ-glutamates and monoglutamate in all the investigated treatments is presented in Fig. 2. A similar trend regarding the changes in the poly-γ-glutamate profile was observed in both florets and stalks.

Crushing unblanched broccoli resulted in deconjugation of the predominantly occurring hepta- and hexa-γ-glutamates into shorter folate poly-γ-glutamates (mainly tri-γ-glutamates) and monoglutamate. The poly-γ-glutamate profile of the unblanched crushed (pH 6.5) floret and stalk samples was similar to that of raw samples discussed in the previous section.

Regarding the influence of different crushing-blanching sequences, crushing broccoli prior to blanching led to deconjugation of folate poly-γ-glutamates as indicated by a higher percentage of monoglutamate, di- and tri-γ-glutamates and a lower percentage of longer poly-γ-glutamates. However, changes in the poly-γ-glutamate profile of broccoli blanched in the intact form (prior to crushing) were dependent on the temperature of the blanching process. HTST-crushed florets samples had a higher percentage of hepta- and hexa-γ-glutamates but lower percentage of shorter chain poly-γ-glutamates and monoglutamates, a profile similar to that of heat shocked samples discussed above. In comparison to HTST-crushed samples, LTLT-crushed samples contained lower concentration of hepta- and hexa-γ-glutamates but higher concentration monoglutamate di- and tri-γ-glutamates indicating

Table 2
The native folate poly-γ-glutamate profile (percentage distribution) and total folate content (nmol/g DM) of heat shocked (95 °C/10 min, HTST) and non-heat shocked (raw) broccoli.

	HK HTST (%)	HK raw (%)	BS HTST (%)	BS raw (%)
Hepta-γ-glutamate	56.5 ± 6.0[a]	20.1 ± 6.0	52.9 ± 10.7	7. ± 0.9
Hexa-γ-glutamate	20.4 ± 1.7	10.6 ± 2.6	23.7 ± 6.0	3.1 ± 0.6
Penta-γ-glutamate	6.8 ± 1.8	4.4 ± 0.9	6.8 ± 2.9	3.1 ± 0.2
Tetra-γ-glutamate	2.4 ± 0.5	13.7 ± 3.8	3.7 ± 1.6	13.7 ± 3.5
Tri-γ-glutamate	7.7 ± 2.9	27.8 ± 7.1	9.1 ± 3.3	47.0 ± 12
Di-γ-glutamate	4.2 ± 2.0	13.4 ± 3.2	2.9 ± 0.8	16.2 ± 7.6
Monoglutamate	1.8 ± 0.5	10.0 ± 3.9	1.0 ± 0.3	9.4 ± 2.6
Total folate (nmol/g DM)	49.9 ± 3.4	48.1 ± 7.8	67.9 ± 27.4	58.7 ± 7.5

[a] Mean ± SD (n = 3).

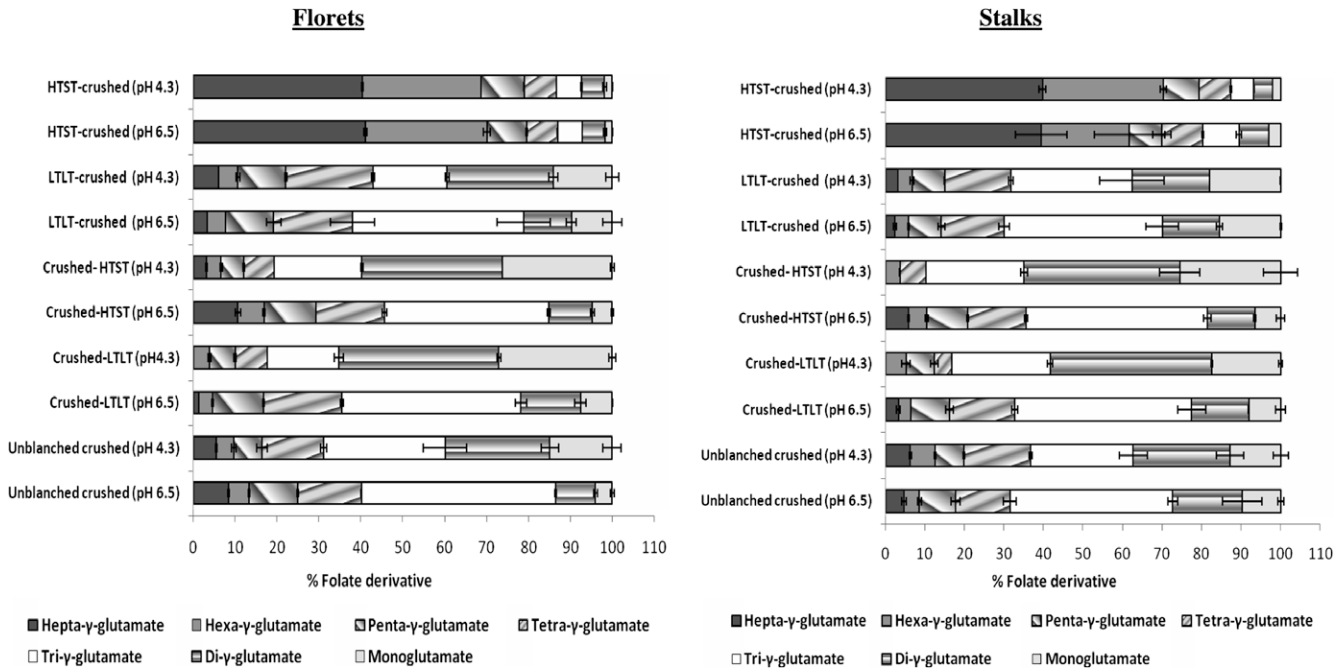

Fig. 2. The influence of crushing, acidification and blanching on the percent distribution of different folate derivatives in broccoli florets and stalks. Error bars indicate the standard deviation ($n = 2$).

that there was deconjugation during LTLT blanching of intact broccoli.

Regarding the influence of blanching temperature, the changes in the polyglutamate profile were identical for LTLT blanched and HTST blanched samples when broccoli was crushed prior to blanching. However, when broccoli was blanched prior to crushing, LTLT blanching resulted in deconjugation of polyglutamates, while HTST blanching maintained the native profile of poly-γ-glutamates.

Acidification had a significant influence on the folate poly-γ-glutamate profile. In general, crushing broccoli at pH 4.3 prior to blanching resulted in a higher percentage of monoglutamate and di-γ-glutamate in comparison to crushing at pH 6.5. The highest concentrations of monoglutamate and di-γ-glutamates were observed in crushed (pH 4.3)-LTLT and crushed (pH 4.3)-HTST samples. In samples crushed at pH 4.3 with subsequent blanching, hepta-γ-glutamate was almost completely deconjugated to shorter poly-γ-glutamates and monoglutamate. The obtained results indicated that GGH was responsible for the deconjugation and that pH 4.3 favored the activity of endogenous broccoli GGH compared to pH 6.5. Lowering the pH of broccoli in samples where the GGH was inactivated (e.g., HTST-crushed (pH 4.3)) did not result in deconjugation. This observation is in agreement with results of Verlinde et al. (2008) who observed that non-enzymatic folate poly-γ-glutamates deconjugation did not occur during thermal and combined thermal-high pressure treatments of 5-CH_3–H_4PteGluγ_5.

Several isozymes of GGH were reported to occur in plant materials, but the number of isozymes may vary depending on the plant species. It was recently demonstrated that two of these isozymes (AtGGH1 and AtGGH2 proteins from *Arabidopsis* but expressed in *Escherichia coli*) could cleave $PteGlu_5$ in different ways. AtGGH1 resulted mainly in $PteGlu_1$, $PteGlu_2$ and $PteGlu_3$ while AtGGH2 resulted mainly in $PteGlu_1$ (Orsomando et al., 2005). Results from the current study indicated that conditions favoring the activity of endogenous broccoli GGH resulted mainly in monoglutamate, di- and tri-γ-glutamates. Leichter et al. (1979) made a similar observation whereby they noted that the free folate content of vegetables increased when vegetables were homogenized before boiling. Similarly, Melse-Boonstra et al. (2002) observed that processing conditions leading to plant cell damage (freezing (−18 and −80 °C) and high pressure treatments) without prior heat treatment contributed to higher folate monoglutamate content in leeks, cauliflower and green beans while blanching and steaming resulted in high amounts of folate poly-γ-glutamates. However, Leichter et al. (1979) and Melse-Boonstra et al. (2002) did not show the folate profile of the vegetables. Verlinde et al. (2008) reported that broccoli predominantly contained tri-γ-glutamates but also noted that the proportion of hepta- and hexa-γ-glutamates was greater in broccoli treated at 70–90 °C for 30 min compared to the untreated samples and samples treated at lower temperatures. The current study has proved that hepta- and hexa-γ-glutamates are the predominant folate poly-γ-glutamates in broccoli. However, hepta- and hexa-γ-glutamates can be enzymatically deconjugated by active GGH to give mainly tri-γ-glutamates.

Literature on the properties of endogenous plant GGHs is limited. A few earlier studies showed that GGH from green leaves of soybean and pollen has an optimum pH of 4.5 (Iwai, 1957; Nielson & Holmström, 1957). However, other authors reported higher pH values for optimum GGH activity. Zheng et al. (1992) reported that broccoli leaves GGH optimally converted $PteGlu_3$ and $PteGlu_6$ to $PteGlu_1$ at pH 5.0. Orsomando et al. (2005) reported an optimum pH between 6.5 and 7.0 for AtGGH1 and pH 6.5 for AtGGH2. Results from the current study indicate that the optimum pH for broccoli GGH could be located below pH 6.5 since the rate of deconjugation was higher at pH 4.3 than at pH 6.5. However, further investigations need to be carried out to confirm the optimal pH of endogenous broccoli GGH. Finding the optimal pH of broccoli GGH might be useful in processing broccoli to products with higher monoglutamate content with the aim of increasing folate bioavailability.

Most vegetables are thermally treated prior to consumption. A survey carried out by McKillop et al. (2002) in the UK showed that 49% of people boil vegetables while 45% steam them. Observations

made in the current study indicate that the most abundant folate forms in thermally treated broccoli (and probably in other thermally treated vegetables) are poly-γ-glutamates. However, acidification, mild heating and crushing of vegetables prior to thermal treatments can be useful strategies of increasing folate monoglutamates without significantly decreasing stability.

3.4. Effect of treatments on stability of folates in broccoli

A total folate content of 690 ± 29 and 303 ± 30 nmol/100 g FW, respectively, was found in unblanched crushed (pH 6.5) florets and stalks in the current study. The values were calculated based on a dry matter content of 11% and 7% for florets and stalks, respectively. The folate content found for florets in the current study was higher than values (231, 258, 261, 401 and 315 nmol/100 g FW) reported by DeSouza and Eitenmiller (1986), Vahteristo, Lehikoinen, Ollilainen, and Varo (1997), McKillop et al. (2002), Stea et al. (2006) and Verlinde et al. (2008), respectively. The higher values reported in this study could be attributed to seasonal and varietal differences. The folate content in broccoli stalks is not widely investigated. In this study, broccoli stalks were found to contain 56% less folates compared to florets.

The influence of different treatments on the stability of folates is shown in Tables 3 and 4. The unblanched crushed (pH 6.5) sample was used as a reference to compare the effect of different treatments on folate stability. In this study, crushing resulted in a limited effect on folate stability in florets since, for example, there was no significant difference in crushed-HTST and HTST-crushed florets. However, stalks crushed prior to blanching (except crushed (pH 6.5)-LTLT) seemed to retain less folates compared to stalks blanched prior to crushing. This apparent difference in folate stability in florets and stalks crushed prior to blanching could be due to lower antioxidants (e.g., ascorbic acid) content in stalks (Nishikawa et al., 2003) than in florets.

Concerning the effect of blanching temperature on folate stability, HTST-blanched samples retained more folates compared to LTLT-blanched samples. The lower folate stability during LTLT blanching could be due to slower and probably incomplete oxidative enzymes (such as ascorbate oxidase, phenol oxidase and peroxidase) inactivation and longer heating time. Slower and probably incomplete oxidative enzyme inactivation during LTLT could have lowered the antioxidant status in LTLT blanched samples leaving folates exposed to oxidation. On the other hand higher temperature during HTST blanching could have resulted in faster oxidative enzyme inactivation thus rendering the folates more protected against oxidation.

The stability of folates was found to be lower at pH 4.3 than at pH 6.5. The lowest stability was observed in treatments involving both pH 4.3 and LTLT blanching. For example, crushed (pH 4.3)-LTLT florets samples showed the highest folate loss (36%) among all treatments investigated. The lower folate stability during treatments involving LTLT blanching and pH 4.3 could be attributed to higher heating intensity and acidic pH. Except for the low folate stability observed in treatments involving LTLT blanching and pH 4.3, lower folate losses compared to losses reported in literature were probably due to exclusion of leaching effects. Most folate losses reported in literature during thermal processing of vegetables were attributed to leaching in the cooking water. DeSouza and Eitenmiller (1986) reported losses of 70% for water blanched (4 min) and only a loss of 9% for steam blanched (4 min) broccoli. In another study by McKillop et al. (2002), boiling broccoli for 10 min caused 51% folate loss compared to raw broccoli while steaming for 10 min caused non-significant folate losses. Stea et al. (2006) observed 27% folate losses after water blanching broccoli in comparison to steaming. Although there is not much data on the effect of pH on folate stability during vegetable processing, low

Table 3
Effect of crushing, acidification and blanching on individual and total folate content in broccoli florets. Letters in parenthesis indicate which treatment resulted in significantly different concentration of each derivative compared to the unblanched crushed (pH 6.5).

Treatment/folate derivative	Unblanched crushed (pH 6.5)	Unblanched crushed (pH 4.3)	Crushed (pH 6.5)-LTLT	Crushed (pH 4.3)-LTLT	Crushed (pH 6.5)-HTST	Crushed (pH 4.3)-HTST	LTLT-crushed (pH 6.5)	LTLT-crushed (pH 4.3)	HTST-crushed (pH 6.5)	HTST-crushed (pH 4.3)
Hepta-γ-glutamate	5.32 ± 0.34[A] (a)	3.09 ± 0.93 (a)	1.37 ± 0.00 (b)	0.00 ± 0.00 (b)	5.41 ± 0.14 (a)	1.43 ± 0.00 (a)	2.08 ± 0.01 (a)	2.58 ± 0.31 (a)	22.76 ± 1.45 (b)	19.31 ± 0.09 (b)
Hexa-γ-glutamate	3.01 ± 0.05 (a)	2.36 ± 0.50 (a)	1.65 ± 0.13 (b)	1.54 ± 0.07 (b)	3.16 ± 0.04 (a)	1.54 ± 0.18 (b)	2.12 ± 0.71 (a)	1.92 ± 0.34 (a)	16.05 ± 0.38 (b)	13.62 ± 0.02 (b)
Penta-γ-glutamate	7.28 ± 0.18 (a)	3.61 ± 0.54 (b)	6.18 ± 0.24 (a)	2.45 ± 0.07 (b)	6.34 ± 0.21 (a)	2.41 ± 0.03 (b)	5.43 ± 2.51 (a)	4.82 ± 0.30 (a)	5.28 ± 0.35 (a)	4.91 ± 0.00 (a)
Tetra-γ-glutamate	9.53 ± 0.44 (a)	8.40 ± 3.10 (a)	9.48 ± 0.37 (a)	3.09 ± 0.21 (b)	8.34 ± 0.52 (a)	3.22 ± 0.17 (b)	9.05 ± 0.49 (a)	8.78 ± 0.66 (a)	4.08 ± 0.17 (b)	3.71 ± 0.01 (b)
Tri-γ-glutamate	29.07 ± 1.39 (a)	17.63 ± 5.05 (a)	21.68 ± 0.22 (a)	6.87 ± 0.88 (b)	19.91 ± 0.43 (a)	9.39 ± 0.37 (b)	19.03 ± 5.63 (a)	7.46 ± 0.86 (b)	3.24 ± 0.21 (b)	2.88 ± 0.08 (b)
Di-γ-glutamate	5.96 ± 0.07 (a)	13.92 ± 3.49 (b)	7.21 ± 0.53 (a)	15.34 ± 1.26 (b)	5.33 ± 0.40 (a)	15.03 ± 0.78 (b)	5.56 ± 1.22 (a)	10.70 ± 1.42 (a)	3.01 ± 0.33 (a)	2.61 ± 0.22 (a)
Monoglutamate	2.51 ± 0.38 (a)	8.25 ± 1.61 (b)	3.84 ± 0.11 (a)	10.86 ± 0.45 (b)	2.43 ± 0.20 (a)	11.74 ± 0.82 (b)	4.58 ± 3.23 (a)	5.93 ± 0.12 (a)	0.96 ± 0.04 (a)	0.89 ± 0.05 (a)
Total folates (nmol/g DM)	62.68 ± 2.71 (a)	57.26 ± 12.54 (a)	47.84 ± 1.06 (a)	42.19 ± 2.80 (b)	55.37 ± 1.59 (a)	47.93 ± 2.34 (a)	50.73 ± 11.27 (a)	40.15 ± 3.77 (b)	50.92 ± 2.93 (a)	44.76 ± 0.03 (b)

[A] Mean ± SD ($n = 2$).

Table 4
Effect of crushing, acidification and blanching on individual and total folate content in broccoli stalks. Letters in parenthesis indicate which treatment resulted in significantly different concentration of each derivative compared to the unblanched crushed (pH 6.5).

Treatment/folate derivative	Unblanched crushed (pH 6.5)	Unblanched crushed (pH 4.3)	Crushed (pH 6.5)-LTLT	Crushed (pH 4.3)-LTLT	Crushed (pH 6.5)-HTST	Crushed (pH 4.3)-HTST	LTLT-crushed (pH 6.5)	LTLT-crushed (pH 4.3)	HTST-crushed (pH 6.5)	HTST-crushed (pH 4.3)
Hepta-γ-glutamate	2.02 ± 0.07[A] (a)	2.80 ± 0.23 (a)	1.53 ± 0.00 (a)	0.00 ± 0.00 (a)	2.08 ± 0.28 (a)	0.00 ± 0.00 (a)	0.82 ± 0.01 (a)	1.17 ± 0.11 (a)	15.10 ± 1.17 (b)	15.70 ± 2.13 (b)
Hexa-γ-glutamate	1.68 ± 0.05 (a)	2.82 ± 0.26 (a)	1.47 ± 0.09 (a)	1.32 ± 0.39 (a)	1.61 ± 0.13 (a)	1.17 ± 0.07 (a)	1.18 ± 0.11 (a)	1.32 ± 0.26 (a)	9.16 ± 2.61 (b)	12.01 ± 1.08 (b)
Penta-γ-glutamate	3.95 ± 0.09 (a)	3.24 ± 0.51 (a)	4.55 ± 0.27 (a)	1.78 ± 0.09 (b)	3.73 ± 0.57 (a)	0.00 ± 0.00 (b)	2.92 ± 0.15 (a)	3.07 ± 0.29 (a)	3.07 ± 0.13 (a)	3.56 ± 0.40 (a)
Tetra-γ-glutamate	5.89 ± 1.10 (a)	7.58 ± 0.78 (a)	7.77 ± 0.08 (a)	2.03 ± 0.00 (b)	5.25 ± 1.01 (a)	2.06 ± 0.07 (b)	5.49 ± 0.39 (a)	6.20 ± 0.71 (a)	4.07 ± 0.92 (a)	3.17 ± 0.27 (a)
Tri-γ-glutamate	17.83 ± 1.22 (a)	11.70 ± 3.05 (b)	20.97 ± 0.84 (a)	6.26 ± 0.75 (b)	16.40 ± 2.41(a)	7.88 ± 0.04 (b)	14.14 ± 3.63 (a)	11.46 ± 3.96 (a)	3.53 ± 0.62 (b)	2.32 ± 0.30 (b)
Di-γ-glutamate	7.69 ± 2.71 (a)	10.96 ± 1.01 (b)	6.83 ± 2.64 (a)	10.27 ± 1.80 (b)	4.30 ± 0.76 (a)	12.52 ± 0.43 (b)	5.03 ± 0.69 (a)	7.22 ± 1.26 (a)	2.98 ± 0.98 (b)	1.87 ± 0.22 (b)
Monoglutamate	4.25 ± 0.60 (a)	5.68 ± 0.12 (a)	3.78 ± 0.51 (a)	4.39 ± 0.89 (a)	2.36 ± 0.83 (a)	8.12 ± 0.39 (b)	5.40 ± 0.47 (a)	6.57 ± 1.06 (a)	1.19 ± 0.21 (b)	0.80 ± 0.12 (b)
Total folates (nmol/g DM)	43.31 ± 4.21 (a)	44.78 ± 5.71 (a)	46.91 ± 1.91 (a)	25.03 ± 2.30 (b)	35.73 ± 5.99 (a)	31.75 ± 0.92 (b)	34.98 ± 5.47 (a)	37.02 ± 2.99 (a)	39.11 ± 5.38 (a)	39.43 ± 4.52 (a)

[A] Mean ± SD ($n = 2$).

pH has been reported to reduce folate stability during folate extraction from vegetables (Vahteristo et al., 1997; Zhang, Storozhenko, Van Der Straeten, & Lambert, 2005) and in model systems (Indrawati et al., 2004).

4. Conclusion

Acidification and thermal treatment (depending on the heating intensity) result in different impacts on the stability and the poly-γ-glutamate profile of folates in broccoli. Lowering pH resulted in lower folate stability but enhanced the activity of endogenous GGH. In addition, the sequence of conducting crushing and thermal treatments also played an important role in facilitating enzymatic and chemical reactions resulting in differences in the stability and poly-γ-glutamate profile of folates.

These observations imply that different process strategies of crushing, acidification and thermal treatment could result in fruit and vegetable based products with similar folate content but different folate poly-γ-glutamate profiles which could eventually affect the bioavailability. This study has demonstrated for example that crushing (pH 6.5) followed by HTST blanching resulted in a folate content similar to that of HTST blanching followed by crushing (pH 6.5) but in a totally different folate poly-γ-glutamates profile. Therefore, it is clear that different sequences of processes could be intelligently designed and controlled to achieve a specific targeted profile of poly-γ-glutamate folate while maintaining folate stability. With regard to process optimization, it should be taken into consideration that the activity and the stability of undesired enzymes, e.g., ascorbate oxidase should also be controlled in parallel with enhancing the activity and the stability of desired enzymes such as endogenous GGH during processing.

So far, information on the processing effect on the stability and the activity of undesired enzymes affecting folate stability, e.g., ascorbate oxidase in food matrix and on the effect of folate poly-γ-glutamate profile on bioavailability is still limited in literature. Hereto, further research addressing these issues is still needed.

Acknowledgements

We acknowledge and appreciate the support of the following organizations in accomplishment of this work: Interfaculty Council for Development Co-operation (IRO-Doctoral scholarship, K.U. Leuven), Specific Targeted Research Project (EU Project, Healthy Structuring 2006-023115), Institute for the Promotion of Innovation through Science and Technology in Flanders (Doctoral Grant, IWT-Vlaanderen) and Research Foundation Flanders (Postdoctoral Grant, FWO-Vlaanderen) The kind donation of (6S)-5-methyl-5,6,7,8-tetrahydrofolic acid calcium salt by Merck Eprova AG was also appreciated.

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Food Chemistry 117 (2009) 577–581

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Effect of soya protein on the dialysability of exogenous iron and zinc

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ARTICLE INFO

Article history:
Received 20 February 2008
Received in revised form 13 April 2009
Accepted 14 April 2009

Keywords:
Soya flour
Soya concentrate
Soya isolate
Iron dialysability
Zinc dialysability

ABSTRACT

The investigation aimed at studying the influence of soya proteins on *in vitro* dialysability of exogenous iron and zinc. Soyabean was processed into various components like soya flour (SF), concentrate (SC) and isolate (SI) and two peptides (<30 kDa and >30 kDa). The protein contents of SF, SC and SI were 51, 62 and 78 g/100 g, respectively. Their iron contents ranged between 9.7 and 15.8 mg/100 g and zinc contents from 3.3 to 6.7 mg/100 g. Phytic acid contents (mg/100 g) of the soya components were SF 339, SC 226, SI 186 and peptides 18–20. In SF, percent dialysable iron and zinc were 12.6 and 50, respectively, this reduced to one-third and one-fifth in SC and SI. Dialysability of exogenously added minerals was found to be significantly lower in all soya protein components. However, the inhibitory effect was less pronounced in peptides. The protein component/matrix effect needs to be investigated to counteract the inhibitory effect of soya on iron dialysability.

1. Introduction

Soyabeans are the richest sources of protein among the plant foods. Good quality and functionality of its proteins, hypoallergenicity, low cost and surplus availability are other added features that motivated the researchers to explore its utility in a wide range of foods including the complementary foods. With these attributes, soyabeans have been considered as an ideal food for meeting the protein needs of the population. Soyabeans in the form of full fat flour, concentrate, isolate and texturised proteins have been used in a wide range of food products. However, the impact of soya based formulas on the growth and development of infants were shown to be contradictory. Some studies have shown that infants fed with soya based formula achieved a similar growth to their peers who received cow's milk (Fomon, 1993), while others have shown that infants fed with soya based formulas showed poor growth rates (Golden & Golden, 1981). The presence of phytic acid at different levels in soya based products has been shown to inhibit trace mineral absorption (Davidson, Zeigler, Kastenmayer, Dael, & Barclay, 2004; Derman et al., 1987; Sandstorm, Cederblad, & Lonnerdal, 1983).

Iron deficiency anaemia is one of the major nutritional problems at global level with a major thrust from the developing world, zinc deficiency is another emerging nutritional problem that needs immediate attention. The main aetiology in these micronutrient deficiencies was found to be low intake accompanied by poor bioavailability (Charlton & Bothwell, 1983; Layrisse et al., 1990). Bioavailability of the minerals in any food is influenced by the proportion of inhibitors and enhancers present in it. Since phytic acid in soyabean was found to be the major inhibitor of both iron and zinc absorption, attempts were made to use dephytinised soya in products. The extent of improvement in enhancing the mineral bioavailability was found to be proportional to the degree of dephytinisation (Davidson, Almgren, Sandstorm, & Hurrell, 1995; Davidson et al., 2004; Hurrell et al., 1992; Lonnerdal, Jayawickrama, & Lien, 1999; Zeigler, Janghorbani, Nelson, & Edwards, 1990). In addition, protein in soyabeans was also reported to be inhibitory for iron absorption (Lynch, Dassenko, Cook, Luillerat, & Hurrell, 1994). The inhibition of absorption of one mineral on the absorption of another has been studied by many investigators, but the results are quite conflicting as each study has used a different food matrix and different ratios of minerals (Sandstorm, 2001).

Food fortification has been considered as one of the effective strategies for combating both macro and micronutrient deficiencies that are co-existent in the developing countries. A recent comprehensive review by Parada and Aguilera (2007) has clearly brought out the fact that food microstructure has a major role to play in affecting the bioavailability of minerals (Moretti et al., 2006). Hence it becomes essential to study the effect of food matrix on the bioavailability of both endogenous and exogenously added micronutrients. The investigation was undertaken to study the effect of soya proteins on the dialysability of endogenous and exogenous iron and zinc.

Abbreviations: $FeSO_4$, ferrous sulphate; ZnO, zinc oxide; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; kDa, kiloDaltons; AAS, atomic absorption spectroscopy.

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0308-8146/$ - see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2009.04.035

2. Materials and methods

2.1. Materials

Ferrous sulphate and zinc oxide were procured from Sigma–Aldrich Ltd. The enzymes used for the study were Pepsin (Hi media, Batch no.; 06-1315) and Pancreatin (Hi media, Batch no.; 5-1497). All the chemicals used for the study were of analytical grade. The dialysis tubing of 10 kDa was procured from Spectrum, USA. Triple distilled water was used for the entire analysis. All the analysis was done in triplicates. Soyabeans [*Glycine hispida* (Moench) maxim] was procured from the local market. The soya protein components namely flour, concentrate and isolate processed at the pilot scale in the laboratory (in a single large-scale batch) were used for the entire analyses cited below.

2.2. Preparation of soya flour components

(i) *Defatted soya flour*: Soyabean seeds were cleaned, dehulled and flaked. The flakes were defatted with n-hexane and the fat content was determined by soxhlet method. Defatting was continued until the fat content was less than 1%. The defatted flakes were dried and powdered in a Quadrumat mill (Buisberg, Germany). The fractions were obtained using sieves, defatted flour was the component having a pore size below 100 μ.

(ii) *Soya protein concentrate: Soya* protein concentrate was prepared by eliminating soluble carbohydrate fractions and some flavour compounds from defatted meal. The process involved acid leaching at isoelectric pH wherein the proteins were insolubilised (a small portion of the carbohydrates remained soluble), and were separated by centrifugation. Solids containing mainly proteins and insoluble carbohydrate were then dispersed in water, neutralised to pH 7 and were spray dried.

(iii) *Soya protein isolate: Soya* protein isolate was prepared from defatted soya meal using mild alkali extraction (pH 8–10) of proteins. The insoluble residue (mostly carbohydrate) was removed by centrifugation, followed by precipitation of soya protein at its isoelectric point. The precipitated protein was separated by mechanical decanting, washed, and neutralised to a pH of 7.0 and then spray-dried.

(iv) *Peptides:* Two peptides of different molecular weight; one of <30 kDa and the other >30 kDa was prepared by ultrafilteration. In brief, the defatted flour was hydrolysed using the enzyme fungal protease for 2 h. The hydrolysates were clarified by tangential flow and the filtrate was fractionated by tangential flow through a Satacon Slice Cassette, which had a cut off of 30,000. The ultra filtrate (concentrate) composed of soya peptides of <30 kDa and retentate composed of fraction having a molecular weight of >30 kDa. The two fractions with different molecular weight were spray dried and desiccated until further use.

Molecular weight of the prepared peptides was determined using 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmili, 1970). The molecular weight markers used were, bovine serum albumin (BSA), 66 kDa; ovalbumin chicken egg, 45 kDa; glyceraldehyde 3-P dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen bovine, 24 kDa; trypsin inhibitor soya, 20 kDa; α-lactalbumin bovine, 14.2 kDa; aprotinin bovine lung, 6.5 kDa.

Molecular weight was further confirmed by fast protein liquid chromatography (FPLC) using a superdex-75 HR column from Amersham Pharmacia (Uversky, 1993). Sample was prepared in 0.02 mM phosphate buffer with a pH of 7.9, 200 μl was loaded and eluted with the same buffer at a flow rate of 0.5 ml/min, elution was monitored at 280 nm.

2.3. Analysis

Moisture: The moisture content of soya flour and its components was estimated by AOAC method (AOAC, 1999).

Protein: The estimation of nitrogen was done by micro Kjeldhal method using automated nitrogen distiller. The protein content was determined by multiplying the nitrogen value with 6.25 (Raghuramulu, Nair, & Kalyanasundram, 2003).

Total ash: The greyish white residue formed after charring and incineration in a muffle furnace at 600 °C for 6–8 h comprised of the total mineral content. Iron and zinc content of the soya protein components were analysed by atomic absorption spectroscopy (AAS) (Raghuramulu et al., 2003).

Dialysable iron and zinc: The dialysable fraction of iron and zinc contents was estimated by the method of equilibrium dialysis (Luten et al., 1996). Iron present in the dialysate predicts non-heme absorption *in vivo,* it contains other minerals that are in the absorbable form. It is expressed as dialysable minerals (iron and zinc).

Phytin phosphorus: Phytic acid was extracted and determined according to the supernatant difference method (Thompson & Erdman, 1982).

The effect of protein on the availability of exogenous iron and zinc was studied by adding ferrous sulphate and zinc oxide for the respective minerals. The ratios of protein and minerals were based on the recommended dietary allowances for adults given by Indian Council of Medical Research (ICMR) (Gopalan, Sastri, & Balasubramanian, 2000). RDA of protein for adults is 55 g/day, iron is 29 mg/day and zinc is 15 mg/day (zinc values are taken from US RDA). Based on protein content of soya products exogenous minerals were added. It was 27 mg iron and 19 mg zinc for 100 g flour, 32.9 mg iron and 17 mg zinc for concentrate and 41.2 mg iron and 21.3 mg zinc for isolate and peptides.

Statistical analysis: the analysed data were subjected to statistical analysis using the statistical package OriginPro, version 7.0, the probability level was set to $P < 0.05$. Analysis of variance (ANOVA) was adopted to study the differences in the dialysable iron and zinc contents between different soya protein products and also on the externally added minerals in the same protein products.

3. Results and discussion

The soya protein products namely flour, concentrate and isolate were prepared as described under methodology. Short peptides with characteristic amino acid composition and defined molecular weight are highly desirable in nutrition and food science for both functional and nutritional purposes. Based on the molecular weight two peptides, one <30 kDa and the other >30 kDa were fractionated by ultra filtration. The molecular weights of the peptides were determined by electrophoresis. Fig. 1 shows the SDS–PAGE gel electrophoretic pattern of control and ultra filtered soya protein in 12% acrylamide gel. The control protein showed twelve bands, ultra filtered protein <30 kDa had two bands below 20 kDa and the filtrate protein >30 kDa had eight bands. The purity of isolated peptides were further confirmed by FPLC given in Fig. 1a and b. The fraction >30 kDa contained seven peaks and peptide fraction <30 kDa had four peaks.

The related chemical components of the soya protein fractions are presented in Table 1. Defatted soya flour comprised of 50 g protein/100 g flour, preparation of the concentrate and isolate further enhanced the protein content by 11 and 27 g/100 g of products, respectively (Table 1). The protein content in the flour and concentrate was found to be similar to those reported by other investigators (Gopalan et al., 2000; Sandstorm, Kivisto, & Cederblad, 1987). However, in the isolate it was found to be slightly lower than that cited in the literature. This could be attributed to the processing

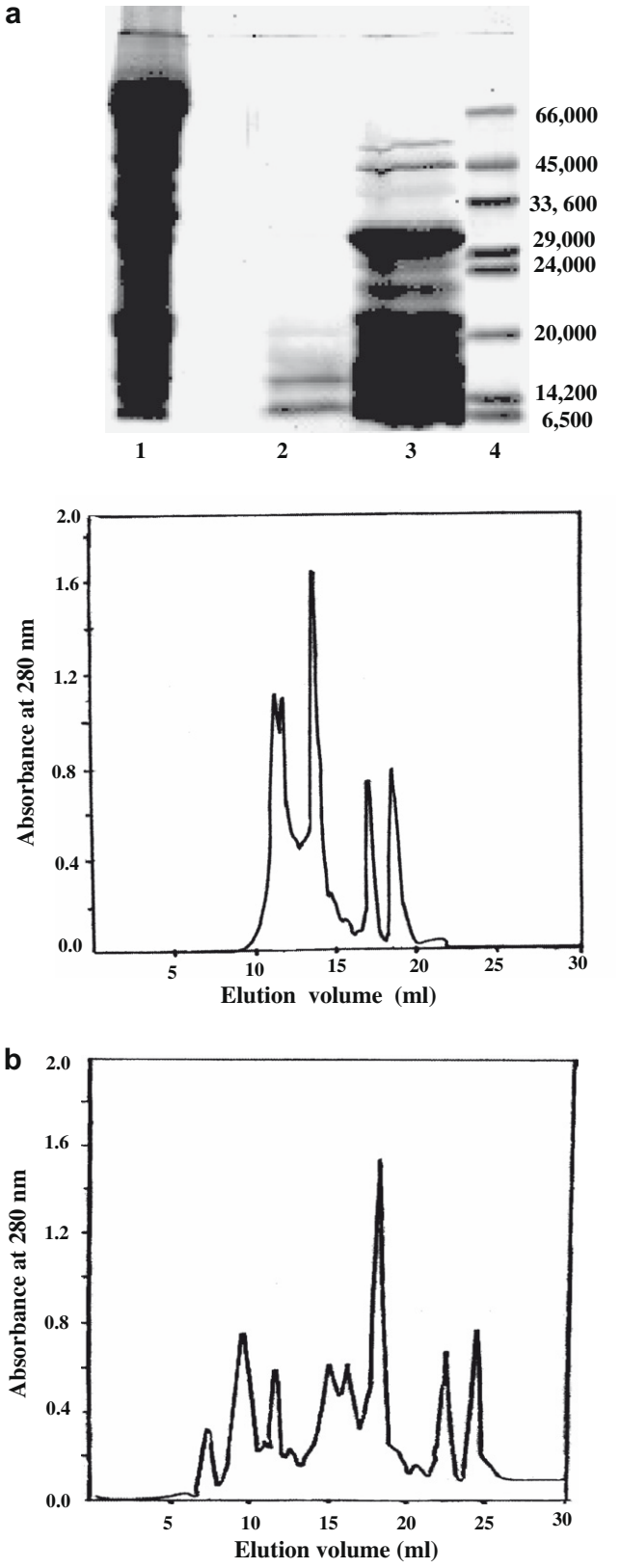

Fig. 1. Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS–PAGE) of soya protein peptides. Lane 1: Total soya proteins; Lane 2: Soya protein fractionless than 30 kDa; Lane 3: Soya protein fractiongreater than 30 kDa; Lane 4: Molecular markers. (a) FPLC pattern of soya peptides below 30 kDa. Superdex-75 HR column equilibrated with 0.02 mM phosphate buffer pH 7.9 and eluted with the same buffer at 0.5 ml/min and monitored at 280 nm. (b) FPLC pattern of soya peptides above 30 kDa. Superdex-75 HR column equilibrated with 0.02 mM phosphate buffer pH 7.9 and eluted with the same buffer at 0.5 ml/min and monitored at 280 nm.

variables. The mineral content of the flour was 8 g/100 g and this was found to reduce by almost 44% on concentration and isolation of the protein. Soya flour apart from being a rich source of protein also contains fairly good amount of iron and zinc. With increase in the concentration of the protein content, a 62–63% increase in iron and 81–106% increase in zinc contents was evident. The extent of differences in the iron and zinc contents among the flour components was found to be extremely significant ($F = 115.14^{***}$ for iron and $F = 188.02^{***}$ for zinc). The estimated iron content for soya flour and soya concentrate of the present study matched well with an earlier report (Sandstorm et al., 1987). The iron content in the isolate was found to be lower in comparison with values reported in the literature which could be attributed to the differences in the processing techniques and the extent of extraction. The estimated zinc of soya flour was found to be half of what was reported earlier (Sandstorm et al., 1987). However, it was two and three times higher in the concentrate and the isolate respectively than that reported by the other researchers. Phytic acid content of different soya products ranged 399–186 mg/100 g, it decreased with increasing concentration of protein from flour to isolate. Phytic acid content was 17.7 mg/100 g and 20.3 mg/100 g in <30 kDa and >30 kDa peptides, respectively, which was ten times lower than that present in isolate.

The proportion of the minerals that is biologically available is more important from the nutritional point of view. As evident from Table 1, around 13% of the iron present in the soya flour was dialysable. In concentrate and isolate the dialysability of the mineral ranged between 4 and 5% of the total content. Though the total iron content was higher by 50% in the concentrate and isolate compared to the flour its dialysability reduced to around 1/3rd of its concentration. The differences in the dialysable iron content of the soya flour components was found to be extremely significant as per ANOVA ($F = 87.36^{***}$). Dialysable zinc of soya flour was found to be 50%, it reduced by almost fivefolds and more in concentrate and isolate. The differences in the dialysability of the element among the soya protein products were found to be extremely significant ($F = 184.8656^{***}$). However, the dialysability of zinc was measurably higher compared to iron in the soya protein products. It is well established that phytic acid negatively affects the dialysability of the minerals in any food matrix. When the dialysable iron and zinc contents were associated with the phytic acid content in different soya protein products, a high negative correlation was observed for both iron ($r = -0.97$) and zinc ($r = -0.99$). Though a linear reduction was evident in the phytic acid content from flour to isolate no positive (rather a negative) impact was evident in the iron or zinc dialysabilities. Our results confirm the reports of other investigators that factors other than phytic acid in soya protein components affect the dialysability of the minerals. Research has shown that dephytinisation partially improved iron dialysability only when the phytic acid content was below 30 mg/100 g in the flour and <1 mg/100 g in the isolate (Hurrell et al., 1992).

3.1. Protein mineral interaction and percent dialysability of iron and zinc

Among the absorption promotive components, the amino acid cysteine is also shown to enhance non-heme iron absorption in humans. In human subjects all sources of proteins do not have an equivalent effect on non-heme iron absorption; the effect may be different in different matrices having varied composition. The present study investigates the effect of soya proteins on exogenous iron and zinc. A model system comprising of anhydrous ferrous sulphate (as source of iron), zinc oxide (as source of zinc) and both in combination with different soya protein products was used.

Table 1
Chemical composition of soya protein components per 100 g.

Soya flour components	Moisture (%)	Protein (g)	Mineral content (g)	Iron (mg)	Dialysable iron* (mg)	Zinc (mg)	Dialysable zinc* (mg)	Phytin phosphorus (mg)
Soya flour	5.33	51.4 ± 0.04	8.0 ± 0.94	9.74 ± 1.05	1.23 ± 0.04 (12.6)	3.27 ± 0.16	1.64 ± 0.03 (50.1)	399.4 ± 5.5
Soya concentrate	7.00	62.3 ± 0.03	4.5 ± 0.15	15.84 ± 1.07	0.65 ± 0.01 (4.1)	5.92 ± 0.46	0.57 ± 0.04 (9.6)	226.3 ± 6.8
Soya isolate	7.00	78.2 ± 0.04	4.5 ± 0.23	15.21 ± 0.56	0.74 ± 0.05 (4.8)	6.74 ± 0.28	0.56 ± 0.02 (8.3)	186.4 ± 4.3

* Figures in parenthesis indicate percent dialysability.

3.1.1. Addition of $FeSO_4$ to soya protein products

Percent dialysability of exogenous iron was found to be very low in all soya protein products, it ranged from around 2.4–0.9% (Table 2). A twofold decrease in the dialysable iron was observed from flour to isolate. The differences between the three protein components analysed were found to be highly significant ($F = 23.019^{**}$), a similar pattern was observed in the dialysability of endogenous iron in all soya protein components wherein iron dialysability was inversely proportional to protein content. Evidences suggest that addition of soya flour to test/semi synthetic meals reduced bioavailable iron and extent of reduction was proportional to concentration of protein (Bodwell, 1983; Schricker, Miller, & Campen, 1982). In the fraction <30 kDa, 9.7% of the iron was dialysable. This was 4–10 times higher than that observed for flour and isolate matrices. The percent dialysable iron for the high molecular weight fraction was found to be 3.1% lower than the low molecular weight fraction. However, it was comparatively higher than flour, concentrate and isolate.

On addition of iron to the soya matrices, zinc dialysability was higher than the endogenous or native zinc in three components of soya. The extent of inhibition decreased with increase in concentration of the protein. In peptides 32–56% inhibition in zinc dialysability was observed in presence of iron. The process of preparation of the peptides reduced the phytic acid content by 9–15 times compared to flour, concentrate, and isolate. This is very well reflected in corresponding increase in iron and zinc dialysability. This confirms the earlier findings wherein the researchers have reported that phytic acid content <30 mg/100 g was less inhibitory to the dialysability of minerals (Lynch et al., 1994). The results of present study show that reducing the phytic acid content is sufficient for enhancing zinc dialysability and protein has no role to play in modifying the zinc dialysability.

3.1.2. Addition of ZnO to soya protein products

Addition of exogenous zinc to the soya protein products resulted in a varied level of zinc dialysability (Table 3). Percent dialysability was found to be 19% for the flour and 5.5% for the concentrate and isolate which was 3.5 folds lower than that of the soya flour. The difference in the dialysability of zinc in the three components was found to be extremely significant ($F = 188.02^{***}$). An inverse relationship was evident between the concentration of the protein and the dialysability of zinc. The percent dialysability of exogenous zinc was lower than that of endogenous zinc, it was lower by 2.6 times in flour, and 1.5–1.7 times in concentrate and isolate. This confirms the findings of several other investigators who studied the dialysability of zinc from zinc carbonate in presence of full fat soya flour, freeze dried soya beverage and soya concentrate. The dialysable fraction in presence of soya products was reported to range between 20% and 40% compared to egg protein (77–94%). The authors indicated that the intrinsic zinc in soya proteins and the zinc in the exogenous form did not form a common absorptive pool (Forbes & Parker, 1997; Whittaker, 1996). The dialysability of the exogenous mineral is influenced by the food matrix used and hence a varied dialysability is observed with different protein fractions of soya flour. In case of peptides the dialysability of zinc was 75–87% which was comparatively higher than other soya protein products. Among the peptides the one with high molecular weight showed higher dialysability. On addition of zinc to the soya flour components, a drastic reduction was observed in the dialysability of iron in all three components analysed. The extent of inhibition was found to increase from flour to isolate (without exogenous minerals). Apart from protein in soya products zinc also interfered in iron dialysability. However the difference in the extent of inhibition was not statistically significant ($F = 0.4747^{ns}$). The fraction <30 kDa showed the least dialysability compared to all the three soya protein fractions. In case of high molecular weight fraction, the dialysability was comparable to soya isolate. On the whole it can be said that zinc in isolation showed an inhibitory effect on iron dialysability.

3.1.3. Addition of $FeSO_4$ and ZnO in combination to soya protein products

Foods/medicinal supplements contain zinc and iron in different proportions, the interaction between the two decides the dialysability of the minerals. The interaction between the trace elements is primarily antagonistic. It is expected that when two chemically similar ions are present in the intestinal lumen the one having molar excess will tend to exclude the other. In terms of recommendations the quantity of zinc is half of iron. The percent dialysability of iron on addition of both minerals in combination was lower in all the three soya protein components (Table 4). The extent of dialysability was lower by 8% in flour and by 2–4% in concentrate and isolate compared to that of endogenous dialysability. The extent of inhibition posed by the combination of minerals was of a lesser magnitude compared to that added in isolation in same protein matrices. The differences in the dialysability of the mineral among the soya protein components was found to be insignificant ($F = 0.4748^{ns}$). Among the peptides the lower molecular weight fraction exhibited around 2% dialysability whereas the higher molecular weight fraction showed four times higher dialysability.

Table 2
Dialysable iron and zinc of soya protein products on addition of ferrous sulphate – mg/100 g soya product.

Soya protein products	Exogenous iron added (mg/100 g)	Dialysable iron	Dialysable zinc
Soya flour	27.1	0.66 ± 0.05 (2.4)	0.53 ± 0.02 (16.4)
Soya concentrate	32.9	0.73 ± 0.03 (2.2)	0.23 ± 0.01 (3.91)
Soya isolate	41.2	0.38 ± 0.02 (0.9)	0.36 ± 0.02 (5.34)
Peptides <30 kDa	41.2	3.98 ± 0.03 (9.7)	4.60 ± 0.56 (68.5)
Peptides >30 kDa	41.2	2.62 ± 0.03 (6.4)	2.95 ± 0.32 (43.8)

Figures in parenthesis indicate percent dialysability.

Table 3
Dialysable zinc and iron of soya protein products on addition of zinc oxide – mg/100 g soya product.

Soya protein products	Exogenous zinc added (mg/100 g)	Dialysable zinc (mg/100 g)	Dialysable iron
Soya flour	14.1	3.46 ± 0.83 (19.0)	0.10 ± 0.00 (0.66)
Soya concentrate	17.0	1.20 ± 0.15 (5.5)	0.12 ± 0.04 (0.73)
Soya isolate	21.3	1.46 ± 0.19 (5.6)	0.10 ± 0.00 (0.38)
Peptides <30 kDa	21.3	16.03 ± 1.05 (75.3)	0.10 ± 0.01(0.04)
Peptides >30 kDa	21.3	18.63 ± 1.53 (87.5)	0.12 ± 0.03 (0.77)

Figures in parenthesis indicate percent dialysability.

Table 4
Dialysable iron and zinc of soya protein products on combined addition of ferrous sulphate and zinc oxide – mg/100 g soya product.

Soya protein products	Exogenously added		Dialysable iron (mg/100 g)	Dialysable zinc (mg/100 g)
	Iron (mg/100 g)	Zinc (mg/100 g)		
Soya flour	27.1	14.1	1.04 ± 0.04 (3.8)	4.35 ± 0.91 (30.9)
Soya concentrate	32.9	17.0	0.50 ± 0.02 (1.5)	1.67 ± 0.25 (9.8)
Soya isolate	41.2	21.3	0.32 ± 0.02 (0.8)	2.60 ± 0.15 (12.2)
Peptides <30 kDa	41.2	21.3	0.67 ± 0.13 (1.6)	14.95 ± 1.05 (70.0)
Peptides >30 kDa	41.2	21.3	3.28 ± 0.33 (7.9)	15.00 ± 1.53 (70.4)

The presence of zinc in both the fractions showed a contradictory effect wherein it inhibited the iron dialysability to a larger extent in the low molecular weight fraction than in the high molecular weight fraction. Research has shown that high concentration of iron (iron:zinc in a ratio of 1:1 in water) can have a negative effect on zinc absorption (Boccio et al., 2002; Whittaker, 1996). However, in foods such as corn tortillas, weaning cereals, white wheat flour and infant formula there was no effect of iron on zinc absorption (Davidson et al., 2005). The dialysability of exogenous zinc was lowered in presence of iron in the soya flour and concentrate but not in isolate (compared to the endogenous zinc). In the isolate the inhibition was reversed to enhancement (though marginal) in combination. In the peptide matrices 70% of the exogenous fraction of zinc was dialysable irrespective of the molecular weight showing the non-interference of iron. Literature suggests that iron reduces zinc absorption in water and in model systems. Contrasting to this our results suggest iron to have a beneficial effect on zinc dialysability when present in combination with zinc in the soya protein fractions.

Soya flour as a whole comprising of protein by half of its weight is a good source of iron and moderate source of zinc. The process of concentrating the protein enhanced the iron and zinc contents between 60% and 100% with a corresponding decrease in the phytic acid content, but dialysability of the minerals decreased by one-third to one-fifth. The dialysability of the exogenous minerals added in the soya protein matrices was negligible. The addition of zinc in isolation significantly lowered the dialysability of iron but it was beneficial for zinc dialysability on addition of iron. The inhibitory effect lowered when both the minerals were added in combination. The dialysability of the minerals was comparatively higher in the peptide matrix, which can be attributed to low phytate content and hydrolysis of protein. As concentrate and isolate are much extensively used in food formulations, the matrix needs to be researched to achieve optimum dialysability of the essential minerals for combating the highly prevalent devastating micronutrient deficiencies.

Acknowledgement

The authors thank the Director, CFTRI for providing the necessary facilities for carrying out the work.

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Food Chemistry 117 (2009) 582–588

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

ACE-inhibitory activity of tilapia protein hydrolysates

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ARTICLE INFO

Article history:
Received 31 December 2008
Received in revised form 13 April 2009
Accepted 14 April 2009

Keywords:
Angiotensin converting enzyme inhibition
Tilapia
Ultrafiltration
Cryotin
Flavourzyme

ABSTRACT

Fish processing wastes can be used for preparing bioactive peptides with various functionalities. Our objective was to evaluate the in vitro angiotensin converting enzyme (ACE) inhibitory activity of tilapia protein hydrolysates and its corresponding fractionates. Tilapia protein was alkali-solubilised at pH 11.0 and recovered at pH 5.5 to obtain a stable substrate. This substrate was hydrolysed using two enzymes, Cryotin-F or Flavourzyme, to 7.5% and 25% degree of hydrolysis (DH). The hydrolysates were ultra-filtered into three fractions: >30 kDa fraction, 10–30 kDa fraction, and <10 kDa fractions. Both hydrolysates and fractionates were tested for ACE inhibition. Results showed that both Cryotin and Flavourzyme hydrolysates with 25% DH gave maximum ACE inhibitory activity. Low MW peptides showed higher ACE inhibition than high MW peptides. The inhibitory activity of fractionates was lower than that of the corresponding hydrolysates, possibly due to separation and removal of synergistic peptides by ultrafiltration. Amongst fractionates, all the 7.5% DH Cryotin fractions and 25% DH Flavourzyme fractions exhibited optimum % ACE inhibition. The results of this research could be used for optimising enzyme parameters to obtain peptides from tilapia with optimum in vitro ACE inhibitory activity.

1. Introduction

Angiotensin-I converting enzyme (ACE) plays an important role in the regulation of blood pressure and hypertension (Pfeffer, 1993). ACE catalyzes the conversion of inactive angiotensin-I into a potent vasoconstrictor, angiotensin-II (Goodfriend, Elliott, & Catt, 1996). ACE also inactivates the vasodilator, bradykinin (Kon, Fogo, Ichikawa, Hellings, & Bills, 1993; Witherow, Helmy, Webb, Fox, & Newby, 2001). Inhibitors of ACE are often used to treat myocardial infraction (Flather et al., 2000), hypertension (Pahor et al., 2000), and other cardio-related diseases. Studies have shown that several food derived proteins and related peptides may have potent ACE inhibitory activities (Ariyoshi, 1993). These peptides are derived from casein hydrolysates (Miguel, Manso, Lopez-Fandino, Alonso, & Salaices, 2007), whey proteins (FitzGerald & Meisel, 1999; Vermeirssen, Van Camp, & Verstraete, 2005), soy proteins (Wu & Ding, 2001), seed proteins (Aluko & Monu, 2003), and fish proteins (Bougatef et al., 2008; Fujita & Yoshikawa, 1999).

Over the last decade, an increase in the consumption of aquatic food products has resulted in an increase in fish processing wastes. Several research groups around the world have been working on converting these wastes into useful food and bioactive ingredients (Kitts & Weiler, 2003; Raghavan & Kristinsson, 2008; Raghavan, Kristinsson, & Leeuwenburgh, 2008; Theodore & Kristinsson, 2007). Some of these groups have been doing research on the ACE inhibitory activities of fish derived proteins and on fish processing wastes (Bougatef et al., 2008; Theodore & Kristinsson, 2007; Tsai, Lin, Chen, & Pan, 2006). Researchers have identified and reported specific peptides from animal and fish proteins responsible for ACE inhibition (Vercruysse, Van Camp, & Smagghe, 2005). However, preparation of cheap and high quality ACE inhibiting protein hydrolysates on a commercial scale poses several challenges including (a) the quality of raw material, and (b) the economy of separation and fractionation to obtain specific peptides.

The nature of fish species and the quality of the raw material are some of the many factors which would affect the bioactive potential of fish protein hydrolysates (Kristinsson, 2005). Hydrolysates from muscle protein sources can have several pro-oxidants such as heme and unstable oxidised lipid substrates. Contamination with these pro-oxidants may not only decrease the stability of protein hydrolysates but may affect their bioactivity as well. An isoelectric precipitation method developed by Hultin and Kelleher (2000) has often been used to prepare protein isolates with improved functional properties (Kristinsson & Hultin, 2003) and a low amount of unstable lipid substrates (Undeland, Kelleher, & Hultin, 2002). In brief, this method involves solubilising myofibrillar proteins at high (alkaline) pH and then recovering the solubilised proteins by isoelectric precipitation at pH 5.5. Utilisation of isoelectric

Abbreviations: DH, degree of hydrolysis; MW, molecular weight; ACE, angiotensin converting enzyme.

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doi:10.1016/j.foodchem.2009.04.058

precipitation will hence be a new and cheap method for preparing high quality bioactive hydrolysates from fish by-products and wastes. In our current study, we used tilapia (*Oreochromis niloticus*) protein isolates prepared using the above mentioned alkali process as a substrate for enzyme hydrolysis. We chose tilapia, a commercially important aquatic species, since large quantities of processed wastes are generated every year. Utilisation of the tilapia waste will not only add value to the fish industry, but would also provide a new source of bioactive compounds. Two commercial enzymes, (a) Cryotin-F, a mixture of trypsin, chymotrypsin and elastase, and (b) Flavourzyme, a mixture of endoproteases and exopeptidases from *Aspergillus oryzae*, were used to hydrolyse tilapia protein to different degrees of hydrolysis. These two enzymes were chosen, as our previous studies showed that hydrolysates prepared using these enzymes have significantly high antioxidant activity (Raghavan & Kristinsson, 2008) and a high ability to quench stimulated mononuclear cells (Raghavan et al., 2008).

The second challenge for the utilisation and commercialisation of fish wastes and by-products is the process of separation and fractionation. Although researchers have identified specific peptides responsible for ACE activity (Vercruysse et al., 2005), commercialisation of these specific peptides would involve either discarding the remaining hydrolysate and peptides or synthetically preparing the ACE inhibiting peptides. Both these methods do not solve the problem of fish waste utilisation. Also, separation and purification using column chromatography on a large-scale poses scale-up problems. Ultrafiltration is a technique used commonly both on laboratory and on commercial scale to fractionate, purify and concentrate proteins (Ghosh, 2003). Although it is possible to obtain very pure protein fractions using suitable columns as in column chromatography, ultrafiltration is a low cost and easy to scale-up method used widely in food industries for quick separation and concentration of food proteins. In our current research we used ultrafiltration to fractionate tilapia hydrolysate into a range of molecular weight fractions.

To summarise, the first objective of our current work is to use alkali-solubilisation technique for preparing fish protein hydrolysates and test their ACE inhibition activity. The second objective is to use ultrafiltration for separating and isolating hydrolysate fractions and compare the ACE inhibition potential of these fractionates with the whole hydrolysates.

2. Materials and methods

2.1. Materials

Tilapia fillets were purchased locally in Gainesville, FL and transported to the laboratory on ice. Chemicals were purchased from Fisher Scientific (Fair Lawn, NJ). All reagents were of ACS grade. Flavourzyme enzyme was given by Novozymes A/S Denmark. Cryotin-F was a gift from North Ltd. (Reykjavik, Iceland). Lyophilized powder of rabbit lung angiotensin converting enzyme, and substrate for ACE (FAPGG or *N*-[3-(2-Furyl) acryloyl]-L-phenylalanyl-glycyl-glycine) were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Methods

2.2.1. Preparation of protein isolates using alkali solubilisation method

The white muscle from tilapia was separated and used for preparing protein isolates. The protein isolates were prepared by alkali solubilisation method as described in Hultin and Kelleher (2000). According to this method, the white muscle was first minced using a Waring PRO professional meat grinder (Model MG800, Waring Products, East Windsor, NJ) through a 3/16-in diameter sieve. The minced muscle was homogenised with 9 parts of cold deionized water using a Biohomogenizer (M133/1281-0, Bio Spec Products Inc., Bartlesville, OK) at high speed for 1 min. Myofibrillar proteins present in the homogenised sample were solubilised by adjusting the pH to 11.0 using 2 N sodium hydroxide. The pH adjusted sample was incubated at 4 °C for 30 min after which the mixture was centrifuged at 10,000*g* for 20 min at 4 °C in a Sorvall RC-5B Superspeed Refrigerated centrifuge (DuPont Instruments-Sorvall, DuPont Co. Newtown, CT). The supernatant was separated and filtered through a double layered cheese cloth. The pH of this supernatant was adjusted to 5.5 using 2 N HCl for precipitating the myofibrillar proteins. The pH adjusted mixture was again centrifuged at 10,000*g* for 2 min at 4 °C for recovering the myofibrillar proteins as sediment. Protein isolate obtained as sediment was used as a substrate for enzyme hydrolysis. The amount of protein in the isolates was determined using Kjeldahl method (AOAC Official Method 991.20).

2.2.2. Enzymatic hydrolysis of protein isolates

Enzymatic hydrolysis of tilapia protein isolates were performed using two different enzymes, Flavourzyme and Cryotin-F. The optimum hydrolysis conditions used for these enzymes were as follows: Flavourzyme (50 °C, pH 7.0), and Cryotin (45 °C, pH 8.0). The protein isolate was added to deionized water and homogenised for 1 min at maximum speed using a Biohomogenizer. The concentration of the homogenate was then adjusted to 2% w/v. The homogenate was equilibrated to the optimum pH and temperature conditions corresponding to the enzyme used for hydrolysis. The enzyme preparation was then added to the homogenate at 1% (w/v) level, and the degree of hydrolysis (DH) was calculated using the following equation (Adler-Nissen, 1986):

$$\%\ \text{Degree of hydrolysis (DH)} = \frac{B \times N_{\text{base}}}{\alpha \times h_{\text{tot}} \times \text{MW}} \times 100$$

where, B = volume of base used, N_{base} = normality of base used, α = degree of dissociation, MW = amount of protein used, h_{tot} = total number of peptide bonds per mass unit. The degree of dissociation (α) was determined by the following equation:

$$\alpha = \frac{10^{\text{pH}-\text{p}K_a}}{1 + 10^{\text{pH}-\text{p}K_a}}$$

where, pH is the value at which enzyme hydrolysis was performed. The pK_a values were calculated using the equation (Steinhardt & Beychok, 1964):

$$\text{p}K_a = 7.8 + \frac{(298 - T)}{298 \times T} \times 2400$$

where, T is the temperature of enzyme hydrolysis in Kelvin degrees. Using each of the two enzymes, tilapia protein isolates were hydrolysed to achieve 7.5% and 25% DH. Once the desired % DH was achieved, the homogenate was heated to 90 °C for 10 min to inactivate the enzymes, followed by cooling on ice. Hydrolysed samples were stored at −20 °C until further use.

Tilapia protein hydrolysates were also fractionated using ultrafiltration (Amicon Model 202 stirred ultrafiltration cell, Amicon Corp., Danvers, MA) into three different fractions (Fig. 1). A 30 kDa pore-sized membrane was used first to separate the hydrolysate into >30 kDa retentate and <30 kDa filtrate. The >30 kDa retentate was the first fraction used for ACE inhibition studies. The <30 kDa filtrate was again ultra-filtered using a 10 kDa pore-sized membrane to obtain a 10–30 kDa retentate and <10 kDa filtrate. These two 10–30 kDa and <10 kDa fractions were used for ACE inhibition studies. The three fractions, >30 kDa, 10–30 kDa and <10 kDa peptides, were stored at −20 °C until further use.

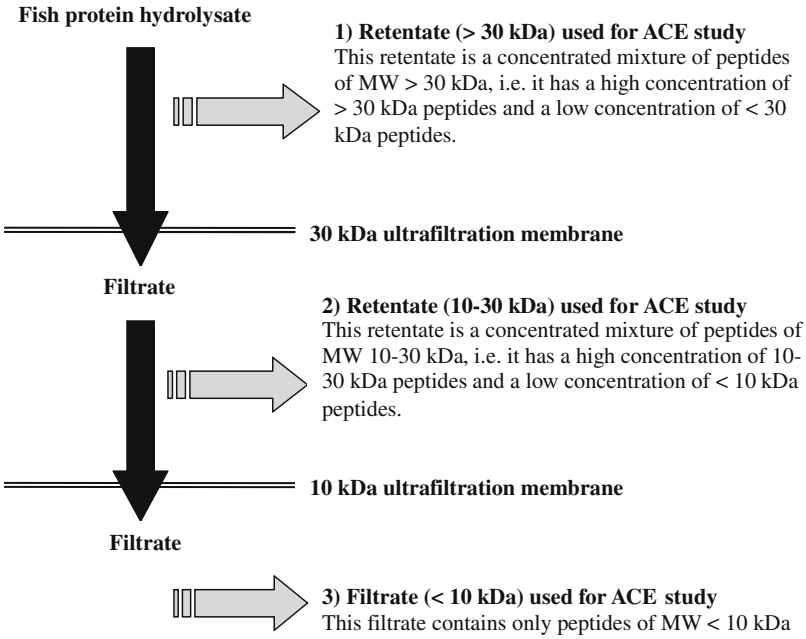

Fig. 1. Flow diagram for ultrafiltration of protein hydrolysates into different molecular weight fractionates. Fractionates used for ACE studies were (1) >30 kDa fraction which is a concentrated mixture of peptides of MW >30 kDa, (2) 10–30 kDa fraction, which is a concentrated mixture of peptides of MW 10–30 kDa, and (3) <10 kDa fraction which contains peptides of MW <10 kDa.

2.2.3. ACE inhibitory activity of tilapia protein hydrolysates

Both Flavourzyme and Cryotin hydrolysed tilapia protein isolates, as well as the three fractions from each hydrolysate, were tested for ACE inhibitory activity using FAPGG as the synthetic substrate for ACE and using a combination of methods of Murray, Walsh, and FitzGerald (2004) and Theodore and Kristinsson (2007) as mentioned below. Samples (100 μL of 0.2% w/v protein concentration), enzyme (50 μL of 15 mU enzyme) and substrate (1 mL of 0.5 mM FAPGG substrate) were mixed and incubated for 60 min at 25 °C. At the end of 60 min incubation period, absorbance at 340 nm was measured using an UV–Vis spectrophotometer. Hydrolysis of FAPGG by ACE will result in a decrease in absorbance at 340 nm. ACE inhibitory activity was measured by the ability of tilapia protein hydrolysates to decrease the hydrolysis of FAPGG as mentioned in Theodore and Kristinsson (2007). A sample containing FAPGG substrate and the ACE enzyme was used as a control. A 100% ACE inhibitory activity would indicate complete inhibition of the enzyme and no decrease in absorbance. All experiments were done in duplicates:

$$\%\ \text{ACE inhibition} = \frac{(\text{Abs}_{\text{no sample}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{no sample}}} \times 100$$

where, $\text{Abs}_{\text{no sample}}$ is the absorbance of the enzyme-substrate mixture in the absence of tilapia protein hydrolysates, and $\text{Abs}_{\text{sample}}$ is the absorbance of the enzyme-substrate mixture in the presence of protein hydrolysates.

2.2.4. SDS–PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was performed on the protein hydrolysates using a 10–20 % tris–tricine gel (Schagger & von Jagow, 1987) to characterise the hydrolysates based on their molecular weights (MW). The MW of the hydrolysates was calculated with reference to the migration of SDS–PAGE wide range molecular weight standard. After electrophoresis, the gels were stained using Sigma Brilliant Blue perchloric acid staining solution. Once the desired staining was achieved, gels were scanned using an Epson Stylus CX5400 scanner. Images were analysed using Scion Image 4.0.2 (Scion Co., Frederick, MD) software. The bands in the samples were compared with known bands of protein standards.

2.2.5. Statistical analysis

Experiments were performed in duplicates and repeated at least 2 times. Statistical analyses on the samples were performed using JMP™ Statistical Discovery Software (version 5.0). Tukey's multiple-comparison tests were used to compare the differences amongst the means. Dunnet's multiple-comparison test was used for comparing the effect of different treatments to the control. Analysis of variance was employed to examine the difference amongst treatments at $p < 0.05$ level.

3. Results and discussion

3.1. Characterisation of tilapia hydrolysates using SDS–PAGE

SDS–PAGE was used to characterise the molecular weight of both tilapia protein hydrolysates (Fig. 2). At 7.5% DH, both Cryotin and Flavourzyme yielded hydrolysates with MW ranging from 3.5 kDa up to 110 kDa (Fig. 2). These may represent various intact and hydrolysed myofibrillar proteins. Increasing hydrolysis to 25% significantly ($p < 0.05$) increased the amount of low molecular weight peptides. At 25% DH, both Cryotin and Flavourzyme hydrolysates showed some light bands near 40 and 60 kDa, but a majority of the peptide bands were less than 40 kDa. The ability of various enzymes such as Protamex, Neutrase and Alcalase to yield low molecular weight peptides at high % DH in species such as catfish, cod and salmon has been well documented (Lalasidis, Bostrom, & Sjoberg, 1978; Liaset, Lied, & Espe, 2000; Panyam & Kilara, 1996; Theodore, 2005).

3.2. Characterisation of tilapia fractionates using SDS–PAGE

The hydrolysates were ultra-filtered using 10 and 30 kDa membranes and were characterised using SDS–PAGE (Fig. 3). When a

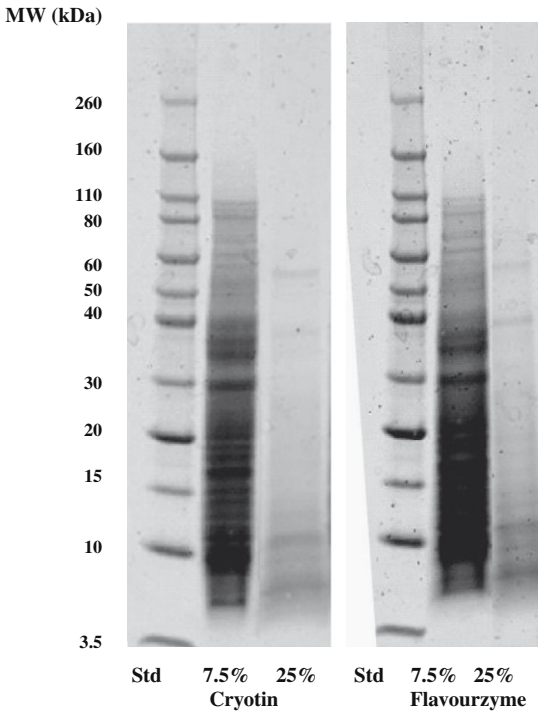

Fig. 2. SDS–PAGE of tilapia protein hydrolysates performed using 10–20% tris–tricine gels. Two enzymes, Cryotin and Flavourzyme were used for hydrolysing the tilapia isolate to 7.5% and 25% DH (degree of hydrolysis). The lane indicated as Std was the molecular weight (MW) marker with its corresponding molecular weights indicated as a column to its left. The wells adjacent to the marker from the left are Cryotin 7.5% DH, Cryotin 25% DH, Flavourzyme 7.5% DH and Flavourzyme 25% DH.

30 kDa membrane was used, >30 kDa retentate fraction and <30 kDa filtrate were obtained. Although ultrafiltration is a fast and easy technique for separating peptides based on their MW, it would require numerous washings and prolonged separation times to completely get rid of low MW fractions (i.e. <30 kDa fractions) from the >30 kDa retentate. However, as the majority of <30 kDa fractions are removed during ultrafiltration, the >30 kDa retentate will be concentrated in high MW fractions rather than with low MW fractions. In the SDS gel, this was indicated by strong bands of MW greater than 30 kDa (Fig. 3). Also, 25% DH hydrolysates obtained before ultrafiltration (Fig. 2) showed weak bands of MW >30 kDa, while the corresponding fractionates obtained after ultrafiltration (Fig. 3) showed strong bands of high MW indicative of the ability of ultra-filtration to concentrate the retentate samples. Between the 7.5% and 25% ultra-filtered samples, 7.5% ultra-filtered samples showed significantly higher number of strong bands between 30 kDa and 110 kDa than 25% DH samples. This could be due to the breakdown of high MW peptides at 25% DH. The filtrate obtained using the 30 kDa membrane had MW <30 kDa. This <30 kDa filtrate was again ultra-filtered as mentioned in the methods section, using a 10 kDa membrane filter to yield two fractions, a retentate fraction of MW 10–30 kDa, and a filtrate fraction of MW <10 kDa. An analysis of 10–30 kDa fractionates showed higher amount of peptides in 10–30 kDa region for 7.5% DH samples compared to 25% DH samples. This could be again due to the break down of proteins by 25% DH resulting in small peptides which could not be characterised in the gel. The <10 kDa fractions show a light band just below the 10 kDa range. Otherwise no band was visible indicating the presence of low MW peptides which could not be detected using SDS–PAGE.

3.3. ACE inhibitory activity of tilapia protein hydrolysates

The hydrolysates samples obtained after enzyme hydrolysis were tested for ACE inhibitory activity (Fig. 4). Both Cryotin and Flavourzyme hydrolysates exhibited ACE inhibition. For Cryotin hydrolysates, the ACE inhibitory activity was around 62% and 71% for 7.5% DH and 25% DH, respectively. Cryotin, as mentioned earlier, is a mixture of trypsin, chymotrypsin and elastase. It has already been shown that hydrolysis of yellowfin sole frame proteins (Jung et al., 2006) and whey proteins (Mullally, Meisel,

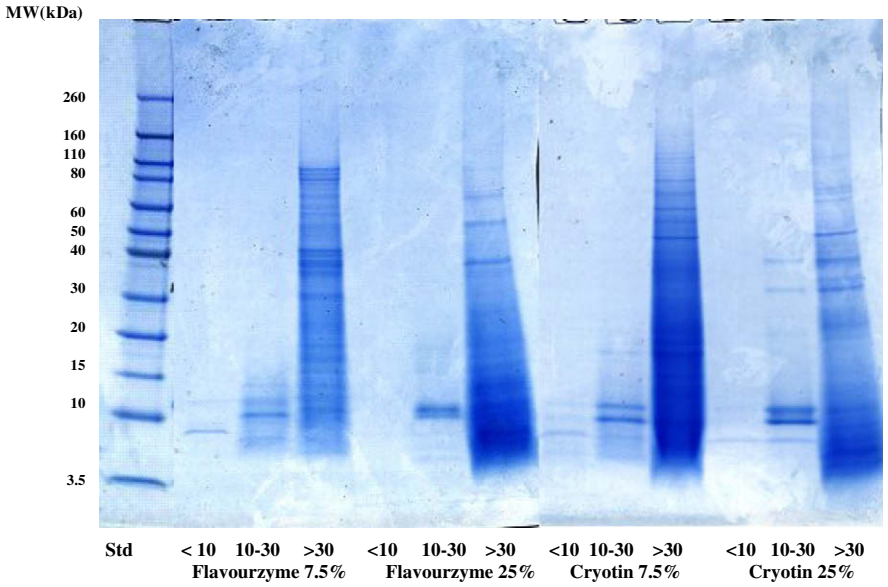

Fig. 3. SDS–PAGE of tilapia protein fractionates performed using 10–20% tris–tricine gels. Two enzymes, Cryotin and Flavourzyme were used for hydrolysing the tilapia isolate to 7.5% and 25% DH (degree of hydrolysis). Both these hydrolysates were first fractionated using 30 kDa membranes to obtain two samples: >30 kDa and <30 kDa. The latter sample was fractionated using 10 kDa membrane to obtain a third, <10 kDa sample. The lane indicated as Std was the molecular weight (MW) marker with its corresponding molecular weights indicated as a column to its left. The wells adjacent to the marker from the left are Flavourzyme 7.5% DH fractionates <10 kDa, 10–30 kDa and >30 kDa, Flavourzyme 25% DH fractionates <10 kDa, 10–30 kDa and >30 kDa, Cryotin 7.5% DH fractionates <10 kDa, 10–30 kDa and >30 kDa and Cryotin 25% DH fractionates <10 kDa, 10–30 kDa and >30 kDa.

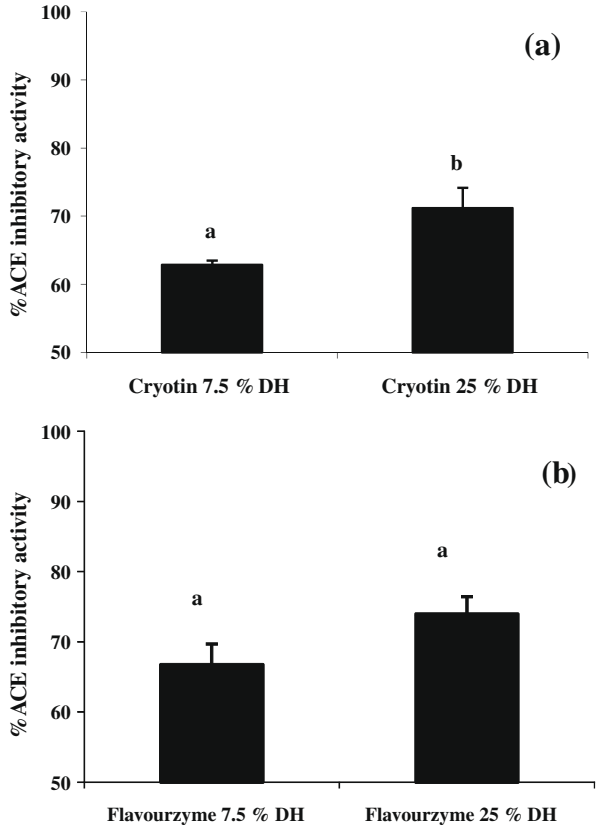

Fig. 4. ACE inhibitory activity of tilapia protein hydrolysates. Two enzymes, Cryotin and Flavourzyme were used for hydrolysis. (a) Cryotin hydrolysates with 7.5% and 25% DH. (b) Flavourzyme hydrolysates with 7.5% and 25% DH. DH is the degree of hydrolysis. Different letters in each figure represent a significant difference amongst the treatments ($p < 0.05$).

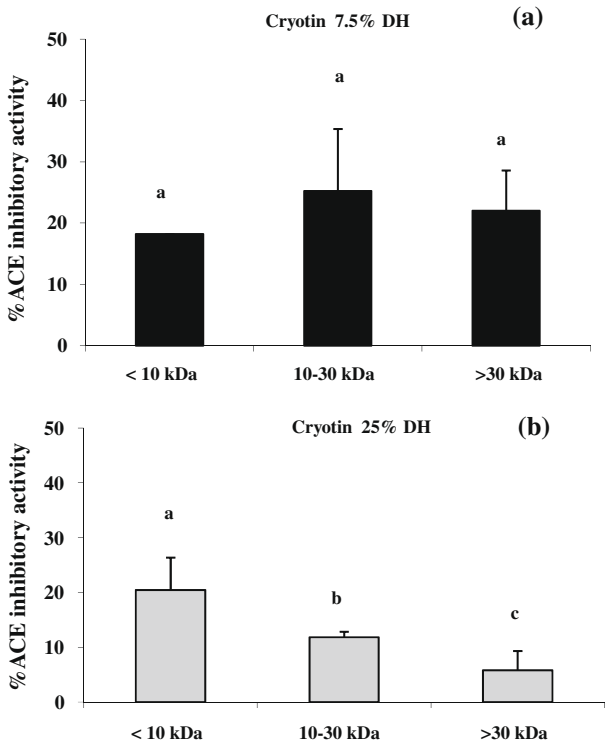

Fig. 5. ACE inhibitory activity of fractionates obtained from Cryotin-hydrolysed tilapia protein. Fractionates with molecular weights <10 kDa, 10–30 kDa and >30 kDa were obtained by ultrafiltration of tilapia hydrolysates. (a) Cryotin-hydrolysed sample with 7.5% DH. (b) Cryotin-hydrolysed sample with 25% DH. DH is the degree of hydrolysis. Different letters in each figure represent a significant difference amongst the treatments ($p < 0.05$).

& FitzGerald, 1997) using pancreatic enzymes yield peptides with high ACE inhibitory activity. For Flavourzyme hydrolysates, the ACE inhibitory activity was 66% and 73% for 7.5% DH and 25% DH, respectively. Flavourzyme is a complex mixture of fungal proteases and peptidases with un-specific peptide cleavage activity. The results from Fig. 4 indicate that the type of enzymes did not significantly ($p > 0.05$) affect ACE inhibitory activity. Also, in general, 25% DH hydrolysates showed higher ACE inhibition compared to 7.5% DH hydrolysates indicating that low MW peptides are better ACE inhibitors than high MW peptides. The ability of low MW peptides to inhibit ACE activity has also been reported for peptides derived from a variety of fish species such as bonito (Masaaki et al., 2000), yellowfin sole (Jung et al., 2006), Alaska pollock (Je, Park, Kwon, & Kim, 2004) and sea bream (Fahmi et al., 2004).

3.4. ACE inhibitory activity of tilapia protein fractionates

The three fractions obtained using ultrafiltration was tested for their ACE inhibitory activity. In general, ultrafiltration significantly decreased ($p < 0.05$) the ACE inhibitory activity of fractionates (Figs. 5 and 6) compared to the whole hydrolysates (Fig. 4). For example, Cryotin and Flavourzyme hydrolysates with 25% DH showed more than 70% inhibition compared to their corresponding ultra-filtered fractionates which showed less than 25% inhibition. The lower activity of fractionates compared to whole hydrolysate indicates a possible synergistic action amongst all the peptides when present together in the whole hydrolysate. Amongst the different samples, the inhibitory activity of >30 kDa fractionates (for both enzymes) was significantly lower ($p < 0.05$) than that of whole hydrolysates,

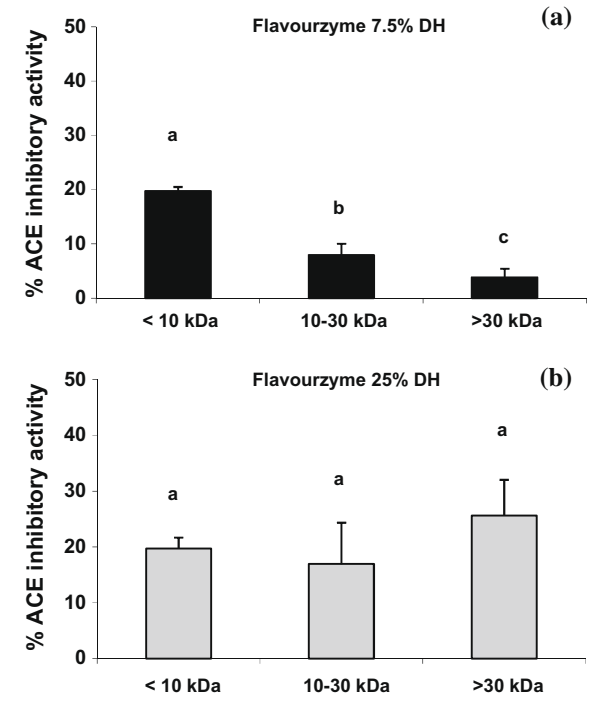

Fig. 6. ACE inhibitory activity of fractionates obtained from Flavourzyme-hydrolysed tilapia protein. Fractionates with molecular weights <10 kDa, 10–30 kDa and >30 kDa were obtained by ultrafiltration of tilapia hydrolysates. (a) Flavourzyme-hydrolysed sample with 7.5% DH. (b) Flavourzyme-hydrolysed sample with 25% DH. DH is the degree of hydrolysis. Different letters in each figure represent a significant difference amongst the treatments ($p < 0.05$).

although both hydrolysates and fractionates had peptides in the MW range 3.5–110 kDa (Figs. 2 and 3). The >30 kDa fractionates obtained from Cryotin 7.5% DH and Flavourzyme 25% DH showed ~25% ACE inhibition while their corresponding whole hydrolysates showed around 65% and 75% ACE inhibition. As explained in the previous section under 'characterisation of tilapia fractionates using SDS–PAGE', the whole hydrolysate contain peptides of all molecular weights, while the >30 kDa fractionates are concentrated with >30 kDa fractions with a small amount of <30 kDa peptides. Hence, the difference in ACE inhibition between >30 kDa fractionates and the whole hydrolysates could be either due to the high concentration of large MW peptides and the absence of low MW peptides in the >30 kDa fraction.

At 7.5% DH, Cryotin fractionates showed no significant ($p > 0.05$) variation in ACE inhibitory activity (Fig. 5a), but Flavourzyme fractionates (Fig. 6a) showed a significant decrease in inhibitory activity with an increase in MW of fractions. The ACE inhibition of Cryotin fractionates was around 20%, and for Flavourzyme fractionates the inhibitory activity decreased from 20% for <10 kDa fraction to less than 5% for >30 kDa fraction. At 25% DH, Cryotin fractionates showed a decrease in % ACE inhibitory activity with an increase in MW of the fractions (Fig. 5b), while Flavourzyme fractionates showed no significant difference ($p > 0.05$) in their inhibitory activity (Fig. 6b). For Flavourzyme, the % ACE inhibition for various fractions was around 20–25%, while the inhibitory activity for Cryotin fractionates was around 10% for a >30 kDa fraction and the % inhibition increased to 20% for <10 kDa fractions. A similar increase in the ACE inhibitory activity of ultra filtered low MW fractions was also reported for whey protein digests (Pihlanto-Leppala, Koskinen, Piilola, Tupasela, & Korhonen, 2000) and for hydrolysates prepared from the protein of yellowfin sole frames (Jung et al., 2006).

These results show that tilapia hydrolysed using Cryotin or Flavourzyme achieved its optimum ACE inhibitory activity at 25% DH (Fig. 4). Ultrafiltration of 25% DH Cryotin hydrolysate into various fractions did not improve the ACE inhibition efficacy of fractionates compared to the whole hydrolysates. When Cryotin hydrolysates were hydrolysed to 25% DH, the inhibition activity of the low MW (<10 kDa) fractions increased while that of high MW (>30 kDa) fractions decreased. However, the inhibition activity of the low MW (<10 kDa) fraction was not significantly ($p > 0.05$) different from Cryotin 7.5% DH fractionates. When Flavourzyme was used at 7.5% DH, the low MW fraction (<10 kDa) showed optimum ACE inhibitory activity while the high MW (10–30 kDa and >30 kDa) fractions showed lower activity. Increasing hydrolysis to 25% DH increased the activity of high MW (10–30 kDa and >30 kDa) fractions, but all the fractions at 25% DH had similar efficacy as Flavourzyme 7.5% DH, <10 kDa fraction.

4. Conclusion

Hydrolysates prepared from alkali solubilised tilapia white muscle exhibited significant ACE inhibitory activity. The inhibitory activity did not differ significantly between the types of enzymes used for hydrolysis, i.e. Cryotin and Flavourzyme, but increased with the % DH. Our results indicate the potential for converting the wastes from tilapia industry by enzyme hydrolysis into useful bioactive food ingredients. Fractionating whole tilapia hydrolysates using ultrafiltration into various molecular weight fractions did not improve the ACE inhibitory activity of fractionates compared to the whole hydrolysates. Hence, it would be economical to enzyme hydrolyse and utilise whole tilapia protein hydrolysates for their bioactive potential (without fractionation).

Acknowledgment

This work was supported by the Cooperative State Research, Education and Extension Service, US Department of Agriculture by Grant 2004-35503-14119 of the USDA NRI Competitive Grants Program.

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Food Chemistry 117 (2009) 589–598

Contents lists available at ScienceDirect

Food Chemistry

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Correlation between plasma antioxidant capacity and verbascoside levels in rats after oral administration of lemon verbena extract

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ARTICLE INFO

Article history:
Received 28 January 2009
Received in revised form 1 April 2009
Accepted 14 April 2009

Keywords:
Verbascoside
Antioxidant capacity
TEAC
ORAC
MDA
HPLC-MS/MS
SOD
FRAP
Pharmacokinetics
Malondialdehyde
Biological membranes

ABSTRACT

Phenylpropanoids are the main class of compounds from lemon verbena which have shown a wide biological activity, verbascoside being the most abundant one. In this work, the composition of a lemon verbena extract was elucidated by HPLC-ESI-MS/MS and one flavone and one methoxylated phenylpropanoid were found in this source for the first time. The antioxidant activity of the lemon verbena extract was fully characterised by several methodologies. Unexpectedly, the extract was especially active in lipophilic environments and lipid peroxidation inhibition assay, as it was found for pure verbascoside. The lemon verbena extract, containing verbascoside as its major bioactive compound, was acutely administered to rats and verbascoside was the only metabolite detected in plasma samples as measured by HPLC mass spectrometry. The correlation between the highest verbascoside concentration in plasma and maximum plasma antioxidant capacity was observed at 20 min as measured by different techniques, i.e. minimum malondialdehyde (MDA) generation, highest ferric-reducing ability of plasma (FRAP value) and maximum superoxide dismutase activity (SOD). Therefore, the *in vitro* measurements of the antioxidant activity of lemon verbena extract may significantly support the antioxidant activity observed *in vivo* in this work. Moreover, neither evidence of acute oral toxicity nor adverse effects were observed in mice when the lemon verbena extract containing 25% verbascoside was used at a dosage of 2000 mg/kg.

1. Introduction

Lemon verbena (*Aloysia triphylla* or *Lippia citriodora*) is an herb mainly used as a spice and a medicinal plant. It grows spontaneously in South America and is cultivated in North Africa and Southern Europe. Lemon verbena leaves are used to add a lemony flavour in many culinary purposes such as fish and poultry dishes, vegetable marinades, salad dressings, jams, puddings, and beverages. It is also used to make herbal teas and refreshing sorbets. Therefore, lemon verbena products and their compounds can be considered into the food category.

The leaves of these species are reported to possess digestive, antispasmodic, antipyretic, sedative and stomachic properties. It has traditionally been used in infusions for the treatment of asthma, cold, fever, flatulence, colic, diarrhoea and indigestion (Newall, Anderson, & Phillipson, 1996). Phenylpropanoids represent the main class of compounds of this plant as verbascoside being the most abundant one (Fig. 1) (Bilia, Giomi, Innocenti, Gallori, & Vincieri, 2008; Laporta et al., 2004). Several properties have been described for this compound like anti-inflammatory (Deepak & Handa, 2000; Diaz et al., 2004; Hausmann et al., 2007), antimicrobial (Avila et al., 1999) and antitumor (Ohno, Inoue, Ogihara, & Saracoglu, 2002). Among several factors, verbascoside antioxidant activity may suppose a significant contribution to its protective effects (Liu et al., 2003; Siciliano et al., 2005; Valentao et al., 2002; Wong, He, Huang, & Chen, 2001).

Abbreviations: AAPH, 2,2′-azobis (2-methyl-propionamine) dihydrochloride; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt; ANOVA, analysis of variance; AUC, area under curve; BHT, butylated hydroxytoluene; BPC, base peak chromatogram; DAD, diode-array detector; EIC, extracted-ion chromatogram; ESI, electrospray ionisation; EtOH, ethanol; EYPC, egg yolk phosphatidylcholine; FL, fluorescein; FRAP, ferric-reducing ability of plasma; HPLC–MS, high-performance liquid chromatography–mass spectrometry; LD_{50}, lethal dose 50%; LOD, limit of detection; LOQ, limit of quantification; MDA, malondialdehyde; ORAC, oxygen radical absorbance capacity; SOD, superoxide dismutase; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TE, Trolox equivalents; TEAC, Trolox equivalent antioxidant capacity; TEP, 1,1,3,3-tetraethoxypropane; TPTZ, 2,4,6,-tripyridyl-S-triazine; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; UV, ultraviolet–visible wavelength.

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doi:10.1016/j.foodchem.2009.04.059

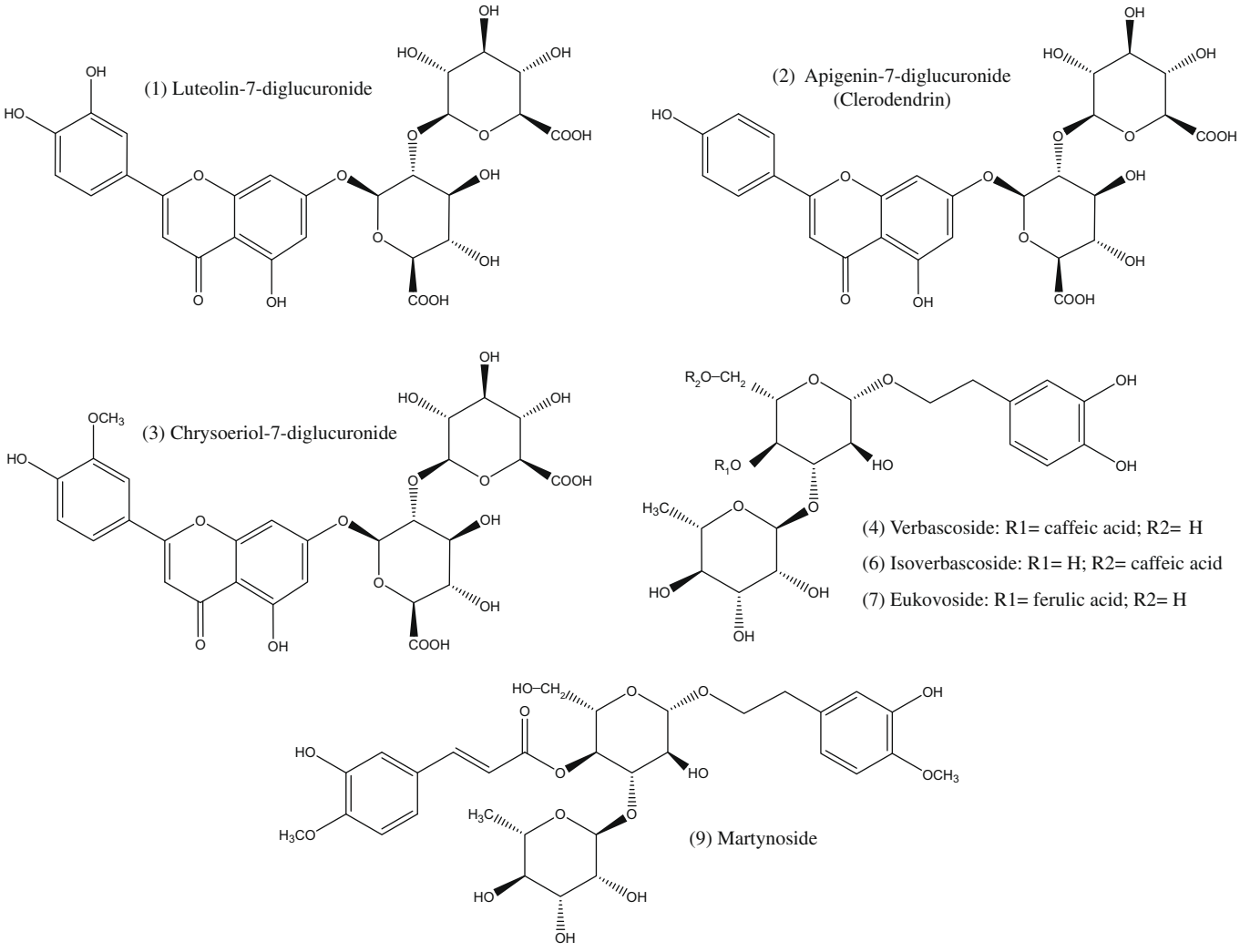

Fig. 1. Chemical structures of the major compounds found in lemon verbena leaves extract.

Due to the presence of lemon verbena in food products, it may be necessary to know its pharmacokinetics and its possible antioxidant effects *in vivo*. So far, most of the previous results on lemon verbena derive from *in vitro* assays. Till now, detailed information about the pharmacokinetics of verbascoside is very limited and derives from plants which are different from lemon verbena (Wu, Lin, Sung, & Tsai, 2006; Wu, Tsai, Lin, & Tsai, 2007). Only one report has shown pharmacokinetics of this compound after oral administration and its bioavailability was found to be very low (Wu et al., 2006).

In this work, the composition and *in vitro* antioxidant activity of an extract from lemon verbena standardised in 25% verbascoside (w/w) was determined. Moreover the pharmacokinetics of this compound in rats after oral administration of the above mentioned extract was studied. Significant amounts of verbascoside were found in plasma of rats which was readily available 20 min after oral administration. In addition, a correlation of the plasma antioxidant status in rats, as measured by different antioxidant methods, and the concentration of verbascoside is shown for the first time. Finally the toxicity of the extract in mice was also studied.

2. Materials and methods

2.1. Reagent and chemicals

Sulphatase and glucuronidase (EC 232-772-1) from *Helix pomatia* type H-1, butylated hydroxytoluene (BHT); trichloroacetic acid (TCA); thiobarbituric acid (TBA); 1,1,3,3-tetraethoxypropane (TEP); 2,4,6, tripyridyl-S-triazine (TPTZ); ferric chloride ($FeCl_3$); Trolox (6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid); 2,2′-azino-bis(3-ethylbenzothiazoline-6 sulphonic acid) (ABTS); 2, 2′-azobis (2-methyl-propionamine) dihydrochloride (AAPH); fluorescein (FL), xanthine; xanthine oxidase and cytochrome *c* were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). Natural lipid egg yolk phosphatidylcholine (EYPC) was obtained from Avanti Polar Lipids (Birmingham, AL, USA), dissolved in chloroform/methanol (1:1, v/v) and stored at −20 °C. Lemon verbena extract (25% verbascoside, w/w), *Citrus aurantium* peel extract (25% naringin, 25% neohesperidin, w/w) and olive leaf extract (25% oleuropein, w/w) were kindly provided by Monteloeder, S.L. (Elche, Spain). All other compounds were of analytical, spectroscopic or chromatographic reagent grade and were obtained from Merck KGaA (Darmstadt, Germany). Double-distilled and deionized water was used throughout this work.

2.2. Animals

Twenty-eight male Wistar rats (280–380 g) were housed in standard cages at room temperature with free access to food and water for two weeks. Throughout the experiments, animals were processed according to the suggested ethical guidelines for the care of laboratory animals (Morton et al., 2001).

2.3. Treatment of animals

Rats were orally treated with lemon verbena extract (2180 mg/kg, corresponding to 545 mg/kg of verbascoside) via gastric gavage. For the administration, the extract was suspended in saline serum (3 mL). The control group received only saline serum. Rats were subjected to ketamine/xylazine anesthesia and the blood samples were withdrawn via cardiac puncture into heparinized tubes at 20, 40, 55, 75, and 100 min post dosing. All blood samples were centrifuged at 1000*g* for 15 min at 4 °C, and then plasma was stored at −80 °C.

2.4. Measurement of the Trolox equivalent antioxidant capacity (TEAC)

The Trolox equivalent antioxidant capacity (TEAC) assay, which measures the reduction of the radical cation of ABTS by antioxidants, was performed as previously described (Laporta, Pérez-Fons, Mallavia, Caturla, & Micol, 2007). Compounds were assayed at five different concentrations determined within the linear range of the dose–response curve. A calibration curve was prepared with different concentrations of Trolox (0–20 μM). Results were expressed in mM of Trolox per mM pure compound or g of extract.

2.5. Radical-scavenging capacity by measurement of inhibition of malondialdehyde (MDA) generation

The quantitative evaluation of the antioxidant capacity of the compounds/extracts against lipid peroxidation was determined through the measurement of the inhibition of MDA generation by using small unilamellar vesicles formed by sonication of multilamellar vesicles of egg yolk phosphatidylcholine (EYPC). Oxidation conditions, extraction and quantitation of TBA–MDA chromogen were performed as previously described (Laporta et al., 2007). Briefly, the chromogen was determined using HPLC coupled to fluorescence detection. The analysis were conducted by injecting 20 μL of sample into a reverse phase column LiChrospher® 100 RP-18 (5 μm, 250 x 4 mm i.d.) from Merck KGaA (Darmstadt, Germany) using isocratic mode with methanol-50 mM potassium phosphate buffer, pH 6.8 (40:60, v/v), and a flow rate of 1 mL/min. The TBA–MDA product was monitored by fluorescence detection with excitation at 515 nm and emission at 553 nm. Results were expressed in mg/L for extracts or μM of pure compound which were able to inhibit 50% of the generation of MDA (nmol MDA/mg EYPC).

2.6. Assay of the oxygen radical absorbance capacity (ORAC)

To assay the capacity of the compounds/extracts to scavenge peroxyl radicals a validated ORAC method, which uses fluorescein (FL) as the fluorescent probe ($ORAC_{FL}$), was utilised (Ou, Hampsch-Woodill, & Prior, 2001) with minor modifications (Laporta et al., 2007). ORAC values were expressed as micromole Trolox equivalents per gram of antioxidant substance.

2.7. Enzymatic hydrolysis and determination of verbascoside in plasma

Plasma (50 μL) was mixed with 50 μL of enzyme mixture in 100 mM acetate buffer (pH 5.0) containing 12.5 units of sulphatase and 270 units of β-glucuronidase activity. The mixture was incubated at 37 °C for 45 min. Soon after 200 μL of 0.83 M acetic acid in methanol were added. The mixture was vortexed, sonicated, and centrifuged (7400*g* for 5 min at 4 °C), and the supernatant was analysed by HPLC-DAD-MS/MS system. The LC/MS system consisted of an Agilent LC 1100 series (Agilent Technologies Inc., Palo Alto, CA, USA) controlled by the Chemstation software. The HPLC instrument was coupled to an Esquire 3000 + (Bruker Daltonics, GmbH, Germany) mass spectrometer equipped with an ESI source and ion trap mass analyser, and controlled by Esquire control and data analysis software. A Merck LiChrospher 100 RP-18, 5 μm, 250 × 4 mm (i.d.) column was used for analytical purposes. For the analysis of verbascoside, a 20 min linear gradient from 5% to 30% acetonitrile in 1% formic acid in water was used. For the accurate performance of the LC–MS pump, 10% of organic solvent was premixed in the water phase. The flow rate was 0.5 mL/min (Maatta, Kamal-Eldin, & Torronen, 2003). Diode-array detection was set at 280 and 330 nm. Mass spectrometry operating conditions were optimised in order to achieve maximum sensitivity values. The ESI source was operated in negative mode to generate $[M-H]^-$ ions using the following conditions: desolvation temperature at 350 °C and vapourizer temperature at 400 °C; dry gas (nitrogen) and nebulizer were set at 5 L min^{-1} and 65 psi, respectively. The MS data were acquired as full scan mass spectra at 150–1000 m/z by using 50 ms for collection of the ions in the trap.

2.8. Peaks identification and validation of the verbascoside assay method

Identification of all constituents was performed by HPLC-DAD- and –MS/MS analysis, comparing the retention time, UV and MS spectra of the peaks in the samples with those authentic reference samples or data reported in the literature. The purity of peaks was checked comparing the UV spectra of each peak with those of authentic reference samples and by examination of the MS and MS/MS spectra.

The accuracy of the method for the determination of verbascoside in plasma was further assessed with recovery studies by spiking verbascoside into blank plasma and water in triplicates. The linearity range of the responses was determined on eight concentration levels with three injections for each level. Calibration graphs for HPLC were recorded with sample amount ranging from 0.25 μg/mL to 0.25 mg/mL ($r^2 > 0.9999$). Quantitative evaluation of verbascoside was performed by means of a six-point regression curve ($r^2 > 0.996$) in a concentration range between 0.25 μg/mL and 0.1 mg/mL, using verbascoside as reference external standard and evaluated at 330 nm. LOD (Limit of Detection) was 0.10 μg/mL and Limit of Quantification (LOQ) was 0.25 μg/mL.

2.9. HPLC analysis of plasma MDA

Briefly, 50 μL of plasma were mixed with 50 μL of 0.05% BHT in ethanol and 50 μL of TCA 20% in HCl 0.6 M. The samples were incubated 15 min on ice and then were centrifuged at 5000*g* during 15 min at 4 °C. Then 100 μL of TBA 0.6% in water were added to 100 μL of supernatant. At follow, the mixture was incubated at 97 °C for 1 h, let to cool down and extracted with 300 μL of n-butanol through vigorous shaking, and then samples were centrifuged at 10000*g* for 3 min. The TBA–MDA chromogen was determined using an HPLC and fluorescence detection system as previously described in paragraph 2.5 of this section.

2.10. Ferric-reducing ability of plasma

To assess plasma antioxidant capacity, the ferric-reducing ability of plasma (FRAP) was measured as previously described (Benzie & Strain, 1996). Briefly, 40 μL of diluted plasma samples (1:2) were mixed on a 96-well plate with 250 μL of freshly prepared FRAP reagent. Samples were incubated for 10 min at 37 °C and then absorbance at 593 nm was recorded during 4 min on a microplate reader (SPECTROstar Omega, BMG LabTech GmbH, Offenburg, Germany). FRAP values were calculated using $FeSO_4 \cdot 7H_2O$ as standard.

2.11. Superoxide dismutase (SOD) activity in plasma

SOD activity was measured through an adaptation of the method of McCord and Fridovich (1969). The xanthine/xanthine oxidase system was used to generate the superoxide anion. This anion produced the reduction of cytochrome *c*, which was monitored at 550 nm. The SOD activity in the sample removed the superoxide anion and produced an inhibition of the cytochrome *c* reduction. The activity was determined with a SPECTROstar Omega microplate reader at 37 °C.

2.12. Acute oral toxicity assessment

Ten-week-old male and female ICR strain mice were used for the study. The animals were housed in cages in a temperature-controlled animal room (23 ± 1 °C) with a relative humidity of 55 ± 5% and were fed a standard diet. The animal care and handling was done according to the regulations of Council Directive 86/609/EEC about Good Laboratory Practice (GLP) on animal experimentation. The animals were allowed to fast by withdrawing the food and water for 18 h and divided into four groups of eight individuals each (two groups for the control and two groups for the test substance) before the administration. Briefly, the powder (lemon verbena extract, 25% verbascoside) was dissolved in 0.9% NaCl sterile solution, pH 7.4 at a concentration of 80 mg/ml and centrifuged to eliminate insoluble particles. The test substance was administered by oral gavage in a total volume of 0.5 mL at a single dose of 2000 mg/kg. Weight control and observations were continued for 14 days. On day 14, the mice were anesthetized, killed by exsanguinations, and examined by necropsy. The assay was performed following the recommend regulations (OECD Test Guideline 420; Fixed Dose Procedure).

2.13. Statistical analysis

Statistical analysis was performed using the statistical package SPSS 13.0 for Windows. The effects of lemon verbena extract consumption on the investigated parameters were evaluated by comparing the values obtained at different time intervals with the control (0 time) using one way ANOVA test. Depending on the results, significance values of $p < 0.01$, $p < 0.05$ and $p < 0.10$ were considered statistically significant.

3. Results and discussion

3.1. Composition of lemon verbena extract by HPLC–MS/MS

The composition of the lemon verbena extract utilised in this work was determined by means of HPLC-DAD-ESI-MS/MS. The identification of the peaks was based on the analysis of their retention time, UV spectra and MS/MS data as mentioned in the Materials section. Fig. 2 shows the high-performance liquid chroma- tography profile at 330 nm of lemon verbena extract (Fig. 2A) and its corresponding base peak chromatogram (BPC) (Fig. 2B), which is indicative of peak purity detection in data generated with LC–MS.

We were able to identify seven major phenolic compounds in the extract (see Fig. 1 and Table 1), namely four phenylpropanoids, and three glycosylated flavones. Nevertheless, no iridoid derivatives were identified in the extract although other authors have found them in lab-scale ethanolic extractions from lemon verbena leaves (Bilia et al., 2008). Among the phenylpropanoids, verbascoside was clearly the most abundant compound (around 25%, w/w). In contrast to previously reported (Bilia et al., 2008), its positional isomer, isoverbascoside, was found in very low amounts (Fig. 2). It has been reported that isoverbascoside can be obtained from the deacetylation and caffeoyl migration of verbascoside under hydrolytic environment, so it might derive from drastic extraction conditions (Kawada, Asano, Makino, & Sakuno, 2002). The methoxylated phenylpropanoid eukovoside was also present in the lemon verbena extract, as previously reported (Bilia et al., 2008; Shuya, Shengda, Xingguo, & Zhide, 2004; Sticher, Salama, Chaudhuri, & Winkler, 1982). Martynoside, another methoxylated phenylpropanoid (Kirmizibekmez et al., 2005) was indentified for the first time in the lemon verbena extract.

Three flavones were also identified in minor quantities in the lemon verbena extract (Fig. 1), all of them in their diglucuronide form, luteolin-7-diglucuronide (Carnat et al., 1995) and apigenin-7-diglucuronide (Bilia et al., 2008; Ragone, Sella, Conforti, Volonte, & Consolini, 2007), both previously reported in ethanolic extracts of lemon verbena. Moreover, one methoxylated flavone, chrysoeriol-7-diglucuronide is reported for the first time in lemon verbena. Total mass data and fragmentation patterns of these flavones were identical to those ones reported in other plant sources (Wang et al., 2008).

3.2. Antioxidant activity of lemon verbena extract

The antioxidant activity of lemon verbena extract was fully characterised *in vitro* by using several antioxidant measurements. First, the antioxidant activity by means of the Trolox equivalent antioxidant capacity (TEAC) assay was studied and compared to other antioxidant extracts and pure compounds. Quercetin, one of the most potent compounds under this type of assay, was used as a reference, which showed TEAC values that agreed to those found by other authors (Miller & Begoña Ruiz-Larrea, 2002; Rice-Evans, Miller, & Paganga, 1996; Soobrattee, Neergheen, Luximon-Ramma, Aruoma, & Bahorun, 2005) (Table 2). Lipophilic TEAC value obtained for lemon verbena extract was considerably high, i.e., 1.15 ± 0.07 (mM Trolox/g). Pure verbascoside showed a lipophilic TEAC value of 1.28 ± 0.11 mM Trolox/mM (i.e., 1.28 mmol Trolox/mmol or 2.05 mmol Trolox/g), which was consistent with the value previously found by other authors (Siciliano et al., 2005). In spite of the extract only contained 25% verbascoside and minor quantities of other phenylpropanoids and flavones (Fig. 2), TEAC value found for lemon verbena extract accounted for more than the half of the value determined for pure verbascoside per gram. The theoretical TEAC value of the extract due to verbascoside contribution would have been 0.51 mM Trolox/g but unexpectedly more than double of this value was obtained. From this result it could be postulated that other minor components of the extract such as the flavones could contribute to enhance the antioxidant capacity of the extract probably in a synergistic manner. Furthermore, lemon verbena extract showed stronger TEAC values than other strong antioxidant extracts such as olive leaf extract (25% oleuropein) or orange flavonoid extract (25% naringin, 25% neohesperidin).

Surprisingly, lemon verbena extract showed a higher TEAC value in a lipophilic environment than in water, which has been shown for other hydrophilic and glycosylated phenolic antioxidants such as hypoxoside (Laporta et al., 2007). As it has been previously reported, this fact may be related to its stronger capacity to scavenge free radicals in a lipophilic environment. This behaviour was also observed for the orange flavonoid extract, which also contains minor quantities of flavones that could contribute to this antioxidant behaviour. In contrast, olive leaf extract showed similar TEAC value in a lipophilic environment (ethanol) than in a hydrophilic medium (water).

In order to determine the contribution of the radical scavenging activity of the single compounds which are part of the verbascoside molecule, i.e., caffeic acid and hydroxytyrosol, TEAC and MDA inhibition values of these compounds were also evaluated in comparison to verbascoside. Lipophilic and hydrophilic TEAC and MDA

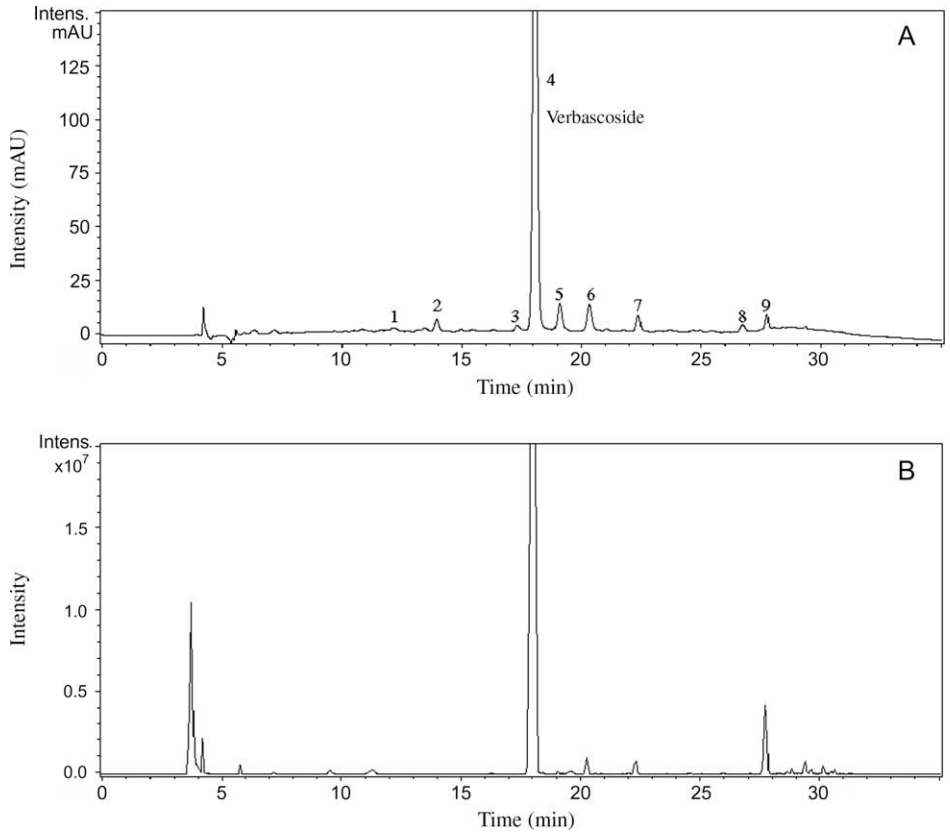

Fig. 2. High performance liquid chromatography profile of lemon verbena extract (A) at 330 nm, and (B) base peak chromatogram of 50–800 m/z. Intensity scale was magnified to show the less abundant compounds.

Table 1
List of the main phenolic compounds in the lemon verbena extract identified by HPLC-DAD-ESI-MS/MS.

Retention time (min)	Peak no. Lemon verbena extract	Compound	UV_{Max} (nm)	Mol. ion $[M-H]^-$	Primary fragment (MS/MS)
12.1	1	Luteolin-7-diglucuronide	348	637	351, 285
14.0	2	Apigenin-7-diglucuronide (Clerodendrin)	340	621	351
17.3	3	Chrysoeriol-7-diglucuronide	340	651	351
18.0	4	Verbascoside	330	623	461
19.0	5	Not identified	350	566	–
20.2	6	Iso-verbascoside	330	623	461
22.3	7	Eukovoside	330	637	351
26.7	8	Not identified	340	711	–
27.7	9	Martynoside	330	651	475

Table 2
Lipophilic and hydrophilic TEAC values and IC_{50} values for MDA generation inhibition of several extracts and pure compounds. Quercetin is shown as reference value.

Extract or compound	Antioxidant capacity		
	TEAC mM (H_2O)[a]	TEAC mM (EtOH)[b]	MDA inhibition (IC_{50}[c])
Lemon verbena	0.81 ± 0.04[d]	1.15 ± 0.07	6.91 ± 0.21[f]
Olive leaf	0.92 ± 0.12	0.94 ± 0.11	7.00 ± 1.20
Orange flavonoid	0.51 ± 0.01	0.71 ± 0.08	15.6 ± 1.64
Verbascoside	1.03 ± 0.15[e]	1.28 ± 0.11	3.82 ± 0.5[g]
Caffeic acid	0.59 ± 0.03	0.73 ± 0.14	7.41 ± 1.10
Hydroxytyrosol	0.66 ± 0.12	0.80 ± 0.10	15.42 ± 2.70
Quercetin	3.00 ± 0.30	2.26 ± 0.07	3.66 ± 0.20

[a] mM Trolox equivalent antioxidant capacity determined in H_2O ($n = 3$).
[b] mM Trolox equivalent antioxidant capacity determined in EtOH ($n = 3$).
[c] Concentration corresponding to the 50% inhibition of lipid peroxidation determined by HPLC–MDA measurement ($n = 3$).
[d] mM Trolox/g of extract.
[e] mM Trolox/mM of pure compound.
[f] mg/L of extract.
[g] μM of pure compound which were able to inhibit the 50% of the production of MDA (nmol MDA/mg lipid).

inhibition values of hydroxytyrosol were in agreement to previously reported (Laporta et al., 2007). Among the tested pure compounds, verbascoside showed higher TEAC values than caffeic acid or hydroxytyrosol, but it was less potent than quercetin. Verbascoside exhibited the same behaviour shown before, i.e., a higher TEAC value in EtOH than in water, what was consistent with the behaviour observed for the lemon verbena extract. Caffeic acid and hydroxytyrosol also showed a similar behaviour, but it was less pronounced. This fact might reveal the efficacy of verbascoside to scavenge free radicals within a hydrophobic environment. In contrast, quercetin evidenced a lower TEAC value in a lipophilic environment (ethanol) than in a hydrophilic medium (water).

The capacity of the lemon verbena extract to inhibit lipid peroxidation was also determined by HPLC determination of MDA generation (Table 2). Lemon verbena extract showed an IC_{50} value of 6.91 ± 0.21 mg/L, which revealed stronger antioxidant activity than other antioxidant extracts, such as olive leaf and tea catechins, determined in the same conditions (Laporta et al., 2007), and similar potency to the previously reported African potato extract, i.e., 7.5 mg/L (*Hypoxis hemerocallidea*). This fact may also be in relation to the stronger capacity showed by this extract in the lipophilic TEAC assay, what may also reveal the capability of lemon verbena extract (and therefore verbascoside) to scavenge free radicals under a specific environment such as the biological membranes. Some apparently hydrophilic antioxidants such as hypoxoside (Laporta et al., 2007), have shown to be effective antioxidants in lipophilic environments and exerted strong effects in biological membranes (Laporta, Funes, Garzón, Villalaín, & Micol, 2007). When the capacity to inhibit lipid peroxidation was measured by MDA inhibition assay, verbascoside was much stronger than hydroxytyrosol or caffeic acid, and as potent as quercetin (Table 2). This result reveals that even though verbascoside is a water-soluble compound, it seems to be quite efficient to avoid lipid peroxidation, probably establishing some kind of molecular interactions with the lipid membrane surface, as it has been proposed for other hydrophilic antioxidants (Laporta et al., 2007).

The antioxidant capacity of the lemon verbena extract was also determined by using the well-known ORAC-FL assay (Ou et al., 2001), which accounts for the scavenging of peroxyl radicals. The antioxidant dose–response behaviour of this extract was determined through the measurement of the area under the fluorescence decay curve (AUC) of the sample as compared to that in the absence of antioxidant. Lemon verbena extract showed an $ORAC_{FL}$ value of 4075 ± 234 TE/g dw, which is a significant antioxidant value compared to other antioxidant powdered extracts measured in the same conditions (Ou et al., 2001) and similar to the values previously reported for green tea extract (70% catechins) and olive leaf extract (25% oleuropein), and performed in identical conditions (Laporta et al., 2007).

Since verbascoside was the major compound present in lemon verbena extract, we might assume that the *in vitro* antioxidant capacity of the extract shown in the previous assays was mainly due to this compound, although synergistic effects of verbascoside with the rest of minor compounds (other phenylpropanoids and flavones) cannot be discarded.

3.3. Pharmacokinetic study of verbascoside from lemon verbena in rats

A pharmacokinetic study after oral administration of lemon verbena extract was performed in order to determine the presence of the compounds from the extract in the plasma of rats. Verbascoside was detected in rat plasma using LC–MS/MS with electrospray ionisation. Fig. 3 shows the HPLC-DAD-MS profile of a rat plasma sample 20 min after the oral administration of lemon verbena extract and the product ion scan spectrum using electrospray negative-ion mode. Retention time of verbascoside in plasma samples was 17.3 min. The mass spectrum revealed a base peak at *m*/*z* 623 corresponding to $[M-H]^-$, which was corroborated with the use of a commercially available verbascoside standard (Fig. 3B). Moreover, a fragment ion at *m*/*z* 461 ($[M-H-163]^-$) was consistent with the loss of the rhamnosyl moiety.

The pharmacokinetic study was performed using five individuals for each of five times data collection plus controls (Table 3). The pharmacokinetic parameters revealed an AUC of 3085 ± 1400 ng min/mL for an oral administration of total extract corresponding to a verbascoside dose of 545 mg/kg. The maximum verbascoside concentration found in plasma after oral administration was reached within the first 20 min, meaning that this compound was quickly absorbed from the gastrointestinal tract. In this study, the maximum verbascoside concentration found in plasma samples was 1431 ± 300 ng/mL (around 2.3 μM), which means 15 times more than that one previously reported by another study which used a different plant source containing verbascoside (Wu et al., 2006). Nevertheless, it can be concluded that oral bioavailability of verbascoside in rats is still rather low. However, the presence of other metabolites deriving from verbascoside or flavones and still non-determined in plasma could contribute to the *in vivo* biological activity of the lemon verbena extract.

Absorption and pharmacokinetics of verbascoside have been proposed to be similar to other phenolic compounds such as rosmarinic acid or some tea catechins (Wu et al., 2006). The plasma concentration of these and other polyphenolic compounds such as phenolic acids, flavonoids, stilbenes and lignans in plasma, as determined in different mice and human studies have been estimated to be in the low micromolar range (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004; Manach, Williamson, Morand, Scalbert, & Remesy, 2005; Rice-Evans, Miller, & Paganga, 1997; Yang, Sang, Lambert, & Lee, 2008). Nevertheless, a large body of evidences has demonstrated the biological activity of several phenylpropanoids at the low micromolar range (Korkina, 2007). The antiinflammatory activity of several phenylpropanoids has been proven in several cellular models at low micromolar values (Diaz et al., 2004; Matsuda, Morikawa, Managi, & Yoshikawa, 2003; Matsuda, Pongpiriyadacha, Morikawa, Ochi, & Yoshikawa, 2003). In addition, several phenylpropanoids, verbascoside among them, have shown strong antioxidant capacity at low micromolar concentrations in microsomal and LDL lipid peroxidation assays (Cos et al., 2002; Wong et al., 2001). Moreover the antitumour activity of phenylpropanoids has been shown in the micromolar range in cultured cells (Chen et al., 2005; Inoue, Sakuma, Ogihara, & Saracoglu, 1998; Zhang et al., 2002) and some phenylpropanoids have been able to suppress the growth and metastasis of tumour xenografts in nude mice *in vivo* (Chung et al., 2004). Moreover, the *in vivo* hepatoprotective effect of verbascoside against CCl_4 in mice and the antioxidant effects in rats were achieved at relatively low dosages, i.e., 30 and 3 mg/kg, respectively (Lee et al., 2004). Therefore it seems reasonable to postulate that low verbascoside concentrations, i.e., within the low micromolar range, maintained for a long term in plasma might be able to exert some effects at their cellular targets.

3.4. Plasma antioxidant capacity in rats after lemon verbena oral administration

The antioxidant activity of verbascoside has been previously documented in several *in vitro* and *ex vivo* systems (Liu et al., 2003; Seidel et al., 2000; Siciliano et al., 2005; Valentao et al., 2002; Wang et al., 1996; Wong et al., 2001). Therefore, in this work, the *ex vivo* antioxidant activity of plasma samples of rats previously fed with lemon verbena extract was determined. Several measurements were performed in the same samples in which the pharmacokinetic study was done: determination of the level

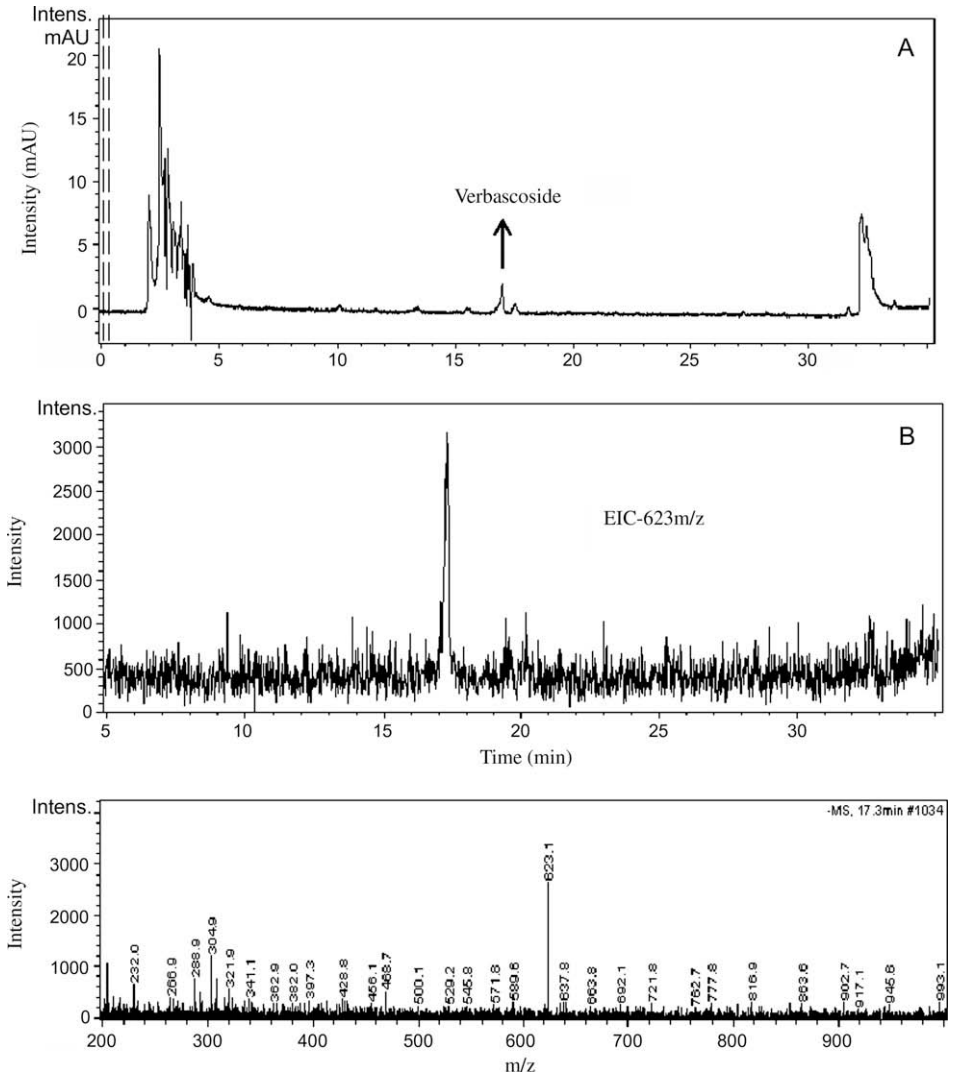

Fig. 3. HPLC-DAD-ESI-MS profile of rat plasma sample containing verbascoside collected 20 min after oral administration of lemon verbena (A) at 330 nm and (B) extracted-ion chromatogram (EIC) at 623 m/z and MS spectra of the peak corresponding to the same sample.

of plasma malondialdehyde (MDA) by HPLC, the ferric-reducing ability of plasma (FRAP value) and the superoxide dismutase activity (SOD).

Fig. 4A shows the level of MDA determined by HPLC with fluorescence detection in rat plasma samples corresponding to the different time data points of the pharmacokinetic study (bars). The figure also shows the concentration of verbascoside determined in the same samples as measured by HPLC-DAD-ESI-MS/MS. The figure shows a clear correlation between the maximum peak of verbascoside concentration at 20 min (1431 ± 300 ng/mL) and the minimum MDA value. The differences of MDA values determined at 20 and 40 min were statistically significant compared to the control.

Table 3
Pharmacokinetic data of verbascoside after oral administration of lemon verbena extract in rats.

Parameter	545 mg/kg, p.o.
T_{max} (min)	20
C_{max} (ng/mL)	1431 ± 300
AUC_{0-100} (ng min/mL)	3085 ± 1400
K_{el} (ng min/mL)	0.016 ± 0.005

T_{max} and C_{max} were obtained from experimental observation. The AUC_{0-t} was calculated using trapezoidal rule to the last point, and elimination rate constant (K_{el}) was calculated by the residual method.

To further characterise the antioxidant activity of the plasma samples of rats subjected to a single oral dose of lemon verbena extract, the ferric-reducing ability of plasma, FRAP, was determined *in vitro* (Fig. 4B). In this case, maximum antioxidant activity was observed for samples corresponding to 20 and 40 min extractions, with a significance of $p < 0.01$ compared to control. In addition, the capability of rat plasma samples to scavenge superoxide radicals was determined by using the xanthine/xanthine oxidase system coupled to superoxide dismutase (Fig. 4C). The results showed that the maximum capacity to inhibit the formation of the superoxide anion radical, i.e., SOD activity, also correlated to the rat plasma sample obtained at 20 min after the oral ingestion of lemon verbena extract, at which it was also observed the highest verbascoside concentration by LC–MS/MS.

Therefore, all these results obtained using three different complementary antioxidant measurements lead to the assumption that the antioxidant activity shown in the plasma samples of rats subjected to oral ingestion of lemon verbena extract must be mainly due to the presence of verbascoside or its derivatives, which may also exert other biological effects after reaching the appropriate

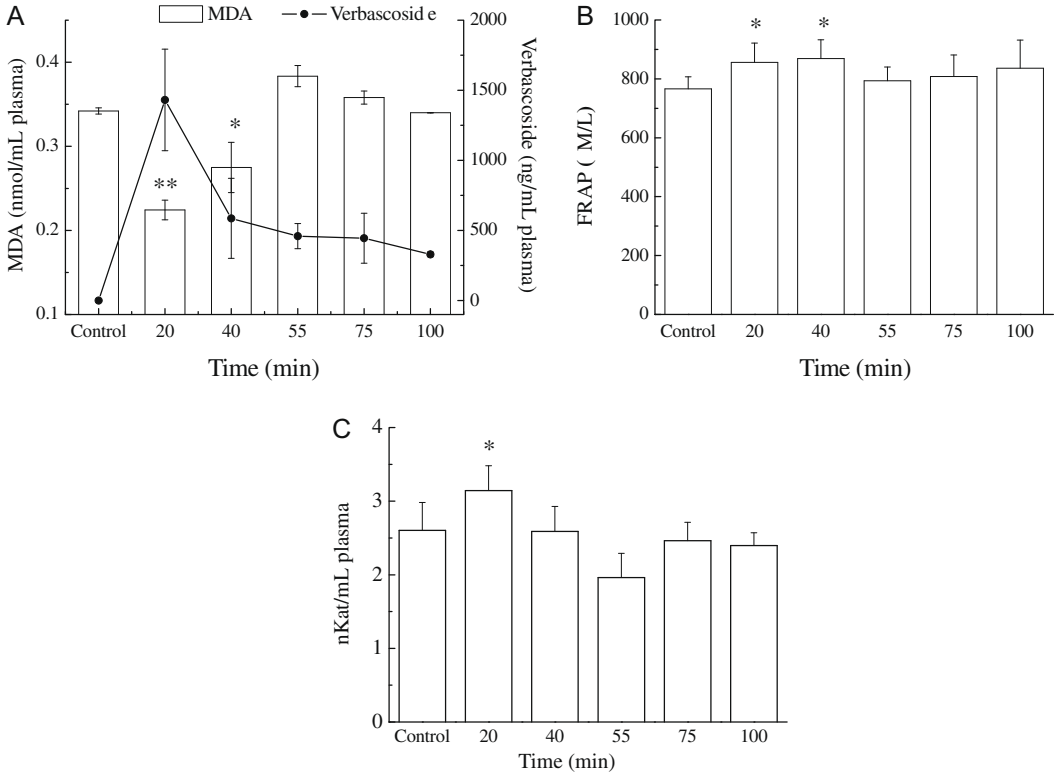

Fig. 4. (A) Inverse correlation between MDA values determined by HPLC-fluorescence (bars) and plasmatic levels of verbascoside determined by HPLC-DAD-ESI-MS/MS (filled circles) in the same rat plasma samples. Each bar represents the mean ± SD ($n = 8$); ** ($p < 0.05$) and * ($p < 0.1$) indicate statistically significant differences compared to control. (B) Ferric-reducing capacity of plasma (FRAP) in rats at 20, 40, 55, 75 and 100 min after being orally treated with lemon verbena extract. Each bar represents the mean ± SD ($n = 12$); * ($p < 0.01$) indicates statistically significant differences compared to control. (C) Superoxide dismutase activity in plasma of rats at the same time intervals than those in B after the oral ingestion of lemon verbena extract. Each bar represents the mean ± SD ($n = 8$); * ($p < 0.05$) indicates statistically significant differences compared to control.

cellular targets. As deduced from the *in vitro* antioxidant measurements, verbascoside might be also able to reach targets on lipophilic environments. It could also be postulated that the presence of verbascoside, or other related but non-detected metabolites, could increase the activity of antioxidant enzymes such as catalase, superoxide dismutase or glutathione peroxidase at post-translational level. Moreover, any change at the level of gene expression regulation must be discarded due to the short time of the observed effect. However, further research is needed in order to find any possible cellular metabolites from phenylpropanoids and to determine the presence of the metabolites deriving from lemon verbena in humans.

3.5. Oral Acute Toxicity of lemon verbena extract

Although lemon verbena is an edible plant and is commonly used to prepare infusions and spices, the oral acute toxicity of an extract containing a high content of verbascoside was performed in mice in order to establish the approximate oral LD_{50}. A single dose of 2000 mg/kg b.wt. of lemon verbena extract (25% verbascoside) was utilised and mice were observed through a 2-week period. No deaths occurred in either the control or the lemon verbena groups during treatment. No significant alterations in the body weight of the lemon verbena-treated groups (male and female) compared with their respective controls were observed (not shown).

Once the assay was completed, two individuals (one from each sex) treated with the extract were sacrificed and compared with their respective controls through postmortem and pathological examinations. Observation comprised examination of the external surface of the body (skin), all orifices, mucous membranes and the cranial, thoracic and abdominal cavities and their contents. The postmortem analysis of either male or female individuals subjected to treatment did not show abnormalities on vital organs such as brain, heart, lungs, liver, spleen, kidneys or intestines. Digestive system (stomach, duodenum, ileum, etc.) was also completely normal compared to controls. In addition, no particular gender-related effects were observed since no toxicity was noticed in male or female groups. A complete absence of toxicity at a concentration as high as 2000 mg/kg b.wt. was observed meaning that the substance may be classified either as a very low toxicity substance, i.e., GHS Category 5 (Globally Harmonized System) or unclassified. Considering that the lemon verbena extract used contained 25% verbascoside, an LD_{50} value for the pure compound, verbascoside, of $\geqslant$500 mg/kg b.wt. might be extrapolated.

4. Conclusions

To conclude, lemon verbena extract containing 25% verbascoside showed strong antioxidant capacity, especially in a lipophilic environment, which was higher than expected as concluded from the antioxidant capacity of pure verbascoside, probably due to synergistic effects. The capacity of verbascoside to act as an effective radical scavenger in lipophilic environments was also shown.

Moreover, verbascoside was found as the only metabolite identified by HPLC-ESI-MS/MS in the plasma samples deriving from rats fed with the lemon verbena extract, obtaining a C_{max} at 20 min of 2.3 μM. Verbascoside peak correlated to maximum plasma antioxidant activity as measured by malondialdehyde (MDA)

determination, ferric-reducing ability of plasma (FRAP value) and superoxide dismutase (SOD) measurements. In addition, no signs of toxicity were observed in mice at a verbascoside dosage of 500 mg/kg. Therefore, lemon verbena extract may suppose a non-toxic source of verbascoside.

Verbascoside or acteoside is present in many vegetable materials and by-products. For instance, recent work has found verbascoside to be the most potent antioxidant compound in olive mill waste extract (Obied, Prenzler, & Robards, 2008), then verbascoside-enriched extracts, such as lemon verbena extract, might have interesting applications in cosmetic, nutraceuticals or functional food.

Acknowledgements

This investigation has been supported by Grants AGL2007-60778, AGL2007-62806 and AGL2008-05108-C03-03, and FPI fellowship to L. Funes from MEC. We also thank MONTELOEDER, S.L. for providing us with the lemon verbena extract.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2009.04.059.

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Food Chemistry 117 (2009) 599–607

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Germination as a process to increase the polyphenol content and antioxidant activity of lupin seeds (*Lupinus angustifolius* L.)

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ARTICLE INFO

Article history:
Received 13 January 2009
Received in revised form 14 April 2009
Accepted 15 April 2009

Keywords:
Lupin seed
Germination
Polyphenols
Flavonoids
Antioxidant activity

ABSTRACT

In this work, the effect of germination of lupin seeds (*Lupinus angustifolius* L., c.v. Zapatón) on bioactive phenolic compounds as well as on the antioxidant activity was studied. Phenolic compounds were analysed by HPLC-PAD-ESI/MS. The antioxidant activity was determined by spectrophotometry, evaluating the free radical scavenging activity of the samples. Germination produced significant changes in flavonoids and non-flavonoid phenolic compounds. In the analysed samples, isoflavones, flavones and dihydroflavonols in free and conjugated forms were identified. The results obtained indicate that germination modifies the quantitative and qualitative polyphenolic composition of lupin (*Lupinus angustifolius* L.) seeds during the different days of the process, with a significant increase of flavonoids. An increase in the antioxidant activity was also observed as a consequence of the process. Germination was shown to be a good process to increase the phenolic content of lupin seeds as well as their antioxidant activity.

1. Introduction

Germination of seed legumes is conducted extensively in many countries in which legume sprouts have great appeal to consumers. During germination, some of the seed reserve materials are degraded and used for respiration and synthesis of new cell constituents of the developing embryo, thus causing significant changes in the biochemical characteristics. Germination of legume seeds to produce sprouts is one of the processing methods with the potential to increase nutritive value and health qualities of the seeds (Urbano et al., 2005; Zanabria, Katarzyna, De Jong, Birgit, & Robert, 2006). This process also raises the levels of free amino acids, available carbohydrates, dietary fibre and other components and augments also the functionality of the seeds due to the subsequent increase in the bioactive compounds (Frías, Miranda-Zárate, & Vidal-Valverde, 2005; Fernández-Orozco et al., 2006; López-Amorós, Hernández, & Estrella, 2006).

It has been reported that germination processes caused a significant increase in the antioxidant capacity of lupin seeds due to the enhancement of vitamin C, E and tocopherols' content (Fernández-Orozco et al., 2006; Frías et al., 2005). In germinated pea and bean flours, the changes of phenolic compounds seem to be associated with an increase in the free radical scavenging capacity (López-Amorós et al., 2006).

Lupin (*Lupinus*) is a legume valued primarily for its high protein content, similar to that of soybean, and for its full range of essential amino acids, and important dietary minerals. In contrast to soy, lupin can be grown in more temperate to cool climates and it can be considered as the strongest potential competitor of soybean (*Glycine max*). In addition the sweet lupin varieties *Lupinus albus* and *Lupinus angustifolius* present low content in fats, and high content of dietary fibre (Górecka, Lampart-Szczapa, Janitz, & Sokolowska, 2000) and are more palatable (Vilariño, Mareggiani, Grass, Leicach, & Ravetta, 2005). Germination, among other processes, has been widely used for its ability to decrease levels of antinutritional factors present in legume seeds, at the same time improving the concentration and bioavailability of their nutrients. Other studies have demonstrated that the germination of lupin seeds modifies the composition of the protein fraction, and minimises the presence of alkaloids, improving the nutritional quality of legume and obtaining more palatable seeds (Gulewicz et al., 2008; Sanchez et al., 2005).

One of the most abundant groups of secondary metabolites in legumes are phenolic compounds (Dueñas, Estrella, & Hernández, 2004; Dueñas, Fernández, Hernández, Estrella, & Muñoz, 2005; Dueñas, Hernández, & Estrella, 2002; López-Amorós et al. 2006; Amarowicz, Estrella, Hernández, & Toroszynska, 2008; Amarowicz & Pegg, 2008), which are considered to be natural antioxidants, representing an important group of bioactive compounds in foods, which may prevent the development of many diseases such as arteriosclerosis, cancer, etc.

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.04.051

The protection against disease of phenolics may be associated with the powerful antioxidant and free radical scavenging properties of these compounds (Dueñas, Hernández, & Estrella, 2006; Amarowicz & Pegg, 2008). This property affects the auto-immune system of the consumer organism (Nakagami, Nanaumi-Tamura, Toyomura, & Shgehisa, 1995). The content of phenolics depends not only on the species and variety but also on the degree of ripeness, processes, etc. (López-Amorós et al., 2006; Dueñas, Hernández, & Estrella, 2007a, 2007b). The different phenolic compounds found in lupin seeds can influence the taste and colour (Oomah, Tiger, Olson, & Balasubramanian 2006) and they can also act as fungicides, bacteriostats, etc.

Phenolics such as flavones and isoflavones are present in several parts of lupin, including the seeds (García-López, Kachlicki, Zamora-Natera, Ruiz-Moreno, & Stobiecki, 2006; Tsaliki, Lagouri, & Doxastakis, 1999), which are related to high antioxidant activity, although the presence of some amino acids and peptides can also contribute to this activity (Frías et al., 2005).

The greatest therapeutic effect of phenolics, such as antioxidant activity, depends on their structure, including the number of hydroxyl groups and their position. In general the flavonoid compounds present a stronger antioxidant activity than non-flavonoids (Jovanovic, Steenken, Simic, & Hara, 1998) and the combined forms, such as glycosides, have lower activity than free forms (Rice-Evans, Miller, & Paganga, 1996).

The aim of the present study was to identify the phenolic compounds in the extracts of raw and germinated lupin (*Lupinus angustifolius* L., c.v. Zapaton) and to know the effect of different germination periods on the phenolic composition. The antioxidant activity of raw and germinated seeds' flours was also evaluated in order to determine the optimal conditions of germination for attaining flours with higher functionality.

2. Materials and methods

2.1. Samples

Sweet lupin seeds (*Lupinus angustifolius* L., c.v. Zapatón) were provided by the Agrarian Research and Technology Development Service from the Agriculture and Commerce Council of the Junta de Extremadura (Spain). Seeds were cleaned and stored in polyethylene containers at 4 °C in a cool room until use.

2.2. Germination process

Two hundred grams of lupin seeds were treated for 30 min with 1000 ml of 0.07% sodium hypochlorite. Then, the seeds were washed with distilled water until the pH was neutral. Afterwards, seeds were soaked with 1000 ml of distilled water for 5 h and 30 min and shaken every 30 min. The hydrated seeds were placed on a germination tray, where a wet laboratory paper was extended and they were then covered with the same wet paper. The tray was introduced into the germination machine (G-120 Snijders International S.L., Holland) and the wet paper was in contact with the circulating water of the germinator; therefore the seeds were maintained wet by capillarity (Frías et al., 2005). The seeds were germinated in darkness at 20 °C for 2, 3, 4, 5, 6 and 9 days. The germination process was evaluated by percentage of germinated seeds and the sprouts were freeze-dried, ground and passed through a sieve of 0.5 mm. The flours obtained were stored in darkness, under vacuum conditions in desiccators at 4 °C until further analysis.

2.3. Preparation of samples and extraction of phenolic compounds

The lupin samples (10 g) were macerated with 3 × 80 ml of a solution of methanol-HCl ($1^0/_{00}$)/water (80:20 v/v) using an orbital shaker at room temperature, separating the supernatants by centrifuging. The three combined supernatants were taken to a fixed volume (240 ml) of the methanol solution, yielding a methanol extract whose total phenolic content was evaluated by Folin–Ciocalteu reaction (Singlenton & Rossi, 1965).

An aliquot of the methanolic solutions (100 ml) was extracted three times with 3 × 30 ml of diethyl ether and 3 × 30 ml of ethyl acetate and evaporated to dryness under vacuum at 30 °C. The residue was dissolved in methanol/water (1:1 v/v), and filtered through a 0.45 μm cellulose acetate filter (Millipore) and kept at 4 °C until HPLC analysis. The samples were prepared and extracted in duplicate.

2.4. HPLC–PAD and HPLC–MS analysis

The chromatographic system was comprised of an auto-injector, a quaternary pump, a photodiode-array detector 2001 and Millennium 32 chromatography manager software (Waters, Milford, Mass, USA). Separation of phenolic compounds was achieved on a reverse phase C_{18} column (Nova-Pak, 300 × 3.9 mm, particle size 4 μm). The mobile phase was comprised of two solvents, A: water/acetic acid (98:2, v/v) and B: water/acetonitrile/acetic acid (78:20:2, v/v/v). The gradient profile was 0–55 min, 100–20% A; 55–70 min, 20–10% A; 70–80 min, 10–5% A; 80–110 min, 100% B. The flow rate was 1 ml/min from the beginning to 55 min and 1.2 ml/min from this point to the end. The column was re-equilibrated between injections with 10 ml of acetonitrile and 25 ml of the initial mobile phase. Detection was performed by scanning from 210 to 400 nm with an acquisition speed of 1s. A volume of 25 μl was injected. The samples were analysed in duplicate.

Mass spectra were obtained using a Hewlett Packard 1100MS (Palo Alto, CA) chromatograph equipped with an API source, using an ESI interface. The solvent gradient and column used were the same as for HPLC–PAD but with a flow rate of 0.7 ml/min. ESI conditions were as follows: negative mode, nitrogen was used as the 40 psi nebulizing pressure drying gas, 10 l/min at 340° C; voltage at capillary entrance, 4000 V; and variable fragmentation voltage, 100 V (m/z < 200), 200 V (m/z 200–1000), 250 V (m/z 1000–2500). Mass spectra were recorded from m/z 100 to m/z 2500.

Chromatographic peaks were identified by comparison of the retention times, UV spectra and data of UV spectral parameters with those of standards and confirmed by HPLC–MS. The standards, protocatechuic, *p*-hydroxybenzoic, *trans p*-coumaric, *p*-hydroxyphenyl acetic and *trans*-ferulic acids, flavones apigenin 7-*O*-glucoside, luteolin 7-*O*-glucoside, diosmetin 7-*O*-rutinoside, diosmetin 7-*O*-neohesperidoside, isoflavones genistein and genistein 7-*O*-glucoside and the flavanone eriodictyol, were obtained from Extrasynthèse (France). Other compounds, for which no standards were available, were identified based on the study of data of UV spectral parameters and by HPLC–MS (ESI) (Dueñas et al., 2002, 2004).

Quantification was carried out using the external standard method with commercial standards. The calibration curves were made by injection of different volumes from the stock solutions over the range of concentration observed for each of the compounds, using a linear regression for the relationship of area sum versus concentration under the same conditions as for the samples analysed. The unknown non-flavonoid and flavonoid derivatives were quantified with the calibration curves of the most similar compounds.

2.5. Antioxidant activity

Antioxidant activity (EC_{50}) of the methanol extracts was determined by the Brand-William, Cuvelier, & Berset (1995) method with 2,2′diphenyl-1-picrylhydrazyl (DPPH) radical, adapted to the

legume samples by López-Amorós et al. (2006). The reaction was carried out with 2 ml of a methanol solution of DPPH$^{\cdot}$ (0.025 g/l) and solutions of different concentrations from the different samples. The absorbance was measured at 1 min intervals at 515 nm until the reaction reached a plateau (time at the steady state). The percentage of remaining DPPH against the sample concentration was plotted to obtain the amount of antioxidant (mg of legume flours) necessary to decrease DPPH by 50%. The antioxidant capacity was expressed as Trolox units. The samples were prepared and measured separately in duplicate.

2.6. Statistical treatment

Analyses were performed in duplicate and the data are presented as mean ± SD. Analysis of variance and comparison of treatment means (LSD, 5% level) and principal components were performed using Statgraphics Plus 5.0 (Statistical Graphics Software System, Rockville, MD, USA).

3. Results and discussion

Values of total phenols, in which a general increase was found, of raw and germinated seeds (expressed as mg of gallic acid) are shown in Table 1. An individualised knowledge of the different phenolic compounds is necessary to reach a more specific conclusion.

3.1. Identification of phenolic compounds in lupin flours

The UV spectra provided a complete overview of the main flavonoids in all samples studied. Thus, for structural elucidation of different phenolic compounds, the UV spectra were considered (Fig. 1) in accordance with the data from Markham (1982). Dihydroflavonols (Fig. 1A) presented a maximum at 289 nm and a shoulder at 310 nm; flavones showed a UV spectrum (Fig. 1B) with two maxima at 254 and 348 nm, the latter more pronounced. The UV spectrum of isoflavones (Fig. 1C) had a maximum at 260 and a shoulder at 320 nm. Most structural information was obtained from HPLC–MS analysis in negative mode.

Among the analysed sample extracts several phenolic compounds, non-flavonoids hydroxybenzoics, hydroxycinnamics and flavonoids, such as flavones, isoflavones and dihydroflavonols, were identified.

Figs. 2 and 3 show the chromatograms of the raw and germinated lupin at 9 days, respectively and Tables 2 and 3 present the wavelength of maximum UV absorption and the molecular ions of identified compounds from HPLC–MS, grouped in accordance with similarity in phenolic structure. Analysis of MS spectra recorded for each peak together with comparison of MS^2, UV spectra and retention times led to identification of some of the compounds from the chromatographic conditions.

3.1.1. Hydroxybenzoic and hydroxycinnamic compounds

Peaks 1, 2, 3, 4, 5, 8, 9 and 11 presented UV spectra which correspond to hydroxybenzoic acids and aldehydes. Some of them, peaks 2, 3, 8, 9 and 11 were identified by comparison of retention times and UV spectra with those of corresponding standards (Table 2).

Peak 1 showed a UV spectrum similar to that of protocatechuic acid and it presented an $[M-H]^-$ at *m/z* 315 (Table 2) corresponding to protocatechuic acid linked to a hexose and one fragment ion $[M-H]^-$ at *m/z* 153.1 from protocatechuic acid. This compound was identified as protocatechuic acid glycoside, an infrequent form found in legumes. This compound was described in pea seeds by the same authors (Dueñas et al. 2004). Protocatechuic acid in esterified form was also identified in cowpeas by Cai, Hettiarachchy, and Jalaluddin (2003).

Peak 4 and 5 presented UV spectra similar to hydroxybenzoic acid, but with different retention times, therefore these compounds were identified as hydroxybenzoic derivatives, since data of their MS spectra could not be obtained.

The UV spectra presented by peaks 7, 10, 14, 16, 21 and 26 were characteristic of hydroxycinnamic compounds and they are, therefore, considered as derivatives of these compounds. Peaks 16 and 21 were identified by comparison of retention times and UV spectra with those of standards (Table 2).

The UV spectrum of peak 7 was similar to that of *trans p*-coumaric acid. In the HPLC–MS (ESI) analysis it presented an $[M-H]^-$ at *m/z* 295.0 which corresponds to *p*-coumaric acid linked to a glutaric acid and a fragment $[M-H]^-$ at *m/z* 163 from *p*-coumaric acid. This compound was identified as *trans p*-coumaroyl-glutaric acid and is reported for the first time in lupin. It was also reported in cowpea seeds by Cai et al. (2003) and Dueñas et al. (2005).

Peak 10 showed a UV spectrum similar to that of ferulic acid, with an $[M-H]^-$ at *m/z* 355 (Table 2) from ferulic acid linked to a hexose and one fragment ion: $[M-H]^-$ at *m/z* 193.1 from ferulic acid. It was identified as a ferulic acid glycoside. Peak 14 was identified by the UV spectrum as a ferulic acid derivative; it presented a molecular ion $[M-H]^-$ at *m/z* 308 from the ferulic acid linked to malic acid. This peak corresponding to a feruloyl malic acid was also identified in the pea seed coat (Dueñas et al., 2004). Peak 26 showed a molecular ion $[M-H]^-$ at *m/z* 178, corresponding to coniferylaldehyde.

3.1.2. Dihydroflavonols

Peaks 6, 15, 23 and 43 presented UV spectra corresponding to dihydroflavonols (Fig. 1A). Peaks 6 and 43 had a fragment ion $[M-H]^-$ at *m/z* 303 from dihydroquercetin and were identified as dihydroquercetin derivatives.

Peak 15 had a molecular ion $[M-H]^-$ at *m/z* 507 which is associated with dihydroquercetin linked to an acetyl glucoside, and a fragment ion $[M-H]^-$ at *m/z* 303 from dihydroquercetin. It was identified as dihydroquercetin acetyl glucoside. Peak 23 generated a molecular ion $[M-H]^-$ at *m/z* 491 corresponding to dihydrokaempferol acetyl glucose.

3.1.3. Flavones

The UV spectra of peaks 17, 18, 28, 29, 32, 33, 34, 39, 41, 42, 44, 47, 48, 49, 50, 51, 52 and 53 corresponded to those of flavones (Fig. 1B).

Peaks 17 and 39 were identified as apigenin apioglucoside and apigenin neohesperidoside, respectively, by comparison of retention times and UV spectra with those of standards (Table 2). Some others, 18, 28, 29 and 50 were identified as apigenin derivatives due to the fragment of molecular ion $[M-H]^-$ at *m/z* 269, corresponding to apigenin.

Table 1
Total phenols* (mg/g dry matter) of raw lupin and lupin sprouts.

Raw	2 days	3 days	4 days	5 days	6 days	9 days
8.56 ± 0.85	10.60 ± 1.02	13.67 ± 1.01	15.46 ± 1.25	11.22 ± 0.97	16.61 ± 1.13	24.37 ± 1.31

* Expressed as gallic acid.

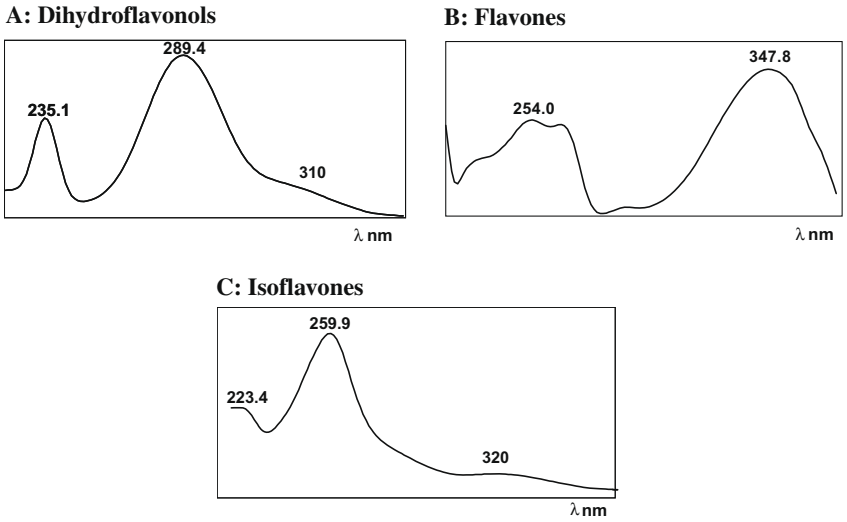

Fig. 1. UV spectra of dihydroflavonols, flavones and isoflavones.

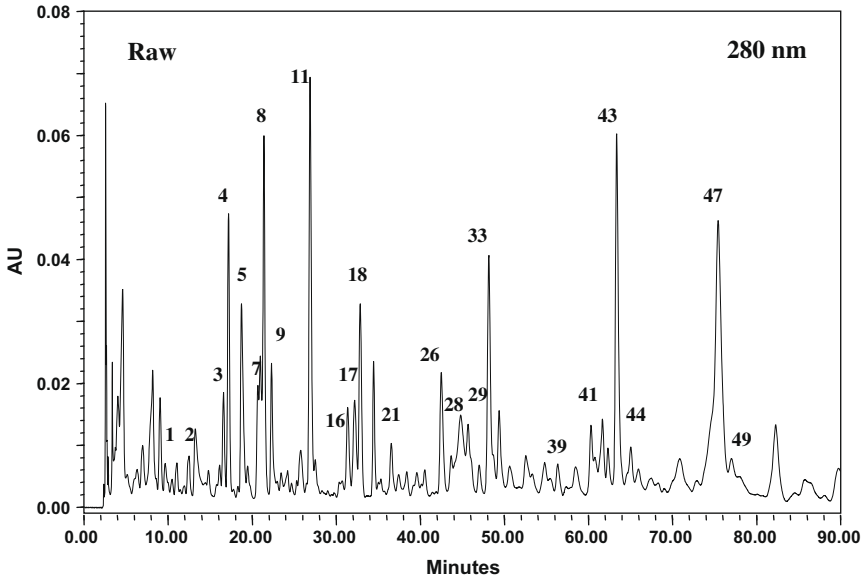

Fig. 2. HPLC chromatogram of raw lupin.

Peaks 33, 34, 41, 48 and 49 presented a fragment ion $[M-H]^-$ at *m/z* 285 which corresponded to luteolin, therefore they were designated luteolin derivatives. Peak 32 was identified as luteolin 7-glucoside by comparison with the standard.

Peak 42 showed a λ_{max} at 347.0 characteristic of luteolin derivatives. This compound presented hypsochromic shifts of their absorption spectra with regard to the luteolin glucoside due to the acetyl moiety. It showed a negative molecular ion $[M-H]^-$ at *m/z* 489, which corresponds to the luteolin glycoside acetylate and two fragments ions $[M-H]^-$ at *m/z* 456 ($[M-42]^-$, loss of acetyl residue) and at *m/z* 285 ($[M-204]^-$, loss of acetyl glycoside residue). It was identified as luteolin glucopyranosyl acetate.

Peaks 44 and 47 were identified as diosmetin 7-rutinoside and diosmetin 7-neohesperidoside, respectively, by comparison with the standards. Peak 52 presented a UV spectrum (252–346 nm) of diosmetin. In the analysis MS showed a negative molecular ion $[M-H]^-$ at *m/z* 635 and two fragments ions $[M-H]^-$ at *m/z* 461 ($[M-174]^-$, (loss of acetyl pentose residue) and at *m/z* 299 $[M-336]^-$, corresponding to diosmetin aglycone. It was identified as diosmetin apiofuranosyl acetyl glycoside. Peaks 51 and 53 presented an $[M-H]^-$ at *m/z* 299 from diosmetin and they were identified as diosmetin derivatives.

Peak 35 presented a UV spectrum (λ_{max} at 285 nm) corresponding to the flavanone eriodictyol with a fragment ion at *m/z* 287 from free eriodictyol; it was identified as an eriodictyol derivative.

3.1.4. Isoflavones

Peaks 12, 13, 19, 20, 22, 24, 25, 27, 30, 31, 36, 37, 38, 40, 45, 46 and 54 were considered isoflavones taking into account their UV characteristics (Fig. 1C).

The following compounds, genistein 7-glucoside (peak 30) and genistein (peak 54) were identified by comparison of retention times and UV spectra to those of standards and confirmed by HPLC–MS (ESI) analysis (Table 3).

Peaks 12, 24, 27, 31 and 46 showed a λ_{max} at 259.0 nm characteristic of genistein; they were considered genistein derivatives based on the fragment ion $[M-H]^-$ at *m/z* 269 in the analysis by HPLC–MS. Peak 22 together with the fragment ion from genistein

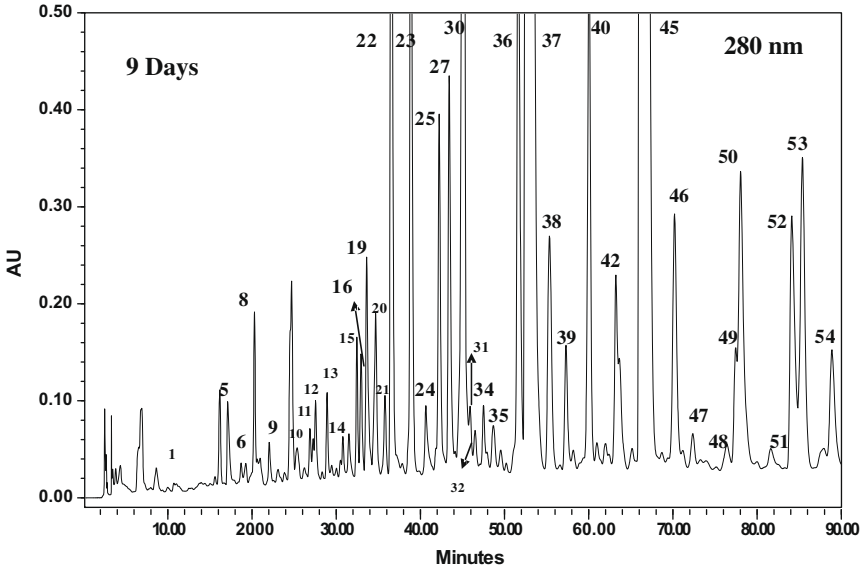

Fig. 3. HPLC chromatogram of 9 day lupin sprouts.

Table 2
Wavelength of maximum UV absorption and molecular ions of identified compounds in raw lupin and nine days lupin sprouts.

Peak		Raw	9 days	λ (nm)	[M−H] (*m/z*)	MS2 (*m/z*)
Hydroxybenzoic and hydroxycinnamic compounds						
1	Protocatechuic acid glucoside	x	x	257, 297	315	153
2	Protocatechuic acid	x		297	153	
3	*p*-Hydroxybenzoic acid	x		255	137	
4	Hydroxybenzoic acid derivative	x				
5	Hydroxybenzoic acid derivative	x	x			
8	*p*-Hydroxybenzoic aldehyde	x		286	121	
9	*p*-Hydroxyphenyl acetic acid	x	x		151	
11	*p*-Vanillin	x	x	277, 308	151	
7	*trans p*-Coumaroyl glutaric acid	x		322	295	163
16	*trans p*-Coumaric acid		x		163	
10	*trans* Ferulic acid glycoside		x	323	355	193
14	*trans* Feruloyl malic acid		x	322	308	193
21	*trans* Ferulic acid	x	x	323	193	
26	Coniferaldehyde	x			178	
Dihydroflavonols and flavones						
6	Dihydroquercetin derivative		x	286		303
43	Dihydroquercetin derivative	x		286		303
15	Dihydroquercetin acetyl glycoside		x	286, 320sh	507	303
23	Dihydrokaempferol acetylglycoside	x	x	284, 383	491	
17	Apigenin 7-apioglucoside	x		268, 337	563	269
18	Apigenin derivative	x		269, 337		269
28	Apigenin derivative	x		260, 340–360		269
29	Apigenin derivative	x		260, 340–360		269
50	Apigenin derivative		x	260–337		269
39	Apigenin 7-neohesperidoside	x	x	267–337	577	268
32	Luteolin 7-glucoside		x	254–349	447	285
33	Luteolin derivative	x		254–345		285
34	Luteolin derivative		x	260–344		285
41	Luteolin derivative	x		262–343	621	284.9
48	Luteolin derivative		x	260–343	488	285
49	Luteolin derivative	x	x	262–343		285
42	Luteolin glucopyranosyl acetate		x	259–347	489	285–456
44	Diosmetin rutinoside	x	x	252. 8–346.6	607	299
47	Diosmetin neohesperidosido	x	x	252.8–346.6	607	299
51	Diosmetin derivative		x	255–350		299
53	Diosmetin derivative		x	255–350		299
52	Diosmetin apiofuransyl acetylglucoside		x	253–347	635	299–461
35	Eriodictyol derivative		x	285		287

x: Presence.

presented a molecular ion $[M-H]^-$ at *m/z* 431 from the genistein linked to a hexose and it was identified as a genistein glycoside.

Peak 13 had a UV spectrum of genistein derivative and a molecular ion $[M-H]^-$ at *m/z* 725 from genistein apiofuranosyl diglycoside with two fragment ions $[M-H]^-$ at *m/z* 593 from genistein diglucoside and $[M-H]^-$ at *m/z* 431 from genistein glucoside.

Peak 38 showed a UV spectrum characteristic of genistein derivatives. In the analysis by HPLC–MS it showed a negative

Table 3
Wavelength of maximum UV absorption and molecular ions of isoflavones identified in raw lupin and nine days lupin sprouts.

Peak		Raw	9 days	λ (nm)	[M-H] (*m/z*)	MS^2 (*m/z*)
12	Genistein derivative		x	259		269
24	Genistein derivative	x	x	259		269
27	Genistein derivative		x	259		269
31	Genistein derivative		x	259		269
46	Genistein derivative		x	260		269
22	Genistein glucoside	x	x	260	431	269
13	Genistein apiofuranosyl diglycoside		x	259	725	431–593
38	Genistein apiofuranosyl glycoside		x	262–288	563	431–269
25	Genistein acetylglycoside	x	x	253	473	431–269
36	Genistein acetylglycoside		x	263–328	473	431–269
45	Genistein acetylglycoside		x	327	473	269
37	Genistein acetyldiglycoside		x	262–332	575	473–431
40	Genistein acetyldiglycoside		x	324	575	473–431
30	Genistein glucoside (genistin)		x	259.9–327	431	269
19	Hidroxygenistein -7 glucoside		x	260–292	447	285
20	Hidroxygenistein -7 acetylglucoside		x	262–288–327	489	447–284
54	Genistein		x	262	269	

x: Presence.

molecular ion $[M-H]^-$ at *m/z* 563, which corresponded to the genistein linked to pentose and hexose and two fragment ions $[M-H]^-$ at *m/z* 431 ($[M-132]^-$, loss of a pentose moiety) and at *m/z* 269 ($[M-294]^-$, loss of pentose-hexose moiety). It was identified as genistein diglycoside.

Peak 25, 36 and 45 showed a λ_{max} at 259.0 nm characteristic of genistein derivatives. These compounds showed a negative molecular ion $[M-H]^-$ at *m/z* 473, which corresponded to the genistein glycoside acetylate and two fragments ions $[M-H]^-$ at *m/z* 431 ($[M-42]^-$, loss of acetyl residue) and at *m/z* 269 ($[M-204]^-$, loss of acetyl glycoside residue). They were identified as genistein acetyl glycosides in different positions.

Peaks 37 and 40 showed a negative molecular ion $[M-H]^-$ at *m/z* 575, which corresponded to the genistein linked to acetyl and pentose and hexose and two fragments ions $[M-H]^-$ at *m/z* 431 ($[M-132]^-$, loss of pentose residue) and at *m/z* 269 ($[M-204]^-$) corresponding to genistein. These compounds were identified as genistein acetyl diglycosides.

Peaks 19 and 20 corresponded to isoflavones, and they presented a fragment ion at *m/z* 285 assigned to hydroxygenistein (Bednarek et al., 2003). These compounds were identified as hydroxygenistein 7-*O*-glucoside and hydroxygenistein 7-*O*-acetyl glucoside, respectively.

3.2. Changes occurring during the germination process

From the results obtained in the evaluation of total phenols (Table 1) it can be deduced that germination caused an increase of total phenols (expressed as mg of gallic acid) in sprouts of *Lupinus angostifolius* var. Zapatón. An increase was observed at four days and a more pronounced one at nine days, where a rise of up to 63% was found. These results agree with those reported by Fernández-Orozco et al. (2006) who also found an increase of 53% of total phenols (expressed as mg of catechin) in lupin sprouts after nine days. A rise in total phenols of chickpea was also reported after five days of germination (Khattak, Zeb, Bibi, Khalil, & Khattak, 2007).

Moreover, greater qualitative and quantitative differences in the identified phenolic compounds were observed between the raw and germinated lupin seeds (Tables 2 and 3). Due to the high number of identified phenolic compounds in the samples, Table 4 shows the total concentration of the compounds grouped in different families (hydroxybenzoics, hydroxycinnamics, flavones, dihydroflavonols and isoflavones) in order to achieve a better clarification of the results.

The hydroxybenzoic compounds represented 18% of the identified phenolics in raw lupin seeds. In general, there was a loss of these compounds, corresponding to a reduction of 65% at 9 days. Several variations were observed during the different days of germination with the maximum value at day 4 and the minimum being produced on day 6 (Table 4).

The hydroxycinnamic compounds in raw lupin seeds represented 1.2% of the identified phenolic compounds. During germination this group increased its content considerably, with two maxima at 4 and 9 days, augmenting by 91% at the end of the process (Table 4). This group included hydroxycinnamic compounds in free forms (*trans p*-coumaric and *trans*-ferulic acid) and those linked to other compounds (hydroxyacids and sugars) (Table 2). In general, *p*-coumaric and ferulic acids increased their contents, as well as other derivatives from these acids, some of them not being identified in raw lupin. Other authors also observed that with germination there was the novo synthesis of hydroxycinnamic acids such as *p*-coumaric and ferulic acids (Diaz-Batalla, Widholm, Fahey, Castaño-Tostado, & Paredes-López, 2006; López-Amorós et al., 2006).

The flavones group presented the highest percentage in all samples (Table 4), in raw (76%) and germinated lupin seeds (68–84%). During germination, variations in the behaviour of this group were observed with minimum values at 2, 4 and 5 days. Two increases were observed, one at 3 days and from day 6 until day 9 of germination where an increase of 86% with respect to raw seeds was observed (Table 4). The flavones mainly identified were glycosides of luteolin, apigenin and diosmetin (Table 2). Germination for 9 days gave rise to an increase in the number of identified compounds from luteolin and diosmetin derivatives, in contrast to apigenin derivatives. This could be attributed to the biochemical changes of seeds during germination, which might produce some secondary plant metabolites, such as flavonoids or release aglycones from conjugated glycosides due to enzymatic activation (Ribeiro et al., 2006).

The flavanone identified as an eriodictyol derivative was only found in germinated lupin seeds (Table 2). The dihydroflavonols represented the lowest percentage (0.4%) in raw lupin seeds and, in the germinated samples at different times, ranged from 0.6–1.5%, which means an increase during the time of germination. It was at 9 days when lupin showed the highest rise (93%) (Table 4).

The content of isoflavones in the raw lupin seeds represented 4% of identified phenolics (Table 4). The isoflavones significantly increased during the germination from 2 days, with a high value at 3 days, but it was at 9 days when lupin sprouts attained the highest increase (95%).

Table 4
Concentrations (μg/g) in raw and germinated lupin, of identified phenolic compounds, grouped in hydroxybenzoics, hydroxycinnamics, flavones, dihydroflavonols and isoflavones.

Compounds	Raw	2 days	3 days	4 days	5 days	6 days	9 days
Hydroxybenzoics	4.60 ± 0.51^{d}	1.90 ± 0.08^{b}	1.40 ± 0.05^{ab}	2.70 ± 0.11^{c}	1.30 ± 0.03^{ab}	0.80 ± 0.02^{a}	1.60 ± 0.05^{b}
Hydroxycinnamics	0.30 ± 0.01^{a}	0.80 ± 0.02^{b}	1.20 ± 0.03^{b}	4.20 ± 0.21^{e}	0.90 ± 0.02^{b}	2.20 ± 0.11^{c}	3.60 ± 0.09^{d}
Flavones	19.10 ± 1.95^{b}	10.20 ± 0.98^{a}	31.50 ± 1.12^{c}	13.20 ± 0.85^{ab}	12.40 ± 0.91^{ab}	42.60 ± 2.10^{d}	135.30 ± 5.21^{e}
Dihydroflavonols	0.10 ± 0.01^{a}	0.09 ± 0.02^{a}	0.40 ± 0.03^{c}	0.20 ± 0.05^{b}	0.30 ± 0.04^{b}	0.50 ± 0.04^{c}	1.60 ± 0.09^{d}
Isoflavones	0.90 ± 0. 03^{a}	2.10 ± 0.07^{ab}	8.60 ± 1.01^{c}	3.10 ± 0.21^{b}	2.50 ± 0.12^{ab}	9.30 ± 0.54^{c}	18.20 ± 0.91^{d}

Values are mean ± SD (n = 2); means followed by the superscript letters in a row are not significantly different (LSD, 5%).

The elevated number of isoflavones identified as genistein derivatives in the sprouts is remarkable in comparison to raw lupin (Table 3). Several authors have also observed in other legumes, such as soybean or beans that the isoflavones content increased, during the process of germination (Lin & Lai, 2006; Diaz-Batalla et al., 2006). After germination for 72 h an increase of isoflavones in the cotyledon of soybeans was also found which was accompanied by a rise of β-glucosidase activity (Ribeiro et al., 2006).

It is important to point out the presence of the aglycon genistein in the germinated lupin, considering its antioxidant capacity. It is important to note that the isoflavones, such as genistein, are known as phytoestrogens that have been related to a risk reduction of cardiovascular disease and cancer, particularly breast and prostate cancer (Mathers, 2002). This would make the germinated common lupin an alternative source of phytoestrogens.

Genistein aglycone showed a considerable increase (data not shown) after 9 days of germination. It is well known that during germination, enzymatic activity increases, such as that of glucosidases (Ribeiro et al., 2006); therefore, if glucosidase activity increased, it would increase the content of aglycones during the germination. At the beginning of germination not only aglycones are released from glucosides through the catalysis of activated glucosidase in seeds, but also isoflavones are produced through the biosynthesis in the malonate and phenylpropanoid pathways (Hahlbrock & Scheel, 1989). Genistein derivatives were the most prominent compounds found in lupin sprouts after 9 days.

During the germination of *Lupinus angustifolius* studied in this work, different behaviours of flavonoids and non-flavonoid phenolic compounds was observed. Flavonoids presented two maximum concentrations at 3 and 9 days of germination, showing the biggest increase at 9 days. Differences were observed in non-flavonoids compounds, such as hydroxycinnamics and hydroxybenzoics. These compounds presented a maximum at 4 days. Hydroxybenzoic compounds decreased at the end of the process.

Previous studies carried out by our research group in legumes after different processes, such as fermentation, addition of enzymes and germination reported similar behaviour to that found in this work, with increases and decreases being observed through the study period. This great variation of these compounds could be explained by the complex biochemical metabolism of seeds during treatment.

At the end of the germination (9 days) a considerable increase (84%), with respect to raw lupin, in the total concentration of identified phenolic compounds was observed. However, there were few differences between the groups, hydroxycinnamics (92%), flavones (86%), isoflavones (95%) and dihydroflavonols (94%).

3.3. Antioxidant activity of raw lupin and lupin sprouts

The increase of phenolic compounds with the germination process could have an influence on the free radical scavenging capacity. For this reason, the evaluation of antioxidant activity in the methanolic extracts of all samples was carried out to discover the changes in relation to the days of germination, in order to know the influence of germination on the antioxidant activity, taking into account the correlation of phenolic compounds with the free radical scavenging activity.

Table 5 shows the antioxidant activity values determined as mg Trolox/g sample. It was observed that at 3, 6 and 9 days of germination antioxidant activity increased considerably with respect to raw lupin (59%, 61%, and 63%, respectively) and a lesser increase was observed in samples of 4 and 5 days.

Comparing the content in total phenolics (Table 1) with the values of antioxidant activity (Table 5) for the different samples, we can observe no coincidence among them. A low correlation between the total phenolics and antioxidant activity was obtained (R^2 = 0.7195), which suggests that other compounds different than phenolics, which also react with the Folin–Ciocalteu reagent. However, an increase in total phenolics did not always bring about a corresponding increase in antioxidant activity as seen for example, from day 3 to day 4.

The improvement of antioxidant activity during the germination process has also been observed by other authors. Fernández-Orozco et al. (2006) observed that TEAC values changed slightly up to 5 days of germination but after 6 and 9 days significant increases (25% and 28%, respectively) were found in lupin seeds. Frías et al. (2005) also observed that germination of lupin (*Lupinus albus*) after 9 days produced an enhancement in the antioxidant activity that corresponded to an increase in content of vitamins E and C.

The variations in the antioxidant activity could be better explained in relation to the changes in the different identified phenolics. In both cases (antioxidant activity and levels of identified phenolics) there was an increase at 9 days of germination in the groups of the identified compounds, with the exception of hydroxybenzoic compounds.

López-Amorós et al. (2006) reported an increase in the antioxidant activity of beans and peas germinated for 6 days, but an opposite behaviour was observed for lentils. The changes observed in these legumes were related to the increase of phenolics in peas and beans as a consequence of germination and the decrease in the case of lentils. An increase of antioxidant activity after germination was also observed in soybean, azuki bean and mung bean (Lin & Lai, 2006), attributed to the biochemical metabolism of seeds during germination.

The antioxidant activity of phenolic compounds is related to their chemical structure, thus in order to relate antioxidant activity to the identified polyphenolic compounds in the lupin samples on the different days of germination an analysis of principal components was carried out. Principal component analysis was performed on the values of the antioxidant activity (Table 5) and the concentrations of the groups of identified compounds (Table 4).

Table 5
Antioxidant activity (mg trolox/g sample) of raw and germinated lupin.

Raw	2 days	3 days	4 days	5 days	6 days	9 days
7.1 ± 0.3	8.3 ± 0.2	17.5 ± 3	12.3 ± 0.2	11.4 ± 0.2	18.2 ± 3	19.1 ± 0.2

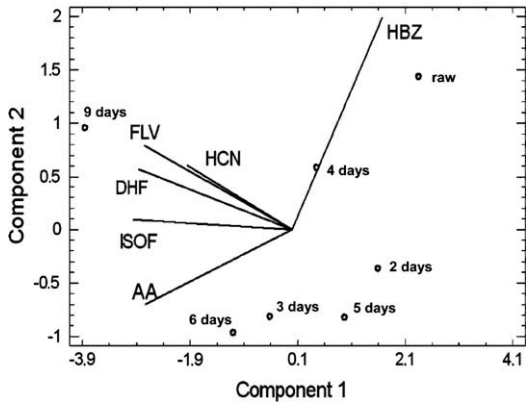

Fig. 4. Plot of the principal components of the polyphenolic compounds and antioxidant activity (Trolox) of raw and germinated lupin seeds during 2, 3, 4, 5, 6 and 9 days. HBZ, hydroxybenzoics; HCN, hydroxycinnamics; FLV, flavones; DHF, dihydroflavonols; ISOF, isoflavones; AA, antioxidant activity.

Six components were obtained of which the first two accounted for 86.51% of the total variance. Fig. 4 shows the graphic representation of the first two components. Four groups of compounds, hydroxycinnamics, isoflavones, flavones and dihydroflavonols were more positively related to the antioxidant activity and at the same time (9 days of germination), which means that these compounds produced an increase in this activity when compared to raw lupin. Hydroxybenzoics were the only phenolics that had a high relation with raw lupin and they are not related to antioxidant activity.

It is well known that non-flavonoid compounds present less antioxidant activity than flavonoids (Baderschneider & Winterhalter, 2001). However, hydroxycinnamic compounds show a relationship with the antioxidant activity; thus, in previous work carried out by our group with lentils and peas, these also showed a relationship between the hydroxycinnamic compounds and the antioxidant activity (Dueñas et al., 2006; López-Amorós et al., 2006).It has also been reported that compounds such as ferulic and *p*-coumaric acids are correlated with antioxidant activity in cooked soybeans (Kim et al., 2006). Isoflavones are the phenolic compounds present in lupin which most influence the antioxidant activity, especially considering the presence of the aglycone genistein in lupin seeds at the end of the germination process, with a more potent antioxidant activity than the conjugated forms (Arora, Nair, & Strasburg, 1998).

4. Conclusions

Germination caused significant changes in the phenolic composition of lupin, due mainly to endogenous enzymes' activation and the complex biochemical metabolism of seeds during this process. During germination non-flavonoids presented a maximum of concentration at four days. This differs from flavonoids, whose maximum appeared at three days of germination, which demonstrates different behaviours for the two types of compounds during this process. Germination during nine days gave rise, in general, to an increase in the content of the majority of phenolic compounds mainly flavonoids, such as flavones, isoflavones and dihydroflavonols with respect to raw lupin.

Therefore, germination may be a good process to obtain a functional food with a high antioxidant capacity.

Acknowledgements

This work was supported by the financed project ALGL2002–02905ALD, through the Spanish "Comisión Interministerial de Ciencia y Tecnología".

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Food Chemistry 117 (2009) 608–614

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Metal oxide semiconductor sensors for monitoring of oxidative status evolution and sensory analysis of virgin olive oils with different phenolic content

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ARTICLE INFO

Article history:
Received 8 January 2009
Received in revised form 19 February 2009
Accepted 15 April 2009

Keywords:
Electronic nose
Oxidative stability
Phenols
Sensory analysis
Storage
Virgin olive oil

ABSTRACT

An electronic nose based on an array of six metal oxide semiconductor sensors was used to monitor the oxidative status of virgin olive oils (VOO) during storage. VOO samples, with and without phenolic compounds, were stored at 60 °C for 7 weeks. Once a week, absorbance at 232 and 270 nm, oxidized stability index, electronic nose, and sensory analysis were evaluated. Linear discriminant analysis models were constructed in order to classify samples according to oxidative levels. Based on these models, VOO samples with and without phenolic compounds at different storage times, divided in eight categories, were correctly classified also achieving a good correlation for sensory analysis. The method is a fast and economical tool for on-line monitoring of VOO oxidation status.

1. Introduction

Among edible oils, virgin olive oil (VOO) is a basic component of the Mediterranean diet and has several characteristics that render it unique (Bendini et al., 2007a). The importance of VOO is mainly attributed to its high oxidative stability and its characteristic sensory and nutritional properties (Aparicio, Roda, Albi, & Gutiérrez, 1999; Bendini et al., 2007a; Cerretani, Salvador, Bendini, & Fregapane, 2008). The high oxidative stability of VOO with respect to other vegetable oils is mainly due to its fatty acid composition, and in particular to the high monounsaturated-to-polyunsaturated ratio and the presence of minor compounds, that play a major role in preventing oxidation. However, in spite of its high stability, VOO is also susceptible to suffer oxidative processes such as enzymatic oxidation (which occurs when the oil is in the fruit and during the technological extraction process), photo-oxidation (which occurs when the oil is exposed to light) and auto-oxidation (mainly produced during processing and storage when the oil is in contact with oxygen) (Bendini, Cerretani, Salvador, Fregapane, & Lercker, submitted for publication; Frankel, 1985). In these latter cases, the presence of antioxidant compounds can modify the oxidation process. A classification of the antioxidants based on their mechanism of action includes primary antioxidants, synergistic, and secondary antioxidants (Rajalakhmi & Narasimham, 1996). Phenolic compounds are one of the major groups in the polar fraction and act as primary antioxidants (AxH) to inhibit oxidation in VOO. These compounds act as chain breakers by donating a radical hydrogen to the alkylperoxyl radicals ($ROO^{\cdot}$) formed during the initiation step of lipid oxidation, and subsequently form a radical ($Ax^{\cdot}$) that is stabilized by resonance forms during the reaction (Carrasco-Pancorbo et al., 2005):

$$ROO^{\cdot} + AxH \rightarrow ROOH + Ax^{\cdot}$$

The polar extracts of VOO contain phenolic compounds that belong to different classes comprising phenolic acids, phenyl ethyl alcohols, hydroxy-isochromans, flavonoids, lignans, and secoiridoids. The secoiridoid family is characteristic of Oleaceae plants, being the main compounds of the phenolic fraction of VOO (Bendini et al., 2007a). The shelf-life of VOO is longer than other vegetable oils, mainly due to the presence of phenolic molecules possessing a catechol group, such as hydroxytyrosol and its secoiridoid derivatives. Three different assays (antiradical test, electrochemical method and a forced oxidation model system) have been used to establish the antioxidant activity of these phenolic compounds (Carrasco-Pancorbo et al., 2005).

VOO is also a unique, edible vegetable oil with characteristic sensory properties. The sensory attributes of VOO mainly depend on the content of minor components such as phenolics and volatile compounds. The individual components contribute to different sensory perceptions (Cerretani et al., 2008). Approximately 180

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doi:10.1016/j.foodchem.2009.04.052

compounds belonging to several chemical classes such as aldehydes, alcohols, esters, ketones, acids, ethers, hydrocarbons and terpens have been separated from the volatile fractions of VOO (Angerosa et al., 2004). The oxidation process leads to the production of several products of varying volatility. These compounds, mainly saturated and unsaturated aldehydes generated by autoxidation, are the major cause of the sensory perception of the rancid defect in vegetable oils (Vichi, Pizzale, Conte, Buxaderas, & López-Tamames, 2003). Concerning gustatory perceptions, it is well established that phenolic compounds are responsible for bitterness (Beltrán, Ruano, Jiménez, Uceda, & Aguilera, 2007; Cerretani et al., 2008; Esti, Contini, Moneta, & Sinesio, 2009; Gutierrez-González, Albi, Palma, Rios, & Olias, 1989; Gutierrez-Rosales, Rios, & Gomez-Rey, 2003) and pungency in VOO (Andrewes, Busch, De Joode, Groenewegen, & Alexandre, 2003; Cerretani et al., 2008; Esti et al., 2009; Tovar, Motilva, & Romero, 2001). In particular, Andrewes et al. (2003) found that decarboxymethyl-ligstroside aglycon, also called oleocanthal (Beauchamp et al., 2005), is the major phenolic molecule responsible for the burning pungent sensation of VOO. Recently, Beauchamp et al. (2005) found that oleocanthal inhibits enzymes related to inflammation and also shows ibuprofen-like activity. Moreover, the phenolic content has been associated with protection against several chronic and degenerative diseases (Menendez et al., 2007).

For commercial classification of VOO, the European Union Commission has provided 26 chemical–physical and organoleptic parameters (EC Reg. 1989/03; EC Reg. 640/08; EEC Reg. 2568/91). The latter are important for quality analysis in order to identify sensory defects (mainly winey, fusty, mouldy, muddy, and rancid) present in VOO. Among these defects, only rancidity is linked with aging of VOO.

The official and/or routine chemical and sensory methods used for these determinations are expensive, time-consuming, labor intensive, and require large amounts of solvent and reagents (Bendini et al., 2007c). The use of spectroscopic techniques together with multivariate analysis (Bendini et al., 2007b,c; Maggio et al., 2009; Sinelli, Cosio, Gigliotti, & Casiraghi, 2007) can minimize these disadvantages and offer potentially rapid methods that can screen large numbers of samples. More recently, metal oxide semiconductor (MOS) sensors have been shown to be valid instruments that are applicable in many fields of food control; these sensors have a low cost and can work on-line without sample pretreatment (Escuderos, Uceda, Sánchez, & Jiménez, 2007; Esposto et al., 2009). Regarding control of aroma in VOO, electronic noses have been used to detect a variety of sensory defects (Aparicio, Rocha, Delgadillo, & Morales, 2000; Camurati, Tagliabue, Bresciani, Sberveglieri, & Zaganelli, 2006; García-González & Aparicio, 2002; García-González & Aparicio, 2003) and to authenticate VOOs according to varietal or geographical origin of olives (Tena, Lazzez, Aparicio-Ruiz, & García-González, 2007). In this regard, the oxidation level of VOO has been recently studied (Buratti, Benedetti, & Cosio, 2005; Cosio, Ballabio, Benedetti, & Gigliotti, 2007).

To date, only limited research has been carried out using MOS sensors coupled with linear discriminant analysis (LDA) to detect the oxidation level of VOO (Cosio et al., 2007). In this study, only three oxidation levels were identified.

The aim of the present investigation was to establish a non-destructive method based on MOS sensors, in combination with LDA, for the classification of the oxidative level of VOO. The application of this system allowed the classification of VOOs with and without phenolic compounds sampled at different storage times in eight oxidation levels. This approach is a fast and economical tool for on-line monitoring of the oxidation status of VOO.

2. Materials and methods

2.1. Reagents and sample preparation

The following reagents were used: sodium hydroxide (NaOH), sodium chloride (NaCl), potassium hydroxide (Carlo Erba, Milan, Italy), hexane, methanol, diethyl ether (Sigma–Aldrich, St. Louis, MO, USA), chloroform, acetonitrile, hydrochloric acid (HCl), anhydrous sodium sulfate, formic acid, ethanol, phenolphthalein, sodium thiosulfate (Merck, Darmstadt, Germany), iso-octane, potassium iodide, 3,4-dihydroxy phenyl acetic acid (3,4-DHPAA) and acetic acid (3, and acetic acid (Fluka, Buchs, Switzerland).

An extra-virgin olive oil (EVOO) sample from the olive fruit variety Brugnola produced at San Marino (picked on October 03, 2008) was used. The olives were processed using an Oliomio 150 extraction machine (Tem, Tavernelle Val di Pesa, Florence, Italy) in order to obtain EVOO. For evaluation of EVOO oxidation, the oil sample was divided in two aliquots: EVOO and EVOO without phenols (EVOOP). Phenolic compounds were removed from EVOO according to the procedure described by Bonoli-Carbognin, Cerretani, Bendini, Almajano, and Gordon (2008). Briefly, 35 g EVOO were washed with several aliquots of 0.5 M NaOH (4 × 15 mL). To eliminate the aqueous phase, the mixture was centrifuged (1000*g*, 5 min) after each washing. Combined olive oil fractions were then washed with 0.5 M HCl (2 × 10 mL) and saturated NaCl solution (5 × 10 mL), centrifuged at 1000*g* for 5 min, dried with anhydrous sodium sulfate and finally filtered under vacuum. Dried olive oil free of phenolic compounds (EVOOP) was obtained.

According to Bendini, Cerretani, Vecchi, Carrasco-Pancorbo, and Lercker (2006) and Bonoli-Carbognin et al. (2008), both samples, EVOO and EVOOP, were divided in eight aliquots each, stored in glass bottles and kept in the dark at 60 °C for 7 weeks. Every week an aliquot of each sample was analyzed for diene and triene, oxidized stability index, electronic nose, and sensory analysis.

Moreover, an additional 25 VOO samples were used to evaluate the model. To assure the robustness of the model, samples from different geographical origins and genetic varieties, collected during different years, were also analyzed.

2.2. Instrumentation

2.2.1. MOS sensor array

An electronic olfactory system (EOS 507, Sacmi Imola S.C., Imola, Bologna, Italy) composed of a measuring chamber with six MOS sensors and a personal computer was used for the acquisition and analysis of the data generated by the EOS 507. The sensors used were: sensor 1 (SnO_2), sensor 2 (SnO_2 + SiO_2), sensor 3, 4 and 5 (catalyzed SnO_2 with three different metals) and sensor 6 (WO_3). During the analysis, sensors were maintained at a temperature range of 350–450 °C. The EOS 507 was controlled by an integrated PDA equipped with proprietary software, and was connected to an automatic sampling apparatus (Model HT500H) which had a carousel of 10 sites for loading samples. Samples were kept at controlled temperature (37 °C) and placed in a chamber provided by a system that removes humidity from the surrounding environment.

2.2.2. Experimental procedure

After samples were removed from the oven, 15 g were placed in 100 mL Pyrex vials equipped with a pierceable silicon/Teflon cap. Samples were incubated at 37 °C for 7 min before injection. The oil headspace, sampled with an automatic syringe, was then pumped over the sensor surfaces for 2 min during which the sensor signals were recorded. The sensors were then exposed to filtered air at a constant flow rate of 50 sccm (standard cubic cm per min) to obtain the baseline. After this, another 7 min period was

then applied to restore the original MOS conditions. Ambient air filtered with activated silica and charcoal was used as a reference gas during the recovery phase of the measurement cycle. The previous conditions ensured that the baseline reading had indeed been recovered before the next analysis was performed.

The experimental conditions adapted from Camurati et al. (2006) were used, and each sample was evaluated in duplicate.

2.2.3. Oxidized stability instrument

An eight-channel oxidative stability instrument (OSI) (Omnion, Decatur, IL, USA) was used. The instrument was set at 110 °C with an air flow rate of 120 mL min^{-1}. The OSI index was determined twice for each sample and the mean value was expressed as OSI time in hours.

2.3. Qualitative parameters

The chemical parameters measured were: acidity, which is indicative of the free fatty acid content of the oil expressed as the percentage of oleic acid; peroxide value (PV), which is a measure of the amount of hydroperoxides expressed as meq O_2 kg^{-1}; UV absorbance at 232 and 270 nm (k_{232}, k_{270}), which provide a measurement of the state of oxidation of the oils and fatty acid (FA) composition, expressed as percentage of saturated, monounsaturated, and polyunsaturated FAs (as methyl esters). The ratio of oleic/linoleic acids was also calculated. All chemical analyses were performed according to the official methods of the European Commission (EEC Reg. 2568/91). The content in total phenols expressed as mg of 3,4-DHPAA kg^{-1} oil was measured, and the extraction procedure, high performance liquid chromatography (HPLC) with diode array detector (DAD) analysis and quantification were adapted from Carrasco-Pancorbo et al. (2007). The oxidative stability index of the samples was also monitored according to Bendini et al. (2007a).

2.4. Sensory analysis

Sensory analysis was performed according to the European normative reported in Annex XII of EEC Reg. 2568/91. Ten trained assessors working as a professional panel from the Dipartimento di Scienze degli Alimenti (panel recognized by Italian Ministry-Mipaaf on 20 July 2006) used a scorecard to provide a quantitative-descriptive analysis (Cerretani, Biasini, Bonoli-Carbognin, & Bendini, 2007) of orthonasal perceptions. A set of positive (green or ripe fruity and other pleasant attributes such as leaf, grass, artichoke, tomato, almond, apple, others) and negative (winey-vinegary, fusty, mouldy, muddy, rancid, others) sensory attributes were evaluated. Oil samples were graded for each positive and negative attribute using a numerical scale from 0 to 5 related to the perception of flavor stimuli, according to the judgement of assessors. The median, mean, and robust standard deviation (EC Reg. 640/08) were calculated for each attribute. If the value of the robust standard deviation was higher than 20%, the analysis was repeated. All samples were subjected to a panel test.

2.5. Data treatment and statistical analysis

The data from the electronic nose was extracted and analyzed with the statistical package "Nose Pattern Editor" (Sacmi Imola S.C.). A feature extraction algorithm called "classical feature" was applied to the data before other statistical treatments. The response extracted by each sensor was defined by:

$$X = R/R_0$$

where R_0 was the initial resistance of the sensor balanced in the air, R was the resistance of a sensor in the presence of the volatile compounds emitted from the VOO headspace (which decreased respect to R_0), and X was the response of each sensor recorded. This algorithm defined several conditions for evaluation of R_0 and R reported in the software package.

Successive statistical analyses were performed using SPSS (v. 11.0, Statistical Package for the Social Sciences, Chicago, IL, USA).

3. Results and discussion

3.1. Evaluation of VOOs during storage

The chemical composition and oxidative parameters of the EVOO and EVOOP from the Brugnola variety are reported in Table 1. The free acidity percentage of EVOO and EVOOP ranged from 0.24% to 0.18%, while the PV varied from 11.96 to 12.44 meq O_2 kg^{-1} oil (Table 1). These values (free acidity and PV) were below the limits set by the EC Regulation for extra-VOO (EC Reg. 1989/03).

The main groups of FA of samples at storage time zero (t_0) are reported in Table 1. EVOO and EVOOP showed very similar values in terms of FA composition, which demonstrate that the phenol-removing procedure does not affect this fraction. The oils were characterized by high values in terms of MUFA and the oleic/linoleic acid ratio, confirming the contribution of FA composition of these oils towards oxidative stability (Aparicio et al., 1999). On the contrary, a significant variation in total tocopherols was observed, and EVOOP showed a slight decrease with respect to EVOO.

EVOO and EVOOP were also analyzed by HPLC-DAD to quantify individual phenols. Considering the sum of all identified and quantified phenolic compounds, their total concentration in EVOO was high with a value of 162 mg DHPAA kg^{-1} oil. The stripping of phenolic compounds from the oil by alkaline washing reduced the concentration of these minor compounds to a very low level; in fact, the decrease was higher than 99.5% (Table 1). A similar effect has been previously observed by Bonoli-Carbognin et al. (2008). The oxidative status of oils was assessed by OSI time values, which underlined that phenols have a major contribution to the oxidative stability of VOO (Aparicio et al., 1999). In fact, as shown in Table 1, EVOOP showed a 73.4% reduction in terms of oxidative stability compared to EVOO.

The changes in the oxidative status of EVOO and EVOOP stored at 60 °C are shown in Fig. 1 as conjugated dienes (A) and trienes (B). The two samples at t_0 showed k_{232} and k_{270} values that were below the limits established by the EC Regulation for extra-VOO category (EC Reg. 1989/03), which corresponded to 2.50 and 0.22, respectively. However, after 1 week of storage (t_1), EVOO and EVOOP exceeded the limit for k_{232}, reaching values of 9 and

Table 1
Chemical parameters of EVOO and EVOOP samples at storage time zero.

Parameter	EVOO	EVOOP
Acidity (%)	0.24	0.18
PV (meq O_2 kg^{-1})	11.96	12.44
C18:1/C18:2[a]	7.87	7.85
MUFA[b] (%)	73.80	73.75
PUFA[c] (%)	9.67	9.68
SFA[d] (%)	16.53	16.57
k_{232}	2.23	2.45
k_{270}	0.19	0.16
Tocopherol (mg kg^{-1})	172.46	120.17
Total phenol (mg 3,4-DHPAA kg^{-1})	162.22	0.69
OSI time (h)	33.65	10.80

[a] Ratio oleic acid/linoleic acid.
[b] Monounsaturated fatty acids.
[c] Polyunsaturated fatty acids.
[d] Saturated fatty acids.

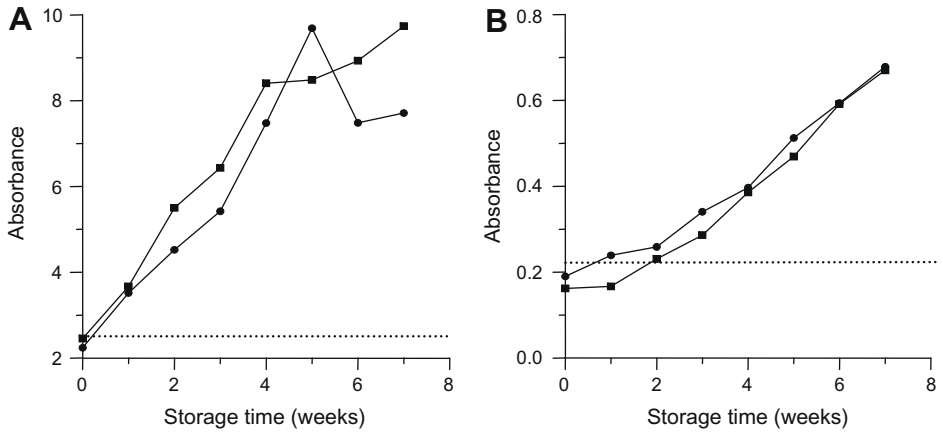

Fig. 1. Changes in conjugated diene at 232 nm (A) and triene at 270 nm (B) contents of the extra-VOO (●) and EVOOP (■) samples during storage at 60 °C. The horizontal line indicated the legal limit established by the EC Regulation for extra-VOO category.

7 for EVOOP and EVOO, respectively, after 7 weeks (t_7) of storage (Fig. 1). Concerning k_{270}, after 1 week of storage (t_1) only EVOO exceeded the EC limit, but 7 days later EVOOP also surpassed the legal value. k_{270} showed a similar trend respect to k_{232} and after 7 weeks (t_7) of storage final values of 0.7 for both types of samples were seen (Fig. 1). Similar trends were also found by Bendini et al. under comparable experimental conditions (Bendini et al., 2006).

Starting from t_0 to the end of the storage time (t_7), both EVOO and EVOOP samples showed a substantial decrease in terms of OSI time that was more evident for EVOO, also if this oil was characterized by a higher OSI time at t_0 (Fig. 2). This difference in terms of oxidative stability between EVOO and EVOOP, especially at the beginning of storage time, could be explained by the different amounts of phenolic compounds. For EVOOP, the OSI value at t_0 close to 10 h could only be attributed to the high oleic acid content, the low amounts of polyunsaturated FA, and thus to the high oleic/linoleic acid ratio.

3.2. Construction of the data matrices and LDA models

LDA, a supervised classificatory technique, is widely recognized as an excellent tool to obtain vectors showing the maximal resolution between a set of previously defined categories. In LDA, vectors minimizing the Wilks' lambda, λ_w, are obtained (Vandeginste et al., 1998). This parameter is calculated as the sum of squares of the distances between points belonging to the same category divided by the total sum of squares. Values of λ_w approaching zero are obtained with well resolved categories, whereas overlapped categories approach a λ_w of one. Up to N-1 discriminant vectors are constructed by LDA, and N has the lowest value for either the number of predictors or the number of categories.

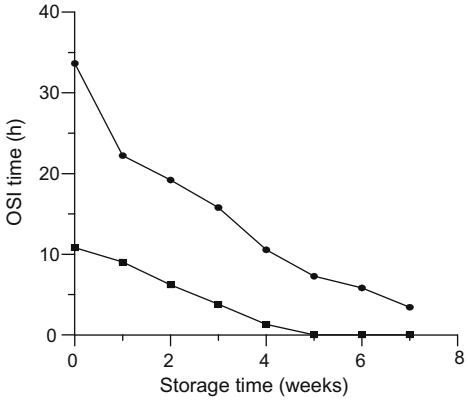

Fig. 2. Oxidized stability index of EVOO (●) and EVOOP (■) samples.

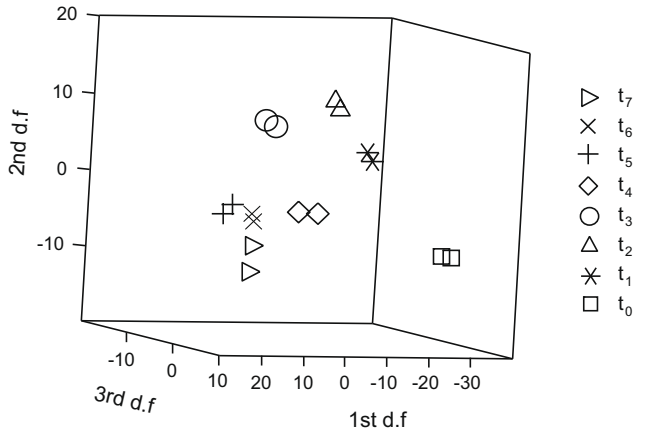

Fig. 3. Score plot on an oblique plane of the 3-D space defined by the three first discriminant functions of the LDA model constructed with EVOO samples. d.f., discriminant function.

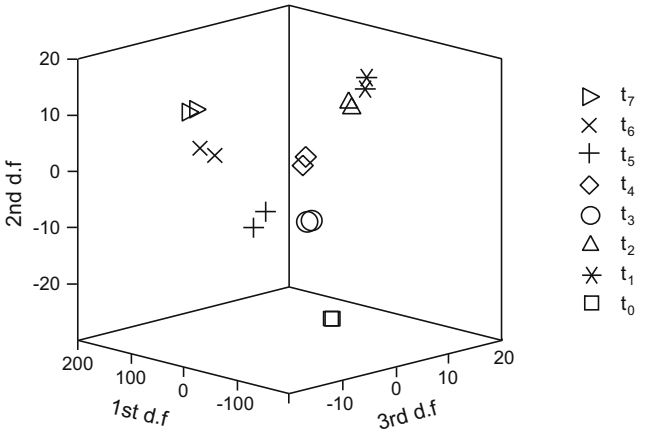

Fig. 4. Score plot on an oblique plane of the 3-D space defined by the three first discriminant functions of the LDA model constructed with EVOOP samples. See Fig. 3 for additional comments.

Table 2
Predictors selected and corresponding standardized coefficients of the three LDA models constructed to predict the storage time.

Predictor	EVOO				EVOOP				EVOO and EVOOP			
	f_1	f_2	f_3	f_4	f_1	f_2	f_3	f_4	f_1	f_2	f_3	f_4
Sensor 1	1.75	0.50	−1.46	3.58	−2.36	1.99	0.50	−2.58	−2.83	−4.84	−2.15	4.06
Sensor 2	−1.01	−0.00	1.27	1.47	−14.51	0.74	−1.62	0.03	−5.80	−0.90	−1.31	0.33
Sensor 3	0.53	−0.56	2.90	−3.62	–	–	–	–	–	–	–	–
Sensor 4	−1.22	1.06	−2.39	−1.30	10.73	−2.30	2.35	0.03	4.50	0.96	2.77	−0.36
Sensor 5	–	–	–	–	6.40	−0.12	−0.30	2.93	4.16	5.45	1.01	−3.25

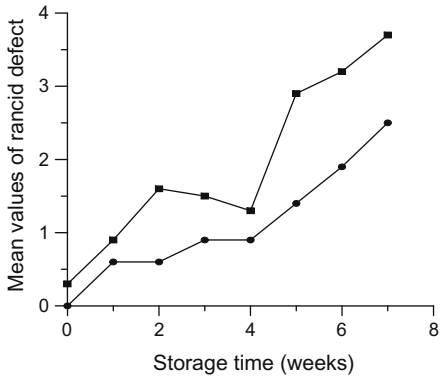

Fig. 5. Evolution of the rancid defect expressed as the mean of the sensory analysis values as a function of storage time for EVOO (●) and EVOOP (■) samples.

Using the 6 MOS sensors as predictors, LDA models capable of classifying the oil samples according to storage time were constructed. Three matrices containing the six predictors were constructed: one containing EVOO samples (8×2 replicates), another containing EVOOP samples (8×2 replicates), and the third containing both types of samples (16×2 replicates). A response column containing the eight categories corresponding to the different storage times was added to the matrices. To construct the LDA models, the SPSS stepwise algorithm was used. According to this algorithm, a predictor is selected when the reduction of λ_w produced after its inclusion in the model exceeds F_{in}, the entrance threshold of a test of comparison of variances or *F*-test. However, the entrance of a new predictor modifies the significance of those predictors which are already present in the model. For this reason, after the inclusion of a new predictor, a rejection threshold, F_{out}, is used to decide if one of the other predictors should be removed from the model. The process terminates when there are no predictors entering or being eliminated from the model. Probability values of F_{in} and F_{out}, 0.05 and 0.10, respectively, were adopted.

First, an LDA model was constructed using the matrix containing the EVOO samples, and excellent resolution between the category pairs was obtained (Fig. 3, $\lambda_w = 0.049$). This value was very low taking into account that a large number of categories were simultaneously distinguished. Next, another LDA model was constructed using the matrix constructed with the EVOOP samples. With this model, resolution between the category pairs was also excellent (Fig. 4, $\lambda_w = 0.068$). Finally, another LDA model was constructed using all the samples, and good resolution between all the category pairs was achieved ($\lambda_w = 0.892$). In this case, for the same category, two different groups were observed, one for EVOO and the other for EVOOP samples (data not shown). This dispersion within the same category could explain the high λ_w value achieved. With this LDA model, it is possible to conclude that electronic nose data discriminated between samples with and without phenolic compounds. The variables selected by the SPSS stepwise algorithm, and the corresponding standardized coefficients of the three

Table 3
Median values for the sensory attributes and predicted category (t_x) for the 25 samples used to evaluate the LDA model.

Sample	Fruity (type)	Other pleasant attributes	Defects	Predicted category
N1	3 (Green)	2 (Grass, tomato)	0	t_0
N2	2 (Green)	2 (Grass, artichoke, almond)	0	t_0
N3	2 (Green)	2 (Grass, artichoke)	0	t_0
N4	2 (Green)	2 (Leaf, almond)	0	t_0
N5	2 (Green)	2 (Leaf, grass, tomato, almond)	0	t_0
N6	3 (Green)	2 (Grass, artichoke, tomato)	0	t_0
N7	3 (Green)	3 (Grass, artichoke, tomato)	0	t_0
N8	3 (Green)	2 (Grass, artichoke, tomato)	0	t_0
N9	2 (Ripe)	2 (Others)	0	t_0
N10	2 (Ripe)	2 (Tomato)	0	t_0
N11	1 (Ripe)	0	1.5 (Fusty)	t_1
N12	1 (Ripe)	0	1 (Winey)	t_1
N13	1 (Ripe)	0	2 (Rancid)	t_1
N14	1 (Ripe)	0	5 (3 of Muddy, 2 of rancid)	t_2
N15	1 (Ripe)	0	2 (Rancid)	t_1
N16[a]	0	0	2 (Winey)	t_0
N17[a]	0	0	3 (Winey)	t_0
N18[a]	0	0	3 (Rancid)	t_3
N19[a]	0	0	5 (Rancid)	t_7
N20[a]	0	0	1 (Fusty)	t_1
N21[a]	0	0	3 (Fusty)	t_3
N22[a]	0	0	1 (Muddy)	t_1
N23[a]	0	0	2 (Muddy)	t_1
N24[a]	0	0	1 (Mouldy)	t_1
N25[a]	0	0	2 (Mouldy)	t_1

[a] Official defects provided by International Oil Council (IOC).

models, showing the predictors with large discriminant capabilities, are shown in Table 2. As can be deduced, the LDA model constructed with the EVOO samples was the only one which uses sensor 4 as a predictor. For this reason, this sensor was surmised to have a response due to volatile compounds formed after oxidation in the presence of phenolic compounds. For the three models, and using the leave-one-out validation, all points were correctly classified (the degree of classification is 100%).

3.3. Sensory analysis and evaluation of the LDA model

EVOO and EVOOP samples were evaluated by the panel of testers. Only the EVOO sample at t_0 was characterized by a green fruity and leaf and grass attributes, while all other samples had a ripe fruity and any pleasant attributes. Only the EVOO sample at t_1 showed a winey defect, probably masked at t_0 by the green fruity aspect, while rancidity was the only defect noted in all samples during the 7 week period. The evolution of the rancid defect is shown in Fig. 5 (mean values were used instead of median to better demonstrate inter-sample variations). As can be observed, rancidity was more pronounced for the EVOOP samples, which could be indirectly attributed to the absence of phenolic compounds. Therefore, the model clearly responded to the rancid defect.

An additional 25 VOOs were also subjected to the panel test assessment, being also used to evaluate the model in order to verify if they were well assigned according to sensory data. The most important sensory attributes of these samples and the predicted category for the model are shown in Table 3. As is evident, the model distinguished all the samples without defects from the others, assigning them to the t_0 category. Moreover, Table 3 shows that the model was able to correctly identify 13 defective samples, with the exception of N16 and N17, which were characterized by a winey defect and fruity absence. In particular, the model responded linearly for the four samples characterized by the rancid defect, quite well for N11, N20, and N21, which presented different intensities of fusty, but less for oils with other defects (mouldy, muddy).

It is interesting to note that slight differences were observed for the real defected samples (from N13 to N15) and those provided by IOC to recognize the rancid defect (N18 and N19). This could be linked to the differences in total volatile compounds between the profile of VOO and the rancid standard (Aparicio et al., 2000). In this respect, the near total absence of volatile components responsible for pleasant fruity note (from the LOX pathway) and the high presence of several saturated and unsaturated aldehydes in the IOC samples could have influenced the MOS response.

4. Conclusions

The possibility of classifying VOOs according to their oxidative level by using MOS sensor data has been demonstrated. In fact, for samples with and without phenols (EVOO and EVOOP) characterized by different storage times under forced oxidation (7 weeks at 60 °C), excellent resolution was achieved by LDA which divided oils into eight categories. Moreover, good correlation was obtained between the model and sensory analysis. In particular, the model distinguished all VOOs without defects from the others, and responded linearly for the four samples characterized by a rancid defect. The method is a fast and economical tool for on-line monitoring of VOO oxidation status, and could be also used to study the oxidative level of other vegetable oils during processing (i.e. refining steps) and distribution phases in addition to industrial uses (i.e. frying).

Acknowledgements

The authors gratefully acknowledge Sacmi Imola S.C. who kindly allowed us to use the MOS system (EOS 507), and Ibanez Riccò for technical assistance. Project CTQ2007-61445 (MEC and FEDER funds) are acknowledged. MJL-G thanks the Generalitat Valenciana for an FPI grant for PhD studies and for a grant to study in a foreign institution.

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Food Chemistry 117 (2009) 615–620

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Antioxidant potential of barley as affected by alkaline hydrolysis and release of insoluble-bound phenolics

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ARTICLE INFO

Keywords:
Antioxidants
Barley
Alkaline hydrolysis
ORAC
TEAC

ABSTRACT

Phenolic constituents of six barley varieties, namely Falcon, AC Metcalfe, Tyto, Tercel, Phoenix and Peregrine were separated into free, soluble conjugate and insoluble-bound fractions. Soluble conjugates and insoluble-bound phenolics were extracted into diethyl ether after consecutive alkaline hydrolysis for 4 h. Total phenolic content of each of the three fractions was determined by using Folin–Ciocalteau method. Total antioxidant capacity of the phenolic fractions was determined by trolox equivalent antioxidant capacity (TEAC) assay. The extracts were evaluated for their efficacy in scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Oxygen radical scavenging capacity ($ORAC_{FL}$) of the extracts was determined using a fluorometric assay. Effectiveness of phenolic extracts in inhibiting oxidation of human LDL cholesterol and radical-induced supercoiled DNA breakage was also evaluated. The contribution of insoluble-bound phenolics toward total phenolic content was significantly ($p < 0.05$) higher than the soluble phenolics for all barley extracts tested. The ratio of soluble to insoluble phenolics ranged from 1:27 to 1:35. TEAC and ORAC values and DPPH radical scavenging capacity of the insoluble phenolic fraction were significantly ($p < 0.05$) higher than those of their insoluble counterparts. A similar trend was observed against inhibition of LDL cholesterol oxidation and radical-induced DNA breakage.

1. Introduction

Cereals contain a myriad of phenolic compounds including benzoic acid and cinnamic acid derivatives, anthocyanins, quinones, flavanols, chalcones, flavones, lignans, proanthocyanidins, and amino-phenolic compounds (Goupy, Hugues, Boivin, & Amoit, 1999; Shahidi & Naczk, 2004). Polyphenols identified in barley include anthocyanins, flavonols, phenolic acids, catechins and proanthocyanidins (Goupy et al., 1999). There are more than 50 proanthocyanidins reported in barley. Proanthocyanidins in barley include oligomeric and polymeric flavan-3-ol, catechin (c), and gallocatechin (gc). The most abundant proanthocyanidin in barley are dimeric proanthocyanin B3 and procyanidin B3. Major trimers include T1 (gc–gc–c), T2 (gc–c–c), T3 (c–gc–c), and T4 or procyanidins C2 (c–c–c) (Friedrich, Eberhardt, & Galensa, 2000).

Cereals contain a wide range of phenolic acids, mainly those belonging to the benzoic acid and cinnamic acid groups. Phenolic acids are different from other phenolic compounds bearing acidic properties due to the presence of a carboxylic acid group. Ferulic acid and *p*-coumaric acid are the major phenolic acids found in many cereals, including barley. A significant proportion of these phenolic acids is known to be linked to lignans and arabinoxylans (Nordkvist, Salomonsson, & Amna, 1984). Ferulic acid is highly concentrated in the cell walls of aleurone layer that is rich in arabinoxylan (McNeil, Albersheim, Taiz, & Jones, 1975; Maillard & Berset, 1999).

Phenolic compounds in cereal grains exist in the free, soluble esters or conjugates and insoluble-bound forms. It is reported that 74% and 69% of total phenolics present in rice and corn, respectively, are in the insoluble-bound form (Adom & Liu, 2002). Most of the studies reported in the literature have ignored insoluble-bound phenolic compounds, hence underestimating the content of phenolic compounds present.

Having both carboxylic acid and hydroxyl groups in their structures, phenolic acids are capable of forming both ester and ether bonds with other compounds leading to the formation of linkages with cell wall polysaccharides. Phenolic acids can be esterified with small molecules such as alcohols, other phenolic acids, phenols, and alkaloids, among others (Yu, Vasnathan, & Temelli, 2001). Non-starch polysaccharides such as xylose, and arabinose units may easily esterify with phenolic acids. Phenolic acids with carboxyl and hydroxyl groups can bind either starch or other polysaccharides through hydrogen bonds, chelation, or covalent bonds (Yu et al., 2001).

Phenolic esters can be hydrolyzed to release phenolic acids by different means. Alkaline, acidic or enzymatic hydrolysis methods are adopted in order to release insoluble-bound phenolics from

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doi:10.1016/j.foodchem.2009.04.055

the cereal matrix (Yu et al., 2001). Generally, alkaline hydrolysis is the method mostly used for extracting esterified or bound phenolics at room temperature. Alkaline hydrolysis was used in this study to release phenolic acids present in the insoluble-bound form.

The objectives of this study were to extract free, esterified, and insoluble-bound phenolic compounds from six barley cultivars using alkaline hydrolysis and to determine antioxidative, and anti-radical efficacies of the phenolic fractions so obtained.

2. Materials and methods

2.1. Chemical reagents

Sodium carbonate, Trisma base, methanol and hexane were purchased from Fischer Scientific (Nepean, ON, Canada). Compounds 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferulic acid, Folin–Ciocalteu reagent, mono- and dibasic sodium and potassium phosphates, trolox, deoxyribonucleic acid (DNA) of pBR 322 (*Escherichia coli* strain RRI), sodium chloride, agarose, ethidium bromide, bromophenol, xylene cyanol, sucrose, human low density lipoprotein (hLDL), copper chloride, ferric chloride, luminol, and fluorescein were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada).

2.2. Samples

Grains of six barley cultivars from 2002 crop year, namely Falcon, AC Metcalfe, Tyto, Tercel, Phoenix and Peregrine (grown under the same conditions) were obtained from Field Crop Development Centre, Lacombe, Alberta, Canada. All cultivars examined were grown under the same climatic and agronomic conditions, in well-drained fertile loam soil under relatively cool temperatures and under full sunlight conditions.

2.3. Separation of phenolic fractions using alkaline hydrolysis

The free phenolics, soluble conjugates and insoluble-bound phenolic acids from AC Metcalfe, Falcon, Phoenix, Tercel, Tyto and Peregrine barley samples were isolated using the procedure explained by Krygier, Sosulski, and Hogge (1982) and Naczk and Shahidi (1989). Barley meals (2 g) were extracted six times with a 40 mL mixture of methanol-acetone-water (7:7:6, v/v/v) using a Polytron homogenizer at 10,000 rpm for 4 min. The resulting mixture was centrifuged at 4000*g* for 5 min and the supernatants were collected and combined. The solvent mixture was evaporated at 30 °C *in vacuo* to approximately 40 mL, followed by extracting six times with diethyl ether at 1:1 (v/v) solvent to supernatant ratio. The combined extracts were evaporated to dryness *in vacuo* at 30 °C in order to obtain free phenolic acid fraction which was subsequently dissolved in methanol. The supernatant containing soluble conjugates was subsequently hydrolyzed with 30 mL of 4 M NaOH under N_2 for 4 h at room temperature. The resultant hydrolysate was acidified to pH 2 using 6 M HCl followed by extraction with diethyl ether. The ether extracts were combined and evaporated to dryness *in vacuo* at 30 °C and subsequently dissolved in methanol to obtain soluble esters. The leftover meal was treated with 20 mL of 4 M NaOH and hydrolyzed for 4 h at room temperature under a stream of N_2 and acidified to pH 2 with 6 M HCl and centrifuged at 4000*g* for 5 min. The supernatant was extracted with diethyl ether six times. The combined extracts were evaporated to dryness at 30 °C *in vacuo* and subsequently dissolved in methanol to obtain insoluble-bound phenolic fraction.

2.4. Determination of total phenolic content

The total content of phenolics was determined according to the procedure explained by Singleton and Rossi (1965) and the results were expressed as mg of ferulic acid equivalents per gram of defatted material.

2.5. Total Antioxidant Capacity by trolox Equivalent Antioxidant Capacity (TEAC) assay

The TEAC assay is based on scavenging of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical anion ($ABTS^{\cdot-}$). A solution of $ABTS^{\cdot-}$ was prepared in 100 mM saline phosphate buffer (pH 7.4, 0.15 M sodium chloride) (PBS) by mixing 2.5 mM 2,2'-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) with 2.0 mM $ABTS^{2-}$. The solution was heated for 16 min at 60 °C, protected from light by covering in a tin foil, and stored at ambient temperature until used. The $ABTS^{\cdot-}$ solution was used within 2 h as the absorbance of the radical itself depletes with time. Barley extracts were dissolved in PBS at a concentration of 3 mg/mL and diluted accordingly to have them fit in the range of values in the standard curve (drop of 0–0.3 optical density unit). For measuring antioxidant capacity, 40 μL of the sample were mixed with 1.96 mL of $ABTS^{\cdot-}$ solution. Absorbance of the above mixture was read at 734 nm after 6-min as the extracts needed a minimum of 6 min for completion of the reaction. The decrease in absorbance at 734 nm after 6 min of addition of a test compound was used for calculating TEAC values. A standard curve was prepared by measuring the reduction in absorbance of $ABTS^{\cdot-}$ solution at different concentrations of trolox. Appropriate blank measurements (decrease in absorbance at 734 nm due to solvent without any compound added) were carried out and the values recorded (van den Berg, Haenen, van den Berg, & Bast, 1999). TEAC values were expressed as μmol trolox equivalents per gram of defatted material.

2.6. DPPH radical scavenging assay

The effect of extracts on DPPH radical was monitored according to the method of Hatano, Kagawa, Yasuhara, and Okuda (1988). The extracts (100 μL at a concentration of 3 mg/mL) were added to a methanolic solution (1.9 mL) of DPPH radical (final concentration of DPPH radical was 5.7 μM). The mixture was shaken vigorously and left standing at room temperature for 20 min; the absorbance was measured spectrophotometrically at 517 nm. DPPH radical scavenging capacity was expressed as μmol ferulic acid equivalents per gram of defatted material based on the depletion of absorbance after 20 min of the assay.

2.7. Determination of oxygen radical absorbance capacity ($ORAC_{FL}$)

The determination of $ORAC_{FL}$ was carried out using a Fluostar Optima plate reader (BMG Labtech, Durham, NC) equipped with an incubator and two injector pumps with fluorecsein as the probe and AAPH as the radical generator. Fluorescein was dissolved in phosphate buffer (0.75 M, pH 7.0) to obtain a final concentration of 32 nM and injected into the wells containing 20 μL of extract or standards using the first pump in the first cycle. The mixture was incubated for 20 min at 37 °C in the built-in incubator and subsequently 60 μL of APPH solution were injected into the wells using the second pump. The plate was shaken for 4 s after each addition at 4 mm shaking width. To optimize signal amplification in order to obtain maximum sensitivity, a gain adjustment was performed at the beginning by manually pipetting 200 μL of fluorescein into a designated well. No more than 35 wells of the 96-well plate (Costar 2650 black plate) were used due to increased

cycle time. Fluorescence was determined and recorded every minute for 60 min and the antioxidant activity of the extracts was calculated as trolox equivalents using a standard curve prepared with 1–10 μM (final concentration) trolox. Fluorescein filters with an excitation wavelength of 485 nm and an emission wavelength of 520 nm were used (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002).

2.8. Determination of metal chelation activity

The Fe^{2+} chelation activity of the barley extracts was measured by using a 2,2′-bipyridyl competition assay explained by Yu, Haley, Perret, and Harris (2002) and Yamaguchi, Ariga, Yoshimura, and Nakazawa (2000). The reaction mixture contained 0.2 mL of 1 mM $FeSO_4$, 1 mL of Tris–HCl buffer (pH 7.4), 0.2 mL of extract (6 mg/mL whole seed extract; 2 mg/mL, pearling fractions) or ethylenediaminetetracetic acid (EDTA) standard, 0.4 mL of 10% hydroxylamine HCl, 1 mL of bipyridyl solution (0.1% in 0.2 M HCl), and 2.5 mL of ethanol. The reaction mixture was mixed well and the intensity of the colour developed was measured at 522 nm. Metal chelation activity was expressed as EDTA equivalents using a standard curve prepared with 75–675 μM EDTA.

2.9. Measurement of cupric ion-induced human low density lipoprotein (LDL) peroxidation

The method explained by Andreason, Landbo, Christensen, Hansen, and Meyer (2001) and Hu and Kitts (2000) was used to measure LDL cholesterol oxidation. Human LDL in saline phosphate buffer solution (PBS) containing 0.01% EDTA was dialyzed against 10 mM saline PBS (pH 7.4, 0.15 M NaCl) for 12 h under nitrogen at 4 °C and EDTA-free LDL was subsequently diluted to obtain a standard protein concentration of 0.2 mg/mL with PBS. The diluted LDL solution (200 μL) was mixed with 1000 μL of PBS and 10 μL of extract (2 mg/mL) in a test tube. Oxidation of LDL was initiated by adding 5.1 mM cupric sulphate solution resulting in a 4 μM copper concentration in the reaction mixture. The mixture was incubated at 37 °C for 100 min and conjugated dienes (CD) of the mixture were measured (at 232 nm) at 5 min intervals over 100 min. Inhibition percentage was calculated based on the CD values at 100 min of incubation.

2.10. Supercoiled strand DNA scission by peroxyl and hydroxyl radicals

Plasmid supercoiled DNA (pBR 322) was dissolved in 10 mM PBS (pH 7.4, 0.15 mM sodium chloride). DNA (25 ng/ μL) was mixed with ferulic acid and barley extracts dissolved in the same PBS. Peroxyl radical was generated using AAPH (dissolved in PBS; pH 7.4, 0.15 mM sodium chloride) to obtain a final concentration of 1 mM and mixed with the DNA and the extract mixture at a total volume of 12 μL. The reactants were incubated at 37 °C for 1 h in the dark (Hu, Zhang, & Kitts, 2000).

The hydroxyl radical generating system consisted of 100 μM ferric chloride, 100 μM ascorbic acid, 100 μM hydrogen peroxide and 100 μM EDTA for non site-specific hydroxyl radical generation (Halliwell et al., 1987). The total volume was adjusted to 12 μL with PBS and the reaction mixture was incubated at 37 °C for 1 h in the dark. Upon completion of incubation, the loading dye (3 μL) (consisting 0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose in distilled water) was added to the sample and loaded to a 0.7% (w/v) agarose gel prepared in Tris–acetic acid–EDTA buffer (40 mM Tris acetate, 2 mM EDTA, pH 8.5). Horizontal gel electrophoresis was performed at 32v for 8 h. DNA strands were stained with 0.5 μg/mL ethidium bromide and visualized under ultraviolet light. Images were analyzed using AlphaEase™ stand alone software (Alpha Innotech Co., San Leandro, CA). The protective effect of extracts and catechin was calculated based on the following equation.

$$\text{DNA retention},\% = \left\{\frac{\text{Intensity of supercoiled DNA in sample}}{\text{Intensity of supercoiled DNA in control}}\right\} \times 100$$

3. Results and discussion

3.1. Total phenolic content

The contents of free, soluble conjugate, and insoluble-bound phenolics of barley extracts are listed in Table 1. The content of insoluble-bound phenolics was significantly higher than those of soluble conjugate and free phenolic fractions among all barley extracts tested. Free phenolic content ranged from 0.18 to 0.42 mg ferulic acid equivalents per gram defatted material with the highest content in Peregrine extracts while soluble conjugates ranged from 0.42 to 0.81 mg ferulic acid equivalents per gram of defatted material. Insoluble-bound fraction ranged from 2.03 for Tercel to 3.36 mg ferulic acid equivalents per gram of defatted Peregrine. The ratio of soluble phenolics (the sum of free and soluble conjugate fractions) to insoluble-bound phenolics ranged from 1:2.74 to 1:3.92 for barley extracts.

Adom and Liu (2002) analyzed a number of cereals, namely corn, wheat, oat and rice and reported that corn had the highest free phenolic content (0.411 mg/g of grain), followed by rice (0.407 mg/g of grain), then wheat (0.368 mg/g of grain), and finally oat (0.343 mg/g of grain). The content of insoluble-bound phenolic was significantly higher among all cereals tested (Adom & Liu, 2002). It is evident that a significantly high proportion of phenolic content is present in the bound form in cereals. Similar results were found for canola seeds by Naczk and Shahidi (1989). Insoluble-bound phenolic compounds are associated with cell wall materials, especially complex carbohydrates such as arabinoxylan. Gastrointestinal enzymes usually are not capable of releasing phenolics from the complexes of phenolic compounds and complex carbohydrates, hence they pass the upper intestinal tract intact.

3.2. Total antioxidant capacity (TAC) as measured by trolox equivalent antioxidant capacity (TEAC)

Total antioxidant activity of free, soluble conjugates and insoluble-bound phenolic fractions is listed in Table 2. It is evident that TAC follows a similar trend to that of TPC among all barley extracts tested. Total antioxidant activity of free, soluble conjugate and insoluble-bound phenolic fractions ranged from 1.89 to 3.11, 1.77 to 3.98, and 7.44 to 9.88 μmol trolox/g defatted material, respectively. The highest TAC was rendered by Peregrine extract while the lowest efficacy was due to Tyto extract.

TEAC value of a compound represents the concentration of trolox (a water soluble vitamin E analogue without the side chain moiety) that has the same antioxidant capacity as the compound or a mixture of compounds of interest (van den Berg et al., 1999). Thus, TEAC value may be considered as a stoichiometric number related to TEAC, for trolox value of 1. Insoluble-bound phenolic fraction contributed the highest proportion towards TAC, followed by soluble conjugate and free phenolics.

3.3. DPPH radical scavenging capacity of phenolic fractions of barley

DPPH radical scavenging capacity of free, soluble conjugate and insoluble-bound phenolic fractions from six barley cultivars is listed in Table 3. DPPH radical scavenging capacity of free phenolic

Table 1
Contents of free, soluble conjugate, and insoluble-bound phenolics of barley cultivars[A].

Barley cultivar	Free phenolics	Soluble conjugate phenolics	Insoluble-bound phenolics	Total phenolic content[B]	Soluble to insoluble-bound phenolics[C] ratio
Falcon	0.34 ± 0.02^{c}	0.63 ± 0.00^{c}	2.66 ± 0.06^{c}	3.63	1:2.74
AC Metcalfe	0.31 ± 0.01^{c}	0.81 ± 0.01^{d}	3.34 ± 0.01^{e}	4.46	1:2.98
Tyto	0.26 ± 0.02^{b}	0.52 ± 0.02^{b}	2.78 ± 0.03^{d}	3.56	1:3.56
Tercel	0.22 ± 0.00^{ab}	0.49 ± 0.01^{ab}	2.16 ± 0.00^{b}	2.64	1:3.04
Phoenix	0.18 ± 0.00^{a}	0.42 ± 0.00^{a}	2.03 ± 0.01^{a}	2.63	1:3.38
Peregrine	0.42 ± 0.02^{d}	0.73 ± 0.04^{d}	3.36 ± 0.02^{e}	4.51	1:3.92

Results are means of three determinations ± standard deviation.
Values in each column having the same superscript are not significantly different ($p > 0.05$).
[A] Phenolic contents are expressed as mg ferulic acid equivalents/g defatted material.
[B] The sum of free, soluble conjugate and insoluble-bound phenolics; expressed as mg ferulic acid equivalents/g defatted material.
[C] Soluble phenolics represent the sum of free and soluble conjugate phenolic fractions.

Table 2
Total antioxidant capacity (TAC) of free, soluble conjugate, and insoluble-bound phenolic fractions of barley cultivars as measured by trolox equivalent antioxidant capacity (TEAC)[1].

Barley cultivar	Free phenolics	Soluble conjugate phenolics	Insoluble-bound phenolics
Falcon	3.11 ± 0.02^{e}	3.46 ± 0.05^{c}	8.98 ± 0.06^{c}
AC Metcalfe	2.67 ± 0.31^{cd}	3.87 ± 0.16^{d}	9.45 ± 0.17^{cd}
Tyto	2.45 ± 0.02^{bc}	2.37 ± 0.22^{b}	7.44 ± 0.23^{a}
Tercel	1.89 ± 0.05^{a}	1.77 ± 0.08^{a}	7.53 ± 0.19^{a}
Phoenix	2.16 ± 0.09^{ab}	2.66 ± 0.04^{b}	8.23 ± 0.06^{b}
Peregrine	3.02 ± 0.02^{de}	3.98 ± 0.09^{d}	9.88 ± 0.26^{d}

Results are means of three determinations ± standard deviation.
Values in each column having the same superscript are not significantly different ($p > 0.05$).
[1] TEAC values are expressed as mg μmol trolox/g defatted material.

Table 3
DPPH radical scavenging capacity of free, soluble conjugate, and insoluble-bound phenolics fractions of barley cultivars[A].

Barley cultivar	Free phenolics	Soluble conjugate phenolics	Insoluble-bound phenolics
Falcon	2.20 ± 0.02^{e}	2.76 ± 0.00^{e}	4.56 ± 0.06^{d}
AC Metcalfe	1.89 ± 0.01^{b}	2.23 ± 0.01^{c}	3.95 ± 0.01^{a}
Tyto	2.11 ± 0.02^{d}	1.99 ± 0.02^{a}	4.76 ± 0.03^{c}
Tercel	1.76 ± 0.00^{a}	2.10 ± 0.01^{b}	4.12 ± 0.00^{b}
Phoenix	2.56 ± 0.00^{f}	2.44 ± 0.00^{d}	4.22 ± 0.01^{b}
Peregrine	1.98 ± 0.02^{c}	2.98 ± 0.04^{f}	5.62 ± 0.02^{e}

Results are means of three determinations ± standard deviation.
Values in each column having the same superscript are not significantly different ($p > 0.05$).
[A] DPPH radical scavenging capacity values are expressed as μmol trolox equivalents/g defatted material.

fraction ranged from 1.76 to 2.56 μmol ferulic acid equivalents per gram defatted material with the highest efficacy rendered by Phoenix extract. The corresponding values for soluble conjugate and insoluble-bound fractions were 1.99–2.98 and 3.95–5.62 μmol ferulic acid equivalents per gram defatted material. In the DPPH assay, antioxidants reduce DPPH·, one of the few stable organic nitrogen radicals that has an absorption maximum at 515 nm. DPPH assay is based on the measurement of reducing ability of antioxidants toward DPPH·, which can be monitored by measuring the decrease in absorbance spectrophotometrically or by electron paramagnetic resonance (EPR) spectrometry (Prior, Wu, & Schaich, 2005).

3.4. Oxygen radical absorbance capacity ($ORAC_{FL}$) of phenolic fractions from barley

Oxygen radical absorbance capacity of free, soluble conjugate, and insoluble-bound phenolic fractions is listed in Table 4. $ORAC_{FL}$ values of free, soluble conjugate, and insoluble-bound phenolic fractions were in the range of 5.99–8.45, 11.78–18.88, and 22.13–34.67, respectively. As observed with TAC and DPPH radical scavenging data, the contribution of insoluble-bound fraction towards the total $ORAC_{FL}$ was significantly higher than free and soluble ester conjugates. Peregrine exhibited the highest total $ORAC_{FL}$ followed by Tyto, Falcon, AC Metcalfe, Phoenix, and Tercel. $ORAC_{FL}$ value is based on the inhibition of the peroxyl radical-induced oxidation initiated by thermal decomposition of azo compounds such as 2,2'-azinobis [3-ethylbenzthiazoline-6-sulphonic acid] (APPH). $ORAC_{FL}$ is the only assay that combines both inhibition time and degree of inhibition into a single quantity (Prior et al., 2005), that is μmol trolox equivalents/g defatted material.

3.5. Inhibition of LDL cholesterol oxidation by phenolic fractions from barley

Percentage inhibition of oxidation of LDL cholesterol by free, soluble conjugate, and insoluble-bound phenolic fractions at a final concentration of 16 μg/mL is listed in Table 5. Percentage inhibition of oxidation of LDL cholesterol was in the range of 5.33–12.32, 14.33–29.87, and 42.99–72.32 for free, soluble conjugate and insoluble-bound phenolic fractions, respectively. As expected, insoluble-bound phenolic fraction rendered the highest antioxidant activity towards inhibition of LDL cholesterol oxidation followed by soluble conjugates. Conjugated dienes (CD) are often used as indicators of the level of peroxidation of LDL in antioxidant studies. Inhibition of LDL cholesterol oxidation of the extracts was

Table 4
Oxygen radical absorbance capacity ($ORAC_{FL}$) of free, soluble conjugate, and insoluble-bound phenolics fractions of barley cultivars[A].

Barley cultivar	Free phenolics	Soluble conjugate phenolics	Insoluble-bound phenolics
Falcon	6.10 ± 0.03^{b}	15.92 ± 0.20^{d}	29.43 ± 0.06^{c}
AC Metcalfe	7.44 ± 0.00^{e}	14.67 ± 0.07^{c}	25.87 ± 0.09^{b}
Tyto	8.45 ± 0.02^{f}	12.88 ± 0.17^{b}	32.75 ± 0.79^{d}
Tercel	6.54 ± 0.03^{c}	16.43 ± .12^{e}	22.13 ± 1.22^{a}
Phoenix	5.99 ± 0.04^{a}	11.78 ± 0.05^{a}	23.98 ± 0.12^{ab}
Peregrine	7.11 ± 0.02^{d}	18.88 ± 0.04^{f}	34.67 ± 1.99^{d}

Results are means of three determinations ± standard deviation.
Values in each column having the same superscript are not significantly different ($p > 0.05$).
[A] ORAC values are expressed as μmol trolox equivalents/g defatted material.

Table 5
Percentage inhibition of Cu(II)-induced human LDL oxidation by free, soluble conjugated, and insoluble-bound phenolic fractions from barley.

Barley cultivar	Free phenolics	Soluble conjugated phenolics	Insoluble-bound phenolics
Falcon	10.32 ± 0.44^{cd}	17.89 ± 0.42^{b}	58.00 ± 4.77^{c}
AC Metcalfe	7.64 ± 1.32^{b}	14.33 ± 0.11^{a}	43.99 ± 2.98^{a}
Tyto	8.97 ± 0.76^{bc}	18.87 ± 1.07^{c}	42.99 ± 1.67^{a}
Tercel	6.97 ± 0.32^{ab}	17.66 ± 2.71^{b}	53.45 ± 4.22^{b}
Phoenix	5.33 ± 0.14^{a}	21.78 ± 1.59^{d}	66.12 ± 2.55^{d}
Peregrine	12.32 ± 0.92^{d}	29.87 ± 0.54^{e}	72.32 ± 6.23^{e}

Results are means of three determinations ± standard deviation.
Values in each column having the same superscript are not significantly different ($p > 0.05$).

Table 6
Percentage inhibition of peroxyl radical-induced supercoiled strand scission of PBR322 DNA by free, soluble conjugated, and insoluble-bound phenolic fractions from barley.

Barley cultivar	Free phenolics	Soluble conjugated phenolics	Insoluble-bound phenolics
Falcon	11.89 ± 1.32^{bc}	22.33 ± 1.61^{cd}	87.95 ± 3.45^{abc}
AC Metcalfe	9.22 ± 0.99^{ab}	17.78 ± 2.22^{ab}	96.66 ± 5.76^{c}
Tyto	8.87 ± 0.87^{a}	15.97 ± 0.01^{a}	82.34 ± 3.12^{ab}
Tercel	12.33 ± 1.21^{c}	19.94 ± 0.65^{bc}	84.34 ± 2.80^{ab}
Phoenix	10.00 ± 1.11^{abc}	24.45 ± 1.40^{d}	78.98 ± 2.55^{a}
Peregrine	12.56 ± 0.67^{c}	25.32 ± 0.09^{d}	92.23 ± 4.88^{bc}

Results are means of three determinations ± standard deviation.
Values in each column having the same superscript are not significantly different ($p > 0.05$).

expressed as percentage inhibition based on the CD value after 100 min of incubation.

3.6. Inhibition of peroxyl radical-induced supercoiled plasmid DNA scission by phenolic fractions

The percentage inhibition offered by free, soluble conjugate, and insoluble-bound phenolic fractions of barley extracts at 1 mg/mL concentration were in the range of 8.87–12.56%, 15.97–25.32%, and 82.34–96.66%, respectively. The inhibitory effect was tested at 0.4–1.2 mg/mL, however, 1 mg/mL concentration was used for calculating the inhibitory effect. Peregrine exhibited the highest inhibition against DNA scission while Phoenix exhibited the lowest inhibition of 78.2% at 4 mg/mL. It is important to note that no pro-oxidant activity was observed at any level for all the extracts tested. All extracts showed a similar pattern of concentration dependence in protecting supercoiled plasmid DNA (see Table 6).

4. Conclusions

A major proportion of phenolic compounds present in balrey kernels existed in the insoluble-bound form. Insoluble-bound phenolic compounds contributed most to the TPC of barley, followed by soluble conjugates, the least contribution was due to the free phenolics. This finding lends support to those in the existing literature for barley and other cereals such as wheat and rye. The antioxidant and antiradical activities of insoluble-bound phenolic fraction, in general, was higher than those of soluble conjugates and free phenolic fractions.

Acknowledgements

We thank Mr. Mike Oro, Field crop specialist, Field crop development center, Lacombe, AB, Canada for providing barley samples. Financial support from the Natural Science and Engineering Research Council (NSERC) of Canada in the form of a discovery grant to FS is acknowledged.

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Food Chemistry 117 (2009) 621–626

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Methyl jasmonate enhances biocontrol efficacy of *Rhodotorula glutinis* to postharvest blue mold decay of pears

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ARTICLE INFO

Article history:
Received 24 December 2008
Received in revised form 24 February 2009
Accepted 16 April 2009

Keywords:
Pear
Blue mold
Postharvest decay
Methyl jasmonate (MeJA)
Rhodotorula glutinis
Natural infection
Quality parameters

ABSTRACT

The effect of *Rhodotorula glutinis* treatment alone or in combination with methyl jasmonate (MeJA) in controlling blue mold decay, the natural fungal decay of pears and the postharvest quality parameters including fruit firmness, total soluble solids, titratable acidity, and ascorbic acid were investigated. The combination of methyl jasmonate (200 μM) and *R. glutinis* (1×10^8 CFU/ml) was a more effective approach to reduce the disease incidence and lesion diameter of blue mold decay of pears than the application of MeJA or *R. glutinis* alone after incubation for 7 d at 20 °C. The natural fungal decay of pears treated with the application of *R. glutinis* combined with MeJA resulted in reduced average decay incidence of 10.42% or 4.16%, respectively, compared with 27.17% or 20.83% in the control fruits following storage at 20 °C for 15 d or 4 °C for 60 d followed by 20 °C for 15 d. The combined treatment did not impair quality parameters of fruits under both conditions.

1. Introduction

Blue mold decay caused by *Penicillium expansum* is one of the important postharvest diseases of pears (Spotts & Chen, 1987; Zhang, Zheng, & Su, 2006) and can be controlled effectively by synthetic chemical fungicides. However, the development of fungicide resistance by pathogens and increasing international concern over fungicidal residues in fruits and vegetables has stimulated a search for alternative measures for disease control (Droby et al., 2002; Fan & Tian 2001; Mlikota Gabler & Smilanick 2001). Microbial biocontrol agents have shown a great potential as an alternative to synthetic fungicides for the control of postharvest decay of fruits and vegetables (Wilson et al., 1991). Recently, an antagonistic yeast strain of *Rhodotorula glutinis* have been reported as an effective biocontrol agent against postharvest decay of apples (Qin, Tian, Liu, & Xu, 2003), pears (Zhang, Wang, Huang, Dong, & Zheng, 2008), strawberries (Zhang et al., 2007), sweet cherries (Tian, Qin, & Xu, 2004) and oranges (Zheng, Zhang, & Sun, 2005). It has been reported that biological control is only effective with high concentrations of antagonistic yeasts (Fan & Tian, 2001). However, higher concentrations of antagonists would increase production costs and make biological control methods less attractive. Moreover, biological control as the alternative to chemical control is not as broad spectrum and is usually not as effective as chemical fungicides; therefore, it is important to find effective methods that could increase the antagonistic activity of yeasts and inhibit pathogenic infections. Enhancement of the biocontrol activity of antagonists could be obtained by many approaches. Combining organic and inorganic additives with antagonistic yeasts is a very effective method for enhancing the biocontrol efficacy of yeasts (Jackson, Whipps, Lynch, & Bazin, 1991).

The use of exogenous substances to potentiate the natural defence of plants represents another alternative, potentially promising approach to disease control (Kessmann et al., 1994). Jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), exist naturally in a wide range of higher plants. MeJA is a potential plant defence activator that has been shown to reduce postharvest disease on a number of horticultural crops (Creelman & Mullet, 1997). Exogenous application of methyl jasmonate (MeJA) has been considered as effective at inducing secondary metabolites in plant cell cultures, resulting in the expression of a set of defence genes and inducing resistance of host against pathogens (Epple, Apel, & Bohlmann, 1997; Kozlowski, Buchala, & Métraux, 1999). Recently, MeJA had shown promise in preventing postharvest disease and disorders in horticultural crops, application of MeJA has been reported to effectively suppress gray mold rot caused by *Botrytis cinerea* in strawberry (Moline, Buta, Saftner, & Maas, 1997), to decrease green mold decay caused by *Penicillium digitatum* in grapefruit (Droby et al., 1999), to reduce microbial contamination of freshcut celery

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doi:10.1016/j.foodchem.2009.04.054

and peppers (Buta & Moline, 1998), to decreased fruit decay infected by *Colletotrichum gleosporioides* and *Alternaria alternata* on papaya fruit (González-Aguilar, Buta, & Wang, 2003), to suppress anthracnose rot caused by *Colletotrichum coccodes* in tomato fruit (Tzortzakis, 2007), to reduce anthracnose rot caused by *Colletotrichum acutatum* infection in loquat fruit (Cao et al., 2008), and to inhibit brown rot caused by *Monilinia fructicola* in sweet cherry fruit (Yao & Tian, 2005a). Furthermore, exogenous application of MeJA has been found to enhance the efficacy of the antagonistic yeast *Cryptococcus laurentii* to control brown rot and blue mould caused by *Monilinia fructicola* and *Penicillium expansum* in peach fruit (Yao & Tian, 2005b). However, to the best of our knowledge, there is little information concerning the effect of a combination of *R. glutinis* and MeJA on the control of the postharvest blue mold decay of pear fruits.

The objective of this study were to evaluate (a) the effects of MeJA and the antagonistic yeast *R. glutinis*, used separately or in combination, on controlling postharvest blue mold decay of pear fruits caused by *P. expansum*; (b) the effects of MeJA on spore germination of *P. expansum* in vitro; (c) the effect of MeJA on population dynamics of the *R. glutinis* on fruits; (d) the efficacy of MeJA and *R. glutinis*, separately or in combination, in controlling of natural decay development of pears and its effects on quality of pears after storage, including firmness, total soluble solids, ascorbic acid and titratable acidity.

2. Materials and methods

2.1. Pathogen inoculum

Penicillium expansum Link was isolated from infected pear fruits. The culture was maintained on potato-dextrose agar medium (PDA: extract of boiled potatoes, 200 ml; dextrose, 20 g; agar, 20 g and distilled water, 800 ml) at 4 °C, and fresh cultures were grown on PDA plates before use, respectively. Spore suspensions were prepared by removing the spores from the sporulating edges of a 7 d old culture with a bacteriological loop, and suspending them in sterile distilled water. Spore concentrations were determined with a hemocytometer, and adjusted as required with sterile distilled water.

2.2. Antagonist

The yeast antagonist *R. glutinis* (Fresenins) Harrison was isolated from the surfaces of strawberries harvested in unsprayed orchards and identified by VITEK 32 Automicrobic system (bioMérieux Company, Marcy l'Etoile, France). *R. glutinis* isolates were maintained at 4 °C on nutrient yeast dextrose agar (NYDA) medium containing 8 g nutrient broth, 5 g yeast extract, 10 g glucose and 20 g agar, in 1 l of distilled water. Liquid cultures of the yeast were grown in 250-ml Erlenmeyer flasks containing 50 ml of NYD broth (NYDB) which had been inoculated with a loop of the culture. Flasks were incubated on a rotary shaker at 28 °C for 20 h. Following incubation, cells were centrifuged at 5000*g* for 10 min and washed twice with sterile distilled water in order to remove the growth medium. Cell pellets were re-suspended in sterile distilled water and adjusted to the concentrations required.

2.3. Fruits

Pear fruits (*Pyrus pyrifolia* Nakai.) cultivar "Shuijing" were harvested at commercial maturity from Zhenjiang of Jiangsu province. Fruits were used immediately after harvest, and selected for uniformity of size, ripeness and removed any fruit with apparent injuries or infections. Fruits were disinfected with 1% sodium hypochlorite for 2 min, washed with tap water, air dried prior to wounding.

2.4. Efficacy of MeJA at various concentrations in controlling of blue mold decay of pears

The pear fruit samples were wounded (3 mm diameter and approximately 5 mm deep) using a sterile borer. Each wound was treated with 30 μl of MeJA at 0, 10, 100, 200 and 500 μM, respectively. Two hours later, 20 μl of suspension of *P. expansum* (5×10^4 spores/ml) was inoculated to each wound. After air drying, the pears were stored in enclosed plastic trays to maintain a high relative humidity (above 95%) and incubated at 20 °C. The number of the infected fruit wounds and the average lesion diameters of the overall treated fruits were examined after storage at 20 °C for 7 d post-inoculation. There were three replicates of 10 fruits for each treatment, and the experiment was conducted three times.

2.5. Efficacy of R. glutinis treatment alone or in combination with MeJA in controlling of blue mold decay of pears

The pear fruit samples were wounded (3 mm diameter and approximately 5 mm deep) using a sterile borer. Each wound was treated with 30 μl of: (1) the cell suspensions of *R. glutinis* (1×10^8 cells ml^{-1}); (2) a solution of MeJA (200 μM); (3) *R. glutinis* suspensions (1×10^8 cells ml^{-1}) amended by MeJA (200 μM); and (4) sterile distilled water as the control. Two hours later, 20 μl of a suspension of *P. expansum* (5×10^4 spores/ml) was inoculated to each wound respectively. After air drying, the pears were stored in enclosed plastic trays to maintain a high relative humidity (above 95%) and incubated at 20 °C. The number of the infected fruit wounds and the average lesion diameters of the overall treated fruits were examined after storage at 20 °C for 7 d post-inoculation. There were three replicates of 10 fruits for each treatment, and the experiment was conducted three times.

2.6. Effects of MeJA on spore germination of P. expansum in vitro

The effects of MeJA on spore germination of pathogen were tested in potato dextrose broth (PDB). Aliquots of 100 μl of *P. expansum* suspension were added into 10 ml glass tube containing 5 ml of PDB to obtain a final concentration of 1×10^6 fungal spores ml^{-1}. The PDB contained different concentrations of MeJA (0, 1, 10, 100, 200 and 500 μM). All tubes were put on a rotary shaker at 100 rpm at 25 °C and incubated for 18 h. Approximately 100 spores of each pathogen were measured for germination rate per treatment within each replicate. Each treatment was replicated three times and the experiment was conducted three times (Zhang et al., 2007).

2.7. Effects of MeJA on population growth of R. glutinis in pear wounds

Pears were rinsed in fresh water, and air dried. A uniform 3 mm wide by 5 mm deep wound was made at the equator of each fruit (put on its side) using the tip of a sterile dissecting needle. Thirty microlitres of a suspension of *R. glutinis* at 1×10^8 CFU/ml alone or in combination with MeJA at 200 μM was applied to wounds on the fruit. Fruits were incubated at 20 °C (90% relative humidity) or at 4 °C (98% relative humidity). *R. glutinis* was recovered from the wounds after incubation at 20 °C for 0 (1 h after treatment), 1, 2, 3, 4, 5, 6 and 7 d, and at 4 °C, for 0 (1 h after treatment), 5, 10, 15, 20, 25 and 30 d, respectively. Wounded tissue was removed with an ethanol-flamed, 7 mm (internal diameter) cork borer and ground with a autoclaved mortar and pestle in 50 ml of sterile 0.85% sodium chloride solution. Serial tenfold dilutions were made and 0.1 ml of each dilution was spread in NYDA. The plates were incubated at 28 °C for 2 d and the colonies were counted. Population densities of *R. glutinis* were expressed as $\log_{10}$ CFU per wound.

There were three single fruit replicates per treatment, and the experiments were repeated twice (Zhang et al., 2007).

2.8. Efficacy of R. glutinis treatment alone or in combination with MeJA on natural infections and postharvest quality of pears

Intact fruits were inoculated by dipping them in the suspension of (1) the cell suspensions of *R. glutinis* (1×10^8 cells ml^{-1}); (2) a solution of MeJA (200 μM); (3) *R. glutinis* suspensions (1×10^8 cells ml^{-1}) amended by MeJA (200 μM); (4) sterile distilled water as the control respectively for 30 s, and air-dried. The treated fruits were sealed in polyethylene-lined plastic boxes to retain high humidity. Fruits were stored at 20 °C for 15 d or 4 °C for 60 d followed by 20 °C for 15 d in order to determine disease development under normal shelf-life conditions. Infection rate was recorded afterwards. There were three replicate trials of 10 fruits with a complete randomisation in each test and experiment was repeated three times.

To evaluate the effect of *R. glutinis* treatment alone or in combination with MeJA on postharvest quality of pears, freshly harvested fruits were treated, and stored as described above to evaluate the effect of *R. glutinis* treatment alone or in combination with MeJA on reducing natural decay development. Quality parameters were measured after storage, on three replicates of five fruits each, and performed at ambient temperature (about 20 °C). The testing methods are described below.

Firmness values of each individual pear were measured at three points of the equatorial region by using the TA-XT2i Texture Analyser (Microstable Instruments, UK) with a 5 mm diameter flat probe. The probe descended toward the sample at 1.0 mm s^{-1}, and the distance that the probe travelled through the sample is 10 mm. The maximum force (N) was defined as firmness (Zhang et al., 2008).

Total soluble solids (TSS) were determined by measuring the refractive index of the same juice with a hand refractometer and the results expressed as percentages (g per 100 g fruit weight) (Manganaris, Vasilakakis, Diamantidis, & Mignani, 2007).

The 2,6-dichloroindophenol titrimetric method was used to determine the ascorbic acid content of pressed fruit juice. Results were expressed as milligrams of ascorbic acid per 100 g sample (Özden & Bayindirli, 2002).

Acidity was measured by titration with 0.1 N NaOH to pH 8.1; 4 g of juice diluted with 20 ml of distilled water was evaluated for each replicate. Titratable acidity was calculated as percent malic acid (Wright & Kader, 1997).

2.9. Statistical analysis

The data were analysed by the analysis of variance (ANOVA) in the statistical program SPSS/PC version II.x, (SPSS Inc. Chicago, Illinois, USA). When the number of means in each group is three or above, the analysis of variance (ANOVA) was performed and Duncan's multiple range test was used for means separation. The statistical significance in this experiment was all applied at the level $P < 0.05$.

3. Results

3.1. Efficacy of MeJA at various concentrations in controlling of blue mold decay of pears

The results showed that MeJA at various concentrations had no effect on the disease incidence of blue mold caused by *P. expansum* (Table 1). Similarly, MeJA at concentrations of 1, 10, and 500 μM had no effect on the lesion diameter of blue mold decay, whereas MeJA at concentrations of 100–200 μM significantly decreased the lesion diameter of blue mold decay after 7 d incubation at 20 °C ($P < 0.05$).

Table 1
Effects of MeJA at various concentrations in control of blue mold decay of pears.

MeJA concentration (μM)	Disease incidence (%)[A]	Lesion diameter (cm)
0	100 ± 0a	1.78 ± 0.16a
1	100 ± 0a	1.63 ± 0.11a
10	100 ± 0a	1.52 ± 0.13a
100	100 ± 0a	1.30 ± 0.13b
200	100 ± 0a	1.17 ± 0.12c
500	100 ± 0a	1.59 ± 0.11a

[A] Means are averaged values of three replications ± the standard error. Values followed by the same letter within column are not significantly different at $P = 0.05$ according to Duncan's multiple range test.

3.2. Efficacy of R. glutinis treatment alone or in combination with MeJA in controlling of blue mold decay of pears

MeJA, as a stand-alone treatment, could not reduce the percentage of blue mold infected wounds (Fig. 1). However, it significantly reduced lesion diameters of blue mold decay. *R. glutinis*, as a stand-alone treatment, significantly reduced the disease incidence and

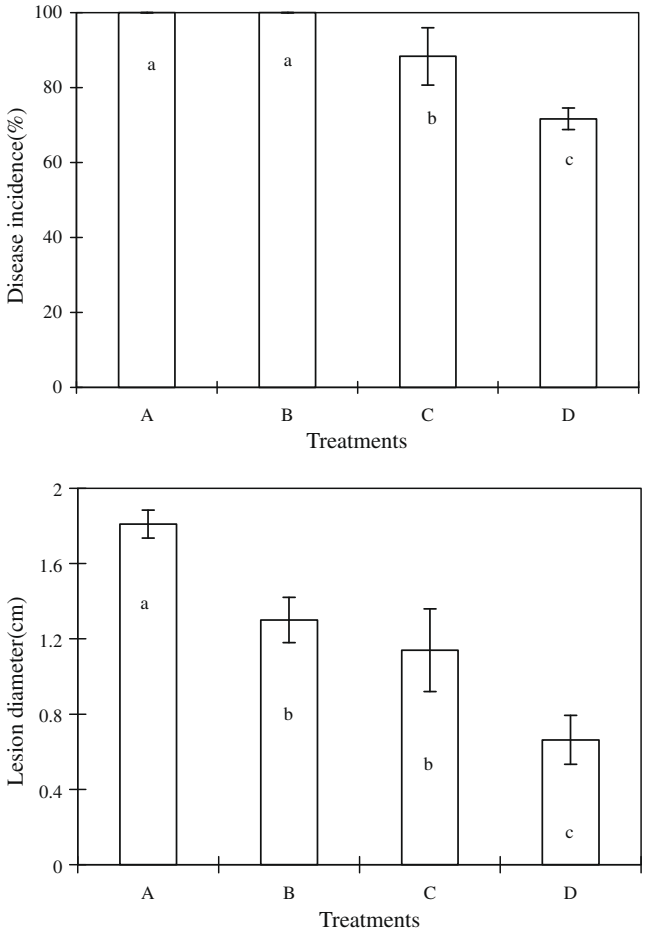

Fig. 1. Effect of *R. glutinis* treatment alone or in combination with MeJA on blue mold rots incidence and lesion diameter in pear wounds. Treatments are as follow: A = Control, B = MeJA (200 μM), C = *R. glutinis* (1×10^8 CFU ml^{-1}), D = *R. glutinis* (1×10^8 CFU ml^{-1}) + MeJA (200 μM). Each value represents the mean of three experiments. Bars represent the standard error of the mean. Data in columns with different letters are statistically different according to Duncan's multiple range test at $P = 0.05$.

lesion diameter of blue mold decay in pears (Fig. 1). The combination of *R. glutinis* with MeJA was a more effective approach to reduce the disease incidence and lesion diameter of blue mold decay of pears than the applications of *R. glutinis* alone. The disease incidence and the lesion diameter of blue mold infected wounds treated with *R. glutinis* combined with MeJA were 71.66% and 0.66 cm, respectively, compared with the 100% and 1.81 cm of the control, respectively.

3.3. Effects of MeJA on spore germination of P. expansum in vitro

Our experiments evaluated the effects of MeJA at various concentrations on spore germination of *P. expansum* in PDB. The result presented in Fig. 2 indicated that MeJA at the concentrations of 1–500 μg ml^{-1} had no significant effect on the spore germination of *P. expansum* compared with the control ($P < 0.05$).

3.4. Population studies of R. glutinis in fruit wounds

Rapid growth of *R. glutinis* in wounds was observed during the first 2 d at 20 °C when *R. glutinis* was applied to wounds in combination with MeJA at 200 μM. However, when *R. glutinis* was applied to wounds alone, rapid growth of *R. glutinis* in wounds was observed during the first 3 d (Fig. 3). At the first 2 d at 20 °C, the population of *R. glutinis* in the fruits treated with MeJA was higher than that of the control. Similarly, on pear wounds kept at 4 °C, rapid growth of the yeast in wounds was observed during the first 5 d after being applied either alone or in combination with MeJA (200 μM), and then the populations stabilised for the remaining storage period. However, the population of *R. glutinis* in the fruits treated with MeJA was higher than that of the no MeJA control for the entire duration of the experiment (Fig. 3).

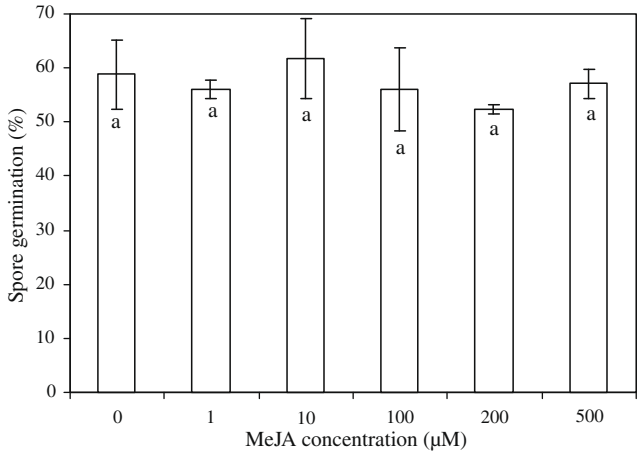

Fig. 2. Effect of MeJA at various concentrations on spore germination of *P. expansum* in potato-dextrose broth medium. Germination rate was measured by electronic microscope after 18 h incubation at 25 °C. Each value is the mean of three experiments. Bars represent the standard error of the mean. Data in columns with different letters are statistically different according to Duncan's multiple range test at $P = 0.05$.

3.5. Efficacy of R. glutinis treatment alone or in combination with MeJA on natural infections and postharvest quality of intact fruits

Our experiments evaluated the efficacy of yeast antagonist and MeJA, as stand-alone treatments or in combination, in reducing the natural infections after storage at 20 °C for 15 d or 4 °C for 60 d followed by 20 °C for 15 d. The result presented in Table 2 indicated that, the application of *R. glutinis* alone significantly reduced the average decay incidence on fruit after storage compared with the control under both storage conditions. Similarly, MeJA (200 μM), as a stand-alone treatment, could reduce the natural fungal decay after storage under both conditions (Table 2). The natural disease incidence of pears treated with the application of *R. glutinis* combined with MeJA resulted in low average decay incidence in 10.42% or 4.16%, respectively, compared with 27.17% or 20.83% in the water-treated control fruit following storage at 20 °C for 15 d or 4 °C for 60 d followed by 20 °C for 15 d.

After 15 d storage at 20 °C, *R. glutinis*, MeJA as a stand-alone treatment or in combination, had no significant effect on total soluble solids, titratable acidity or ascorbic acid (Table 2). Similarly, *R. glutinis* and MeJA as stand-alone treatments had no significant effect on the firmness, however the firmness of pears treated with the combination of *R. glutinis* with MeJA was significantly higher than that of the control. After storage at 4 °C for 60 d followed by 20 °C for 15 d, the firmness, titratable acidity and ascorbic acid of the pears treated with *R. glutinis*, MeJA as stand-alone treatments or in combination were equal or higher than that of the control. The total soluble solids of the pears treated with *R. glutinis* and MeJA as stand-alone treatments or in combination were equal or lower than that of the control.

4. Discussion

In this study, we found that MeJA treatment at 100 or 200 μM could significantly reduce blue mold rot lesion diameter of pear fruits. However, MeJA at various concentrations had no effect on the disease incidence of blue mold. These results suggest that MeJA may have the ability to control postharvest blue mold decay, but the ability is limited, and must be used with other control measures to obtain an improved treatment. The combination treatment of MeJA

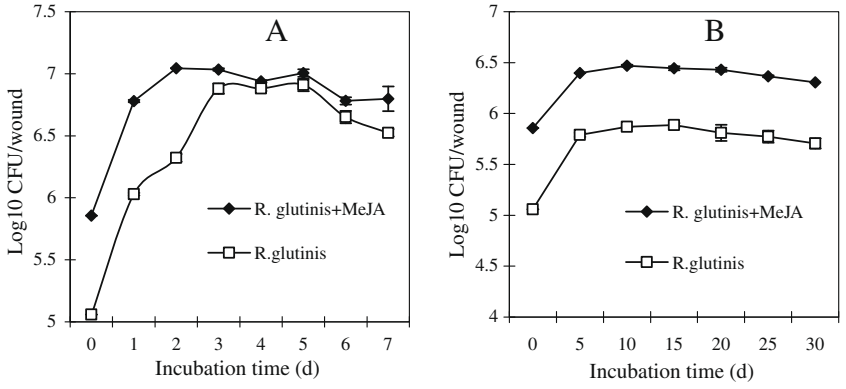

Fig. 3. Population dynamics of *R. glutinis* with and without MeJA in pear wounds at 20 °C (A) and 4 °C (B). Bars represent the standard error of the mean.

Table 2
Effect of *R. glutinis* treatment alone or in combination with MeJA on natural infections and quality parameters of pears.

Storage condition	Treatments	Disease incidence (%)	Firmness (N)	TSS (%)	Titratable acidity (%malic acid)	Ascorbic acid (mg/100 g)
20 °C for 15 d	Control	27.17 ± 2.17a[A]	10.95 ± 0.37b	10.82 ± 0.15a	0.13 ± 0.02a	1.58 ± 0.07a
	MeJA	22.92 ± 2.08b	11.26 ± 0.40ab	10.02 ± 0.14a	0.13 ± 0.01a	1.38 ± 0.14a
	R glutinis	14.58 ± 2.08c	11.25 ± 0.57ab	10.11 ± 0.34a	0.13 ± 0.02a	1.53 ± 0.08a
	R. glutinis + MeJA	10.42 ± 2.08c	12.45 ± 0.41a	10.81 ± 0.25a	0.13 ± 0.01a	1.73 ± 0.06a
4 °C for 60 d followed by 20 °C for 15 d	Control	20.83 ± 2.08a	9.79 ± 0.16b	9.65 ± 0.17a	0.11 ± 0.01b	0.84 ± 0.13a
	MeJA	14.58 ± 2.08b	10.60 ± 0.38ab	8.86 ± 0.21b	0.12 ± 0.01ab	0.74 ± 0.08a
	R. glutinis	7.50 ± 1.25c	10.92 ± 0.22a	9.75 ± 0.11a	0.13 ± 0.01ab	0.88 ± 0.07a
	R. glutinis + MeJA	4.16 ± 2.08c	10.31 ± 0.48ab	9.38 ± 0.10a	0.14 ± 0.02a	0.9 ± 0.09a

[A] Means are averaged values of three trials ± the standard error. Values followed by the same letter are not significantly different at $P = 0.05$ according to analysis by Duncan's multiple range test.

at 200 μM with *R. glutinis* significantly reduced the disease incidence and lesion diameter of blue mold rot of pear fruits, and the control efficacy was better than *R. glutinis* alone at 20 °C. These results suggested that combining MeJA with *R. glutinis* significantly enhanced the biocontrol activity of *R. glutinis* against blue mold decay of pears.

To understand the direct effect of MeJA on the growth of this pathogen, we investigated the effects of MeJA on spore germination of *P. expansum* in vitro. Our results showed that MeJA at all tested concentrations could not suppress the spore germination of *P. expansum*. This is similar with other research results which indicated that MeJA could not directly influence the growth of some fungal pathogens such as *B. cinerea*, *P. digitatum* and *M. fructicola* (Darras, Terry, & Joyce, 2005; Droby et al., 1999; Tsao & Zhou, 2000; Yao & Tian, 2005a). These results suggest that MeJA controls the postharvest blue mold decay and enhances the biocontrol efficacy of *R. glutinis* by mechanisms other than directly effecting pathogen growth/germination. Yao and Tian (2005b) have suggested that the mechanism by which MeJA enhanced the biocontrol efficacy of the antagonistic yeast may be related to its ability to induce resistance in peach fruit by MeJA and antagonistic yeast. However, this mechanism should be further studied.

Our results showed that the populations of *R. glutinis* were greatly increased by the presence of MeJA in the wounded sites at 4 °C and 20 °C. The result was consistent with the result obtained by Yao and Tian (2005b), who observed that MeJA stimulated growth of *C. laurentii* in the wounds of peach fruit at both 25 and 0 °C. This may be because MeJA induces resistance to pathogens in fruits, and pathogens cannot utilise the nutrients of fruits well, so that antagonistic yeast can rapidly colonise the fruit tissues. The biocontrol yeasts have been selected mainly for their capacity to rapidly colonise and multiply in surface wounds, and subsequently to compete with the pathogen for nutrients and space (Droby et al., 2002). The nutritional environment at the wound site may be favourable to *R. glutinis,* which rapidly colonises the fruit tissues and will be competing with the pathogen for nutrients. When MeJA was used with *R. glutinis* in fruits, *R. glutinis* multiplied rapidly in wounds and consumed available nutrients, which may facilitate biocontrol by nutrient competition. These results suggest that MeJA enhances the biocontrol efficacy of *R. glutinis* to postharvest blue mold decay of pears by facilitating the growth of *R. glutinis* in pears and enhancing nutrient competition. The mechanism by which MeJA enhances the biocontrol efficacy of yeast is complex and may be attributed to the influence of MeJA on antagonist, pathogen, and fruits.

Examination of the effects of *R. glutinis* in combination with MeJA on natural infections and postharvest quality parameters of pear fruits revealed that the combined treatments significantly reduced the average decay incidence and did not impair quality parameters of fruits including firmness, total soluble solids, titratable acidity and ascorbic acid under commercial conditions after 15 d storage at 20 °C or 4 °C for 60 d followed by 20 °C for 15 d. There was however minimal difference between *R. glutinis* treatment alone and the *R. glutinis* and MeJA combination treatment in preventing natural fungal decay. These suggest that the combination of *R. glutinis* and MeJA has potential for commercialisation.

In conclusion, the results of this experiment showed that combination of *R. glutinis* with MeJA provided a more effective control for blue mold decay than the application of *R. glutinis* or MeJA alone, which should be considered to be a useful and promising measure for controlling postharvest decay on a commercial scale. We infer that MeJA could enhance the biocontrol activity of *R. glutinis* against blue mold decay in pears by facilitating the growth of *R. glutinis* in fruits thereby reducing nutrient availability, but this mechanism should be further studied. Future research will be aimed at developing the technology to be used under large-scale operations and investigating the mode of action of the induction of resistance and the delay of senescence in pear fruits by these treatments.

Acknowledgements

This research was supported by the grants from China Postdoctoral Science Foundation (200801362), the Nature Science Foundation of Jiangsu Provincial Education Department (06KJB210013), the Foundation for the Eminent Talent of Jiangsu University and the Government Scholarship for Study Abroad of Jiangsu.

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Food Chemistry 117 (2009) 627–633

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Biochemical changes in dry-cured loins salted with partial replacements of NaCl by KCl

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ARTICLE INFO

Article history:
Received 5 December 2008
Received in revised form 23 February 2009
Accepted 17 April 2009

Keywords:
Dry-cured loin
Sodium replacement
Potassium chloride
Proteolysis
Lipolysis
Sensory properties

ABSTRACT

The effect of partial replacement of NaCl by KCl in the dry-curing of loins has been evaluated by biochemical and sensory analysis of the final products. Endoproteolytic enzymes like cathpesins B and B + L appeared to be more active when more KCl and less NaCl were present in the curing salt while cathepsin H remained unaffected. Proteolysis was reflected by SDS–PAGE in the higher density of the sarcoplasmic proteins bands within the range 55.0–28.0 kDa. Alanyl aminopeptidase (AAP) was unaffected while arginyl and leucyl aminopeptidases were more activated by KCl. On the other hand, methionyl aminopeptidase and dipeptidylpeptidase I and III were more inhibited as KCl increased in the curing salt blends. The sensory analysis revealed no significant differences between control loins with 100% NaCl and those with up to 50% of KCl substitution. Furthermore, loins elaborated with 50% of each salt obtained the highest scores.

1. Introduction

During the last decades, several studies have linked high levels of sodium chloride (NaCl) intake with raised blood pressure, also known as hypertension (He & MacGregor, 2003; Obarzanek et al., 2003). This fact has promoted the current tendency to reduce sodium intake, in order to proportionally lower blood pressure levels and confer significant health benefits by contributing to a decreased risk of cardiovascular disease (Matthews & Strong, 2005). For these reasons, consumer demand for a variety of low-salt products, especially meat products has increased considerably (Russunen & Puolanne, 2005).

NaCl is an essential ingredient in processed meat products for its contribution to the water-holding capacity, prevention of microbial growth, reduction of water activity, facilitating the solubilisation of certain proteins and conferring a typical salty taste by enhancing the flavour of such food products. Moreover, salt affects some chemical and biochemical phenomena such as proteolysis, lipolysis and lipid oxidation which contribute to the development of texture and typical flavour in meat products (Andrés, Cava, Ventanas, Muriel, & Ruiz, 2004; Guàrdia, Guerrero, Gelabert, Gou, & Arnau, 2006; Toldrá, 2006a). Nevertheless, consumers are demanding lower sodium chloride content in meat products (Gou, Guerrero, Gelabert, & Arnau, 1996; Guàrdia et al., 2006; Russunen & Puolanne, 2005). However, sodium chloride must be reduced without altering the curing process. In this sense, main approaches are focused towards the reduction of the NaCl added or substitution with other salts like KCl, but these actions may require an alteration of the processing techniques. The reduction of the level of NaCl added has led to some problems such as an excess of proteolysis due to the intense action of endopeptidase enzymes resulting in defective textures like softness, and also exopeptidase enzymes which could overact on polypeptides and proteins generating excessive content of low molecular weight nitrogen compounds (peptides and free amino acids), which could lead to unpleasant flavour by enhancing the bitter and metallic tastes (Martín, Córdoba, Antequera, Timón, & Ventanas, 1998; Toldrá 1998; Toldrá, Aristoy, & Flores, 2000). Further, the decrease of salt content in meat products also seems to have a slight promoting effect on the lipolysis phenomena (Andrés, Cava, Martín, Ventanas, & Ruiz, 2005).

The partial substitution of NaCl by KCl appears to be the best alternative to reduce sodium content in meat products. Indeed both salts have similar properties and potassium intake has not been linked to the development of hypertension and cardiovascular diseases (Buemi et al., 2002; Geleijnse et al., 2007; Kimura et al., 2004), although its addition to meat products is mainly limited by its bitter and astringent taste (Reddy & Marth, 1991).

In this sense, numerous attempts have been made during the last decades by several researchers to develop acceptable low salt meat products using NaCl/KCl mixtures. Thus, curing salts with up

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.04.056

to 75% of KCl, in fresh pork sausages (Pasin et al., 1989) or 50% in frankfurters (Whiting & Jenkins, 1981) were used with no negative effect on their sensory properties. Similar results were obtained in cured products like dry-cured ham (Hand, Terrel, & Smith, 1982; Keeton, 1984), dry-cured loin (Gou et al., 1996) and fermented sausages (Gelabert, Gou, Guerrero, & Arnau, 2003). These results indicate that the substitution up to 50% of NaCl with KCl is adequate to obtain an acceptable meat product without affecting its texture, flavour, colour and also microbiological characteristics. Recently, the effect of different chloride salts, including KCl, on porcine muscle proteases has been evaluated (Armenteros, Aristoy, & Toldrá, 2009). However, there is no available information about the effect of a partial or total replacement of NaCl by KCl on the proteolytic and lypolitic phenomena taking place during the dry-curing process. Consequently, the basic aim of this study was to evaluate the effect of the partial NaCl substitution by KCl on the biochemical and sensory characteristics at the end of the ripening stage in dry-cured loins. This meat product may be considered as a model system because of its homogeneous shape and structure and the short time required for ageing.

2. Materials and methods

2.1. Samples

Fifteen fresh loins (2.6 ± 0.8 kg) from *Landrace x Large White* pigs were obtained from a local slaughterhouse. Loins were vacuum-packed and immediately frozen at −40 °C and stored for a minimum of 5 days at −20 °C. Then, the frozen loins were thawed in a cold chamber at 3–4 °C for 5 days, similarly to the industrial process. Three of the loins were used as a control of the raw material. The remaining 12 loins were divided into four groups with three loins each. Loins from one group (batch I) were salted by rubbing and kneading with the traditional NaCl content (100% NaCl, treatment I). The other groups were salted using the same processing conditions, but with a partial substitution of NaCl by KCl. So, the second batch (batch II) was salted with 65% NaCl and 35% KCl (treatment II), the third batch (batch III) was salted with 50% of both salts (treatment III) and finally the fourth batch (batch IV) with 30% NaCl and 70% KCl (treatment IV). All the salting experiments were carried out at 3 ± 1 °C and 90% of air relative humidity (RH) for 6 days and each loin was weighed every day. The amount of the salt mixture was about 2% the weight of the loin and also 150 ppm of KNO_3 and 150 ppm of $NaNO_2$ were added to the mixture as curing agents (Toldrá, 2006b).

After the salting stage, the loins were stuffed into artificial casings and immediately hung in a chamber for ripening at 3 °C and 90% RH for 5 days. Subsequently, the loins were transferred to a drying chamber at 10 °C and 85% RH for 7 days and afterwards at 12 °C and 75% RH until the loins reached 47% of commercial humidity. Dry-cured loins were immediately vacuum-packed and frozen at −20 °C until analysis.

2.2. Sodium and potassium analysis

Briefly, 5 g of minced dry-cured loin was homogenised 1:10 (*p*/*v*) with 50 ml Milli®-Q water in a stomacher (IUL Instruments, Barcelona, Spain) for 10 min at 4 °C, and then centrifuged at 10.000*g* for 20 min at 4 °C. Supernatant was filtered through glass wool and immediately frozen at −20 °C until used. Previous to the analysis, thawed samples were filtered through nylon membrane filters (0.45 μm) and injected using the Metrohm 838 Advanced Sample Processor (Metrohm Ltd., Switzerland). Subsequently, an Advanced Compact IC 861 (Metrohm® Ltd., Herisau, Switzerland) ion chromatograph (IC) was used to determinate sodium (Na^+) and potassium (K^+) cations in sample solutions. Guard column S-Guard and analytical column Metrosep C3–250A (4.0 × 250 mm) were from Metrohm® Ltd. (Herisau, Switzerland). The mobile phase consisted of 3 mM/l nitric acid with 50 ml/l acetone. The separation was monitored using a conductivity detector and a software IC Net 2.3 (Metrohm® Ltd., Herisau, Switzerland) was used for data collecting and processing. The concentration of each cation was determined by interpolation in the corresponding calibration curve. The calibration for the assay was established using a triplicate set of standard solutions of Na^+ and K^+ (Fluka, Switzerland, Sigma, St. Louis, MO). The results were means of three determinations and were expressed in mg/g of sample.

2.3. Sensory analysis

After the drying stage (~2 months), the dry-cured loins from batches I, II, III and IV were subjected to sensory analysis, in order to evaluate the influence of the partial replacement of NaCl by KCl on sensory characteristics. Batch I (100% NaCl, treatment I) was used as a control. The dry-cured loin casing was removed and the loin cut in approximately 5 mm thick slices, using a commercial slice machine. Slices were left to rest for 30 min on white plastic plates at room temperature (20–23 °C) before serving. A glass with about 100 ml of water at 12 °C and a toast of bread without salt were provided for each assessor after each sample. All sessions were held in a sensory panel booth room at 22 °C equipped with white fluorescent lighting (220–230 V, 35 W) and a personal computer (Hewlett–Packard). The samples were assessed by a non-trained panel consisting of 48 members in two different sessions. A preference test (ISO-5495, 1983) was carried out, in which assessors were asked to indicate their preference within each pair consisting of the control and the respective batch (II, III and IV), regarding colour, aroma, texture, taste and overall quality. Sensory evaluations were recorded by computer software using the Compusense® *five* release 4.6 (Compusense Inc., Guelph, ON, Canada).

2.4. Preparation of enzyme extracts for cathepsin/peptidases assays

Minced dry-cured loin (2.5 g sample) was homogenised in 25 ml of 50 mM sodium citrate buffer, pH 5.0, containing 1 mM EDTA and 2% (*v*/*v*) Triton X-100 (for cathepsins) or 4.0 g of sample was homogenised in 20 ml of 50 mM phosphate buffer, containing 5 mM EGTA, pH 7.5 (for peptidases). In both cases, the homogenisation was achieved by 3 × 10 s at 27.000 rpm in ice using a polytron homogenizer (Kinematica AG, Switzerland) and the obtained extracts were centrifuged at 10.000*g* for 20 min at 4 °C. The resulting supernatants were filtered through glass wool and used for the enzyme assays.

2.5. Assay of enzyme activities

Muscles enzyme activities were measured by fluorometric assays using aminoacyl-7-amido-4-methyl coumarin (AMC) as substrate. Cathepsin activities were assayed as previously described by Rico, Toldrá, and Flores (1991) using N-CBZ-Arg-Arg-AMC, N-CBZ-Phe-Arg-AMC and L-Arg-AMC (Sigma, St. Louis, MO) as substrates for cathepsin B, B + L and H, respectively, which were added to the reaction buffers (40 mM sodium phosphate buffer, at pH 6.0 for cathepsin B and B + L or pH 6.8 for cathepsin H, containing 0.4 mM EDTA, 10 mM cysteine, and 0.05 mM substrate).

Aminopeptidases were analyzed by following the method described by Flores, Aristoy, and Toldrá (1997a). Thus, alanyl aminopeptidase (AAP) activity was assayed in 100 mM phosphate buffer, pH 6.5, containing 2 mM β-mercaptoethanol and 0.1 mM Ala-AMC as substrate. Arginyl aminopeptidase (RAP) was assayed in 50 mM phosphate buffer, pH 6.5, containing 200 mM NaCl, 0.25 mM puromicyn and 0.1 mM Arg-AMC as substrate. Leucyl aminopeptidase

(LAP) activity was assayed in 50 mM borate–NaOH buffer, pH 9.5, containing 5 mM magnesium chloride and 0.25 mM Leu-AMC as substrate. Methionyl aminopeptidase (MAP) activity was analyzed as described by Flores, Marina, and Toldrá (2000) in 100 mM phosphate buffer, pH 7.5, containing 10 mM dithiothreitol (DTT), 0.05 mM bestatin and 0.15 mM Ala-AMC as substrate.

Dipeptidyl peptidase activities (DPP) I, II, III and IV were assayed as previously described by Sentandreu and Toldrá (2001). DPP I activity was assayed in 50 mM sodium acetate buffer, pH 5.5, containing 5 mM DTT and 0.5 mM Gly-Arg-AMC as substrate; whereas DPP II was assayed in the same buffer containing 0.032 mM bestatin and 0.5 mM Lys-Ala-AMC as substrate. DPP III activity was assayed in 50 mM sodium tetraborate buffer, pH 8.0, containing 0.05 mM cobalt chloride and 0.5 mM H-Arg-Arg-AMC as substrate and DPP IV was assayed in 50 mM tris-base buffer, pH 8.0, containing 5 mM DTT and 0.25 mM Gly-Pro-AMC as substrate.

In all cases, 50 μl of extract was diluted with 250 μl of the corresponding reaction buffer containing the substrate, and incubated in a multiple plate at 37 °C for 15 min. The fluorescence was determined using excitation and emission wavelengths of 355 and 460 nm, respectively by using a multiscan fluorometer (Fluoroskan Ascent FL, Labsystems, Helsinki, Finland). Four replicates were performed for each enzyme assay, except when determining dipeptidyl peptidase activity, where three replicates were done. One unit of enzyme activity (U) was defined as the amount of enzyme which hydrolyses 1 μmol of substrate per min at 37 °C.

2.6. Proteolysis analysis

The extractions of the sarcoplasmic and myofibrillar proteins were performed as described by Molina and Toldrá (1992). The samples were ground, homogenised 1:10 (*w*/*v*) with 30 mM phosphate buffer, pH 7.4 in a stomacher for 3 min at 4 °C and centrifuged at 10.000*g* for 20 min at 4 °C. The supernatant containing the sarcoplasmic proteins was filtered through glass wool and kept at 4 °C. This operation was repeated with the pellets up to three times to recover all sarcoplasmic proteins by pooling the supernatants which were stored at 4 °C until use. The resulting pellet was finally homogenised with nine volumes of 100 mM phosphate buffer at pH 7.4, containing 0.7 M potassium iodide and 0.02% sodium azide using a stomacher for 8 min and then centrifuged at 10.000*g*, for 20 min at 4 °C. The supernatant containing myofibrillar proteins was collected. The protein concentration of both, sarcoplasmic and myofibrillar proteins extracts, was determined by the method of Smith et al. (1985), using bicinchoninic acid as a reagent and the bovine serum albumin as a standard (Sigma, St. Louis, MO).

The study of the proteolysis was done by sodium dodecyl sulphate-polyacrylamine gel electrophoresis (SDS–PAGE) as described by Toldrá, Miralles, and Flores (1992). Proteins from the sarcoplasmic and myofibrillar extracts were denaturalised by mixing in the ratio 1:1 (*v*/*v*) with 50 mM Tris buffer, pH 6.8, containing 8 M urea, 2 M thiourea, 75 mM DTT, 3% (*w*/*v*) SDS and 0.05% bromophenol blue (sample buffer). The mixture was heated at 100 °C for 4 min and stored at −20 °C until use. Thawed samples were diluted with the sample buffer up to 1 mg/ml protein concentration and then, 12 μl of these samples were injected, respectively, in each line into the gels. SDS–PAGE was carried out using 12% polyacrylamide gels and stained with coomassie brilliant blue R-250. Standard proteins from BioRad (Hercules, CA, USA) were simultaneously run for molecular mass estimation. Finally, the gels were scanned with Fujifilm Las 1000 Intelligent Dark Box II (Fujifilm, SA, Spain).

2.7. Free amino acids analysis

Samples for free amino acid analysis were extracted and deproteinised following the method described by Aristoy and Toldrá (1991). Dry-cured loin samples were cut, minced and 5 g subsequently homogenised with 0.01 M HCl 1:5 (*v*/*v*) in a stomacher for 8 min at 4 °C and then centrifuged at 10.000*g* during 20 min at 4 °C. The supernatant was filtered through glass wool and stored at −20 °C until required. Next, 200 μl of thawed samples plus 50 μl of an internal standard solution (1 mM norleucine in 0.01 M HCl), were deproteinised with 600 μl of acetonitrile. Free amino acids in the supernatant were derivatised to their phenylthiocarbamyl derivates according to the method of Bidlingmeyer, Cohen, Tavoin, and Frost (1987) and analyzed by reversed-phase HPLC (1100 Agilent, Palo Alto, CA, USA) in a waters Nova Pack® C18 column (3.9 × 300mn) (Waters Corporation, Milford, MA, USA) and ultraviolet detection (254 nm), as described by Flores, Aristoy, Spanier, and Toldrá (1997b).

2.8. Free fatty acids analysis

Total lipids were extracted from dry-cured loin samples according to the method of Folch, Lees, and Sloane Stanlye (1957), using dichloromethane:methanol 2:1 (*v*/*v*) instead of chloroform:methanol 2:1 (*v*/*v*) as solvent. Free fatty acids were purified from the total lipids as described by Countron-Gambotti and Gandemer (1999) using an anion exchange resin (Amberlyst A26, Rohm & Haas, Delf, Nederland) and determined by means of gas chromatography of the corresponding methyl esters (Needs, Ford, Owen, & Tuckley, 1983). The derivatives were obtained according to the method of Berry, Cevallos, and Wade (1965) and the analysis of the fatty acid methyl esters (FAME) was carried out as described by Navarro, Nadal, Nieto, and Flores (2001) using a Fison 816 gas chromatograph (Fisons Instruments, San Carlos, CA, US) equipped with a flame ionisation detector. The split ratio used was 1:50. The capillary column was a DB-225 (J&W Scientific, Barcelona, Spain; 30 m long, 0.25 mm i.d., 0.25 μm film thickness) and the detector and injector temperatures were both set to 240 °C. The individual fatty acids were identified by comparing their retention times with those of standard fatty acids using the araquidonic acid as internal standard for quantification.

2.9. Statistical analysis

The effect of the partial replacement of NaCl by KCl on the studied variables (enzyme activities, free amino acids, free fatty acids and sodium and potassium content) was done by analysis of variance using the STATHGRAPHICS Plus 5.1 version software (Manugistics Inc., Rockville, MD, USA). In those cases where the effects were significant, the means were compared using Fisher's least significant difference (LSD) procedure.

3. Results and discussion

3.1. Sodium and potassium content

The residual content of sodium and potassium cations was determined in the dry-cured loins at the end of the ripening stage. As expected (Table 1), the dry-cured loin salted with a mixture of NaCl and KCl (treatments II, III and IV) showed an enhanced amount of residual K^+ which increased proportionally to the added KCl (taking into account the naturally content of this cation in muscle (0.16 mEq in treatment I). This confirms the correct penetration of this chloride salt. Furthermore, as observed in Table 1 the use of NaCl/KCl mixtures effectively reduced the Na^+mEq:K^+mEq ratio in dry-cured loins from 7.62 to 0.79 for 0 to 70% of substitution, respectively.

Table 1
Sodium and potassium content in dry-cured loins submitted to four types of salting treatments: I: Control, 100% NaCl; II: 65% NaCl–35% KCl; III: 50% NaCl–50% KCl and IV: 30% NaCl–70% KCl.

Parameter	I	II	III	IV
Na^+ (mg g^{-1})	27.82	24.54	15.55	12.30
K^+ (mg g^{-1})	6.21	17.07	18.59	26.31
Na^+:K^+ ratio	4.48	1.44	0.84	0.47
mEq Na^+	1.21	1.07	0.68	0.53
mEq K^+	0.16	0.44	0.48	0.67
Na^+mEq:K^+ ratio	7.62	2.44	1.42	0.79

mEq Na = mg g^{-1} Na^+/23.
mEq K = mg g^{-1} K^+/39.

3.2. Effect of the salting treatments on sensory analysis

The results of the sensory analysis of the dry-cured loin submitted to the four types of salting treatments are shown in Table 2. Non significant differences ($p < 0.05$) were found with respect to aroma, texture and taste attributes between the control (batch I, 100% NaCl) and batches II and III salted with substitutions up to 50% of NaCl by KCl. Furthermore, the dry-cured loins salted with the treatments II and III were preferred by assessors with respect to the colour and overall quality. Whereas, the dry-cured loins salted with 70% of KCl (treatment IV) received lower scores than the control respect to all attributes, except colour, which did not show significant differences ($p < 0.05$). These results are in agreement with those reported by Gelabert et al. (2003) and Gou et al. (1996) for fermented sausages and dry-cured loins and indicated that substitutions up to 50% of NaCl by KCl did not affect the texture or colour and also provided an acceptable flavour without a noticeable bitter taste. In fact, the best scores in our study corresponded to the dry-cured loin salted with 50% of NaCl substitution by KCl (treatment III).

3.3. Effect of the salting treatments on enzyme activities

The cathepsin activity in the dry-cured loins submitted to different salting treatments was studied for its relevance among the proteolytic changes taking place during the processing of these products. Previous studies had demonstrated that salt (NaCl) induces an inhibitory effect on cathepsins B and B + L activities (Rico et al., 1991; Toldrá, Rico, & Flores, 1993). Nevertheless, the presence of 50–70% KCl (treatment III and IV) in the mixture of curing salts favoured a higher cathepsin B and B + L activities ($p < 0.05$) in comparison to that with 100% NaCl (treatment I) as shown in Table 3, while significant differences ($p < 0.05$) were not found between the activity of these enzymes in the dry-cured loin salted with the treatments I and II. The cathepsin H activity was not seriously affected by any of the experimental salting treatments (II, III and IV). These results suggested that the partial replacement of 50% NaCl by KCl or higher could led to a higher cathepsin B and B + L activities and consequently it may have resulted in a prolongation of proteolysis.

The activity of dipeptidyl peptidases in dry-cured loins was different depending on the type of salted treatments used (Table 3). The exception was DPPII, that was not influenced by the type of treatment employed ($p < 0.05$). DPPI activity was significantly higher ($p < 0.05$) in the dry-cured loin salted with 100% NaCl (treatment I) in comparison to the other. However, DPPIII activity decreased ($p < 0.05$) when the substitution of KCl increased. Finally, DPPIV activity was not affected by the salting treatment except by the treatment II, which exerted a slight inhibitory effect ($p < 0.05$).

Finally, the aminopeptidase activities were affected by the salting treatments employed as follows (Table 3). RAP showed higher activity in treatments III and IV and LAP in treatment IV. On the contrary, MAP was more inhibited in the presence of any concentration of potassium salt. The effect of potassium substitution on AAP activity was variable and no clear relationships between experimental salting treatments (II, IIII and IV) and traditional treatment could be found.

3.4. Effect of the salting treatments on proteolysis

Sarcoplasmic and myofibrillar proteins were extracted from the dry-cured loins salted with four different salt formulations. As shown in Figs. 1 and 2, the most relevant differences were observed in the sarcoplasmic proteins while the myofibrillar proteins patterns were very similar. Indeed, the electropherograms of myofibrillar proteins (Fig. 1) in all samples showed an intense degradation of the myosin and actin bands throughout the ripening stage, as already observed in dry-cured ham (Toldrá et al., 1993).

On the other hand, the electrophoretic profile of sarcoplasmic proteins showed more density of the bands in the dry-cured loin salted with partial replacement of NaCl (treatments II, III and IV) than in those salted traditionally (treatment I), as shown in

Table 2
Sensory analysis (preference test) of the dry-cured loins at the end of the ripening stage.

Sensory traits	I[a]	II[a]	III[a]	IV[a]	*P*-values[b]
Colour	8[c]	40			<0.001
	5		43		<0.001
	23			25	n.s
Aroma	28	20			n.s
	29		19		n.s
	34			14	<0.01
Texture	18	30			n.s
	19		29		n.s
	31			17	<0.01
Taste	18	30			n.s
	21		27		n.s
	37			11	<0.001
Overall quality	14	34			<0.01
	17		31		<0.1
	38			10	<0.001

[a] Salting treatments: I (Control,100% NaCl); II = dry-cured loins salted with the treatment II (65% NaCl–35% KCl); III: dry-cured loins salted with the treatment III (50% NaCl–50% KCl); IV: dry-cured loins salted with the treatment IV (30% NaCl–70% KCl).
[b] Significance levels: n.s ($p > 0.05$).
[c] Number of assessors that preferred each treatment (total number equal to 48).

Table 3
Proteolytic enzyme activity ($U_x\ g_x\ 10^{-3}$) in the dry-cured loins with the four types of salting treatments: I: Control, 100% NaCl; II: 65% NaCl–35% KCl; III: 50% NaCl–50% KCl and IV: 30% NaCl–70% KCl.

Enzyme	I	II	III	IV	Pooled SE[A]
Cathepsin B	1.26[a]	1.33[a]	2.29[b]	2.23[b]	0.10
Cathepsin B + L	3.68[a]	4.46[a]	7.92[b]	8.63[b]	0.41
Cathepsin H	0.08	0.24	0.02	0.12	0.09
Dipeptidyl peptidase I	4.64[a]	2.35[b]	1.00[c]	2.42[b]	0.18
Dipeptidyl peptidase II	0.28	0.31	0.36	0.37	0.08
Dipeptidyl peptidase III	5.76[a]	5.02[ab]	4.10[b]	1.63[c]	0.28
Dipeptidyl peptidase IV	1.19[a]	0.39[b]	0.99[a]	1.25[a]	0.11
Alanyl aminopeptidase	8.48[a]	9.45[ab]	6.52[c]	10.23[b]	0.42
Arginyl aminopeptidase	0.71[a]	0.61[a]	1.08[b]	0.92[c]	0.05
Leucil aminopeptidase	4.37[a]	4.97[a]	4.73[a]	6.41[b]	0.13
Methionyl aminopeptidase	2.11[a]	1.22[b]	0.86[b]	1.14[b]	0.21

Means with different superscripts letters differ significantly ($p < 0.05$).
[A] SE: standard error.

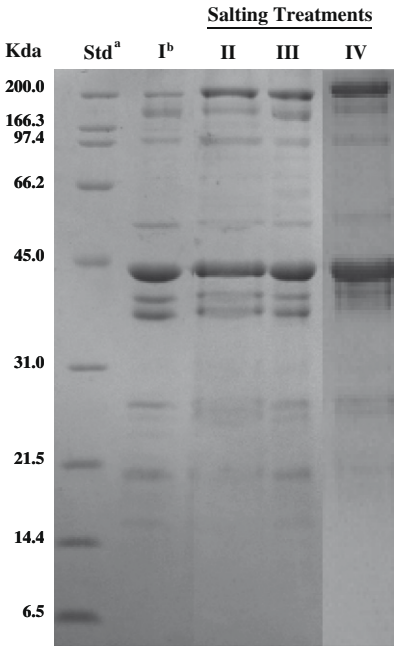

Fig. 1. Twelve percent SDS–PAGE gel of myofibrillar proteins in the dry-cured loin salted with the four types of treatments. [a]Std: BioRad molecular weight standards. [b]Treatment I: 100% NaCl, treatment II: 65% NaCl–35% KCl, treatment III: 50% NaCl–50% KCl and treatment IV: 30% NaCl–70% KCl.

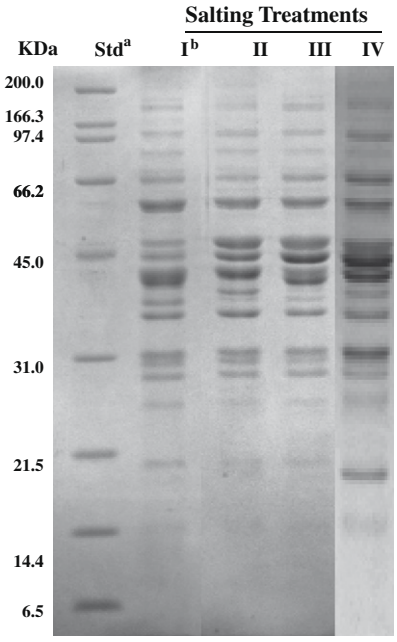

Fig. 2. Twelve percent SDS–PAGE gel of sarcoplasmic proteins in the dry-cured loin salted with the four types of treatments. [a]Std: BioRad molecular weight standards. [b]Treatment I: 100% NaCl, treatment II: 65% NaCl–35% KCl, treatment III: 50% NaCl–50% KCl and treatment IV: 30% NaCl–70% KCl.

Fig. 2. More concretely, the dry-cured loins salted with the treatments II, III and IV showed a progressive increase in the intensity of bands between 55 kDa and 28 kDa. This is also related to the higher cathepsin B and B + L activity in those loins with larger amount of potassium. The exception was the 36 kDa band corresponding to glyceraldehydes-3-phosphate dehydrogenase, which remained in all samples with a similar density.

Free amino acids which are final products of proteolysis, are of great importance not only for their attribution to specific taste but also for their involvement in degradation reactions that generate volatile compounds which contribute to the flavour in meat products (Toldrá et al., 2000). The results of the free amino acids concentrations in the dry-cured loins submitted to the different treatments are presented in Table 4. There was no statistically significant effect of the salting treatment on the release of total free amino acids ($p < 0.05$), which indicated a similar aminopeptidase activity. Similar results were found in fermented fish sauce (Sanceda, Suzuki, & Kuruta, 2003) and Feta cheese (Katsiari, Alichanidis,

Table 4
Free amino acids concentration (mg/100 g sample) in the dry-cured loins salted with the four types of salting treatments.

Free amino acids (FAA)	I	II	III	IV
Asp	7.36	7.98	12.21	5.39
Glu	34.86	35.34	44.40	27.23
Ser	41.34	36.32	46.17	36.94
Asn	15.93	12.27	14.12	10.78
Gly	32.13	30.87	36.48	30.21
Gln	34.39[a]	33.31[a]	37.23[ab]	45.32[b]
ß-ala[A]	3.55	3.38	4.21	3.73
Ala	70.94	75.39	79.90	68.74
Arg	36.41[a]	25.85[b]	33.48[ab]	28.90[ab]
Pro	35.92[a]	34.73[a]	58.36[b]	37.45[a]
Tyr	49.63[a]	46.70[ab]	45.84[ab]	36.83[b]
His	17.91	13.63	18.62	14.67
Thr	36.65	33.61	41.01	32.45
Val	54.56[a]	52.34[a]	69.33[b]	44.22[a]
Met	32.78	30.80	30.17	27.67
Ile	49.88	45.40	48.2	39.74
Leu	92.36	75.50	83.00	74.15
Phe	55.34	50.08	49.66	46.06
Lys	47.94	33.65	44.99	33.35
Total FAA	749.88	677.16	797.40	643.83

Results are expressed as means of the three replicates. Means in the same line with different letters are significantly different ($p < 0.05$%). Treatment I: Control, 100% NaCl; treatment II: 65% NaCl–35% KCl; treatment III: 50% NaCl–50% KCl and treatment IV: 30% NaCl–70% KCl.
[A] ß-Alanine.

Table 5
Free fatty acids composition (expressed as mg/100 g sample) in the dry-cured loin salted with the four types of salting treatments: I: Control, 100% NaCl; II: 65% NaCl–35% KCl; III: 50% NaCl–50% KCl and IV: 30% NaCl–70% KCl.

Free fatty acids (FFA)	I	II	III	IV
C14:0	2.88[ab]	4.14[b]	1.81[a]	2.66[ab]
C16:0	95.30	103.05	92.37	105.98
C18:0	59.49	60.80	57.58	62.06
SFA[A]	157.67	167.99	152.21	170.70
C16:1	8.52	12.20	8.00	13.35
C18:1	136.81	150.98	119.25	177.39
C20:1	2.25[ab]	2.24[ab]	1.62[a]	3.00[b]
MUFA[B]	147.58	165.42	128.88	193.74
C18:2	145.13	158.85	144.84	164.48
C18:3	3.53	3.74	3.41	4.46
C20:2	2.06[a]	1.69[a]	2.46[ab]	3.29[b]
C20:3	4.88	3.93	5.18	5.48
C20:4	42.98	30.24	43.75	44.60
C22:4	2.10	1.01	1.65	2.59
PUFA[C]	203.47	165.41	215.64	224.91
Total FFA	508.72	498.82	496.73	589.36

Results are expressed as means of three replicates.
Means in the same line with different letters are significantly different ($p < 0.05$).
[A] SFA: total saturated fatty acids amount.
[B] MUFA: total monounsaturated fatty acids amount.
[C] PUFA: total polyunsaturated fatty acids amount.

Voutsinas, & Roussis, 2000), in which the proteolysis was not influenced by the type of the salting treatment employed during their manufacture.

On the other hand, the concentrations of some amino acids, like Glu, Pro and Val in the loins salted with the treatment III, were significantly different ($p < 0.05$). The good taste found by sensory analysis in the dry-cured loins submitted to treatment III could be due in part to the contribution of these amino acids (Kato, Rhue, & Nishimura, 1989; Nishimira & Kato, 1988).

3.5. Effect of the salting treatments on free fatty acids content

Free fatty acids (FFA) were analyzed at the end of the ripening stage as indicators of the lipolysis process in the dry-cured loins (Table 5). The treatments did not promote significant differences ($p < 0.05$) in either total amounts of saturated (SFA), monounsaturated (MUFA) or polyunsaturated (PUFA) fatty acids content in the dry-cured loins. These results are consistent with those reported by other authors in cheese (Katsiari, Voutsinas, Alichanidis, & Roussis 2001; Lindsay, Hargett, & Bush, 1982), where the replacement up to 50% of NaCl by KCl did not affect the lipolysis phenomena or in dry-cured ham (Countron-Gambotti, Gandemer, Rousset, Maestrini, & Casabianca, 1999) where salt content did not affect the fatty acid compositions.

4. Conclusions

These results indicate that the replacement of up to 50% NaCl by KCl in the salting stage of the processing of dry-cured loins did not significantly affect the proteolysis and lipolysis phenomena taking place. Also, the sensory analysis of the final products did not reveal marked differences among loins with up to 50% of substitution and even those with 50% KCl were the most valued. These results may be useful as a starting point for the study of NaCl reduction in other dry-cured meat products such as dry-cured ham.

Acknowledgements

This work was supported by grant AGL2004-05064-C02-01 from Ministry of Education and Science (Madrid, Spain) and FEDER. FPI/MEC scholarship to M. Armenteros is also acknowledged. Work prepared within the Unidad Asociada IAD (UPV)-IATA (CSIC) framework.

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Food Chemistry 117 (2009) 634–640

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

The fate of fungicide and insecticide residues in Australian wine grape by-products following field application

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ARTICLE INFO

Article history:
Received 8 April 2008
Received in revised form 15 April 2009
Accepted 17 April 2009

Keywords:
Grape seed
Grape marc
Pesticide residues
Systemic pesticides
Gas chromatography
Liquid chromatography–tandem mass spectrometry

ABSTRACT

Pesticides applied to grape vines before harvest may concentrate in the grape seed due to their high oil solubility. Twenty-four samples of grape marc, representing a range of red and white wine grape varieties, were collected and analysed for selected fungicides and insecticides. Fifteen of the 24 samples were matched with insecticide and fungicide application diaries. Residue concentrations of the fungicides, procymidone, iprodione, cyprodinil, fenhexamid, fludioxinil, pyrimethanil and trifloxystrobin, and the insecticides, indoxacarb and tebufenozide, were higher in grape seed oil and grape seed meal than in the fruit and the marc. The relative concentrations were approximately proportional to the octanol to water partition coefficients, log K_{ow}. A range of other fungicides and insecticides were detected but were not significantly concentrated in the oil and seed meal relative to fruit and marc. The presence of pesticide residues in grape seed oil and grape seed meal will impact on the possibility of producing these wine by-products.

1. Introduction

Large quantities of grape marc (solids remaining after juice extraction) are produced by regional wineries in Victoria, Australia (Jordan, 2002). Currently, the marc is either fed to livestock or consigned to land fill as the costs for transport and reprocessing into distilled alcohol, tartaric acid or polyphenolic supplements make these options uneconomic. Alternatively, grape seed oil (for human consumption) and seed meal (for animal feed), could be produced from the marc. However, previous work (Cabras & Angioni, 2000; Navarro, Barba, Oliva, Navarro, & Pardo, 1999; Teixeira, Aguiar, Afonso, Alves, & Bastos, 2004; Tsiropolous, Aplada-Sarlis, & Miliadis, 1999) suggests that the grape seed oil and the seed meal may contain unacceptable levels of systemic pesticide residues that were applied to the vines prior to harvest. Residue levels of five out of six pesticides (penconazole, chlorpyrifos, vinclozolin, fenarimol and metalaxyl) applied to grape vines prior to harvest were higher in grape marc than in the crushed grapes during elaboration of red wines. Also, the residue levels in must, 4 days after vinification, were significantly lower than in crushed grapes for the six pesticides (including mancozeb) (Navarro et al., 1999). Residue levels of 10 out of 12 fungicides applied to vines prior to harvest were lower in must and wine than in the grapes. Six out of eight of these fungicides had reduced levels in clarified must, suggesting that the residues tend to be retained in the solid portion of the must (seeds, skins and stalks). Similarly, the levels of six out of nine organophosphate insecticides were lower in the wine than in the grapes and the levels of five of these organophosphate insecticides were lower in clarified must (after solids removal) than in the must. However, dimethoate and methidathion, the compounds with lowest log K_{ow} (more soluble in water and ethanol), were at higher concentrations in the must and wine than in the marc (Cabras & Angioni, 2000). Another study by Tsiropolous et al. (1999) showed significantly lower levels of the insecticide teflubenzuron in wine than in the grapes. Clarified must residue levels were also significantly lower than in must suggesting that teflubenzuron residues were associated with the solid portion of seed, skins and stalks rather than the juice of the grape. It was suggested by Teixeira et al. (2004) that systemic pesticides, such as oxydixyl, were preferentially found in the grape pulp, whilst contact pesticides, such as folpet, were present only in the skin. The relationship between the levels in different parts of the grape was also thought to be dependent on the time between application and sampling.

This study investigates the potential concentration of applied fungicides and insecticides in grape seed produced from grape marc collected from selected wineries in Victoria, Australia. Additionally, the relationship between the octanol to water coefficient (log K_{ow}) and the concentration of each fungicide/ insecticide in grape seed was explored.

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doi:10.1016/j.foodchem.2009.04.061

2. Materials and methods

2.1. Reagents

Pesticide hexanes (nanograde 95% n-hexane), nanograde dichloromethane (DCM), and HPLC grade methanol, were all purchased from Mallinkrodt, Kentucky, USA. Acetone was redistilled from AR grade solvent (BDH/Merck, Kilsyth, Australia). HPLC grade acetonitrile was purchased from OmniSolv, EM Science NJ, USA. Labchem grade Florisil was purchased from Ajax Fine Chemicals, Sydney Australia, and activated by placing in a muffle furnace at 550 °C for 2 h prior to use. Anhydrous AR grade sodium sulphate and anhydrous AR grade ammonium acetate were purchased from Ajax Fine Chemicals, Sydney Australia. Water was Type 1 (18 Megohm resistivity).

2.2. Certified standards

Cyprodinil (98.5% purity), fenhexamid (99.5%), fludioxinil (98.0%), metalaxyl (99.5%), pyraclostrobin (99.7%), trifloxystrobin (99.0%), quinoxyfen (99.0%), spinosad A + D (95%), spiroxamine (97.5%), captan (99.5%), chlorothalonil (98.5%), procymidone (98.0%) and iprodione (99.0%) were purchased from Dr. Ehrenstorfer-Schafers, Augsburg, Germany. Indoxacarb (99.3%), pyrimethanil (99.6%), triadimenol (97.7%) and tebufenozide (99.9%) were purchased from the National Measurement Institute, Pymble, Australia. Penconazole (99%) and myclobutanil (99.9%) were purchased from Riedel de Haan, Hanover, Germany and tributyl phosphate (TBP) was purchased from Chem Service, PA, USA.

2.3. Sampling and sample preparation

2.3.1. Fruit and by-product samples

Grape marc and, where possible, matching fruit samples were collected from regional Victorian wineries during the autumn, 2004 vintage. Of a total of 24 samples of marc, 15 samples were matched with application diaries. A target list of fungicides and insecticides was developed from examination of the available diaries. Fungicides represented the highest proportion of chemicals applied to the vines prior to harvest. There was no record of organophosphate or synthetic pyrethroid insecticide usage. Central Victorian marc samples were collected fresh and transferred to Stoney Creek Oils (SCO) located at Talbot in central Victoria. The seed was wet-separated immediately at SCO and the seed dried on forced air drying screens, sieved and stored for pressing. Yarra Valley marc samples were collected and immediately frozen prior to transfer to SCO. The frozen marc samples were thawed and the seed wet-separated, dried, sieved and stored for pressing. Where possible, matching fruit samples were collected from harvest fruit bins at the wineries. However, due to harvesting patterns, there is no guarantee that the fruit samples were representative of chemical usage throughout harvest blocks. A standard linseed oil press was used to cold-press the seed. Residue free seed oil was used to clean the oil press between samples. Seed meal samples were also collected from each seed batch processed and analysed for organic residue content.

2.3.2. Fruit and marc extraction

Fifty grammes of marc or homogenised grape berry were extracted with 200 ml of acetone using a probe homogeniser. The sample was vacuum-filtered, the filtrate mixed with 650 ml of saturated aqueous sodium sulphate and extracted with 1×100 ml and 2×50 ml volumes of DCM. The extracts were combined, passed through an anhydrous sodium sulphate drying column and inverted into 10 ml of hexane.

2.3.3. Seed oil extraction

Two grammes of oil were diluted with 25 ml of hexane and extracted with 1×50 ml and a further 2×25 ml of hexane saturated acetonitrile. The acetonitrile layers were combined, dried, and inverted into 2 ml of dichloromethane (DCM).

2.3.4. Seed meal extraction

Approximately 25 g of meal were mixed with 200 ml of 35% water/65% acetone and shaken for 30 min. The sample was vacuum-filtered, the filtrate mixed with 650 ml of saturated aqueous sodium sulphate and extracted with 1×100 ml and 2×50 ml volumes of DCM. The DCM volumes were combined, dried through an anhydrous sodium sulphate drying column and inverted into 10 ml of hexane.

2.4. Apparatus

2.4.1. Gas chromatography–nitrogen phosphorus detection (GC–NPD)

The fungicides, chlorothalonil, penconazole, procymidone, iprodione, triadimenol and myclobutanil were determined using a Varian 3400 CX capillary gas chromatograph fitted with nitrogen–phosphorus detector (Varian, Mulgrave, Australia). An aliquot (2 μl) of hexane (derived for each sample as described above) was simultaneously injected onto parallel columns (15 m, 0.32 ID dimethyl-polysiloxane stationary phase, J&W® DB-1) and a 15 m, 0.32 ID 50% diphenyl-dimethyl-polysiloxane stationary phase (J&W® DB-17) via a split/splitless injector with a split ratio of 1:20. The GC oven was temperature-programmed (120 °C 0–2 min, 120–300 °C at 20 °C/min, held 300 °C for 1 min) for optimum separation efficiency. The injector and detector temperatures were set at 280 and 320 °C, respectively. Helium was used as carrier gas. Varian Star software (V6.0) was used to manage the chromatographic data. The organic residues were quantified by comparison with external standards.

2.4.2. Gas chromatography–electron capture detection (GC–ECD)

The fungicide captan was determined using a Varian 3800 capillary gas chromatograph fitted with an electron capture detector (Varian, Mulgrave, Australia). The hexane extract obtained from sample processing, as described above, was further purified by adding 0.5 ml of the extract to 3.0 g of activated florisil, followed by elution with 25 ml of 1% acetonitrile/49% hexane/50% DCM. This was inverted to 2 ml of hexane. An aliquot (2 μl) of hexane was injected simultaneously onto parallel capillary columns (15 m, 0.32 ID 14% cyanopropyl-phenyl on dimethyl-polysiloxane stationary phase, J&W® DB-1701) and a 15 m, 0.32 ID, 25% cyanopropyl, 25% diphenyl, 50% dimethyl-polysiloxane stationary phase (Restek®, RTX-225) via a split/splitless injector with a split ratio of 1:20. The GC oven temperature was set at 200 °C. The injector and detector temperatures were set at 280 and 350 °C, respectively. Helium was used as carrier gas. Varian Star software (V6.0) was used to manage the chromatographic data. The organic residues were quantified by comparison with external standards.

2.4.3. Liquid chromatography–tandem mass spectrometry (LC–MS/MS)

The fungicides, cyprodinil, fenhexamid, metalaxyl, fludioxinil, pyraclostrobin, quinoxyfen, trifloxystrobin and pyrimethanil, and the insecticides, spinosad, spiroxamine, indoxacarb and tebufenozide, were determined using a Waters 2975 separation module interfaced with a Waters Micromass Quattro Micro tandem mass spectrometer operating in the positive ion electrospray mode (Waters Micromass, Manchester, UK). An aliquot (0.2 ml) of the hexane extract, obtained as described above, was inverted into methanol (4 ml) containing 0.06 μg/ml of tributylphosphate (TBP) as internal standard. The compounds were separated with

a 150 mm × 2.0 mm Luna® 5 μm C18(2) analytical HPLC column fitted with a C18 guard column. The HPLC column was maintained at 25 °C. The mobile phase consisted of (A) 20% methanol in 5 mM ammonium acetate and (B) 90% methanol in 5 mM ammonium acetate with the following gradient: 100% A–100% B (0–15 min), 100% B (15–28 min), 100% B–100% A (28–30 min) with a flow rate of 0.2 ml/min. Masslynx Software (V4.0) was used for data processing. Residues were quantified using external standards and each standard set was assayed a minimum of three times during each sample batch run. Each standard also contained TBP (0.06 μg/ml) as the internal standard. Sample and recovery concentrations were calculated from a linear regression of the standards. Samples and standards were corrected for internal standard (IS) response. The tandem mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. The precursor ions, product ions, confirmatory product ions and retention times for each compound are listed in Table 1.

2.5. Quality control

Spiked recoveries for each residue were included in every sample extraction batch and the recoveries determined as described for samples. Samples were spiked in the range of 0.05–1.2 mg/kg. Mean recoveries for GC residues were; 79% in fruit, 92% in marc, 89% in seed oil and 85% in seed meal. Mean recoveries for LC–MS/MS residues were: 65% in fruit, 91% in marc, 69% in seed oil and 51% in seed meal. The reported data were not corrected for batch recoveries. The lower recovery of LC–MS/MS analytes in grapes is related to the matrix suppression of mass spectrum ion response routinely observed in extracts from fruit high in moisture and soluble sugars, such as grapes and pome fruit. This effect was also observed in LC–MS/MS in the extracts of seed oil and seed meal. Matrix enhancement of the LC.MS/MS response was observed for pyrimethanil, and the strobilurin fungicides, pyraclostrobin and trifloxystrobin.

3. Results and discussion

3.1. General

Table 2 shows the residue levels present in fruit, marc, seed oil and seed meal for fungicides that are routinely used in Victoria, Australia. At the time of the study, the maximum residue limit (MRL) for grapes was taken from the Food Standards Australia and New Zealand (FSANZ) Code (Food Standards Australia New Zealand, 2006). FSANZ MRLs for agricultural produce are applied to derived foods, such as seed oils, where there is no separate MRL listed.

3.2. Cyprodinil and fludioxinil

The anilinopyrimidine systemic fungicide cyprodinil and the phenylpyrrole non-systemic fungicide fludioxinil are applied in a mixture formulated to control grey mould, *Botrytis cinerea* (Australian Pesticide and Veterinary Medicine Authority (APVMA), 2008). The formulated mixture has a withholding period of four weeks and a restriction of a maximum of two applications per season. No samples contained either fungicide above the Australian MRL; however, the residues in the seed oil from sample CH10 may have been above the MRL at the withholding period. Label instructions limit applications prior to grape veraison at the latest, so it is unlikely that any crop would be harvested at the minimum withholding period. The relative concentrations of the fungicides in the samples reflect the composition of the formulation (37.5% cyprodinil and 25% fludioxinil).

3.3. Procymidone

The systemic dicarboximide fungicide procymidone is registered for control of grey mould in wine grapes and has a withholding period of 7 days (APVMA, 2008). Seven out of nine seed oil samples contained residues above the Australian MRL of 2 mg/kg (Food Standards Australia New Zealand (FSANZ), 2006). Four out of nine marc samples also contained residues above the MRL. The residues for grape sample CH4 was more than twice the MRL for grapes, indicating that the withholding period may be too short, especially where multiple applications have been made prior to harvest. There were no application diary records for samples CH8, CH14 and Ref#14. The data suggest that it is likely that sample CH15 had an unrecorded application close to the withholding period. Sample CH15 also reported a grape sample above the MRL, supporting the previous observation that the withholding period was too short for the listed MRL. Results show that procymidone is a high-risk fungicide for seed oil, marc, seed meal and grapes in these samples. In 2005, following numerous international reports of its toxicity, additional application and usage controls for procymidone, to control grey mould in wine grapes, were implemented by the Australian Pesticide and Veterinary Medicine Authority. The revised controls have resulted in the phase-out of procymidone use on wine grapes in Australia.

Table 1
LC–MS/MS precursor ions, product ions, confirmatory product ions and retention times for the fungicides and pesticides measured in this study.

Fungicide/insecticide	Precursor ion (*m*/*z*)	Production quantitative (*m*/*z*)	Production confirmatory (*m*/*z*)	Retention time (min)
Cyprodinil	226.10	92.64	117.86	18.99
Fenhexamid	301.97	96.79	142.70	17.70
Fludioxinil	248.98	157.86	185.00	17.13
Pyraclostrobin	388.06	162.96	132.80	19.04
Quinoxyfen	307.90	196.88	161.85	21.32
Trifloxystrobin	409.18	185.94	144.81	19.62
Pyrimethanil	200.11	106.92	81.90	16.94
Spinosad A	732.36	141.91	na	24.87
Spinosad D	746.67	141.89	na	26.58
Spiroxamine	298.23	143.92	99.97	21.09
Indoxacarb	528.15	202.91	149.82	19.42
Tebufenozide	353.40	297.03	na	18.35
Metalaxyl	280.35	220.10	na	15.47
Tributylphosphate (TBP)[a]	267.44	98.78	na	19.83

na = not available.
[a] TBP is the internal standard.

Table 2
MRL, label withholding periods and fungicide residues in fruit, marc, seed oil and seed meal.

Fungicide (MRL) Label WHP (days)	Application: days prior to harvest	Sample/variety[a]	Fruit (mg/kg)	Marc (mg/kg)	Seed oil (mg/kg)	Seed meal (mg/kg)
Cyprodinil (2 mg/kg)	nr	CH4 (C)	<0.01	<0.01	0.36	0.01
Label WHP = 28 days	72	CH8 (C)	0.01	0.46	0.90	0.48
	81	CH9 (C)	0.08	0.20	0.63	0.41
	88	CH10 (P)	0.20	0.35	1.6	0.84
	nr	CH14 (M)	<0.01	<0.01	0.09	<0.01
	nr	Ref#14 (S)	n/s	0.10	0.18	0.29
Fludioxinil (2 mg/kg)	81	CH9 (C)	0.10	0.12	0.23	Trace
Label WHP = 28 days	88	CH10 (P)	Trace	0.21	0.56	0.19
	nr	Ref#14 (S)	n/s	0.11	0.27	<0.1
Procymidone (2 mg/kg)	9, 30, 84	CH4 (C)	4.5	8.5	17	14
Label WHP = 7 days	28, 105	CH5 (S)	0.46	2.1	11	3.0
	nr	CH8 (C)	<0.05	0.11	1.7	0.10
	34	CH9 (C)	0.39	1.0	7.0	0.64
	40	CH10 (P)	0.90	1.8	11	2.0
	43	CH11 (P)	0.41	2.3	13	2.8
	nr	CH14 (M)	0.10	0.36	3.9	0.48
	77	CH15 (S)	2.2	10	30	9.0
	nr	Ref#14 (S)	n/s	0.78	1.8	0.52
Fenhexamid (10 mg/kg)	115	CH4 (C)	<0.01	0.06	0.49	0.08
Label WHP = 21 days	132	CH5 (S)	0.01	0.05	0.10	0.15
	110	CH8 (C)	<0.01	0.03	0.06	0.04
	95	CH9 (C)	0.03	0.13	0.25	0.14
	101	CH10 (P)	0.03	0.07	0.18	0.10
	nr	Ref#14 (S)	n/s	0.06	0.09	0.13
Pyrimethanil (5 mg/kg)	119	CH4 (C)	0.12	0.47	0.29	1.1
Label WHP = 7 days	140	CH5 (S)	0.04	0.06	0.13	0.18
	120	CH11 (P)	0.03	0.11	0.30	0.25
	nr	CH14 (M)	0.02	0.05	0.11	0.14
	nr	CH15 (S)	0.08	0.15	0.34	0.35
	nr	Ref#14 (S)	n/s	0.01	0.02	0.05
Captan (10 mg/kg)	51, 84, 140	CH4 (C)	0.29	0.16	1.2	0.14
Label WHP = 30 days	72, 105, 161	CH5 (S)	0.16	<0.01	0.10	0.01
	46, 96	CH8 (C)	0.25	1.6	0.45	0.05
	77	CH9 (C)	0.14	0.11	1.0	0.02
	52	CH10 (P)	0.07	<0.01	0.04	<0.01
	90	CH14 (M)	0.06	0.03	0.12	0.02
	77	CH15 (S)	0.66	<0.01	0.11	0.02
Iprodione (20 mg/kg)	nr	Ref#1 (R)	1.1	1.9	16	2.6
Label WHP = 7 days	nr	Ref#6 (S)	n/s	<0.05	2.2	<0.10
	nr	Ref#7 (S)	<0.05	<0.05	0.56	<0.10
	nr	Ref#8 (CS)	<0.05	<0.05	0.60	<0.10
	nr	Ref#9 (M)	<0.05	n/s	0.52	<0.10
	nr	Ref#10 (CS)	n/s	<0.05	2.0	<0.10
	nr	Ref #17 (S)	n/s	n/s	16	4.2
	nr	Ref #18 (S)	n/s	n/s	0.56	<0.10
Trifloxystrobin (0.5 mg/kg)	102	CH9 (C)	0.008	0.011	0.050	0.002
Label WHP = 35 days	108	CH10 (P)	0.005	0.006	0.020	0.008
	120	CH11 (P)	0.006	0.014	0.030	0.005
	nr	Ref#7 (S)	0.011	0.008	0.010	0.01
	145	Ref#8 (CS)	0.012	0.025	0.150	0.027
	nr	Ref#9 (M)	0.016	n/s	0.130	0.020
	nr	Ref#10 (CS)	n/s	0.010	0.068	0.010
	nr	Ref#14 (S)	n/s	0.016	0.042	0.005
	152	Ref#16 (S)	n/s	n/s	0.010	0.003
Pyraclostrobin (2 mg/kg)	110	CH8 (C)	<0.01	0.06	0.10	0.020
Label WHP = 21 days	nr	CH15 (S)	0.02	0.07	0.20	0.09
Metalaxyl (MRL = 1 mg/kg)	68	CH9 (C)	<0.01	<0.01	Trace	<0.01
Label WHP = 7 days	74	CH10 (P)	<0.01	<0.01	0.01	0.01
	64	CH11 (P)	0.01	0.01	0.02	0.04
	nr	Ref#14 (S)	n/s	Trace	<0.01	0.03
Quinoxyfen (2 mg/kg)	nr	CH4 (C)	<0.01	<0.01	0.04	<0.01
Label WHP = 14 days	72	CH8 (C)	<0.01	0.07	0.10	0.02
	115	Ref#10 (CS)	<0.01	Trace	0.030	Trace
	122	Ref#16 (S)	n/s	n/s	Trace	<0.01

n/s = no sample.
nr = no record(diary).
[a] C = chardonnay, P = pinot, M = merlot, S = shiraz, R = Riesling, CS = cabernet sauvignon.

3.4. Fenhexamid

Fenhexamid is a contact (non-systemic) hydroxyanilide fungicide, used for controlling grey mould. Fenhexamid is registered for a 21-day withholding period on grapes (APVMA, 2008). There is a label restriction with a maximum of two applications per season. Application diary records show one application 3–4 months prior to harvest. Seed oil samples showed the highest level of residues but these were less than 10% of the Australian MRL. Given the existing usage patterns, fenhexamid is a low risk fungicide.

3.5. Pyrimethanil

Pyrimethanil is a contact anilinopyrimidine fungicide used for control of grey mould. The withholding period for pyrimethanil application on grapes is 7 days (APVMA, 2008). All samples were reported at less than 25% of the Australian MRL. Three of the six reported samples had application diary records which indicated application four months prior to harvest. It is likely that the three samples without application diary records were also treated 3–4 months prior to harvest. Following this usage pattern, pyrimethanil is a low risk fungicide for residues in seed oil and marc.

3.6. Captan

Captan is a contact multi-site phthalimide fungicide. The registered product has a withholding period of 7 days on grapes and is used for control of grey mould, black spot (*Elsinoe ampelina*), downy mildew (*Plasmopara viticola*) and phomopsis leaf blight (*Phomopsis viticola*) (APVMA, 2008). Up to five applications are allowed per season. Three of the seven samples showed captan use more than once in the season. All applications were at a minimum of 45 days withholding period and reported residues were all less than 20% of the Australian MRL. As expected, the sample with the highest residue level (1.6 mg/kg in seed oil) was the sample with the shortest actual withholding period. Captan is a low risk fungicide in the samples tested with the reported usage patterns.

3.7. Iprodione

Iprodione is a contact dicarboximide fungicide used for controlling grey mould (*B. cinerea*). The withholding period is 7 days with a recommendation to limit application to a maximum of two sprays per season (APVMA, 2008). None of the available application diaries showed applications of iprodione; however, iprodione residues were detected in samples for which application diaries were not available. Two of the reported seed oil samples contained 16 mg/kg of iprodione, or 75% of the Australian MRL. It is likely that iprodione was used with an application pattern similar to procymidone. Results show that iprodione is a high-risk fungicide for seed oil, marc, seed meal and grapes in these samples.

3.8. Trifloxystrobin

Trifloxystrobin is a strobilurin type fungicide, used for controlling powdery mildew (*Uncinula necator*) and suppression of downy mildew. This fungicide is mesostemic in action and it also has translaminar activity and can be redistributed by superficial vapour movement (Tomlin, 2003). The label withholding period is 35 days. Label instructions limit usage prior to grape bunch closure (APVMA, 2008), thus it is unlikely that application would be made three months prior to harvest. This would include the samples for which no application diary records were available. Five samples had application diary records indicating application had actual withholding periods of more than one hundred days prior to harvest. With these application patterns, it is unlikely that grape, marc, seed oil or meal would be at 50% of the Australian MRL, so trifloxystrobin is a low risk fungicide for the production of grape seed oil and seed meal for commercial purposes. The reported levels are lower than for most fungicides, due to the exceptional sensitivity of the LC–MS/MS instrument for the determination of trifloxystrobin.

3.9. Pyraclostrobin ($\log K_{ow} = 4.0$)

Pyraclostrobin is used for controlling powdery mildew and downy mildew. The label withholding period is 21 days. The label stipulates a maximum of three applications at 10–14 day intervals, commencing at flowering (APVMA, 2008), therefore it is unlikely to be applied within three months of harvest. Two samples of seed oil contained pyraclostrobin residues at 10% of the Australian MRL. Pyraclostrobin is a low risk fungicide providing label usage is followed. Pyraclostrobin is considered to be a lower risk fungicide than trifloxystrobin, as the MRL for this similar fungicide is four times lower (0.5 mg/kg).

3.10. Metalaxyl ($\log K_{ow} = 1.75$)

Metalaxyl is a phenylamide–acylalanine systemic fungicide with protective and curative properties with a label withholding period of 7 days (APVMA, 2008). It is registered for use in controlling downy mildew and is absorbed through leaves, stems and roots. Label instructions limit application to a maximum of four per season. Application diary records for three samples showed one application at two months minimum prior to harvest. Residues were less than 10% of the Australian MRL for grapes and by-products. Metalaxyl is low risk given current usage patterns.

3.11. Quinoxyfen ($\log K_{ow} = 4.7$)

Quinoxyfen is registered for protective (not curative) action against powdery mildew and has a withholding period of 14 days (APVMA, 2008). Label advice limits applications to three per season. Reported levels are less than 10% of the Australian MRL. Quinoxyfen is low risk given the current usage pattern.

3.12. Chlorothalonil ($\log K_{ow} = 2.9$)

Chlorothalonil is a multi site-chloronitrile non-systemic foliar fungicide registered for protective action against downy mildew, black spot and grey mould. The label withholding period is 14 days (APVMA, 2008). Application diaries indicate that it was applied to at least seven samples and records show that the minimal actual withholding period was 100 days. It was detected in three of the grape samples at low levels but not detected in seed oil, marc or seed meal samples. It is extremely unlikely that the Australian MRL of 10 mg/kg would be contravened in grapes or grape by-products under these application conditions.

3.13. Penconazole ($\log K_{ow} = 3.7$)

Penconazole is a DMI:triazole systemic fungicide registered for control of powdery mildew. The label withholding period is 14 days. Application is permitted up to three times per season (APVMA, 2008). The Australian MRL is 0.1 mg/kg. It was applied to a minimum of four samples but not detected in any of the samples analysed. Records showed that the minimum actual withholding period was 84 days with no repeat applications. Due to the low MRL, penconazole would represent a risk of contravening MRL residue levels in grape and grape by-products; however, for this reason, growers probably use the chemical with a maximum possible withholding period.

3.14. Triadimenol (log K_{ow} = 3.2)

Triadimenol is a systemic DMI:triazole fungicide with protective, curative and eradicant action used for control of powdery mildew. Its label withholding period is 7 days (APVMA, 2008) and the Australian MRL is 0.5 mg/kg. Application is listed on the label for the period until the fruit is set, so it is unlikely that residues would be reported at harvest. Application diaries indicated that triadimenol was applied to two samples with withholding periods listed as 125 and 146 days, however, triadimenol was not detected in any of the samples analysed.

3.15. Myclobutanil (log K_{ow} = 2.9)

Myclobutanil is a systemic DMI:triazole fungicide with protective and curative action, used to control powdery mildew. The label withholding period is 14 days (APVMA, 2008) and the Australian MRL 1 mg/kg. Applications are restricted to three per season; however, it is unlikely that more than one application was made. Application diaries indicate that myclobutanil was applied to one sample; however, it was not detected in any of the samples analysed.

3.16. Spiroxamine

Spiroxamine is a morpholine:spiroketalamine systemic fungicide with protective, curative and eradicant action, used to control powdery mildew. It is composed of two isomers, A and B, with a log K_{ow} = 2.8 and log K_{ow} = 2.9, respectively. The label withholding period is 28 days (APVMA, 2008) and application was listed in three application diaries at actual withholding periods of 119, 161 and 168 days. Spiroxamine was not detected in any samples.

Table 3 shows data for the diacylhydrazine insecticide, tebufenozide, and the oxydiazine insecticide, indoxacarb. These compounds are used to control light brown apple moth. Withholding periods are 21 days and 8 weeks, respectively.

3.17. Tebufenozide

This was listed in two application diaries (100 and 115 days prior to harvest) and residues were detected in six samples. For the samples with application diaries, the residues were detected in the seed oil up to 10% of the MRL. However, for sample CH14, where the application was not recorded, the residue level in the seed oil is 30% of MRL. It is possible that tebufenozide residues in seed oil for this sample would be at the MRL at the minimum withholding period.

3.18. Indoxacarb

This was listed in four sample application diaries and was reported in these samples in addition to three other samples. The actual withholding periods are listed as 81–140 days, in excess of the minimum label withholding period of 56 days. Two samples of seed oil without application diary records show residues at 40% of the MRL. It is possible that MRL levels of indoxacarb would occur at the minimum withholding period in the seed oil.

3.19. Spinosad

Spinosad is a biopesticide used for controlling light brown apple moth, comprising a mixture of two isomers, A and D. The log K_{ow} is 4.0 for the A isomer and 4.5 for the D isomer at pH 7 (Tomlin, 2003). The log K_{ow} for both isomers decreases with decreasing pH. The Australian MRL is 0.5 mg/kg. Spinosad was identified in two application diaries but was not detected in any of the samples. Applications were at 84 and 105 days prior to harvest.

The data in Table 4 show that residue concentrations, for eight of the listed pesticides with acceptable sample sets, are significantly higher in seed oil than in fruit. The residues are also higher in marc than in fruit and by calculation, higher in seed oil than marc. A similar pattern of residue levels for the fungicides, fludioxinil, quinoxyfen and pyraclostrobin, was also evident for a smaller number of samples. The two systemic fungicides (procymidone and cyprodinil) have the highest concentration factor in seed oil relative to fruit and marc. This suggests that there is a partitioning of the highly oil-soluble (log K_{ow} > 2.5) fungicides into the oily components of the

Table 4
Mean concentration ratios of pesticides in oil/fruit and marc/fruit.

Pesticide	log K_{ow}	Number of samples	Oil/fruit	Marc/fruit
Cyprodinil	3.9	6	27	12
Procymidone	3.1	9	20	3.6
Tebufenozide	4.2	4	10	5.0
Indoxacarb	4.6	7	8.5	2.5
Fenhexamid	3.5	6	8	3.7
Trifloxystrobin	4.5	6/5	6.1	1.6
Pyrimethanil	2.8	5	5	2.7
Captan	2.8	7	2.4	2.1

Table 3
MRL, label withholding periods and pesticide residues in fruit, marc, seed oil and seed meal.

Pesticide (MRL) Label WHP (days)	Application: days prior to harvest	Sample/variety[a]	Fruit (mg/kg)	Marc (mg/kg)	Seed oil (mg/kg)	Seed meal (mg/kg)
Tebufenozide (2 mg/kg)	115	CH4 (C)	0.02	0.13	0.18	0.13
Label WHP = 21 days	nr	CH5 (S)	0.03	0.05	0.14	0.10
	110	CH8 (C)	0.01	0.05	0.21	0.03
	nr	CH9 (C)	<0.01	<0.01	0.03	<0.01
	nr	CH11 (P)	<0.01	<0.01	0.02	<0.01
	nr	CH14 (M)	0.05	0.42	0.63	0.36
Indoxacarb (2 mg/kg)	81	CH9 (C)	0.03	0.06	0.15	0.09
Label WHP = 56 days	88	CH10 (P)	0.05	0.13	0.27	0.19
	107	CH11 (P)	0.02	0.07	0.13	0.17
	nr	Ref#7 (S)	0.05	0.09	0.41	0.01
	nr	Ref#8 (CS)	0.07	0.17	0.94	0.03
	nr	Ref#9 (M)	0.07	n/s	0.82	0.02
	140	Ref#10 (CS)	n/s	0.05	0.27	0.01

n/s = no sample.
nr = no record (diary).
[a] C = chardonnay, P = pinot, M = merlot, S = shiraz, CS = cabernet sauvignon.

grape vine, and that systemic activity facilitates transfer to the seed oil. This supports the hypothesis that oil-soluble pesticides, removed from must by clarification, are present in the seed oil. Alternatively, polar (water-soluble) pesticides are more likely to be retained in the wine and metabolised by fermentation.

For cyprodinil (Table 2), the increase in residue levels from fruit to seed oil ranged from 8 (CH9, CH10) to 64 (CH8). Similarly, for procymidone (Table 2), the increase in residue levels from fruit to seed oil ranged from 4 (CH4) to 40 (CH8, CH14).

4. Conclusion

This study confirms that oil-soluble ($\log K_{ow} > 2.5$) fungicides and insecticides applied to wine grapevines are concentrated in the grape seed oil. The concentrations of the pesticide residues in the seed oil relative to marc and fruit were highest for the systemic fungicides, procymidone and cyprodinil. Thus oil-solubility and systemic action are confirmed as key risk factors for residue contamination in grape marc and seed oil. Thus the pattern of minimal applied pesticide residues in wine reported in the literature is logical, as these mainly oil-soluble pesticides are concentrated in the seeds, a major component of marc.

Acknowledgements

The authors would like to thank Greg Jarratt, Jamie Hewett, Andrew Fleming and Richard Shenfield, of Fosters Ltd., Fred Davies of Stoney Creek Oil Products P/L for supplying samples, Dr. Jun Du, Pei Zhang and Colin Cook of DPI-Werribee for technical support and Dr. Craige Trenerry for assistance in preparing the manuscript. This project was funded by the Department of Primary Industries (Victoria) through the Naturally Victorian Initiative.

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Food Chemistry 117 (2009) 641–646

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Acidic degradation and enhanced antioxidant activities of exopolysaccharides from *Cordyceps sinensis* mycelial culture

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ARTICLE INFO

Article history:
Received 7 January 2009
Received in revised form 22 February 2009
Accepted 17 April 2009

Keywords:
Medicinal fungus
Polysaccharides
Hydrolysis
Molecular weight
Antioxidant activity
Radical scavenging

ABSTRACT

An exopolysaccharide (EPS) fraction isolated from mycelial culture of a *Cordyceps sinensis* fungus, designated EPS-1 with an average molecular weight (MW) of 38 kDa, was hydrolysed in dilute sulphuric acid solution at pH 1 and 90 °C, yielding two major MW fractions, 3.0 kDa and 30 kDa, respectively. While the proportion of lower MW fraction increased with the hydrolysis period (18% in 0.5 h and 92% in 10 h), the polydispersity (M_w/M_n) of EPS decreased steadily (from 1.47 initially to 1.10 in 10 h). The IR spectra of hydrolysed EPS fractions showed changes only in the C–O–C and C–O–H band peaks from that of EPS-1. These results suggest that the hydrolysis of EPS in the acidic solution caused the EPS degradation mainly by cleaving the glycosidic linkage but no change in the primary molecular structure. The hydrolysed EPS fractions had much higher (30–80%) antioxidant and radical-scavenging activities.

1. Introduction

Cordyceps sinensis (Berk.) Sacc., generally called Cordyceps or Dong-Chong-Xia-Cao (winter worm–summer grass) in Chinese, is a precious and famous medicinal fungus in China which has been used as a general tonic and therapeutic herb by the Chinese over hundreds of years. Cordyceps has shown a broad range of health effects on the immune, kidney, liver and cardiovascular functions, and antitumor and antioxidation activities (Li & Tsim, 2004; Russell & Paterson, 2008; Zhu, Halpern, & Jones, 1998). Natural Cordyceps is a rare and endangered species insufficient to meet the demand, and mycelial fermentation is a more economical and sustainable process for production of *Cordyceps* mycelial biomass and polysaccharides. Polysaccharides represent a major class of bioactive compounds in Cordyceps and many other medicinal mushrooms which have anticancer and immunomodulation activities, and other notable health effects such as antioxidation and hypoglycemic activity (Li & Tsim, 2004).

Since oxidative stress evoked by reactive oxygen species (ROS) is harmful to human health and a potential cause of many human diseases (Halliwell & Gutteridge, 1998), the antioxidant properties of functional foods and herbal products are important for health protection and disease prevention. Numerous previous studies have examined the antioxidant and radical-scavenging activities of water extracts of natural Cordyceps and cultivated fungal mycelia (Dong & Yao, 2008; Li, Dong, & Tsim, 2001; Yamaguchi, Kagota, Nakamura, Shinozuka, & Kunitomo, 2000). As the major constituents of water extracts, polysaccharides are attributable to their antioxidant activities. A few studies have also shown the antioxidant activities of purified polysaccharide fractions isolated from *Cordyceps* species, such as the one isolated from *C. sinnesis* mycelia protecting against hydrogen peroxide-induced damage of PC12 cells in culture (Li et al., 2003), and the one from cultivated *C. militaris* fruit bodies scavenging hydroxyl radicals (Yu et al., 2007).

The application and efficacy of polysaccharides for health care is dependent not only on the chemical structure but also on the molecular size or molecular weight (MW), which has a direct influence on solubility and viscosity in solution, and usually also on the bioactivity (Shahidi, Arachchi, & Jeon, 1999). Most of the polysaccharides isolated from various *Cordyceps* species are of high average MW over 10 kDa, usually from 50 to 1000 kDa (Leung, Zhao, Ho, & Wu, 2009). Moderate degradation of the high-MW polysaccharides may increase the water solubility and decrease the viscosity in solution, thus improving the medicinal properties. Moderate degradation has also been shown to improve the antioxidant activities of natural and modified polysaccharides, such as ulvan (from seaweeds, Chorophyta) (Qi, Zhao, Zhang, & Zhao, 2005) and chitosans (Xing, Liu, Guo, & Yu, 2005). While the polymer degradation can usually be accomplished through chemical, enzymatic and

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doi:10.1016/j.foodchem.2009.04.068

thermal processes, the hydrolysis by dilute acids is a simple and effective method, and not destructive to the primary chemical structures of biopolymers (Soldi, 2005).

Cs-HK1 is a fungal species isolated from natural Cordyceps fruiting body in our lab, and is a high producer of exopolysaccharides (EPS) in mycelial liquid culture (Leung, Zhang, & Wu, 2006). The EPS from the Cs-HK1 mycelial culture had a wide range of MW from 5 to over 200 kDa and showed moderate antioxidant activities (Leung et al., 2009). The present study was to examine the degradation of a high-MW EPS fraction isolated from Cs-HK1 mycelial culture by hydrolysis in dilute sulphuric acid. The molecular weight profiles of hydrolysed EPS fractions were analysed, and their antioxidant activities were measured by different assays.

2. Materials and methods

2.1. Chemicals

ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) and Trolox were purchased from Calbiochem/EMD (Gibbstown, NJ, USA), vitamin C (Vc, ascorbic acid) and assay kits for lipid peroxidation, hydroxyl and superoxide anion radicals from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All reagents were of analytical grade. Aqueous media and reagent solutions were all prepared with distilled de-ionised water. Dry EPS fractions were stored in a desiccator at room temperature, and EPS solutions stored at 4 °C before use.

2.2. Preparation of EPS-1 fraction from Cs-HK1 mycelial culture

Cs-HK1 mycelial liquid culture was maintained in a stirred fermentor for 6–7 days; at the end of fermentation, the culture broth was spun down and the liquid medium was collected. Exopolysaccharide (EPS) was isolated from the liquid medium by ethanol precipitation. Details of the Cs-HK1 mycelial culture conditions and fermentation process, and the EPS isolation procedure can be found in Leung et al. (2006, 2009). The crude EPS isolated from the Cs-HK1 fermentation broth by ethanol precipitation contained about 70% carbohydrate and 20–25% protein, and the carbohydrate content was mainly composed of β-glucans.

The crude EPS fraction (ethanol precipitate) from the Cs-HK1 mycelial culture was purified through the following steps. It was first deproteinised with enzyme and Sevag reagent as described by Staub (1956). For enzyme deproteinisation, papain (lyophilised powder, from Sigma–Aldrich Chemical Co.) was applied at 1.5–2 U/mg EPS to the EPS solution at pH 6.4 and incubated at 60 °C for 3 h. The solution was then centrifuged and the supernatant was mixed with Sevag reagent (1-butanol/chloroform at 1:4 v/v) at 25 °C with constant stirring for 2 h. The deproteinised EPS solution (1 g/l) was then mixed with 1.5% (w/v) powdered activated carbon at 80 °C for 40 min and three times to remove the brown pigments. After removal of the activated carbon (by filtration), the EPS solution was dialysed (using 12–14 kDa membrane) against water for 48 h. The purified EPS solution was concentrated by vacuum evaporation and then lyophilised, which was designated as EPS-1 and used as the starting EPS material in the following acid hydrolysis experiments. The total carbohydrate content of EPS was determined by the phenol–sulphuric acid method using glucose as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.3. Acidic hydrolysis of EPS

Acidic hydrolysis of EPS-1 was performed according to the procedure as described by Xu, Andrey, and Lari (2008) with slight modifications. EPS-1 was first dissolved in water at 0.1% (w/v) with constant stirring at 80 °C for 2 h, and then at room temperature overnight. For acid hydrolysis, each 20 ml of the EPS-1 solution was filled in a 50 ml centrifuge tube and the solution pH was adjusted to 1.0 by adding 1 M H_2SO_4. The tubes were capped and immersed in a water bath at 90 °C or other selected temperatures for 0.5–10 h. After the hydrolysis period, the acidic EPS solution was cooled down immediately in an ice-water bath and adjusted to neutral pH with 1 M NaOH solution.

2.4. Determination of intrinsic viscosity

The EPS samples were dissolved in water at a series of dilute concentrations, and the solution viscosity was measured with an Ubbelohde viscometer at 25 °C. The intrinsic viscosity $[\eta]$ of a sample was taken from the intercept of $\ln(\eta_r/C)$ versus C line, where η_r is the relative viscosity and C the concentration of sample solution (Chinese Pharmacopoeia Committee, 2002).

2.5. Measurement of molecular weight and IR spectra

The molecular weights (MW) of EPS samples were analysed by high-pressure gel permeation chromatography (HPGPC). The HPGPC system consisted of a Waters 1525 binary pump and a 2414 RI detector (Waters Co., Milford, MA, USA), and a series of two GPC columns, a TSK-GEL G-5000 PW xL column and a TSK-GEL G-3000 PW xL column (both of 7.8 mm × 300 mm dimension) (TOSOH Co., Japan). A solution of 0.02 M KH_2PO_4 was used as the elution solvent flowing at 0.6 ml/min. The samples were dissolved in the elution solvent at 2 mg/ml and filtered through 0.45 μm membrane before the injection. The MW calibration curve was generated with six linear dextran MW standards of molar masses ranging from 5.2 to 668 kDa (Sigma). The GPC spectra were analysed with the Breeze V3.3 software (Waters Co.) to derive the weight- and number-average MW, M_w and M_n of EPS samples.

The infrared (IR) spectrometry of EPS samples was performed in the 4000–800 cm^{-1} wave number region on a Vector 33 FTIR instrument (Bruker Co., Germany).

2.6. Antioxidant activity tests

The antioxidant properties of EPS fractions were measured with four different assays as described below for more comprehensive evaluation of their activities against lipid peroxidation and various free radicals (Halliwell & Gutteridge, 1998; Oyanagui, 1984). In these assays the EPS samples were predissolved in water and tested at various concentrations in parallel with water as the blank and vitamin C (Vc) as an antioxidant reference (positive control) (or Trolox as a reference in the TEAC assay).

The assays for inhibition of lipid peroxidation, and scavenging hydroxyl and superoxide anion radicals were performed using the commercial kits from Jiancheng Bioengineering Institute (Nanjing) according to the supplier's manuals. The lipid peroxidation assay kit was based on the peroxidation of linoleic acid and formation of malondialdehyde (MDA) with a peak absorbance at 520 nm. The hydroxyl radicals ($OH^{\cdot}$) in the assay kit were generated by the Fenton reaction (exhibiting a red colour with a peak absorbance at 550 nm); the assay reaction was performed at 37 °C in a water bath for 20 min. The superoxide anion radicals ($O_2^{\cdot-}$) in the assay kit were generated by the xanthine/xanthine oxidase reaction with 2,4-iodiphenyl-3,4-nitrophenyl-5-phenyl-tetrazolium chloride to form formazan, a coloured compound with a peak absorbance at 530 nm. After the assay reactions, the absorbance of assay solutions was measured and the inhibition or scavenging activity (in %) was represented by the percentage of absorbance decrease with the sample relative to the blank. TEAC (Trolox equivalent antioxi-

dant capacity) is the activity of a sample equivalent to Trolox in scavenging $ABTS^{\cdot+}$, a stable free radical (Rice-Evans, Miller, & Paganga, 1996), and was measured of the EPS fractions as described in Leung et al. (2009).

2.7. Statistical analysis

All hydrolysis experiments and antioxidant tests were performed in triplicate and the results were represented by mean ± standard deviation (SD). Statistical significance of the antioxidant tests was determined by Duncan's multiple-range test at $p < 0.05$.

3. Results

3.1. Molecular weights of EPS-1 and its hydrolysates

After a series of purification steps (deproteinisation, decolorisation and dialysis) of the crude EPS from the Cs-HK1 mycelial culture, a white, filamentous and water soluble solid was yielded, designated EPS-1 which had a total carbohydrate content of 90% and negligible protein, and an average MW of 38 kDa (M_w). As in our previous study (Leung et al., 2009), the total carbohydrate content (plus protein content) measured of the EPS was lower than 100% which was due probably to the incomplete acid hydrolysis of the EPS in the analytical procedure. Table 1 shows the weight- and number-average molecular weights and MW distribution of EPS-1 before and after the acid hydrolysis at a fixed temperature of 90 °C for various periods of time. The acidic hydrolysis of EPS-1 resulted in two major MW peaks of a higher M_w ranging from 26 to 36 kDa and a lower M_w about 3.0 kDa. The relative proportion (based on the GPC peak area) of lower MW fraction increased with the hydrolysis period, from 0% to 18% in 30 min (EPS-1A), and to 92% in 10 h (EPS-1D), indicating the increasing degree of EPS degradation in the acidic solution.

The polydispersity index M_w/M_n of EPS-1 fraction (Table 1, corresponding to the higher MW peak) decreased steadily and almost linearly with the hydrolysis time; the polydispersity indexes of all hydrolysed fractions were close to 1.0 at the lower MW peaks. This implies that acidic degradation led to a narrower or more uniform MW distribution of EPS fraction.

3.2. Decrease of EPS intrinsic viscosity by hydrolysis

Fig. 1 shows the time course of intrinsic viscosity [η] of EPS-1 undergoing hydrolysis in dilute sulphuric acid at three different temperatures from 25 to 90 °C. The intrinsic viscosity of EPS-1 decreased with time at all temperatures but more rapidly at a higher temperature, e.g., from the initial value of 4.83–1.55 dl/g at 90 °C in 10 h. The decrease in intrinsic viscosity is indicative of an increase in the degree of EPS degradation and the reduction of the average MW by acidic hydrolysis. The degradation rate increased with the hydrolysis temperature.

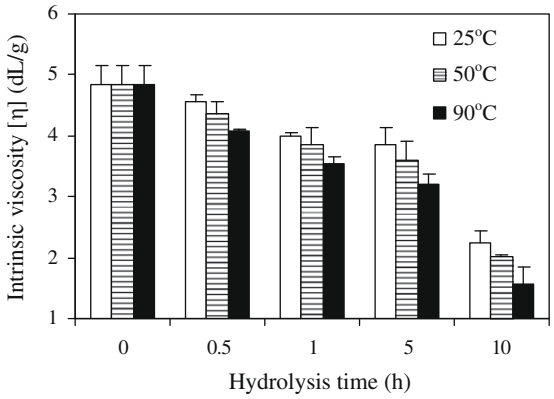

Fig. 1. Intrinsic viscosity time courses of EPS-1 in acidic solution (pH 1) at two different temperatures (significant difference between intrinsic viscosities at 25 °C and 90 °C at $p < 0.05$; error bars for SD, $n = 3$).

3.3. IR spectra of EPS-1 and its hydrolysates

Fig. 2 presents the IR spectra of the original EPS-1 and two hydrolysed fractions. The two EPS-1 hydrolysates (one collected at 0.5 h and the other at 10 h of hydrolysis time) had virtually an identical spectrum, indicating their identical molecular composition. In comparison with the EPS-1 spectrum, the spectra of hydrolysed EPS fractions show a close match in the higher wave number region over 1200 cm^{-1}, but some notable changes in the lower wave number region. In particular, an absorption spike appeared at 1000 cm^{-1} which represents the first order δO–H bond; the band peak at 1144 cm^{-1} which represents the C–O stretching vibration and the second order δO–H in C–O–H was significantly enhanced, due probably to the cleavage of a γC–O–C bond. These spectral data suggest that the major change in the EPS molecule during acid hydrolysis was an alteration of the C–O bond. On the other hand, none of the two characteristic absorption bands associated with a $C–O–SO_3$ group (at 1258 cm^{-1} for the asymmetrical S=O stretching vibration and at 810 cm^{-1} for the symmetrical C–O–S vibration) is present in the spectra of EPS-1 hydrolysates, indicating that no sulphation occurred in the dilute sulphuric acid solution. Overall the IR data (Fig. 2) suggest that the depolymerisation of EPS-1 in the acidic solution mainly caused the breakage of the glycosidic linkages but no significant change of the basic chemical structure of the EPS molecules.

3.4. Enhanced antioxidant activities of EPS-1 by acid hydrolysis

Fig. 3A–C shows the three antioxidant activity indexes of the original and the hydrolysed EPS-1 fractions, inhibition of lipid

Table 1
Average molecular weight and distribution of EPS-1 and hydrolysates from GPC.

EPS fraction[a]	Retention time (min)		M_w (kDa)	M_n (kDa)	M_w/M_n [b]	%Area
EPS-1	Peak 1	26.087	38.0	25.9	1.47	100
EPS-1A	Peak 1	26.350	36.5	24.8	1.46	82.2
	Peak 2	31.050	3.09	2.74	1.13	17.8
EPS-1B	Peak 1	26.400	34.7	24.1	1.44	63.6
	Peak 2	31.053	2.98	2.69	1.11	36.4
EPS-1C	Peak 1	25.554	33.6	27.9	1.21	36.1
	Peak 2	30.983	2.98	2.70	1.10	63.9
EPS-1D	Peak 1	26.583	26.1	24.6	1.06	7.6
	Peak 2	31.100	3.06	2.81	1.08	92.4

[a] EPS-1: the original EPS fraction; EPS-1A, EPS-1B, EPS-1C and EPS-1D: EPS-1 hydrolysed at pH 1 and 90 °C for 0.5, 1, 5, and 10 h, respectively.
[b] Polydispersity index.

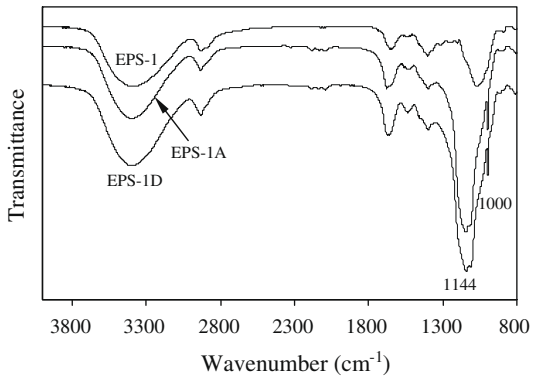

Fig. 2. IR spectra of EPS-1 and two hydrolysed fractions, EPS-1A (0.5 h hydrolysis) and EPS-1D (10 h hydrolysis).

peroxidation (Fig. 3A), scavenging of hydroxyl radicals (Fig. 3B) and superoxide anion radicals (Fig. 3C), which all increased with the concentration from 10 to 120 μg/ml. The three activity indexes exhibited a unanimous and consistent trend of increasing activity with the increasing degree of EPS degradation from EPS-1A to EPS-1D. Similarly, the Trolox equivalent antioxidant capacity (TEAC) of the EPS-1 fractions (Fig. 3D) also showed a steady increase with the degree of EPS degradation, from 18.5 μmol of the original EPS-1 to 30 μmol of EPS-1A and finally to 43.4 μmol Trolox/g of EPS-1D.

Table 2 presents the IC_{50} values derived from the four antioxidant activity assays of the EPS fractions and the antioxidant references. In all assays the hydrolysed EPS fraction EPS-1D has a much lower IC_{50} value and is thus a more potent antioxidant than the original EPS-1. Based on the IC_{50} values, the hydrolysed EPS-1D fraction had more potent activity for scavenging the superoxide anion and $ABTS^{\cdot+}$ radicals, but less potent activity on lipid peroxidation and hydroxyl radicals. The IC_{50} values on superoxide and $ABTS^{\cdot+}$ radicals are close to those of the respective antioxidant references vitamin C and Trolox, suggestive of a great potential of the degraded low-MW EPS fraction as an effective antioxidant for dietary and therapeutic applications.

4. Discussion

The generation of a single low-MW peak from the acidic degradation of EPS-1 is an indication of specific and non-random chain scission in the EPS molecules (Table 1). Such a mode of degradation is favourable for producing a uniform MW polysaccharide fraction. In addition, the change of M_w/M_n during polymer degradation is dependent upon the chain scission mechanisms, of which the central-point scission usually causes a lowering of the polydispersity M_w/M_n but the random chain scission does not change the M_w/M_n (Emsley & Heywood, 1995). According to these rules of thumb, central-point scission is assumed as the chief mode of chain cleavage for the degradation of EPS-1 in the acidic solution.

Because of the relatively mild condition in the weak acid, the degradation only occurred to the most vulnerable points of the polymer chain. The susceptibility of the glycosidic bond to acidic degradation may be attributable to its acetal structure with the glycoside oxygen atom accepting H^+ to form a protonated glycosidic bond, weakening the C–OR bond. Accordingly, we propose the major reaction steps as shown in Fig. 4 for the EPS-1 hydrolysis in the acidic solution. The reaction starts with the cleavage of the reductive C–O bond in the glycosidic linkages, and the resultant fragment is protonated to form a cationic intermediate, which is neutralised by OH^- to form a neutral polysaccharide. After the acidic degradation, more reductive terminals (–O–H) in the PS molecule are exposed which can form hydrogen bonds with other active groups in the solution.

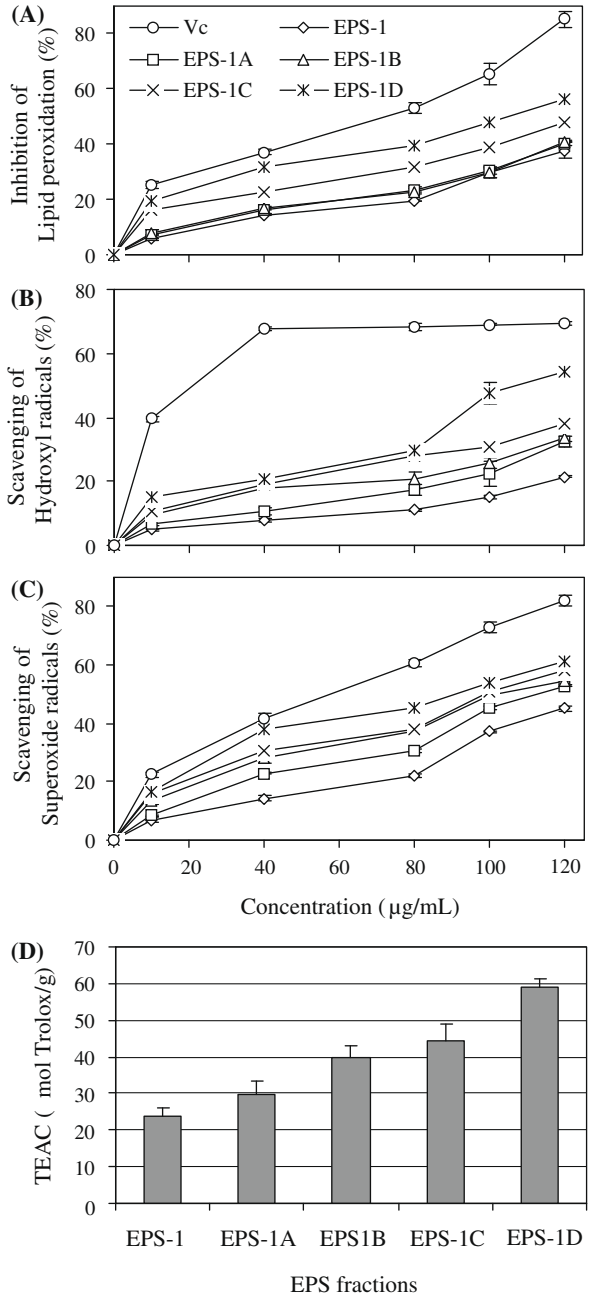

Fig. 3. Antioxidant activity indexes of EPS-1 and hydrolysed fractions: (A) inhibition of linoleic acid peroxidation; (B) scavenging of hydroxyl radicals; (C) scavenging of superoxide anion radicals; (D) Trolox equivalent antioxidant capacity of EPS-1 and its hydrolysed fractions (error bars representing SD, $n = 3$).

It is widely believed that reactive oxygen species (ROS) are involved in the pathogenesis of various diseases (Halliwell & Gutteridge, 1998). Therefore antioxidant activity is of broad significance for the therapeutic and health-promoting functions of many natural and medicinal products. In particular, lipid peroxidation caused by ROS is the culprit of several human diseases such as coronary arteriosclerosis, diabetes mellitus, aging and carcinogenesis (Negre-Salvayre, Coatrieux, Ingueneau, & Salvayre, 2008). Lipid peroxidation in the cell membrane involves the formation and propagation of lipid radicals, the uptake of oxygen, and rearrangement of the double bonds in unsaturated lipids, eventually leading to the destruction of membrane lipids and the production of

Table 2
IC_{50} values of EPS-1, EPS-1 hydrolysates and antioxidant references for inhibiting lipid peroxidation and scavenging free radicals (in μg/ml).

	Linoleic acid peroxidation	Superoxide anion radicals	Hydroxyl radicals	$ABTS^{\cdot+}$ radicals
EPS-1	>120	>120	>120	>120
EPS-1C	>120	101 ± 3.0	>120	104 ± 6.0
EPS-1D	105 ± 2.0	88.9 ± 6.0	>120	89.2 ± 7.0
Vc	63.7 ± 3.0	58.9 ± 5.0	13.5 ± 7.0	n.d.[a]
Trolox	n.d.	n.d.	n.d.	62.7 ± 8.0

[a] n.d.: not determined.

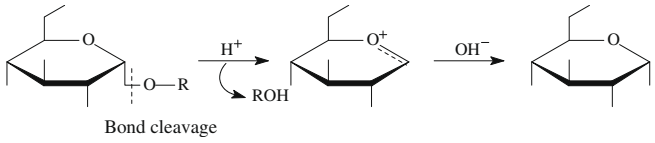

Fig. 4. A hypothesised reaction mechanism for acidic hydrolysis of EPS.

peroxides such as MDA. Antioxidants can prevent or slow down the lipid peroxidation by accepting or reacting with the ROS. Hydroxyl radical ($OH^{\cdot}$) is the most reactive ROS able to induce severe damage to biomolecules; superoxide anion (O_2^{-}) radicals are also harmful to cellular components directly or as a precursor for more active ROS (Chance, Sies, & Boveris, 1979; Halliwell & Gutteridge, 1998).

A variety of assay methods and activity indexes is available for measuring different materials in different systems (Oyanagui, 1984; Schlesier, Harwat, Böhm, & Bitsch, 2002). Because of the multiplicity and complexity of oxidation and antioxidation processes, several assays are needed for a comprehensive and unequivocal evaluation of the antioxidant activity of a sample. In our present study, we employed four different in vitro assays to evaluate and compare the antioxidant activities of the EPS fractions, all of which showed consistently a dramatic enhancement of the antioxidant activities by acidic degradation of the high-MW EPS to lower MW fractions. Similarly, Li et al. (2001) have also found that lower MW polysaccharides fractions isolated from Cordyceps fungi had stronger scavenging effects on superoxide anion radicals. Xing et al. (2005) reported that the hydroxyl radical-scavenging activity of lower MW chitosan fractions (9 kDa) was much higher than that of high-MW fractions (760 kDa) Although the antioxidant properties of natural polysaccharides from various sources such as medicinal fungi and mushrooms have been widely evaluated, the antioxidant activity to molecular structure relationship is still not well understood. Among the functional groups of a polysaccharide molecule, the hydroxyl group is the most possible player in the antioxidation action by donating electrons to reduce the radicals to more stable forms and/or by directly reacts with the free radicals to terminate the radical chain reaction (Lindsay, 1996). In this sense, the lower MW polysaccharide molecules have more reductive –OH terminals (on per unit mass basis) to accept and eliminate the free radicals.

In conclusion, an exopolysaccharide (EPS) about 38 kDa MW isolated from *C. sinensis* Cs-HK1 mycelial culture was hydrolysed in an acidic solution to 3.0 kDa MW. The hydrolysed EPS fraction had a lower polydispersity or narrower MW distribution. The low-MW EPS fraction had much higher antioxidant and radical-scavenging activities due perhaps to an increase in the number of hydroxyl groups available for reacting with the radical species. Further investigation is worthwhile on the degradation mechanism, and the structure to activity relationships. In addition, the antioxidant activity of hydrolysed EPS fractions must be examined in biological systems such as cell cultures and animals to prove and understand their health effects and potential value.

Acknowledgements

This work was supported financially by the Shenzhen Municipal Government for the Shenzhen Virtual University Park (SZVUP), and by the Hong Kong Polytechnic University for the State Key Laboratory of Chinese Medicine and Molecular Pharmacology in Shenzhen.

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Food Chemistry 117 (2009) 647–653

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Effect of air-drying temperature on physico-chemical properties, antioxidant capacity, colour and total phenolic content of red pepper (*Capsicum annuum*, L. var. Hungarian)

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ARTICLE INFO

Article history:
Received 16 December 2008
Received in revised form 4 March 2009
Accepted 17 April 2009

Keywords:
Pepper
Air-drying
Antioxidant properties
Total phenolic content
Radical scavenging activity
Vitamin C

ABSTRACT

Red pepper has been recognised as an excellent source of antioxidants, being rich in ascorbic acid and other phytochemicals. Drying conditions, particularly temperature, leads to pepper modifications that can cause quality degradation. In this work, the effects of process temperatures between 50 and 90 °C on physico-chemical properties, rehydration, colour, texture, vitamin C, antioxidant capacity and total phenolics during the drying of red pepper were studied. The rehydration ratio decreased with temperature and the maximum water holding capacity was achieved at 50 °C. Both vitamin C content and the total phenolic content decreased as air-drying temperature decreased. The radical scavenging activity showed higher antioxidant activity at high temperatures (i.e. 80 and 90 °C) rather than at low temperatures (i.e. 50, 60 and 70 °C). Chromatic parameters (L^*, a^*, b^*, C^* and $H°$), non-enzymatic browning compounds and extractable colour were affected by drying temperature, which contributed to the discolouring of pepper during this process.

1. Introduction

The pepper (*Capsicum annuum*, L.), indigenous to South and Central America, has been introduced worldwide. In particular, red pepper var. Hungarian, also known as Sweet Banana, is a very important product in the manufacture of paprika in different regions of the word. This pepper is highly appreciated for its flavour and colour, being the latter the main quality attribute that determines its overall quality and consequently its final market price (Krajayklang, Klieber, & Dry, 2000). Carotenoids are responsible for the colour of pepper and their contents are related to variety, ripeness and technological factors (Deepa, Kaura, George, Singh, & Kapoor, 2007; Gnayfeed, Daood, Biacs, & Alcaraz, 2001). Carotenoids are natural pigments responsible for the diverse colours in fruits and vegetables and are abundant in peppers. The main pigments in peppers are β-carotene, lutein and capsanthin and they are predominantly provitamin A (Howard, 2001; Rodriguez-Amaya, Kimura, Godoy, & Amaya-Farfan, 2008; Topuz & Ozdemir, 2007). Furthermore, red pepper is an excellent source of vitamin C (Guil-Guerrero, Martinez-Guirado, Rebolloso-Fuentes, & Carrique-Pérez, 2006; Topuz & Ozdemir, 2007) and polyphenols, particularly flavonoids, quercetin and luteolin (Chuah et al., 2008; Materska & Perucka, 2005). The antioxidant activity of phenolics is mainly due to their redox properties which allow them to act as reducing agents, hydrogen donors, single oxygen quenchers and metal chelators (Deepa et al., 2007). Thus, all the mentioned compounds show antioxidant activity as potential action against certain cancers, stimulate the immune system, prevent cardiovascular diseases and delay the aging process, amongst other biological activities (Chuah et al., 2008; Podsedek, 2007).

Dehydration is one of the most widely used methods for fruits and vegetables preservation. Its main objective is the removal of water to the level at which microbial spoilage and deterioration reactions are minimised. However, it is well known that during hot-air drying, vegetables undergo physical, structural, chemical and nutritional changes that can affect quality attributes like texture, colour, flavour, and nutritional value (Di Scala & Crapiste, 2008).

Amongst others, the acceptability of dried products depends mainly on their structural properties, like texture, which is one of the attributes used by consumers in judging their quality. Destruction of the cellular system is one of the most important physical and structural changes that occur during drying (Crapiste,

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0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.04.066

2000; Yadollahinia, Latifi, & Mahdavi, 2009). Moreover, dehydrated pepper must be rehydrated for consumption of prepared foods like soups, sauces, pizzas and other foodstuffs. Rehydration behaviour has been considered as a measure of the induced damage in the material during drying, such as integrity loss and reduction of hydrophilic properties, which decrease the rehydration ability (Marques, Prado, & Freire, 2009). Theoretically, if there are no adverse effects on the integrity of the tissue structure, it should absorb water to the same moisture content as the initial product before drying (Senadeera, Bhandari, Young, & Wijesinghe, 2000, chap. 6). Rapid and complete rehydration is very important for dried products. Rehydration capacity is affected significantly by drying conditions, pretreatments prior to drying and textural characteristics of dried products. Likewise, drying can diminish the osmotic properties of cell walls; as a result, an increase in water absorption and volume occurs due to the swelling of hydrophilic materials such as starch, cellulose, and pectic materials (Kaymak-Ertekin, 2002). Rehydration is maximised when structural disruption at the cellular level is minimized (Crapiste, 2000).

The basic requirements for a vegetable dryer are that it must achieve the required amount of drying in a reasonable time, obtain a product of acceptable quality, minimising operative costs (Kiranoudis, Maroulis, Marinos-Kouris, & Tsamparlis, 1997). Besides, the increasing demand for high-quality shelf-stable dried products requires the optimisation of the drying process conditions, especially temperature, with the purpose of accomplishing not only the efficiency of the process but also the final quality of the dried product (Banga, Balsa-Canto, Moles, & Alonso, 2003). Thus, from an engineering point of view, the control of this variable is a challenging problem that demands the evaluation of many interconnected non-linear mass and heat transport phenomena in a system with variable properties, shrinkage and modifications of quality attributes (Crapiste, 2000).

Therefore, the aim of this work was to study the effect of air-drying temperature on physico-chemical properties, rehydration ability (rehydration ratio (RR) and water holding capacity), colour development, texture (firmness) and antioxidant capacity (phenolic and ascorbic acid content) that occurred during the drying process of red pepper var. Hungarian.

2. Materials and methods

2.1. Sample preparation and drying process

Red peppers were grown and harvested in Salamanca, Chile, and stored at 4 °C before processing for a maximum time period of five days. The samples were selected visually by colour, size and freshness, and with no sign of mechanical damage. Then, they were cut into slabs of 4.0 ± 0.2 mm in thickness. The samples were dried in a pilot-scale convective dryer at five inlet temperatures 50, 60, 70, 80 and 90 °C (Vega-Gálvez, Lemus-Mondaca, Bilbao-Sainz, Yagnam, & Rojas, 2008a). The air flow rate was 2.0 ± 0.1 m/s. Each experiment was carried out in triplicate.

2.2. Physico-chemical analysis

The crude protein content was determined using the Kjeldahl method with a conversion factor of 6.25 (AOAC No. 960.52). The lipid content was analysed gravimetrically following Soxhlet extraction (AOAC No. 960.39). The crude fibre was estimated by acid/alkaline hydrolysis of insoluble residues (AOAC No. 962.09). The crude ash content was estimated by incineration in a muffle furnace at 550 °C (AOAC No. 923.03). The available carbohydrate was estimated by difference. The equilibrium moisture content was determined by means of AOAC method No. 934.06. All methodologies followed the recommendations of the Association of Official Analytical Chemists (AOAC (1990)). The pH was measured using an EXTECH Instruments microcomputer pH-vision 246072 (Waltham, Massachusetts, USA); the level of titratable acidity was expressed as malic acid. The water activity (a_w) was measured at 25 °C by means of a water activity instrument (Novasina, model TH-500, Pfäffikon, Lachen, Switzerland). Soluble solids were measured using a refractometer (ABBE, 1T, Tokio, Japan) which measures refraction indices both of solid and liquid samples in a fast and accurate way and its scale ranges from 0.0 to 95 °Brix. All measurements were done in triplicate.

2.3. Rehydration analysis

The dried pepper slabs were placed in distiled water at 40 °C for 6 h, using a solid to liquid ratio of 1:50. The samples were then removed, drained for 30 s, and weighed. All measures were done in triplicate. The rehydration ratio (RR) was calculated according to Eq. (1) and expressed as grams of water absorbed per gram dry matter. The water holding capacity (WHC) was determined by centrifuging the rehydrated samples at 3500×g for 15 min at 20 °C in tubes fitted with a centrally placed plastic mesh which allowed water to drain freely from the sample during centrifugation. The water holding capacity was calculated from the amount of water removed following Eq. (2), according to Vega-Gálvez, Lemus Mondaca, Bilbao-Sáinz, Fito, and Andrés (2008b):

$$\mathrm{RR} = \frac{W_{\mathrm{reh}} * X_{\mathrm{reh}} - W_{\mathrm{dried}} * X_{\mathrm{dried}}}{W_{\mathrm{dried}} * (1 - X_{\mathrm{dried}})} \quad (1)$$

$$\mathrm{WHC} = \frac{W_{\mathrm{reh}} * X_{\mathrm{reh}} - W_l}{W_{\mathrm{reh}} * X_{\mathrm{reh}}} * 100 \quad (2)$$

where W_{reh} is the weight of the sample after the rehydration process, X_{reh} is the corresponding moisture content on a wet basis, W_{dried} is the weight of the sample after the drying process, X_{dried} is the corresponding moisture content on a wet matter and W_l is the weight of the drained liquid after centrifugation.

3. Quality parameters

3.1. Surface colour measurement

Surface colour of the samples was measured using a colorimeter (HunterLab, model MiniScan™ XE Plus, Reston, VA, USA). Colour was expressed in CIE L^* (whiteness or brightness), a^* (redness/greenness) and b^* (yellowness/blueness) coordinates, standard illuminant D_{65} and observer 10° (Vega-Gálvez et al., 2008b). Five replicate measurements were performed and results were averaged. In addition, colour intensity (Chroma), total colour difference (ΔE) and hue angle were calculated using the following Eqs. (3)–(5), where L_o, a_o and b_o are the control values for peppers (Sigge, Hansmanw, & Joubert, 2001).

$$\mathrm{Chroma} = (a^{*2} + b^{*2})^{0.5} \quad (3)$$

$$\Delta E = \left[(a^* - a_0)^2 + (b^* - b_0)^2 + (L^* - L_0)^2\right]^{0.5} \quad (4)$$

$$\mathrm{Hue\ angle} = tg^{-1}(b^*/a^*) \quad (5)$$

3.2. Extractable colour

The determination was carried out according to the methodology proposed by the American Spice Trade Association (ASTA (1995)). Rehydrated red pepper slabs (0.5 g) were kept in about 50 ml acetone (Sigma Chemical CO., St. Louis, MO, USA) in 100 ml screw-cap jars maintained in the dark for 16 h at ambient temperature. An aliquot of this solution was used for the

spectrophotometric measurement (Spectronic® 20 Genesys™, Illinois, USA) at 460 nm. ASTA units were calculated as follows:

$$\mathrm{Cv} = \frac{A * 16,4 * I_F}{W_{\mathrm{reh}}} \quad (6)$$

where A is the absorbance of the acetone extract; I_F is the instrument correction factor calculated from a pattern solution of potassium dichromate and W_{reh} is the sample weight.

The colour loss value (%) was calculated according to the following equation:

$$(\%)\text{Colour loss value} = \frac{Cv_o - Cv_T}{Cv_o} \times 100 \quad (7)$$

Cv_o is the ASTA colour value of the fresh pepper and Cv_T is the ASTA colour value of the rehydrated peppers dried at different temperatures.

3.3. Determination of non-enzymatic browning index

The methodology applied for determination of non-enzymatic browning compounds (NEB) solubilised in the rehydration water was that proposed by Vega-Gálvez et al. (2008b). The rehydration water was first clarified by centrifugation at 3200×g for 10 min. The supernatant was diluted with an equal volume of ethanol (Sigma Chemical CO., St. Louis, MO, USA) at 95% and centrifuged again at 3200×g for 10 min. The browning index (absorbance at 420 nm) of the clear extracts was determined in quartz cuvettes using a spectrophotometer (Spectronic® 20 Genesys™, Illinois, USA). All measurements were done in triplicate.

3.4. Determination of vitamin C

Vitamin C (AA) was determined based upon the quantitative discolouration of 2,6-dichlorophenol indophenol (Merck KgaA, Darmstadt, Germany) titrimetric method as described in AOAC methodology No. 967.21 (AOAC, 2000). Comparative evaluations of vitamin C stability in fresh and rehydrated pepper were carried out, where 5.0 ± 0.1 g of each sample was weighed, crushed and diluted in 1 l distiled water. The vitamin C content was expressed as mg AA retained/100 g dry matter. All measurements were done in triplicate.

3.5. Determination of total phenolic content

Total phenolic content (TPC) was estimated as gallic acid equivalents (GAE) as described by Folin–Ciocalteau's (FC) method with modifications (Chuah et al., 2008). An aliquot (0.5 ml) of the pepper extract solution is transferred to a glass tube; 0.5 ml of reactive FC is added after 5 min; 2 ml of Na_2CO_3 (200 g/l) are added and shaken. After 15 min of incubation at ambient temperature, 10 ml of ultra-pure water was added and the formed precipitate was removed by centrifugation during 5 min at 4000×g. Finally, the absorbance was measured in an spectrophotometer (Spectronic® 20 Genesys™, Illinois, USA) at 725 nm and compared to a GA calibration curve. Results were expressed as mg acid gallic/100 g dry matter. All reagents were purchased from Merck (Merck KGaA, Darmstadt, Germany), and all measurements were done in triplicate.

3.6. Determination of DPPH radical scavenging activity

Free radical scavenging activity of the samples was determined using the 2,2,-diphenyl-2-picryl-hydrazyl (DPPH) method (Turkmen, Sari, & Velioglu, 2005) with some modifications. Different dilutions of the extracts were prepared in triplicate. An aliquot of 2 ml of 0.15 mM DPPH radical in ethanol was added to a test tube with 1 ml of the sample extract. The reaction mixture was vortex-mixed for 30 s and left to stand at room temperature in the dark for 20 min. The absorbance was measured at 517 nm, using a spectrophotometer (Spectronic® 20 Genesys™, Illinois, USA). The spectrophotometer was equilibrated with 80% (v/v) ethanol. Control sample was prepared without adding extract. All solvents and reagents were purchased from Sigma (Sigma Chemical CO., St. Louis, MO, USA). Total antioxidant activity (TAA) was expressed as the percentage inhibition of the DPPH radical and was determined by the following equation:

$$(\%)\ \mathrm{TAA} = \left(1 - \frac{\mathrm{Abs}_{\mathrm{sample}}}{\mathrm{Abs}_{\mathrm{control}}}\right) \times 100 \quad (8)$$

where TAA is the total antioxidant activity and Abs is the absorbance.

IC_{50}, which is the concentration required to obtain a 50% antioxidant capacity, is typically employed to express the antioxidant activity and to compare the antioxidant capacity of various samples. IC_{50} was determined from a graph of antioxidant capacity (%) against amount of extract (mg).

3.7. Determination of firmness

The property firmness, i.e. the maximum force applied to puncture the pepper tissue, was measured as an indicator of texture. Firmness of samples was measured using a Texture Analyzer (Texture Technologies Corp., TA, XT2, Scardale, NY, USA). The puncture diameter was 2 mm, with a travel distance of 20 mm and 1.7 mm/s test speed. The maximum force was measured by making one puncture in each rehydrated pepper sample, using 10 slabs per treatment. The mean value of maximum firmness for each treatment was then calculated and the results were expressed as N/mm.

3.8. Statistical analysis

The effect of air-drying temperature on each quality parameter was estimated using Statgraphics® Plus 5 (Statistical Graphics Corp., Herndon, VA, USA). The results were analysed by an analysis of variance (ANOVA). Differences amongst the media were analysed using the least significant difference (LSD) test with a significance level of $\alpha = 0.05$ and a confidence interval of 95% ($p < 0.05$). In addition, the multiple range test (MRT) included in the statistical program was used to demonstrate the existence of homogeneous groups within each of the parameters.

4. Results and discussion

4.1. Effect on physico-chemical properties

Proximate analysis of red pepper (on 100 g of fresh weight) presented an initial moisture content of 89.40 ± 1.40 g; crude protein (nitrogen x 6.25) of 1.20 ± 0.10 g; total lipids of 0.53 ± 0.04 g; crude fibre of 1.20 ± 0.10; crude ash of 2.07 ± 0.16 g; available carbohydrates (by difference) of 4.82 ± 0.44 g.

Table 1 shows the mean values and standard deviations of the moisture content, water activity, soluble solids, % acidity and pH of both fresh and dry-rehydrated samples. Significant differences were found between temperature and the properties mentioned ($p < 0.05$). A maximum value of moisture content of 10.63 ± 0.16 g water/g dry matter was observed at 70 °C; however, products with lower moisture contents were obtained at 50 and 60 °C as well as at 80 and 90 °C due to long drying times and high temperatures, respectively. Values of water activity, which is an indicator of water availability, were high for all the samples, as expected for rehydrated samples. Soluble solids and % acidity exhibited a

Table 1
Physico-chemical properties of fresh and dry-rehydrated red pepper as function of air-drying temperature.

Sample Condition	Water content (g water/g dry matter)	a_w	Soluble solids (°Brix)	Acidity (%)	pH
Fresh	4.52 ± 0.28[a]	0.975 ± 0.002	11.33 ± 0.29[a]	0.358 ± 0.004[a]	4.72 ± 0.01[a]
50 °C	8.75 ± 0.75[b]	0.975 ± 0.001	4.2 ± 0.30[b]	0.113 ± 0.009[b,c]	5.29 ± 0.04[b]
60 °C	9.01 ± 0.05[b]	0.981 ± 0.001	3.8 ± 0.30[b]	0.138 ± 0.009[c,e]	4.97 ± 0.05[c]
70 °C	10.63 ± 0.16[c]	0.979 ± 0.002	2.7 ± 0.30[c]	0.108 ± 0.009[b]	5.13 ± 0.06[d]
80 °C	8.72 ± 0.21[b]	0.981 ± 0.001	2.8 ± 0.30[c]	0.128 ± 0.009[d,e]	4.96 ± 0.04[c]
90 °C	8.88 ± 0.66[b]	0.982 ± 0.003	2.5 ± 0.50[c]	0.123 ± 0.009[c,d]	4.96 ± 0.11[c]

Different letters in the same column indicate that the values are significantly different ($p < 0.05$).

decreasing tendency in all the different treatments respect to the fresh sample. In the same table, pH increased slightly from its original value, showing the same tendency of acidity. Similar results were found by Miranda, Maureira, Rodriguez, and Vega-Gálvez (2009), working with dehydrated Aloe Vera.

4.2. Effect on water holding capacity and rehydration ratio

Rehydration process depends on structural changes in vegetal tissues and cells of food material during drying, which produces shrinkage and collapse (Kaymak-Ertekin, 2002). Fig. 1 presents the behaviour of the rehydration ratio (RR) as well as the water holding capacity (WHC) for each air-drying temperature studied. The WHC decreased as the air temperature increased ($p < 0.05$). The maximum WHC was 47.4 ± 2.8 (g retained water/100 g water) at 50 °C which implies that this drying temperature causes tissue structure damage; thus, the pepper dehydrated at this temperature retained a great amount of water. On the other hand, samples dried at 80 and 90 °C have reduced their WHC, thereby preventing the complete rehydration of the dried product. Similar investigations reported that drying temperature is the main factor affecting the WHC (Vega-Gálvez et al., 2008b). In the same figure, RR was affected by the drying temperatures, since absorbed water decreased with temperature. However, RR showed no significant differences ($p > 0.05$). The lowest RR value was 4.24 ± 0.12 (g absorbed water/g d.m.) at 90 °C, this could be explained due to cellular structure damage resulting in modifications of osmotic properties of the cell as well as lower diffusion of water through the surface during rehydration (Kaymak-Ertekin, 2002). Moreover, pretreatments with saline or sweet solutions such as $CaCl_2$ or saccharose allow to maintain the initial texture, leading to cellular structure stability (Lewicki, 2006; Papageorge, McFeeters, & Fleming, 2003).

4.3. Antioxidant activity

Fig. 2 shows the vitamin C as well as the total phenolic content (TPC) for the fresh and dry-rehydrated samples during the five drying experiments. The initial content of vitamin C in peppers was 188.2 ± 4.5 mg ascorbic acid/100 g fresh sample, which is within the ranges found in other studies for red peppers (75–277 mg/100 g fresh weight; Castro et al., 2008). It can be seen that an increase in drying temperature has an important effect on vitamin C ($p < 0.05$), thus, a maximum loss of 98.2% vitamin C in samples dried at 90 °C is observed. This could be explained due to irreversible oxidative processes either during drying or rehydration water lixiviation of this water-soluble vitamin (Sigge et al., 2001; Vega-Gálvez et al., 2008b). In addition, vitamin C is considered as an indicator of the quality of food processing due to its low stability during thermal processes (Podsedek, 2007). Similar results were obtained by other authors working with peppers (Di Scala & Crapiste, 2008; Sigge et al., 2001). Pretreatments like blanching or additives like SO_2 and $CaCl_2$ can improve the retention of this vitamin (Vega-Gálvez et al., 2008b). Besides vitamin C, foods of plant origin also supply our diet with other antioxidants in large amounts: carotenoids and phenolic compounds which constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators (Tabart, Kevers, Pincemail, Defraigne, & Dommes, 2009). The initial phenolic content was 1359 ± 148 g galic acid/g fresh sample. Sweet peppers contain numerous phenolic compounds, and not all of the genotypes may contain a similar profile or relative proportions of these compounds within the profile. Differences in these profiles may subsequently result in complex changes in antioxidant activity or other bioactivities (Deepa et al., 2007). It can be observed (Fig. 2) that an increase in drying temperature has an important effect on the total phenolic content ($p < 0.05$). The formation of phenolic compounds at high temperatures (i.e. 90 °C) might be because of the availability of precursors of phenolic molecules by non-enzymatic interconversion between phenolic molecules (Que, Mao, Fang, & Wu, 2008).

The radical scavenging activity was investigated based on air-drying temperature ($p < 0.05$) as observed in Fig. 3, where dehydration at high temperatures (i.e. 80 and 90 °C) shows higher antioxidant activity rather than at low temperatures (i.e. 50, 60 and

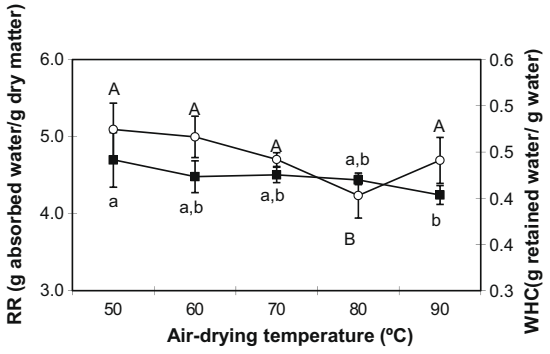

Fig. 1. Effect of air-drying temperature on the rehydration ratio (RR) and the water holding capacity (WHC) for dry-rehydrated pepper samples. Identical letters above the bars indicate no significant difference.

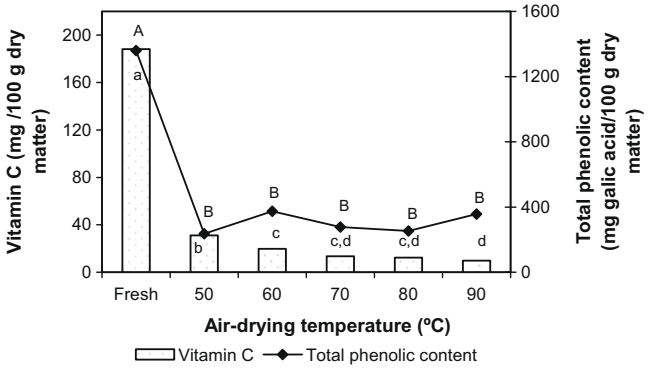

Fig. 2. Effect of air-drying temperature on vitamin C and total phenolic content of fresh and dry-rehydrated pepper samples. Identical letters above the bars indicate no significant difference.

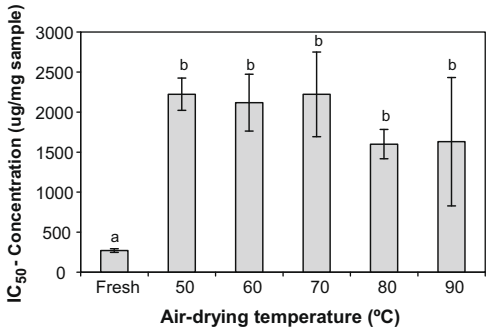

Fig. 3. Effect of air-drying temperature on DPPH free radical scavenging activity of fresh and dry-rehydrated pepper samples. Identical letters above the bars indicate no significant difference.

70 °C). This behaviour could be related to drying process at low temperatures, which implies long drying times that may promote a decrease of antioxidant capacity (Garau, Simal, Roselló, & Femenia, 2007). Furthermore, generation and accumulation of Maillard-derived melanoidins having a varying degree of antioxidant activity could also enhance antioxidant properties at high temperatures (i.e. 80 and 90 °C) (Miranda, Maureira, Rodriguez, & Vega-Gálvez, 2009; Que et al., 2008). Increasing correlation between antioxidant activity and total phenolic content has been reported during food dehydration (Deepa et al., 2007). However, data on the effects of drying on TPC and antioxidant activity of vegetables are conflicting due to several factors, like drying method, type of extraction solvent, antioxidant assays used as well as interactions of several antioxidant reactions (Manzocco, Calligaris, Mastrocola, Nicoli, & Lerici, 2001; Que et al., 2008).

4.4. Effect on colour

Fig. 4 presents the colour changes of pepper related to both non-enzymatic browning (NEB) and the ASTA colour value. It can be observed that an increase in temperature led to an important formation of brown products. This could be explained due to an increase in the kinetic reaction rate that shows a maximum NEB value of 0.19 ± 0.01 Abs/g dry matter at 90 °C. Furthermore, it is well known the effect of water on chemical reactions, via a_w or by plasticising amorphous systems (dehydrated systems) since the inhibitory effect of water seems to be a decisive factor in the NEB reaction rate (Acevedo, Schebor, & Buera, 2008). In addition, the development of the Maillard reaction frequently occurs in concomitance with other events, which can contribute to change both colour and the overall antioxidant capacity of pretreated foods (Manzocco et al., 2001). For example, addition of SO_2 can be applied during drying as an inhibitor of browning due to its capacity of both disinfectant and antioxidant on essential compounds (Miranda, Berna, Salazar, & Mulet, 2009).

Moreover, Fig. 4 shows the profiles of the ASTA colour for the different drying temperatures. The ASTA colour for the fresh samples was 222.83 ± 3.06 ASTA units/ g dry matter. When the results were compared, it could be observed that the highest air-drying temperature (90 °C, 380.90 ± 2.60 ASTA units/g dry matter) presented the lowest value of colour showing a maximum at 70 °C (568.90 ± 6.90 ASTA units/g dry matter). ASTA colour content is mainly attributable to endogenous carotenoids such as capxantine, capsorubin, β-carotene and others (Vega-Gálvez et al., 2008b). In consequence, the behaviour of this parameter is directly related to deterioration in these pigments due to high drying temperatures (Ergunes & Tarhan, 2006). Furthermore, discolouration of carotenoids during processing might occur through enzymatic or non-enzymatic oxidation (Rodriguez-Amaya et al., 2008). Both NEB and ASTA colour presented significant differences ($p < 0.05$).

Fig. 5 shows the average values of the chromatic coordinates L^*, a^* and b^*, for the fresh and dry-rehydrated samples of pepper. It can be observed that coordinate L^* presents the lowest values 27.70 ± 2.65 at high temperatures (90 °C) indicating that fresh peppers presented a darker colour compared to the dry-rehydrated samples. For drying temperatures from 50 to 80 °C, the differences in lightness are not so evident ($p < 0.05$). Although there is a hypothesis related to rehydrated products with high water content having high L^* values, in this case it was not confirmed. Rehydrated peppers become darker probably due to a larger extension of the Maillard reaction, especially at higher drying temperatures (Acevedo et al., 2008; Manzocco et al., 2001). Pretreatments, like addition of different compounds, can enhance the chromatic coordinates (Garau et al., 2007; Sigge et al., 2001).

Modifications in coordinate a^* (greenness–redness) for fresh and dry-rehydrated samples are also presented in Fig. 5, where there was a decrease of this coordinate (13% at 90 °C) respect to fresh samples ($p > 0.05$). This could be explained due to the presence of carotenoids as well as other components (vitamins, carbohydrates, aminoacids, etc.) in the pepper affecting the final product colour (Miranda, Maureira, Rodriguez, & Vega-Gálvez, 2009).

Coordinate b^* (blueness–yellowness) showed a slight increase in its value (15% at 90 °C) as temperature increased (Fig. 5) as a result of generation of brown products due to non-enzymatic reactions ($p > 0.05$). Since total colour difference (ΔE) is a function of the three CIE L^* a* b* coordinates (Eq. (3)), changes from

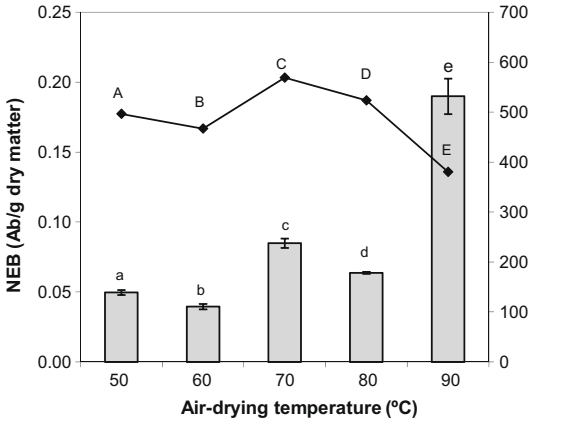

Fig. 4. Effect of air-drying temperature on non-enzymatic browning and ASTA color for dry-rehydrated pepper samples. Identical letters above the bars indicate no significant difference.

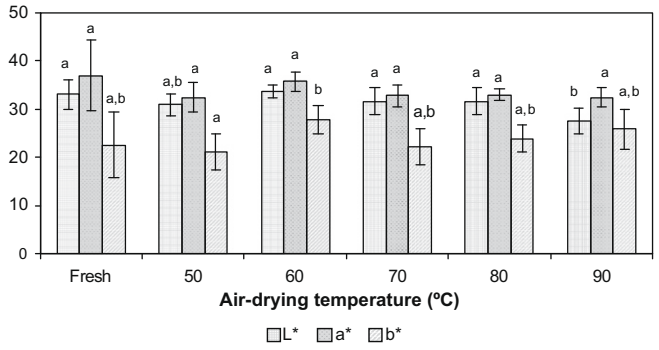

Fig. 5. Effect of air-drying temperature on the chromatics coordinates (L^*, a^* and b^*) of fresh and dry-rehydrated pepper samples. Identical letters above the bars indicate no significant difference.

5.78 ± 2.91 to 7.79 ± 2.16 were estimated for 50 and 90 °C, respectively. Similar results were obtained by Miranda, Maureira, Rodriguez, and Vega-Gálvez (2009), where high ΔE values were found at high drying temperatures due to the effect of high temperatures on heat-sensitive components like proteins and carbohydrates, amongst others.

The saturation index or chroma (C^*) and the hue angle (H^o), as shown in Fig. 6, provide more information about the spatial distribution of colours than direct values of tristimulus measurements (Sigge et al., 2001). The values of C^* and H^o are shown in Fig. 6, where it is observed that both indices are affected by temperature in opposite ways ($p > 0.05$) for both fresh and dry-rehydrated samples. Estimated chroma values retained the 96% of the fresh pepper, the hue angle showed an increase of 23% compared to fresh sample indicating discolouration of the original pepper colour.

4.5. Effect on firmness

Firmness is one of the most desirable attributes in fresh as well as rehydrated peppers (Castro et al., 2008). The behaviour of this physical property affected by drying temperature is illustrated in Fig. 7. It can be observed that air-drying temperature has a negative effect on this textural property presenting a maximum decrease of 50% respect to the fresh sample at 70 °C ($p < 0.05$). This tissue firmness reduction could be explained due to changes in the plant cell wall that occurred during processing at high temperatures (Papageorge, et al., 2003; Castro et al., 2008; Vega-Gálvez et al., 2008b). This behaviour can be minimized adding calcium which has been reported to maintain firmness by crosslinking cell wall and middle lamella pectins, stabilizing cell membranes, and/or affecting cell turgor potential (Castelló, Igual, Fito, & Chiralt, 2009; Vega-Gálvez et al., 2008b).

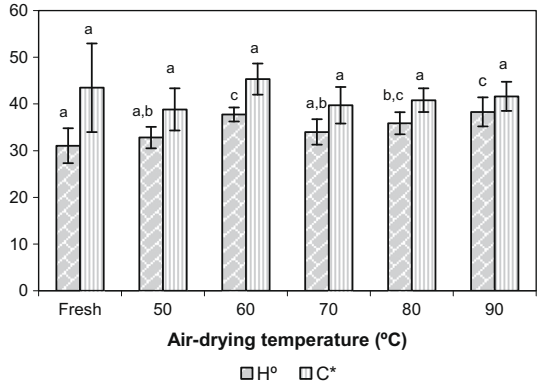

Fig. 6. Effect of air-drying temperature on the saturation index or chroma (C^*) and the hue angle (H°) of fresh and dry-rehydrated pepper samples. Identical letters above the bars indicate no significant difference.

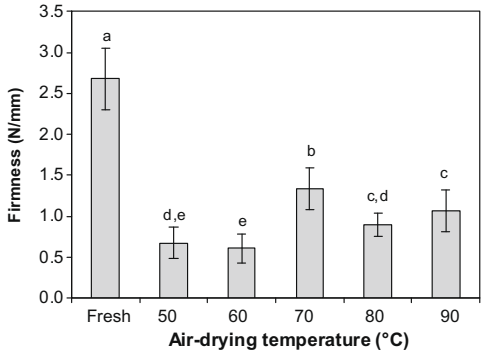

Fig. 7. Effect of air-drying temperature on the firmness of fresh and dry-rehydrated pepper samples. Identical letters above the bars indicate no significant difference.

5. Conclusions

The effect of air-drying temperature on physico-chemical properties, firmness, rehydration, colour and antioxidant activity due to total phenolic content and ascorbic acid of red pepper during hot air-drying between 50 and 90 °C was investigated. The rehydration ratio (RR) decreased with temperature showing a lower RR of 4.24 ± 0.12 (g absorbed water/ g d.m.) at 90 °C. The maximum water holding capacity was 47.4 ± 2.8 (g retained water/100 g water) at 50 °C. Moreover, both vitamin C content and total phenolic content decreased as air-drying temperature increased. A maximum loss of 98.2% in vitamin C was observed in samples dried at 90 °C. The radical scavenging activity showed higher antioxidant activity at high temperatures (80–90 °C) respect to low temperatures (50–70 °C). Non-enzymatic compounds increased with temperature. ASTA colour was affected by temperature and presented the lowest colour value at 90 °C. All chromatic parameters (L^*, a^*, b^*, C^* and H°) were affected by temperature. In addition, the development of the Maillard reaction, which occurs in concomitance with other events, could contribute to change both colour and the overall antioxidant capacity of the pepper. A tissue firmness reduction was observed at high temperatures.

In consequence, the results obtained in this work are essential for the processing of dehydrated pepper in order to obtain the optimum benefits of bioactive compounds present in this food product during drying.

Acknowledgements

The authors acknowledge the Research Department of Universidad de La Serena from Chile, Project DIULS-CD 220-2-06 as well as the Consejo Nacional de Investigaciones Científicas y Tecnicas from Argentina for the financial support given to the publication of this research.

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Food Chemistry 117 (2009) 654–659

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Carbohydrate and protein sources influence the induction of α- and β-galactosidases in *Lactobacillus reuteri*

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ARTICLE INFO

Article history:
Received 17 December 2008
Received in revised form 3 March 2009
Accepted 17 April 2009

Keywords:
α-Galactosidase
β-Galactosidase
Lactobacillus reuteri
Raffinose, Lactose
Yeast extract

ABSTRACT

The induction of α- and β-galactosidases in six strains of *Lactobacillus reuteri* (*L. reuteri*) by six carbohydrate sources and four protein sources was studied. *L. reuteri* grown on raffinose had the highest α-galactosidase activity (10.55 Gal U/ml), while lactose exhibited the highest β-galactosidase activity (43.82 Gal U/ml) when compared to other carbohydrate sources. *L. reuteri* grown on yeast extract exhibited the highest α- and β-galactosidases activity (15.27 and 12.88 Gal U/ml, respectively) when compared to other protein sources. MF14C and SD2112 grown on raffinose had the highest α-galactosidase activity (14.75 and 14.18 Gal U/ml, respectively) followed by CF2-7F (13.38 Gal U/ml). CF2-7F grown on lactose had the highest β-galactosidase activity (82.01 Gal U/ml). SD2112, MM2-3 and CF2-7F grown on yeast extract (20.96, 19.67, 19.67 Gal U/ml, respectively) showed the highest α-galactosidase activity. MM2-3 and CF2-7F grown on yeast extract showed the highest β-galactosidase activity (18.1 and 17.59 Gal U/ml, respectively). Raffinose and lactose were the best carbohydrate sources to produce α- and β-galactosidases, respectively. Yeast extract was the best protein source to produce both enzymes and CF2-7F strain was the best producing strain on all tested conditions.

1. Introduction

α-Galactosidase (EC 3.2.1.22) and β-galactosidase (EC 3.2.1.23) are functional enzymes that catalyse the hydrolysis of oligo- and polysaccharides containing terminal α-galactosyl groups or β-D-galactopyranosides, respectively. Both enzymes have health benefits and industrial applications. α-Galactosidase hydrolyses raffinose and stachyose in leguminous foods and feeds, as these oligosaccharides tend to cause intestinal discomfort, flatulence and low feed utilisation in monogastrites (Ulezlo & Zaprometova, 1982). β-Galactosidase can alleviate the problems associated with whey disposal, lactose crystallisation in frozen concentrated desserts and milk consumption by lactose-intolerant individuals (Kim & Rajagopal, 2000). On the other hand, α- and β-galactosidases are effective for galactooligosaccharides (GOS) synthesis by transglycosylation or reverse hydrolysis reactions (Donkor, Henriksson, Vasiljevic, & Shah, 2007; Hsu, Yu, & Chou, 2005). The formation of GOS can be used as prebiotic functional food ingredients to contribute to human health (Splechtna et al., 2006).

The investigation of the production of α-galactosidase or β-galactosidase from microorganisms is not new (e.g. Donkor et al., 2007; Garro, de Giori, de Valdez, & Oliver, 1994; Hsu et al., 2005; Ibrahim & O'Sullivan, 2000). However, most of the α- and β-galactosidases used in these studies were not approved for food use, because they were not obtained from food grade microorganisms. In addition, production of α- and β-galactosidases from the previously studied microorganisms is low, which hinders its commercial use. To address this need, identification of source microorganisms which are safe for human use and capable of producing high levels of α- and β-galactosidase becomes critically important.

Several studies have shown that Lactic Acid Bacteria (LAB) could be a good source of both enzymes (Akolkar, Sajgure, & Lele, 2005; Alazzeh, Ibrahim, Song, Shahbazi, & AbuGhazaleh, 2009; Bury, Geciova, & Jelen, 2001; Donkor et al., 2007; Garro et al., 1994; Ibrahim & O'Sullivan, 2000) Lactobacilli is part of LAB, which reside normally in the gastrointestinal tract of humans and animals (Xanthopoulos, Litopoulou-Tzanetaki, & Tzanetakis, 2000) meet the Generally Recognized as Safe (GRAS) standard for food processing (Speck, Dobrogosz, & Casas, 1993). *Lactobacillus reuteri* (*L. reuteri*) has a good clinical record and good probiotic characteristics, such as the ability to produce antimicrobial products like reuterin (Gänzle, Hältzel, Walter, Jung, & Kammes, 2000), the ability to prevent and reduce hypercholesterolaemia in mice (Taranto, Medici, Perdigon, Ruiz Holgado, & Valdez, 1998), and the ability to survive gastrointestinal pH (Rodriguez, Arques, Rodriguez, Nunez, & Medina, 2003). Most studies that have investigated LAB for the production of galactosidases have concentrated on *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* and *Lactobacillus fermentum* (Bury et al., 2001; LeBlanc, Garro, & Savoy de Giori, 2004; Sasaki,

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doi:10.1016/j.foodchem.2009.04.065

Samant, Suzuki, Toba, & Itoh, 1993). These studies and others show that the production of α- and β-galactosidases from microorganisms is affected by various culture conditions such as the sources of carbohydrates and proteins and the occurrence of ion metals. For example, α-galactosidase activity with raffinose and melibiose as carbohydrate sources has been demonstrated to be up to 30 folds higher than when glucose is used (Tzortzis, Jay, Baillon, Gibson, & Rastall, 2003; Xiao, Tanaka, Qian, Yamamoto, & Kumagai, 2000). Hsu et al. (2005) found that lactose as a carbohydrate source and yeast culture as a protein source were the best culture conditions to produce β-galactosidase in bifidobacteria. Bury et al. (2001) showed that α-galactosidase activity in *L. delbrueckii* subsp. *Blugaricus* increased threefold when yeast extract was added to a whey media. A few studies have investigated the production of galactosidases from *L. reuteri* and have found that *L. reuteri* could be a good source of galactosidases (Alazzeh et al., 2009; Nguyen, Splechtna, Yamabhai, Haltrich, & Peterbauer, 2007). However, more work is needed to optimise the culture growth conditions of *L. reuteri* to enhance the activities of α- and β-galactosidases. In this study we are trying to establish strains of *L. reuteri* with enhanced α- and β-galactosidases activities. These strains could be added as a food grade additive to achieve added health benefits to food products mainly to those that have indigestible sugars. Our objectives were to optimise the culture conditions of carbohydrate and protein sources of six strains of *L. reuteri* to obtain strains with enhanced α- and β-galactosidases activities that could be good candidates as a food additive or a food supplement.

2. Materials and methods

2.1. Microorganisms

Six strains of *L. reuteri* (CF2-7F, DSM20016, MF14-C, MM2-3, MM7 and SD2112) were used. These strains were obtained from a commercial source (BioGaia, Raleigh, NC). The sources of the strains were child faecal isolate (CF2-7F), mother faecal isolate (MF14C) and mother's milk (DSM20016, MM2-3, MM7 and SD2112).

2.2. Culture conditions

L. reuteri strains were maintained on *Lactobacillus* MRS agar (Difco Laboratories, Becton Dickinson, Sparks, MD) petri dishes at 4 °C. Before use, the strains were activated by taking one bacterial colony of each strain and separately inoculating the colony into *Lactobacillus* MRS broth (Difco Laboratories, Becton Dickinson, Sparks, MD) in anaerobic culture tubes at 37 °C for18 h.

2.2.1. Carbohydrate enzyme induction

The activated strains were harvested by centrifugation (10,000*g* for 10 min at 4 °C) and washed twice with 0.1% sterilised peptone water. To examine the effect of carbohydrate source on induction of α- and β-galactosidases, a modified M17 media (Ibrahim, Ahmed, & Song, 2009) was prepared in 950 ml distilled water comprised of: 37.25 g M17 (Difco Laboratories, Becton Dickinson, Sparks, MD), 5.00 g beef extract, 2.50 g yeast extract and 5.00 g phyto-peptone. This preparation was then sterilised at 121 °C for 15 min and 50 ml of different sterile sugars (dextrose, galactose, lactose, melibiose, raffinose and sucrose solutions (40%) were added separately. The control group consisted of the modified M17 media without added carbohydrate source.

2.2.2. Protein enzyme induction

To test the induction of α- and β-galactosidases activity by different protein sources, the activated strains were harvested by centrifugation (10,000*g* for 10 min at 4 °C) and washed twice with 0.1% sterilised saline water. Fermentation of strains with different protein sources was carried out by transferring 0.1 ml of inoculum into 10 ml culture media samples consisting of 1% g lactose, 1% g raffinose, 0.5% $NaCH_3COO$, 0.05% $MgSO_4{\cdot}7H_2O$, 0.005% $MnSO_4{\cdot}4H_2O$, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.05% L-cysteine (Sigma, St. Louis, MO, USA) and 0.1% Tween 80. Different protein source samples were created by adding either 2% either of beef extract, tryptic soy, tryptone, or of yeast extract. For negative control, the same media was prepared with no added protein source. *Lactobacillus* MRS broth with L-cysteine samples (described earlier) served as a positive control. After 18 h incubation at 37 °C, cultures were harvested for testing for enzymes activities.

2.3. Determination of α- and β-galactosidases activity

Strain samples were prepared as described by Hsu et al. (2005) with slight modification. Overnight cultures were first harvested by centrifugation (10,000*g* for 10 min at 4 °C). After washing twice with phosphate buffer (pH 6.8), the bacteria cells were resuspended in 1 ml of phosphate buffer in Eppendorf tubes containing 0.1 mm glass beads (BioSpec Products Inc., Bartlesville, OK, USA) and maintained in an ice bath. All samples were treated with a bead beater (MiniBead Beater-8, BioSpec Products Inc., Bartlesville, OK, USA) for total of 3 min. After every minute, the samples were rested for 15 s in an ice bath. Samples were then centrifuged (12,000*g*, 30 min) to obtain the supernatant which was preserved at −80 °C until enzyme activity was assayed. The supernatant served as the enzyme source.

2.3.1. α-Galactosidase assay

α-Galactosidase activity was assayed according to the Food Chemicals Codex (2003) protocol. α-Galactopyranoside activity was tested by adding 1 ml of *p*-nitrophenyl-α-D-galactopyranoside substrate to 0.5 ml of each sample and transferring the samples into water bath at 37 ± 0.2 °C for 15 min. All reactions were stopped by adding 2.5 ml of Borax buffer. A blank for each sample was prepared by sequentially adding 0.5 ml sample, 2.5 ml Borax buffer and 1 ml of substrate solution. Absorbance was measured at 405 nm by spectrophotometer (Model Genesys 10Vis, Thermospectronic, Rochester, NY, USA).

2.3.2. β-Galactosidase assay

β-Galactosidase activity was assayed according to the method described by Nagy, Kiss, Szentirmai, and Biró (2001). The reaction mixture was composed of 0.5 ml of enzyme source and 0.5 ml of 15 mM *o*-nitrophenyl-β-D-galactopyranoside in 0.03 M sodium phosphate buffer (pH 6.8). Reactions were maintained on a water bath at 37 ± 0.2 °C with automatic shaking for 10 min, and then 2.0 ml of 0.1 M sodium carbonate was added to stop the reaction. The optical density (OD) for each of the samples was measured at 420 nm with spectrophotometer (Model Genesys 10Vis, Thermospectronic, Rochester, NY, USA). The unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of *o*-nitrophenyl per minute under the assay condition.

2.4. Protein assay

Protein in strain samples was measured by Bradford method with bovine serum albumin as the standard (Bradford, 1976). To calculate α- and β-galactosidases specific activity, activity of each enzyme was divided by its protein content.

2.5. Statistical analysis

Statistical analysis of data was performed using the SAS general linear model (GLM) program (1999). Duplicate samples of each of

the strains under every growth condition were assayed for α- and β-galactosidases activity. Least square means of the duplicate samples were calculated and Duncan's Multiple Range Test was used to determine significant ($P < 0.01$) differences.

3. Results and discussion

3.1. Induction of α- and β-galactosidases by different carbohydrate sources

3.1.1. Effect of carbohydrate source on α-galactosidase activity

Data of each carbohydrate/protein source were averaged to test the effect of single sugar/protein source on the induction of α- and β-galactosidases activity. Results of carbohydrate sources showed that *L. reuteri* grown on raffinose had a significantly higher ($P < 0.01$) α-galactosidase activity (10.55 Gal U/ml) than the control and other carbohydrate (sugar) sources. The next highest producer was melibiose (6.15 Gal U/ml) which was significantly higher ($P < 0.01$) than the remaining carbohydrate sources. Lactose, dextrose, galactose and sucrose induction of α-galactosidase activity was not increased significantly ($P > 0.05$) (Table 1). α-Galactosidase specific activity was also significantly higher ($P < 0.01$) in *L. reuteri* grown on raffinose (11.75 Gal U/mg) than the control and the other carbohydrate sources (Table 1).

The highest levels of α-galactosidase activity being associated with raffinose may be due to its ability to induce the gal operon responsible for LAB production of α-galactosidase (Semsey, Virnik, & Adhya, 2006). Raffinose and melibiose, which are saccharides with an α-galactosyl group linkage between their subunits, might activate genes responsible for the over production of α-galactosidase. The other saccharides used in this study do not have an α-gal linkage, which might explain the lower α-galactosidase production in the media containing these sugars. Our results are in agreement with Xiao et al. (2000) who showed that raffinose followed by melibiose were the most effective sugars to induce α-galactosidase in *Bifidobacterium breve*. Li, Yang, Yan, Zuo, and Jin (1997) found that melibiose followed by raffinose were the best carbohydrate sources for α-galactosidase activity in *Bacillus* sp.

3.1.2. Effect of carbohydrate source on β-galactosidase activity

β-Galactosidase activity (43.82 Gal U/ml) in *L. reuteri* grown on lactose was significantly higher ($P < 0.01$) than for the other carbohydrate sources. Specific activity of β-galactosidase followed the same trend as activity with *L. reuteri* grown on lactose; it had a significantly higher ($P < 0.01$) specific activity (48.25 Gal U/mg) than the other carbohydrate sources (Table 1). The presence of lactose in the media probably triggered β-galactosidase activity by the lac operon mechanism (Miller & Reznikoff, 1978). This interpretation is in agreement with the findings of Akolkar et al. (2005) and Hsu et al. (2005), who showed that the presence of lactose in the media led to an enhancement of β-galactosidase activity for bifidobacteria and *L. acidophilus*, respectively. On the other hand, Kim and Rajagopal (2000) found that galactose is better than lactose as an inducer of β-galactosidase activity in *Lactobacillus crispatus*. In our experiment, the activity of β-galactosidase was minimally higher than lactose only in one strain (MF14C). However, the differences were not significant ($P > 0.05$), and the discrepancies between the two studies could be attributed to the use of different *Lactobacillus* sp.

3.1.3. Between strain comparisons of α-galactosidase activity due to different carbohydrate sources

L. reuteri strains had different responses, in terms of enzyme activity, to the presence of carbohydrate sources in the media. α-Galactosidase activity in MF14C grown on raffinose (14.75 Gal U/ml) was significantly higher ($P < 0.01$) than that in other *L. reuteri* strains, followed by SD2112 (14.18 Gal U/ml) and CF2-7F (13.38 Gal U/ml) both grown on raffinose, which were significantly higher ($P < 0.01$) than the remaining strains (Fig. 1). Among strain enzyme activity was significantly higher ($P < 0.01$) in *L. reuteri* grown on raffinose followed by melibiose (Fig. 1).

3.1.4. Between strain comparisons of β-galactosidase activity due to different carbohydrate sources

CF2-7F grown on lactose showed significantly higher ($P < 0.01$) β-galactosidase activity (82.01 Gal U/ml) than other strains, followed by DSM20016 grown on lactose (73.75 Gal U/ml), which was significantly ($P < 0.01$) higher than the remaining strains (Fig. 2). Results for both enzymes suggest that CF2-7F and DSM20016 strains showed the highest levels. Other strains showed high activity only in one enzyme (MF14C and SD2112 also showed high activity for α-galactosidase).

3.2. Induction of α- and β-galactosidases by different protein sources

3.2.1. Effect of protein source on α-galactosidase activity

L. reuteri grown on yeast extract showed a significantly higher ($P < 0.01$) α-galactosidase activity (15.27 Gal U/ml) than other protein sources (Table 2) followed by tryptone and tryptic soy (11.35 and 9.27 Gal U/ml, respectively), which were significantly higher ($P < 0.01$) than the remaining protein sources. Media containing yeast extract showed also a higher ($P < 0.01$) α-galactosidase specific activity (41.13 Gal U/mg) than other protein sources followed by tryptic soy, tryptone and beef extract (26.7, 22.05 and 18.72 Gal U/mg, respectively) which were significantly higher ($P < 0.01$) than the remaining protein sources. These findings are consistent with Gote, Umalkar, Khan, and Khire (2004) who found that yeast extract was the best protein source for α-galactosidase activity in *Bacillus stearothermophilus*.

3.2.2. Effect of protein source on β-galactosidase activity

β-Galactosidase activity in *L. reuteri* due to different protein sources followed the same trend of α-galactosidase. Yeast extract media showed a higher ($P < 0.01$) β-galactosidase activity (12.88

Table 1
The effect of carbohydrate source on α-galactosidase, β-galactosidase activity and specific activity by six strains of *Lactobacillus reuteri*.

Carbohydrate source	α-Galactosidase		β-Galactosidase		Protein content (mg/ml)
	Activity (Gal U/ml)	Specific activity (Gal U/mg)	Activity (Gal U/ml)	Specific activity (Gal U/mg)	
Dextrose	0.92 ± 0.39^{c}	1.00 ± 0.25^{d}	2.45 ± 0.94^{c}	2.32 ± 0.63^{c}	0.61 ± 0.16^{ab}
Galactose	0.92 ± 0.16^{c}	1.00 ± 0.17^{d}	27.35 ± 5.04^{b}	31.32 ± 5.40^{b}	0.83 ± 0.05^{a}
Lactose	1.6 ± 0.22^{c}	1.83 ± 0.18^{d}	43.82 ± 8.33^{a}	48.25 ± 9.23^{a}	0.80 ± 0.08^{ab}
Melibiose	6.15 ± 1.04^{b}	9.75 ± 1.43^{b}	18.95 ± 5.39^{b}	29.69 ± 8.12^{b}	0.55 ± 0.06^{b}
Raffinose	10.55 ± 1.50^{a}	11.75 ± 1.59^{a}	22.64 ± 7.22^{b}	25.97 ± 8.06^{b}	0.78 ± 0.08^{ab}
Sucrose	1.42 ± 0.30^{c}	1.58 ± 0.37^{d}	1.27 ± 0.21^{c}	1.61 ± 0.28^{c}	$0.76v0.03^{ab}$
Control	1.01 ± 0.17^{c}	6.67 ± 1.05^{b}	0.89 ± 0.13^{c}	6.19 ± 0.85^{c}	0.15 ± 0.02^{c}

Means ± standard error of means in the same column with different superscripts are significantly ($P < 0.01$) different.

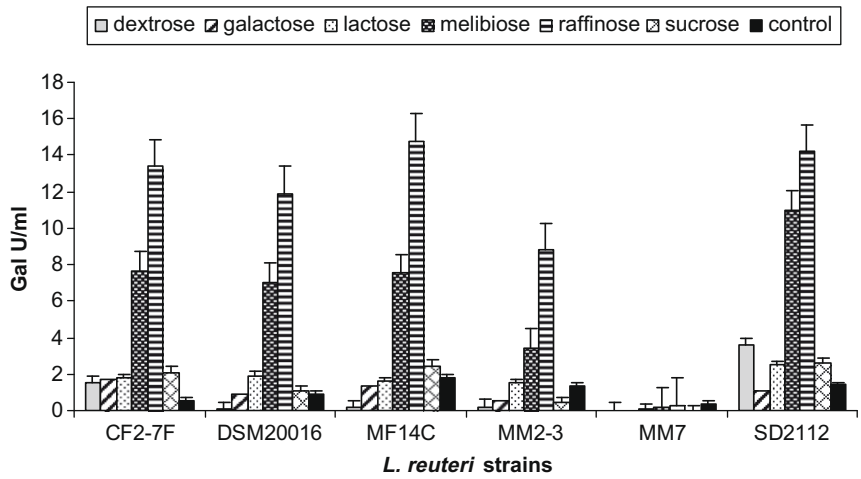

Fig. 1. α-Galactosidase activity (Gal U/ml) of six strains of *L. reuteri* grown on different carbohydrate sources. (Error bars represent a pooled standard error of the means for each carbohydrate source.)

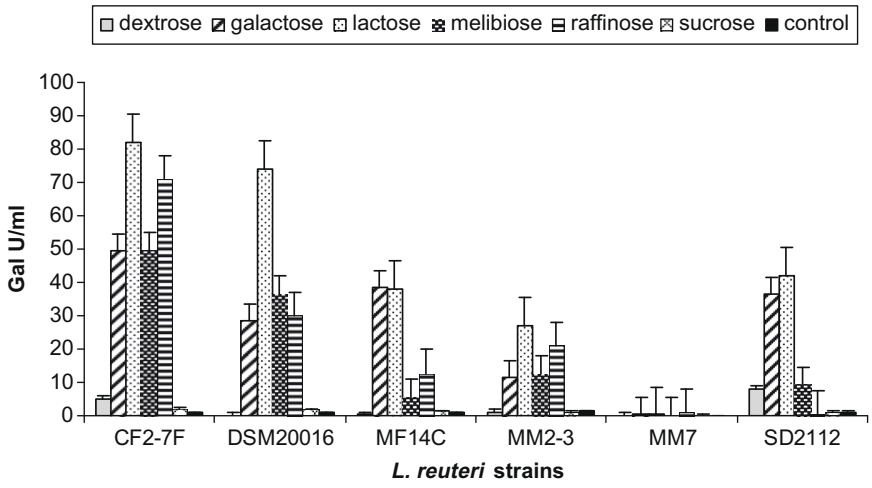

Fig. 2. β-Galactosidase activity (Gal U/ml) of six strains of *L. reuteri* grown on different carbohydrate sources. (Error bars represent a pooled standard error of the means for each carbohydrate source.)

Table 2
The effect of protein source on α-galactosidase, β-galactosidase activity and specific activity by six strains of *Lactobacillus reuteri.*

Protein source	α-Galactosidase		β-Galactosidase		Protein content (mg/ml)
	Activity (Gal U/ml)	Specific activity (Gal U/mg)	Activity (Gal U/ml)	Specific activity (Gal U/mg)	
Beef extract	3.77 ± 0.71^{c}	18.72 ± 3.65^{b}	4.17 ± 0.56^{c}	21.43 ± 4.25^{b}	0.22 ± 0.02^{d}
Tryptic soy	9.27 ± 1.47^{b}	26.70 ± 3.58^{b}	7.91 ± 1.20^{b}	23.99 ± 3.49^{b}	0.34 ± 0.05^{cd}
Tryptone	11.35 ± 1.72^{b}	22.05 ± 3.15^{b}	7.87 ± 1.05^{b}	15.74 ± 1.49^{bc}	0.49 ± 0.06^{ab}
Yeast extract	15.27 ± 2.03^{a}	41.13 ± 4.70^{a}	12.88 ± 0.56^{a}	35.43 ± 4.50^{a}	0.36 ± 0.05^{bc}
MRS	1.20 ± 0.26^{c}	2.51 ± 0.53^{c}	4.04 ± 0.55^{c}	$8.29 \pm 1.32c^{d}$	0.52 ± 0.05^{a}
Negative control	0.04 ± 0.00^{c}	0.04 ± 0.00^{c}	0.01 ± 0.00^{d}	0.24 ± 0.04^{d}	0.05 ± 0.00^{e}

Means ± standard error of means in the same column with different superscripts are significantly ($P < 0.01$) different.

Gal U/ml) than other protein sources, followed by tryptic soy and tryptone (7.91 and 7.87 Gal U/ml, respectively), which were significantly higher ($P < 0.01$) than the remaining protein sources. In addition, yeast extract media produced significantly higher ($P < 0.01$) β-galactosidase specific activity (35.43 Gal U/mg) than the other protein sources followed by tryptic soy and beef extract and peptone (23.99, 21.43 and 15.74 Gal U/mg, respectively. Both Bury et al. (2001) and Hsu et al. (2005) showed that yeast extract was effective in enhancing β-galactosidase activity in *L. delbrueckii* subsp. *bulgaricus* and bifidobacteria, respectively.

3.2.3. Between strain comparisons of α-galactosidase activity due to different protein sources

Between strains comparisons showed that generally, strains grown on yeast extract had the best α-galactosidase activity. SD2112, MM2-3 and CF2-7F grown on yeast extract (20.96, 19.67, 19.67 Gal U/ml, respectively) and SD2112 grown on peptone

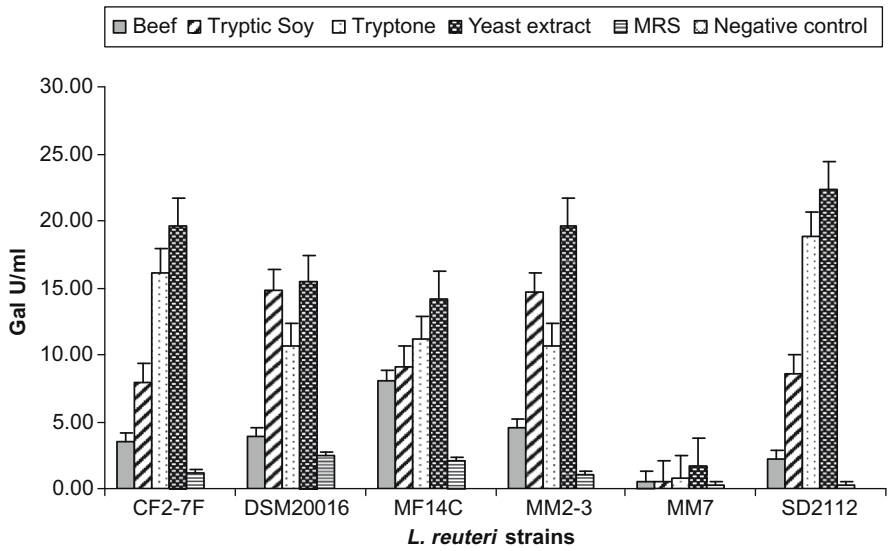

Fig. 3. α-Galactosidase activity (Gal U/ml) of six strains of *L. reuteri* grown on different protein sources. (Error bars represent a pooled standard error of the means for each protein source.)

(18.87 Gal U/ml) were associated with significantly higher ($P < 0.01$) α-galactosidase activity than other tested strains (Fig. 3).

3.2.4. Between strain comparisons of β-galactosidase activity due to different protein sources

β-Galactosidase activity in the six *L. reuteri* strains in response to the different protein sources is shown in Fig. 4. From the figure it can be seen that MM2-3 and CF2-7F strains grown on yeast extract media had significantly higher ($P < 0.01$) activity of the enzyme (18.1 and 17.59 Gal U/ml, respectively). Results from the protein enzyme induction experiment showed that yeast extract lead to a significantly higher ($P < 0.01$) activity and specific activity of both enzymes than the other protein sources. Therefore, this suggests that yeast extract could be a possible protein source in traditional LAB media to enhance the activity of both enzymes. CF2-7F, DSM20016 and SD2112 strains grown on yeast extract showed good results for the activity of both enzymes as a response of different protein sources (Figs. 1 and 2). However, only CF2-7F strain showed high activity of both enzymes either in carbohydrate or protein source experiments.

4. Conclusion

In this study we tried to optimise the growth conditions of *L. reuteri* by testing different carbohydrate and protein sources in the growth media to obtain enhanced activities of α- and β-galactosidases. *L. reuteri* grown on raffinose exhibited the best activity and specific activity of α-galactosidases. MF14C, SD2112 and CF2-7F showed the best α-galactosidase activity when grown on raffinose. *L. reuteri* grown on lactose showed the best activity and specific activity of β-galactosidase. CF2-7F and DSM2112 were the best strains with enhanced activities of this enzyme when grown on lactose. Yeast extract performed as the best protein source in terms of enhanced activity and specific activity of α- and β-galactosidases. SD2112, MM2-3 and CF2-7F grown on yeast extract and SD2112 grown on peptone were the best strains

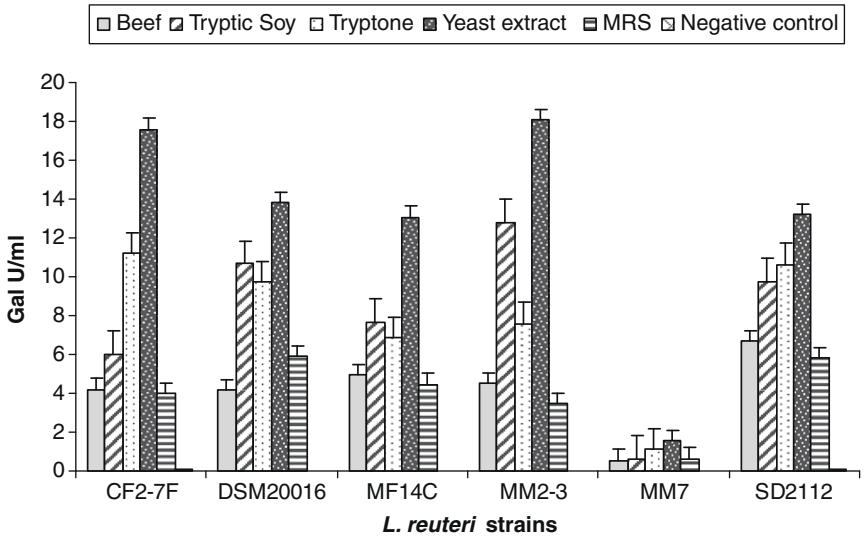

Fig. 4. β-Galactosidase activity (Gal U/ml) of six strains of *L. reuteri* grown on different protein sources. (Error bars represent a pooled standard error of the means for each protein source.)

with enhanced activities of α-galactosidase. MM2-3 and CF2-7F strains grown on yeast extract were the best strains with enhanced activities of β-galactosidase. Yeast extract was the best protein source to enhanced the activities of both enzymes and CF2-7F strain was the only strain with enhanced activities of both enzymes on all tested conditions. Our results showed that CF2-7F grown in a growth medium with raffinose and lactose as carbohydrate sources and with yeast extract as a protein source could provide conditions as a good probiotic candidate. For this reason, *L. reuteri* strain CF2-7F should receive further attention as potentially beneficial food additive or food supplement. With the increasing attention of the role of LAB bacteria in the production of probiotic products for health and nutritional benefits, the use of *L. reuteri* strain CF2-7F with high α- and β-galactosidases activities should be addressed.

Acknowledgments

We like to express our gratitude to Dr. C.S. Turner and Dr. Keith Schimmel (Energy and Environmental Interdisciplinary Studies at NC A&T) for their support and to and Dr. K.J. Gruber from the Agricultural Research Program at North Carolina A&T State University for his review and comments on this manuscript. We would like also to thank Ms. Elizabeth Fitzpatrick for her technical help.

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Food Chemistry 117 (2009) 660–667

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Culinary plants, herbs and spices – A rich source of PPARγ ligands

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ARTICLE INFO

Article history:
Received 7 November 2008
Received in revised form 17 April 2009
Accepted 20 April 2009

Keywords:
Obesity
PPARγ
Diabetes
Plants
SPPARγMs
PPARγ-antagonists

ABSTRACT

Obesity and the related disorders, diabetes, hypertension and hyperlipidemia have reached epidemic proportions world-wide. The influence of 70 plants, herbs and spices on peroxisome proliferators-activated receptor (PPAR)γ activation or antagonism, a drug target for metabolic syndrome, was investigated. Approximately 50 different plant extracts bound PPARγ in competitive ligand binding assay, including pomegranate, apple, clove, cinnamon, thyme, green coffee, bilberry and bay leaves. Five plant extracts transactivated PPARγ in chimeric GAL4-PPARγ-LBD system: nutmeg, licorice, black pepper, holy basil and sage. Interestingly, nearly all plant extracts antagonized rosiglitazone-mediated coactivator recruitment in time resolved fluorescence resonance energy transfer coactivator assay. The five transactivating extracts may function as selective PPARγ modulators (SPPARγMs), and the other extracts seem to be moderate antagonists or undetectable/weak SPPARγMs. As SPPARγMs improve insulin resistance without weight gain and PPARγ antagonists exert antiobesity action, a combination of these plants in diet could reduce obesity and the incidence of metabolic syndrome.

1. Introduction

The prevalence of obesity has increased rapidly over the last decade. According to the World Health Organization (WHO), in 2005 approximately 1.6 billion adults were overweight and at least 400 million adults were obese (WHO, 2006). Obesity is associated with insulin resistance, a key factor of the metabolic syndrome which is defined as the coexistence of hyperglycaemia, hypertension, hyper- and dyslipidemia and central obesity. Cardiovascular diseases such as coronary heart diseases and stroke are more prevalent amongst patients with this syndrome (Alberti, Zimmet, Shaw, & Grundy, 2006).

Diabetes has historically been treated with plants or plant-derived formulations in different cultures (Grover, Yadav, & Vats, 2002; Shapiro & Gong, 2002). Scientific investigations have reported the efficiency of several plants and plant preparations including red clover (*Trifolium pratense*) (Mueller & Jungbauer, 2008), pomegranate (*Punica granatum*) (Huang et al., 2005), garlic (*Allium sativum*), onion (*Allium cepa*), turmeric (*Curcuma longa*), coriander (*Coriandum sativum*) (Grover et al., 2002; Srinivasan, 2005), cinnamon (*Cinnamonum verum*) (Verspohl, Bauer, & Neddermann, 2005), green and black teas (*Camellia sinensis*), allspice (*Pimenta officinalis*), bay leaves (*Laurus nobilis*), nutmeg (*Myristica fragrans*), clove (*Syzygium aromaticum*) (Broadhurst, Polansky, & Anderson, 2000), licorice (*Glycyrrhiza uralensis*) (Mae et al., 2003). The most prominent representatives of plant extracts currently used for treatment of diabetes are cinnamon powder, bitter melon (*Momordica charantia*) and fenugreek. Different mechanisms for the antidiabetic effect of plants have been proposed: increased release of insulin, reduction of intestinal glucose absorption, enhancement of glycogen synthesis (Grover et al., 2002) and PPARγ-activation (Huang et al., 2005; Mae et al., 2003).

One of the drug targets for diabetes is the peroxisome proliferators-activated receptor (PPAR)γ (Guo & Tabrizchi, 2006). Full PPARγ activators increase insulin sensitivity, but simultaneously induce adipocyte differentiation and lipid storage, subsequently causing weight gain. In contrast, selective PPARγ modulators (SPPARγMs) show antidiabetic action without promoting weight gain. These substances function as PPARγ ligands that cause selective cofactor recruitment, meaning that adipogenic cofactors are not recruited (Zhang, Lavan, & Gregoire, 2007). PPARγ antagonists were shown to exert antiobesity and antidiabetic action by the inhibition of adipocyte differentiation, inhibition of adipose tissue accumulation and thus decrease of adipokine secretion (Rieusset et al., 2002).

In this study, a number of edible plants and plant extracts were screened for their PPARγ binding affinity, and PPARγ agonistic and antagonistic activity.

2. Materials and methods

2.1. Materials

Various chemicals were procured from Sigma–Aldrich (St. Louis, MO, USA/ Darmstadt, Germany) and Cayman Chemicals (Ann Arbor, MI, USA).

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doi:10.1016/j.foodchem.2009.04.063

Table 1
Characteristics of the tested plant extracts.

Plant extract, ratio of extraction (standardization)	Species	Plant part	LBA: IC_{50} (μg/ml)	LBA: equivalent IC_{50} (nmol/g)	TR-FRET IC_{50} (μg/ml)	Transactivation EC_{50} (μg/ml) (efficiency)
Rosiglitazone			0.043 ± 0.004		–	0.21 ± 0.05 (100%)
GW 9662			0.0030 ± 0.0005		0.0035 ± 0.0006	–
Pomegranate (40% ellagic acid)	*Punica granatum*	Fruit	0.35 ± 0.04	346171	0.07 ± 0.01	nd
Apple, 200:1 (5% quercetin, 30% phloridzin)	*Malus domestica Borkh.*	Fruit	0.49 ± 0.04	244160	0.15 ± 0.03	nd
Clove	*Syzygium aromaticum*	Flower bud	0.89 ± 0.07	135109	0.50 ± 0.03	nd
Cinnamon 1, 10:1	*Cinnamomum cassia*	Bark	1.9 ± 0.4	61901	0.9 ± 0.2	nd
Thyme 1, 7:1 (5% rosmarinic acid)	*Thymus vulgaris*	Plant	2.4 ± 0.4	50152	60 ± 50	nd
Cinnamon 2 (45% phenolic compounds)	*Cinnamon cassia*	Bark	3.8 ± 0.3	31774		–
Green Coffee	*Coffea Arabica*		4.3 ± 0.4	27738	1.0 ± 0.2	nd
Fenugreek 1	*Trigonella foenum graecum*	Seeds	4.4 ± 0.4	27206	26 ± 3	nd
Bilberry 1 (35% phenols, 15% anthocyanines)	*Vaccinium myrtillus*	Fruit	4.5 ± 0.4	26903	3.5 ± 0.5	nd
Thyme 2	*Thymus vulgaris*	Plant	6 ± 1	20018		–
Rooibos tea	*Aspalathus linearis*	Leaves	6.4 ± 0.9	18907	3.6 ± 0.6	nd
Bay leaf	*Laurus nobilis*	Leaves	9.3 ± 0.7	12971	3.0 ± 1.1	nd
Holy basil 1, 5:1	*Ocimum sanctum*	Plant	16 ± 2	7391	34 ± 4	Active (12.8%)
Holy basil 2, 5:1 (1% ursolic acid)	*Ocimum sanctum*	Plant	16 ± 3	7332		–
Bilberry 2, 5:1	*Vaccinium myrtillus*	Fruit	17.6 ± 0.8	6805		–
Crimson clover	*Trifolium incarnatum*	Flower	18 ± 2	6552	3.3 ± 0.3	nd
Chilli pepper	*Capsicum annuum*	Fruit	18 ± 2	6569	60 ± 10	nd.
Nutmeg	*Myristica fragrans*	Fruit	25 ± 2	4907	11 ± 2	106.1 ± 22.4 (15.3%)
Holy basil 3 (2% ursolic acid)	*Ocimum sanctum*	Plant	25 ± 3	4735		–
Cacao	*Theobroma cacao*	Seed	27 ± 5	4444	22 ± 2	nd
Caraway	*Carum carvi*	Seed	28 ± 5	4234	50 ± 10	nd
Licorice	*Glycyrrhiza glabra*	Root	34 ± 3	3503	27 ± 4	95.0 ± 11.9 (10%)
Sage	*Salvia officinalis*	Leaves	38 ± 4	3217	22 ± 6	active (14.3%)
Marjoram	*Origanum majorana*	Leaves	40 ± 6	3015		–
Holy basil 4	*Ocimum sanctum*	Plant	40 ± 7	2970		–
Rosemary 1	*Rosmarinus officinalis*	Plant	52 ± 8	2323		nd
Maitake mushrooms	*Grifola frondosa*	Plant	54 ± 3	2232		–
Curry plant	*Helichrysum italicum*	Plant	60 ± 10	2185		–
Tarragon	*Artemisia dracunculus*	Plant	60 ± 10	2111		–
Ginseng 1 (15% ginsenosides)	*Panax ginseng*	Root	58 ± 6	2081	nd	nd.
Rosmary 2 (2.4% rosmarinic acid)	*Rosmarinus officinalis*	Plant	60 ± 10	2008		–
Black pepper 1	*Piper nigrum*	Fruit	70 ± 20	1780	>400	active (13.8%)
Kale (1.7% quercetin)	*Brassica oleracea acephala*	Plant	70 ± 10	1762		–
Stevia Tea	*Stevia rebaudiana*	Leaves	70 ± 50	1693	nd	nd.
Black pepper 2 (95% piperine)	*Piper nigrum*	Fruit	80 ± 20	1432	>200	–
Curry powder	*Curcuma longa, Cuminum cyminum, Coriandrum sativum, Capsicum annuum, Foeniculum vulgare, Trigonella foenum graecum*	Root, seeds, fruit, plant	80 ± 20	1423	nd	nd
Onion, red (4.5% quercetin, 2.7% luteolin)	*Allium cepa*	Bulb	90 ± 10	1408		–
Thyme 3	*Thymus officinalis*	Plant	110 ± 40	1098		–
Alfalfa 1	*Medicago sativa*	Leaves	120 ± 10	992		nd
Santolina	*Santolina chamaecyparissus*	Plant	130 ± 10	942		–
Borage	*Borago officinalis*	Flower	130 ± 20	904		–
Dill	*Anethum graveolens*	Plant	200 ± 50	609		–
Nasturtium	*Tropaeolum*	Plant	230 ± 30	531		–
Jiaogulan Tea	*Gynostemma pentaphyllum*	Leaves	230 ± 80	520		–
Mulberry Tea	*Morus* spp.	Leaves	290 ± 70	418		–
White cabbage	*Brassica oleracea var. capitata f. alba*	Plant	300 ± 1300	417		–
Calendula	*Calendula officinalis*	Flower	320 ± 50	370		–
Alfalfa 2	*Medicago sativa*	Sprouts	Ligand			–
Alfalfa 3	*Medicago sativa*	Leaves	Ligand			–
Opuntia	*Opuntia sp.*	Bud	Ligand			–
Sauerkraut juice	*Brassica oleracea var. capitata f. alba*	Fermented fruit juice	Ligand			–
Bear's garlic	*Allium ursinum*	Plant	Ligand			–

(*continued on next page*)

Table 1 (*continued*)

Plant extract, ratio of extraction (standardization)	Species	Plant part	LBA: IC_{50} (μg/ml)	LBA: equivalent IC_{50} (nmol/g)	TR-FRET IC_{50} (μg/ml)	Transactivation EC_{50} (μg/ml) (efficiency)
Bitter melon	*Momordica charantia*	Fruit	Ligand			–
Black currant, 4:1	*Ribes nigrum*	Fruit	Ligand			–
Lovage	*Levisticum officinale*	Plant	Ligand			–
Fenugreek 2, 4:1	*Trigonella foenum graecum*	Seeds	Ligand			–
Fenugreek 3	*Trigonella foenum graecum*	Seeds	Ligand			–
Ginseng 2, 20:1 (15% ginsenoides)	*Panax ginseng*	Root	Ligand			–
Pea	*Pisum sativum*	Fruit	Ligand			nd
Chive (2.3% quercetin)	*Allium schoenoprasum*	Plant	Ligand			–
Parsley	*Petroselinum crispum*	Plant	Ligand			–
Crimson clover	*Trifolium incarnatum*	Leaves	Ligand			–
Lavender	*Lavendula angustifolia*	Flower	Ligand			–
Kudzu	Pueraria montana	Root	nd			–
Cress	*Lepidium sativum*	Plant	nd			–
Celery must	Apium graveolens	Fermented fruit juice	nd			–
Philadendron	*Philadendron sp.*	Fruit	nd			–

Table lists the tested plant extracts, ratio of extraction, standardization according to manufacturer's specifications, the IC_{50} values determined by competitive PPARγ ligand binding assay (= LBA), the equivalent rosiglitazone concentration calculated from ligand binding data, the IC_{50} values (determined by TR-FRET antagonist screening) and the EC_{50} value and efficiency of transactivation (determined by a chimeric GAL4- PPARγ assay). Ligand binding results of $IC_{50} \geqslant 500$ μg/ml were described as "ligands". If no EC_{50} value could be determined in the transactivation assay, compounds were described as "active". Only the most potent ligands were tested by transactivational assay and only one extract per species.

Polar Screen PPAR Competitive Assay and Lantha Screen TR-FRET (time resolved fluorescence resonance energy transfer) PPARγ coactivator assay were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Black U96 microwell plates were obtained from Nunc (Roskilde, Denmark). Fluorescence polarisation was measured with the Genios Pro plate reader from Tecan (Crailsheim, Germany). Dulbecco's minimum essential medium (DMEM) used for cell culture was obtained from Biochrom (Berlin, Germany), foetal calf serum (FCS) was purchased from HyClone (Logan, Utah, USA), SuperFect was obtained from Quiagen (Germantown, Maryland, USA), and the Dual Glo Luciferase Assay System from Promega (Madison, Wisconsin, USA).

2.2. Preparation of plants extracts/powders

Dry powder (100 mg) of plants, herbs or spices was extracted with 1 ml DMSO for 24 h at room temperature. The extract was clarified by centrifugation for 1 h at 13,000 rpm. The clear supernatant was further diluted by DMSO, and used for analysis in the PPARγ ligand binding and transfection assays.

2.3. Polar screen PPAR competitive assay

The PPAR ligand-binding competitive assay was performed with PolarScreen™ PPAR Competitor Assay Green according to the manufacturer's protocol. In brief, the PPARγ ligand-binding domain (LBD) and the fluorescent PPARγ ligand form a complex with a high polarisation value. Displacement of the fluorescent ligand by PPARγ ligands frees the fluoromone in solution to tumble rapidly during its fluorescence lifetime, causing a low polarisation value. The change in polarisation value was used to determine the relative affinity of test compounds for the PPARγ-LBD. Fluorescence polarisation was measured using a Genios Pro plate reader (Tecan, Crailsheim, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Rosiglitazone, a substance with high PPARγ affinity, was used as a standard, and maximal polarisation value of rosiglitazone was defined as 100%.

2.4. Plasmids

The pGAL4-hPPARγ-LBD expression plasmid that expresses the chimeric GAL4 DNA binding domain fused to the human PPAR-LBD (Staels et al., 1998) was provided by Prof. Staels. The luciferase reporter plasmid pFR-Luc containing a firefly luciferase gene controlled by GAL4-binding elements was purchased from Stratagene (La Jolla, California, USA). The Renilla control plasmid pRL-tk coding for *Renilla* luciferase gene and was obtained from Promega (Madison, Wisconsin, USA).

2.5. Transactivation assay

NIH-3T3 cells (German Collection of Microorganisms and Cell Cultures (GSMZ), Ac. No. 59) were seeded at a density of 1×10^4 cells per well. After 24 h of incubation, cells were co-transfected with 30 ng pGAL4-hPPARγ-LBD, 300 ng pFR-Luc reporter plasmid, and 15 ng pRL-TK using SuperFect transfection reagent according to the manufacturer's protocol. After transfection, cells were incubated with standard and test substances dissolved in DMSO solution in DMEM for 24 h; DMSO concentration did not exceed 0.1%. Luciferase assays were performed using the Dual Glo Luciferase Assay System according to the manufacturer's protocol. Renilla and firefly luminescence were measured with the Genios Pro plate reader. The firefly to Renilla ratio was calculated by dividing the firefly luciferase activity by the Renilla luciferase activity, which normalises for transfection efficiency. Rosiglitazone, a strong PPARγ activator, was used as a positive control, and the efficiency of rosiglitazone was defined as 100%.

2.6. TR-FRET coactivator assay

Antagonist screening was performed with the Lantha Screen TR-FRET PPARγ coactivator assay in antagonist mode according to the manufacturer's protocol. In brief, binding of an agonist (in this case rosiglitazone) to PPARγ results in a conformational change to recruit the coactivator peptide TRAP220/DRIP-2. The close proximity of the fluorescently labelled coactivator peptide and the terbium-labelled anti-GST antibody bound to the PPARγ GST fusion protein results in energy transfer from the terbium label to the fluorescein label, which is detected as an emission at 520 nm. Antagonists displace rosiglitazone from PPARγ and the emission signal at 520 nm decreases. Fluorescence intensity was measured using a Genios Pro plate reader at an excitation wavelength of 340 nm and an emission wavelength of 520 and 495 nm with a delay time of 100 μs and an integration time of

200 μs. The emission ratio of 520:495 indicates the extent of coactivator displacement.

2.7. Calculation of concentrations and statistics

Binding affinity, transactivational activity and antagonistic activity were determined by plotting polarisation values, transactivational efficiencies and emission ratio against the concentration of the test compound. Curve fitting was performed using a logistic dose–response model (Eq. (1)) of the Table Curve 2D software (Jandel Scientific, Erkrath, Germany). This non-linear equation uses a Levenburg–Marquard algorithm (Jungbauer & Graumann, 2001)

$$y = a + \frac{b}{1 + (c/x)^d}, \quad (1)$$

where a equals the baseline, b is the difference between the plateau of the curve and the baseline, c is the transition centre of the curve, which is the concentration that causes 50% efficiency (ligand potency), and d is the transition zone and is a measure of positive or negative cooperativity.

The concentration of the test compounds that results in a half-maximal decrease of polarisation value, half-maximal transactivational efficiency, or half-maximal decrease of emission ratio is the IC_{50}/EC_{50} value of the test compound. This value indicates PPARγ binding affinity, transactivational activity or antagonistic activity.

The competitive assay was performed in duplicate, the transactivation assay was performed in quadruplicate and the TR-FRET PPARγ coactivator assay was performed in triplicate. Each assay was performed on independent days, and intervariation was in the same range as intravariation.

The equivalent rosiglitazone concentration corresponds to the theoretical concentration of rosiglitazone in the extract producing the same binding affinity. Rosiglitazone was used as reference substance. The equivalent IC_{50} value was calculated by dividing the IC_{50} value of rosiglitazone by the IC_{50} value of the plant extracts or isoflavones (Eq. (2)):

$$IC_{50}\ (\text{nmol/g}) = \frac{IC_{50(\text{rosiglitazone})}\ (\text{nmol/L})}{IC_{50(\text{extract})}\ (\text{g/L})}. \quad (2)$$

3. Results

The PPARγ binding affinity of various plant extracts was determined by a competitive assay, transactivational activity by a chimeric GAL4-PPARγ-LBD assay system, and antagonistic activity by TR-FRET coactivator assay in antagonist mode. Potencies of the competitive ligand binding assay, the transactivation assay and the TR-FRET coactivator assay were determined by a logistic dose–response curve with the polarisation values, transactivation efficiency or emission ratio (compared to rosiglitazone or GW9662) plotted against the concentration of the test compounds. The potency indicates the concentration necessary to reach half-maximal binding affinity, transactivational efficiency or antagonistic activity.

3.1. Polar screen PPAR competitive assay

Pomegranate fruit extract (*P. granatum*) standardised on 40% ellagic acid exerted the highest binding affinity of the tested extracts, with an IC_{50} value of 0.35 μg/ml (Table 1, Fig. 1). Several extracts showed moderate binding affinity, including, amongst others, apple extract (*Malus domestica*), a 200:1 extract containing 5% quercetin and 30% phloridzin, with an IC_{50} value of 0.49 μg/ml, clove (*S. aromaticum*) (IC_{50} = 0.89 μg/ml), cinnamon (*Cinnamomum zeilanicum*) 10:1 extract (IC_{50} = 1.9 μg/ml), thyme 7:1 extract (*Thymus vulgaris*) (IC_{50} = 2.4 μg/ml), green coffee (*Coffea arabica*)

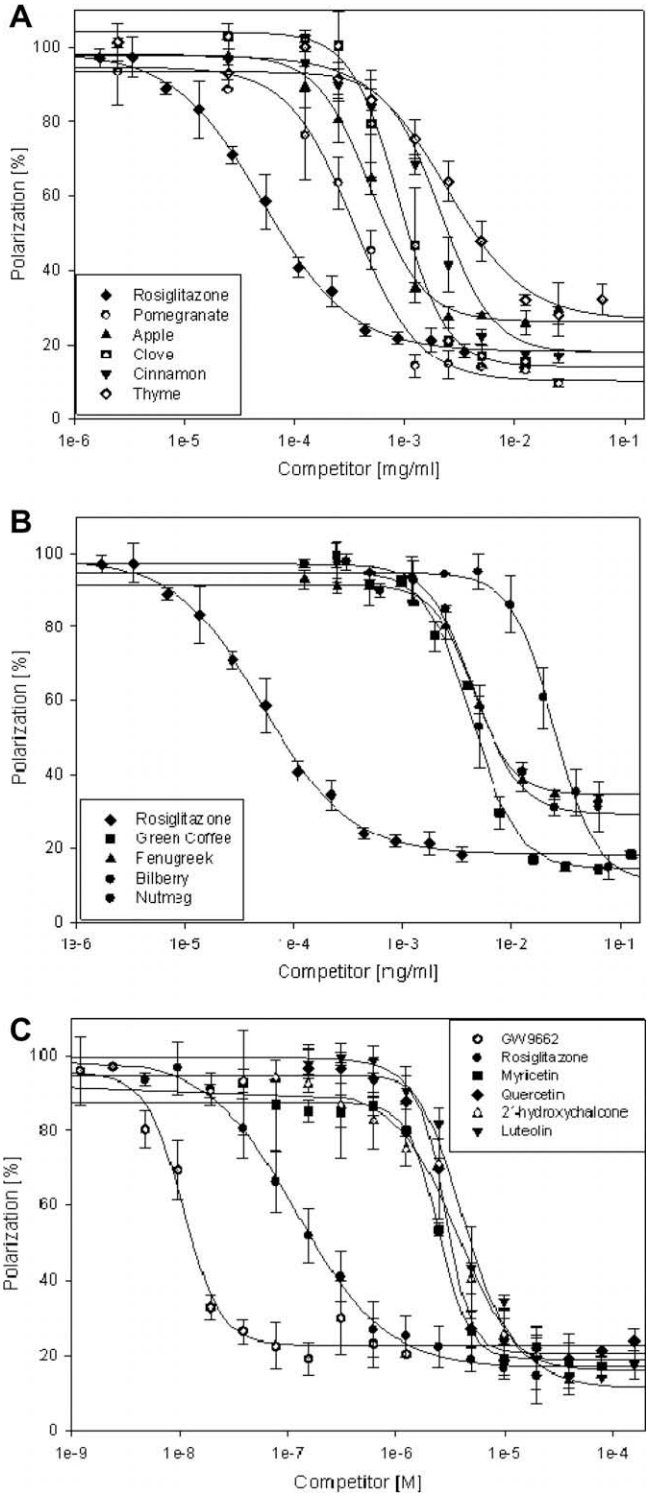

Fig. 1. (A) Logistic dose response curves of rosiglitazone, pomegranate fruit extract (standardised to 40% ellagic acid), apple extract (200-fold concentration and standardised to 5% quercetin and 30% phloridzin), clove, cinnamon extract (10-fold concentration), thyme extract (7-fold concentration) determined by competitive PPARγ ligand binding assay. (B) Logistic dose response curves of rosiglitazone, green coffee extract, fenugreek extract, bilberry extract (standardised to 35% phenols and 10% anthocyanins), nutmeg determined by competitive PPARγ ligand binding assay. (C) Logistic dose response curves of GW9662, rosiglitazone, myricetin, quercetin, 2′-hydroxychalcone, luteolin determined by competitive PPARγ ligand binding assay.

Table 2
Various substances occurring in the tested plant extracts were tested with a PPARγ competitive ligand binding assay (IC_{50}) and a chimeric GAL4-PPARγ assay system (EC_{50}).

Substance	CAS no.	Ligand binding IC_{50} (μM)	Transactivation EC_{50} (μM)	Efficiency (%)	Examples for occurrence (according to literature)
CW9662	22978-25-2	0.011 ± 0.002			
Rosiglitazone	122320-73-4	0.12 ± 0.01	0.21 ± 0.05	100	
Troglitazone	97322-87-7	1.8 ± 0.5			
Myricetin	529-44-2	2.5 ± 0.1	nd		Pomegrante fruit (Naz et al., 2007), bilberry (Ehala et al., 2005)
Quercetin	849061-97-8	2.8 ± 0.2	nd		Pomegrante fruit (Naz et al., 2007), apple (Kahle et al., 2007), tarragon, dill, bay leaf, parsley, chive, lovage (Justesen & Knuthsen, 2001), bilberry (Ehala et al., 2005), onion
2́-Hydroxychalcone	1214-47-7	2.9 ± 0.8	Active	35.8	Cinnamon (in polymeric form) (Jarvill-Taylor, Anderson, & Graves, 2001)
2-Hydroxychalcone	42224-53-3	3.8 ± 0.5	⩾21.3 ± 0.1	47.9	Cinnamon (in polymeric form) (Jarvill-Taylor et al., 2001)
Luteolin	491-70-3	3.9 ± 0.4	nd		Marjoram, sage, rosemary, (Proestos et al., 2005), thyme, tarragon, parsley (Justesen & Knuthsen, 2001), alfalfa (Seguin & Zheng, 2006)
Rosmarinic acid	20283-92-5	16 ± 1	nd		Marjoram, sage, rosemary, lavender (Wang et al., 2004), thyme (Proestos et al., 2005)
Cinnamaldehyde	104-55-2	10 ± 1	nd		Cinnamon (Marongiu et al., 2007)
Coumestrol	479-13-0	11 ± 3	64 ± 7	24.8	Alfalfa (Seguin & Zheng, 2006)
Phloretin	60-82-2	13 ± 1	80 ± 10	43.9	Apple (in glycosidic form) (Crespy et al., 2001; Kahle et al., 2007)
Ciglitazone	74772-77-3	24 ± 6			
Kämpferol	520-18-3	47 ± 6	nd		Pomegrante fruit, tarragon, dill, chive, lovage (Justesen & Knuthsen, 2001)
Resveratrol	501-36-0	62 ± 4	40 ± 5	38.9	Bilberry (Ehala et al., 2005)
Apigenin	520-36-5	80 ± 10	Active	15.7	Marjoram, sage (Proestos et al., 2005), thyme, parsley (Justesen & Knuthsen, 2001), alfalfa (Seguin & Zheng, 2006)
Catechin	18829-70-4	120 ± 40	nd		Apple (Kahle et al., 2007), marjoram, sage, rosemary (Proestos et al., 2005), cinnamon (Anderson et al., 2004), pomegrante fruit, cacao
Eugenol	97-53-0	420 ± 80			Clove (Chaieb et al., 2007), cinnamon (Marongiu et al., 2007)
Safrole	94-59-7	Ligand			Cinnamon, nutmeg, black pepper
Ethylcinnamate	103-36-6	Ligand			Cinnamon, clove (Chaieb et al., 2007)
Phloridzin	7061-54-3	Ligand			Apple (Kahle et al., 2007)
Epicatechin	490-46-0	Ligand			Apple (Kahle et al., 2007), thyme (Proestos et al., 2005), cinnamon (Anderson et al., 2004), cacao
Capsaicin	404-86-4	Ligand			Chili pepper
Indol-3-carbinol	700-06-1	Ligand			White cabbage
Benzylcinnamate	103-41-3	Ligand			Cinnamon, clove (Chaieb et al., 2007)
Cinnamic acid	140-10-3	Ligand			Cinnamon (Marongiu et al., 2007)
Peonidin-Cl	134-01-0	Ligand			Bilberry (Zhang, Kou, Fugal, & McLaughlin, 2004)
α-Humulene	6753-98-6	nd			Clove (Chaieb et al., 2007), cinnamon (Marongiu et al., 2007)
Gallic acid	149-91-7	nd	nd		Pomegrante fruit (Naz et al., 2007)
Curcumin	458-37-7	nd	nd		Tumeric, curry powder
Linalool	78-70-6	nd			Clove (Chaieb et al., 2007), cinnamon (Marongiu et al., 2007)
ß-Caryophyllene	87-44-5	nd			Clove(Chaieb et al., 2007), cinnamon (Marongiu et al., 2007)
p-Cymene	99-87-6	nd			Cinnamon (Marongiu et al., 2007)
2-Heptanone	110-43-0	nd			Clove (Chaieb et al., 2007)
Alliin	556-27-4	nd			Garlic
Diallyl sulphide	592-88-1	nd			Onion, garlic
Theobromine	83-67-0	nd			Cacao
Procyanidin B2	29106-49-8	nd			Apple (Kahle et al., 2007)

Compounds with an IC50 of ⩾ 500 μM were described as "ligand". If transactivation was observed but no EC50 value could be determined, compounds were described as "active". The efficiency describes the maximal induction using chimeric GAL4-PPAR assay and rosiglitazone as the standard. For some substances no binding and transactivation was observed (nd, not determined) when assayed up to a concentration of 2.5 mM (ligand binding assay) or 0.3 mM (transactivation assay).

(IC_{50} = 4.3 μg/ml), fenugreek (IC_{50} = 4.4 μg/ml), bilberry standardised on 35% phenols and 10% anthocyanines (IC_{50} = 4.5 μg/ml), rooibos tea (IC_{50} = 6.4 μg/ml), bay leaves (IC_{50} = 9.3 μg/ml) and holy basil, a 5:1 extract with high tannin content (IC_{50} = 16.2 μg/ml). Overall, approximately 50 plant extracts demonstrated PPARγ binding activity (Table 1, Fig. 1).

Isolated compounds that potentially represented the active substances of different plant extracts were also tested, along with glitazones for comparison (Table 2, Fig. 1). Quercetin showed the highest PPARγ binding affinity (IC_{50} = 2.8 μM), 2-hydroxychalcone and 2′-hydroxychalcone exerted moderate PPARγ binding affinity with IC_{50} values of 3.8 and 2.9 μM, luteolin bound with an IC_{50} value of 3.9 μM, rosmarinic acid bound with an IC_{50} value of 15.7 μM, cinnamaldehyde bound with an IC_{50} value of 10.3 μM and coumestrol moderately bound PPARγ (IC_{50} = 10.5).

3.2. Transactivation assay

Twenty-two plant extracts and several compounds were tested for their induction of PPARγ transactivational activity using a chimeric GAL4- PPARγ assay system. Five plant extracts, nutmeg, holy basil, licorice, sage and black pepper, were moderate PPARγ transactivators (Table 1, Fig. 2). An EC_{50} value could only be determined for nutmeg extract (106.1 μg/ml) as the logistic dose response curve of the other extracts could not be established due to a lack of saturation of receptor activation. Transactivation efficiency compared to rosiglitazone ranged from 10.3% for licorice extract to 15.3% for nutmeg extract.

The 2-hydroxychalcone, 2′-hydroxychalcone, coumestrol, phloretin, resveratrol, and apigenin compounds stimulated PPARγ transactivation (Fig. 2). The EC_{50} values ranged from 21.3

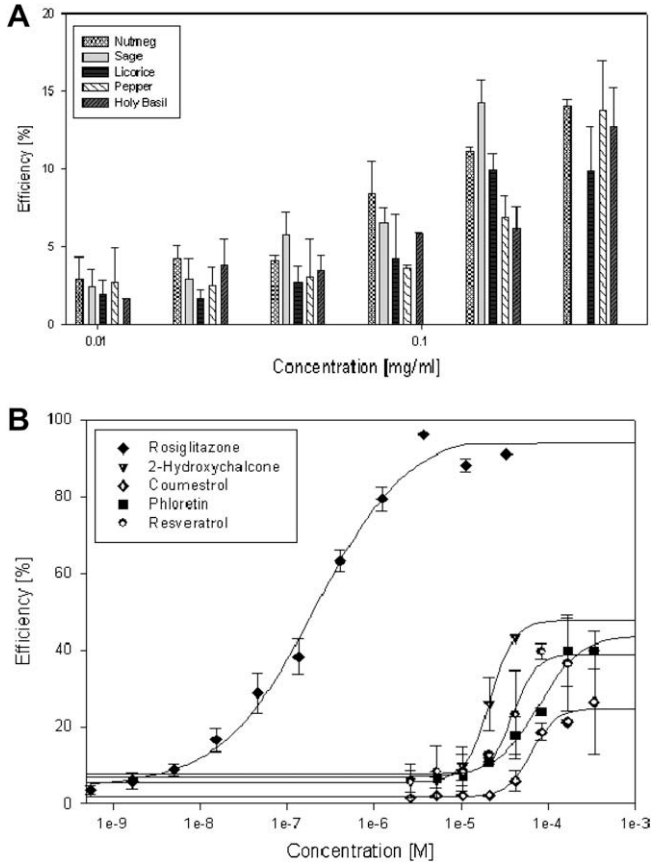

Fig. 2. (A) Nutmeg, licorice, sage, black pepper and holy basil transactivate PPARγ as determined by chimeric Gal4-PPARγ assay system. (B) Logistic dose response curves of rosiglitazone, 2-hydroxychalcone coumestrol, phloretin and resveratrol determined by chimeric Gal4-PPARγ assay system.

(2-hydroxychalcone) to 76.0 (phloretin) and the efficiencies from 15.7% (apigenin) to 47.9% (2-hydroxycachalcone). The strong ligands quercetin, luteolin and rosmarinic acid showed no transactivational activity. Cinnamaldehyde was highly cytotoxic, thus could not be assessed with this assay.

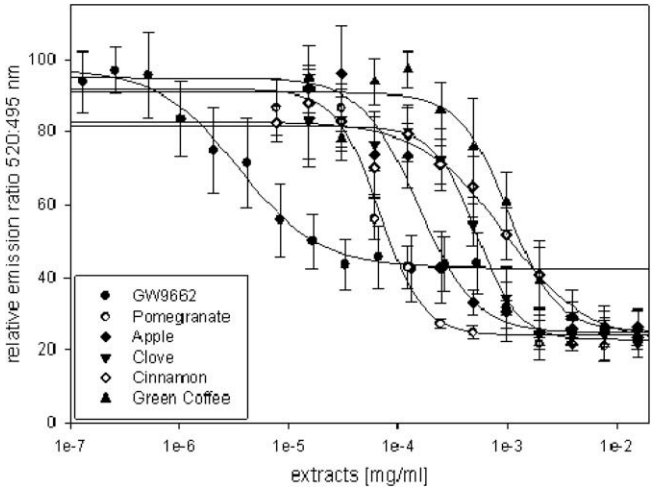

Fig. 3. Logistic dose response curves of GW9662, pomegranate fruit extract (standardised to 40% ellagic acid), apple extract (200-fold concentration and standardised to 5% quercetin and 30% phloridzin), clove, cinnamon extract (10-fold concentration), green coffee extract determined by TR-FRET coactivator assay in antagonist mode.

3.3. TR-FRET coactivator assay

The 22 plant extracts were next assessed with the TR-FRET coactivator assay system, as was the antagonist GW9662 (Table 1, Fig. 3). GW9662 showed strong antagonistic activity, with an IC_{50} value of approximately 0.003 μg/ml. With the exception of ginseng, stevia tea and curry powder, all plant extracts antagonized coactivator recruitment to PPARγ mediated by rosiglitazone. The strongest antagonists were pomegranate fruit extract (IC_{50} = 0.07 μg/ml), apple extract (IC_{50} = 0.15 μg/ml), clove (IC_{50} = 0.5 μg/ml), cinnamon extract (IC_{50} = 0.9 μg/ml), green coffee extract (IC_{50} = 1.0 μg/ml), bay leaves (IC_{50} = 3.0 μg/ml), crimson clover flower (IC_{50} = 3.3 μg/ml), bilberry (IC_{50} = 3.5 μg/ml) and rooibos tea (IC_{50} = 3.6 μg/ml). Interestingly, the extracts with transactivational activity, nutmeg, sage, licorice, holy basil and black pepper, also antagonized PPARγ coactivator recruitment.

4. Discussion

Obesity is a serious world wide health problem reaching nearly epidemic proportions. The health consequences of obesity include hypertension, diabetes, hyper- and dyslipidemia, and consequently an increased risk for cardiovascular morbidity and mortality (Alberti et al., 2006). Treatment of diabetes type 2, a key factor of the metabolic syndrome, with plants has been used a traditional remedy in many cultures (Grover et al., 2002; Shapiro & Gong, 2002), and accordingly, scientific studies have reported many medicinal and culinary plants with anti-diabetic potential (Broadhurst et al., 2000; Grover et al., 2002; Huang et al., 2005; Mae et al., 2003; Mueller & Jungbauer, 2008; Srinivasan, 2005; Verspohl et al., 2005). In this study, we identified many culinary herbs, plants and spices that function as PPARγ antagonists or SPPARγMs, suggesting their possible use in reducing the incidence of obesity and the risk of developing the metabolic syndrome. The main causes for obesity are high calorie intake, high fat intake and a sedentary lifestyle, but PPARγ active compounds may also play an important role in this process.

Approximately 50 different extracts from plants, herbs and spices, surprisingly almost all edible plants, with PPARγ binding affinity were identified. Five extracts (nutmeg, holy basil, licorice, sage and black pepper) showed moderate PPARγ transactivational activity. Our findings are consistent with the previously reported antidiabetic potential of nutmeg, sage and licorice extract via PPARγ agonism (Han et al., 2007; Mae et al., 2003; Rau, Wurglics, Dingermann, Abdel-Tawab, & Schubert-Zsilavecz, 2006).

The binding affinity of several plant extracts can be explained by the affinity of their compounds p. ex. quercetin which is found in pomegranate fruit (Naz, Siddiqi, Ahmad, Rasool, & Sayeed, 2007), apple (Kahle et al., 2007), tarragon, dill, bay leaf, parsley, chive, lovage (Justesen & Knuthsen, 2001), bilberry (Ehala, Vaher, & Kaljurand, 2005), and onion, 2-hydroxychalcone, 2́-hydroxychalcone and cinnamaldehyde which are found in cinnamon (Marongiu et al., 2007), luteolin which occurs in various plants such as sage, rosemary, thyme (Proestos, Chorianopoulos, Nychas, & Komaitis, 2005), tarragon, parsley (Justesen & Knuthsen, 2001), and alfalfa (Seguin & Zheng, 2006), rosmarinic acid, a component of sage, rosemary, lavender (Wang, Provan, & Helliwell, 2004), and thyme (Proestos et al., 2005), or coumestrol, which occurs in alfalfa extract.

Nearly all the tested extracts antagonized rosiglitazone-mediated DRIP205/TRAP220 coactivator recruitment, including the five transactivating extracts. Likely these extracts could function as selective PPARγ modulators (SPPARγMs), which transactivate PPARγ but do not recruit all coactivators.

The extracts that bind PPARγ and antagonize the DRIP205/TRAP220 coactivator recruitment, such as pomegranate, apple, cinnamon, clove or thyme, are moderate antagonists or undetectable

or weak SPPARγMs. We found that compounds of these extracts function as agonists, such as resveratrol and coumestrol, or antagonists, such as quercetin, luteolin and rosmarinic acid. Interestingly, the antagonistic activity exceeds the agonistic activity. Apple extract with a high amount of phloridzin is a putative PPARγ agonist in vivo, as phloridzin was previously shown to be metabolized into aglycone phloretin (Kahle et al., 2007), a moderate PPARγ binder and activator. Although ginseng, stevia and curry powder are PPARγ ligands and did not function as transactivators, they did not antagonize DRIP205/TRAP220 coactivator recruitment. These extracts may have extremely weak and undetectable transactivator activity. According to reports in the literature, ginseng and stevia extract exert antihyperglycemic activity (Jeppesen, Gregersen, Alstrup, & Hermansen, 2002; Vuksan et al., 2008).

Full PPARγ agonists, such as glitazones, are used for treating diabetes type 2; these function by recruiting a specific spectrum of coactivators, and are associated with side effects such as weight gain. Amongst other coactivators, the PPARγ coactivator DRIP205/TRAP220 was classified as a so called "adipogenic factor", indicating that it promotes adipogenesis and adipocyte differentiation in cell culture and weight gain in humans (Rieusset et al., 2002; Zhang et al., 2007). In this study, 19 of 22 tested plant extracts were identified to antagonize DRIP205/TRAP220 coactivator recruitment, and we presume that they may therefore support weight reduction.

Interestingly, this study identified not only herbs and spices, which are consumed in small amounts, as PPARγ activators, but also many edible plants that are consumed in high amounts, such as apple, bilberry, cacao, onion, kale and white cabbage.

There are uncertain parameters in the calculation of a daily effective dose for antiobesity or antidiabetic activity. One challenge is in standardising the plant extracts. Due to the influence of climatic circumstances and growth area, extracts of one species from different suppliers differed significantly in their PPARγ activity. Furthermore, metabolism of compounds can result in more active or less active PPARγ ligand activity, absorption rate of different compounds varies considerably, and the influence of the food matrix must be considered.

A vegetarian or vegetable rich diet is known to reduce the incidence of obesity and other cardiovascular risk factors (Bazzano, Serdula, & Liu, 2003). In addition to exercise, the major factor of weight reduction is still reduction of calorie intake (Bazzano, Serdula, & Liu, 2005). Furthermore a diet rich in herbs and spices may provide pharmacological active compounds that may prevent weight gain (Lee et al., 2008).

With a diet including the above-mentioned culinary plants, a certain amount of PPARγ antagonists and SPPARγMs is simultaneously consumed. Improvement or prevention of insulin resistance and obesity, the main cause of insulin resistance, could be achieved simultaneously. A diet rich in these plants for obese and/or diabetic patients could reduce the dose of hypoglycaemic agents and support weight reduction. Obese patients without manifest diabetes could possibly even avoid those drugs. Obese patients without insulin resistance could prevent developing diabetes.

5. Conclusion

In conclusion, our study identified a wide variety of culinary herbs, plants and spices with PPARγ binding activity, including pomegranate extract, apple extract, clove, cinnamon, thyme, green coffee, fenugreek, bilberry, nutmeg, rooibos tea and bay leaves. The five plant extracts, nutmeg, black pepper, licorice, holy basil and sage, likely function SPPARγMs. Nearly all extracts antagonized DRIP205/TRAP220 coactivator recruitment, which may result in the antagonism of adipocyte differentiation and thus support weight reduction. Inclusion of a combination of these plants in the diet could reduce the incidence of obesity and attenuate developing metabolic syndrome.

Acknowledgements

We thank Prof. Staels (Institut Pasteur, University of Lille, France) for providing the pGAL4-hPPARγ-LBD expression plasmid.

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emulsified shortenings are more suitable for icings, cakes, etc. where the incorporation of air into the shortening system is desired. In terms of emulsifiers, mono- and diacylglycerols possess marked surface activity because of their lipophilic and hydrophilic groups. Improvement in aeration is observed by the addition of mono- and diacylglycerols (2–4% (w/w) of the shortening) (Chrysam, 1985).

The preparation of a shortening through chemical interesterification of high oleic sunflower oil and fully hydrogenated fat is reported here. The functionality of the finished product was related to the chemical composition, polymorphism, morphology, and rheological properties of the fat. Finally, the functionality of the product was compared to that of a widely used commercial shortening in cookie manufacture.

2. Materials and methods

2.1. Raw materials

Blends of fully hydrogenated canola oil (FHCO), fully hydrogenated soybean oil (FHSO) and high oleic sunflower oil (HOSO) in a weight ratio of 17:13:70 were chemically interesterified. Fully hydrogenated canola oil (FHCO) and high oleic sunflower oil (HOSO) were generously provided by Bunge Canada (Toronto, ON, Canada) and Nealanders (Toronto, ON, Canada), respectively. All chemicals and organic solvents were purchased from Fisher Scientific (Ottawa, ON, Canada) and Sigma–Aldrich (Oakville,ON, Canada). Anhydrous citric acid and monoglyceride Dimodan-P-TK-A were generously donated by ADM (Decatur, IL, USA) and Danisco (New Century, KS, USA) Inc., respectively.

2.2. Chemical interesterification

Chemical interesterification was carried out using 0.3% (w/w) sodium methoxide (Sigma–Aldrich, Oakville, ON, Canada) as a catalyst. A 20 kg mixture FHCO, FHSO and HOSO (17:13:70, w/w/w) were melted in a container at 85 °C, under a nitrogen blanket to limit the presence of moisture and air. The chemical reaction was started by the addition of catalyst. The blends were interesterified under agitation and under a blanket of nitrogen for 60 min at 88 ± 2 °C. The start of the reaction was associated with the appearance of a red–brown colour. The addition of 4% acidic water (20% citric acid, w/v) was used to stop the reaction. The product was washed with eight parts hot water (1:8, v/v) to remove soaps, excess citric acid, sodium methoxide and some of the free fatty acids. Residual water and any remaining impurities were then removed by adding bleaching clay (1.5 wt%) and the sample heated again at 88 ± 2 °C for 20 min under nitrogen gas. The interesterified blend was then filtered. The fat was poured into a bucket and stored at 5 °C prior to use.

2.3. Crystallizer equipment

Samples of the interesterified blends were prepared in a Gerstenberg and Agger crystallizer (Copenhagen, Denmark). The unit consists of a melting container, chilling system (three separate chilling units) and pin worker. The CI-sample was heated to 55 °C and mixed with 2% emulsifier (monoacylglyceol, Dimodan-P-TK-A). The homogenous blend at 55 °C was fed at 910 g/min through the chilling unit. A film of solidified, partially crystallized fat was removed from the tube walls by scraper blades. The material exiting from the chilling unit at 10.7 °C was then allowed to enter the pin machine at a speed rate of 350 rpm. The finished product was stored at room temperature in a closed container. For comparison, a commercial all purpose shortening was purchased from a local store.

2.4. Triacylglycerol (TAG) composition

The TAG composition was determined using a high-performance liquid chromatography system (model 1100; Agilent Tech. Palo Alto, CA) equipped with a quaternary pump (C1311A), auto sampler (G1313A), and a Hewlett–Packard Chem Station software system (Version A.10). TAGs were detected with an evaporative light scattering detector (Alltech, MK III, Deerfield, IL, USA) using nitrogen at 50 PSI with neubulization conducted at 50 °C. Solutions of each blend in chloroform (2.5% (w/w)) were filtered (Osmonics magma nylon membrane filter, 0.45 μm pore size, Fisher Scientific, Ottawa, ON, Canada). A 20 μL sample was passed through an Econosil column (C18, 250 × 4.6 mm) (Alltech Associates, Deerfield, IL, USA) in isocratic mode at a flow rate of 1.0 mL/min. The mobile phase was acetonitrile–chloroform (70:30, v/v). TAG species were identified by comparison with standards (Sigma–Aldrich, Oakville, ON, Canada) and they were quantified based on a relative peak area basis.

2.5. Fatty acid composition

Fatty acid composition was determined by gas liquid chromatography (GLC) after preparation of fatty acid methyl esters. Anhydrous 5% HCl/methanol (by weight) for 1 h at 80 °C was used for the methylation reaction. In each case, the resultant fatty acid methyl esters (FAME) were extracted using hexane after the addition of water. Hexane was removed and the FAMEs were purified by TLC on silica gel G plates (Fisher Scientific, Ottawa, ON, Canada) using the developing solvent hexane/diethylether/acetic acid (85:15:1, v/v/v). The FAME band were identified on plate after spraying the plate with 2′,7′-dichlorofluorescein in methanol and the band was visualised under ultraviolet light. The FAME band scrapped off and transferred into a Pasteur pipette and the FAME eluted with hexane (Azizian, Kramer, & Winsborough, 2007). An appropriate concentration of prepared samples were analysed by GC (Hewlett–Packard Model 5890 Series II, Palo Alto, CA, USA) equipped with a flame ionisation detector and an autosampler (Hewlett–Packard, Model 7673). A CP-Sil 88 fused capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness; Varian Inc., Mississauge, ON, Canada) and a Hewlett–Packard Chem Station software program (Version A.10) were used. The operating conditions were: injector and detector temperature both at 250 °C, H_2 as carrier gas (1 mL/min) and for the FID detector (30 mL/min), N_2 make up gas (30 ml/min) and air (300 mL/min). The column temperature program was: initial temperature of 45 °C and held for 4 min, then programmed at 13 °C/min to 175 °C and held for 27 min, then temperature was increased to 215 °C at 4 °C/min and held for 35 min. Individual components were identified by comparison with a GC reference FAME standard (#463, Nu-Chek Prep, Inc., Elysian, MN, USA). Fatty acids were quantified based on relative peak area.

2.6. Physical measurements

Melting profile, storage modulus, Solid fat content, crystal size, texture analysis and polymorphism were performed as described by Ahmadi, Wright, and Marangoni (2008a, 2008b).

2.7. Cone penetrometry

Penetration depth was determined by allowing a metal cone (aluminium) to penetrate into a sample for 5 s. The cone is clamped into an apparatus with which the penetration depth can be measured. Five replicate analyses were performed for each sample and the mean ± SEM is reported. The cone used for determination of the yield values was 15 g cone with an angle = 15°. The yield value [g/cm^2] was calculated as

$$\text{Yield Value} = K\frac{\text{mass of cone(g)}}{(\text{depth of penetration}, 0.1\text{mm})^{1.6}} \quad (1)$$

where K for a cone with an angle of 15° is tabulated as 30700 (Haighton, 1959).

2.8. Use of the shortening in a baking application

To demonstrate the functionality of the CI-shortening, the following formulation was used to make chewy brownie cookies and compared to cookies made with commercial shortening (Table 1).

2.9. Preparation of the cookies and evaluation

Shortening (20.6%), sugar (15.5%), brown sugar (12.6%), and vanilla (0.4%) were mixed in a Kitchen Aid professional HD mixer for 2 min at speed 2 with a flat beater. Beaten egg (9.8%) was added on low speed for 1 min and 30 s until mixture is just combined. Flour (19%), cocoa (5.7%), baking soda (0.3%), and salt (0.3%) were combined and added to the mixture with milk (5%) and beaten on speed 2 for 1 min and 30 s. Finally, chocolate chips (13%) were added and mixed. A 50 g portion of dough was placed on a baking sheet and baked at 350°F for 10 min. Width and thickness of cookies were measured with a caliper and the spread ratio was obtained by dividing width (mm)/thickness (mm). The breaking strength was measured using an SMS Materials Tester model MT-LQ (Stable Micro Systems Ltd., Surrey, England) equipped with a blade cutting probe, 1.4 cm long and 0.4 mm thick. A force–displacement diagram was taken for each sample. The force (N) required to break a cookie was recorded and the average value of 10 replicates was reported (Zoulias, Oreopoulou, & Tzia, 2002).

2.10. Sensory evaluation

The sensory analysis of cookies was carried out by 37 untrained consumer panellists. The selected method was a triangle test. In the triangle test, three coded samples are given to the panellist. In the questionnaire form it is stated that two of the samples are identical and the panellist is asked to identify the odd sample. The sensory evaluation test and analysis of cookies was carried out a day after preparing them (Larmond, 1970).

3. Results and discussion

The fatty acid and triacylglycerol composition of the two samples (CI-sample and commercial sample) are presented in Tables 2 and 3.

The CI-shortening contained high amounts of unsaturated fatty acids (59.7%) in which the major fatty acid was oleic acid (51.5%).

Table 1
Ingredients used for preparation cookies.

Ingredient	Source	Weight (g)
Flour-all purpose	Retail	248.0
Baking soda	Retail	4.0
Sugar, granulated	Retail	200.0
Brown sugar	Retail	163.0
Salt	Retail	4.0
Milk 2%	Retail	60.0
Whole egg	Liquid	101.0
Vanilla	Retail	5.0
Chocolate chips	Retail	170.0
Shortening	Commercial or CI*	267.0
Cocoa powder	Retail	74.0

* CI: Chemical interesterified blend.

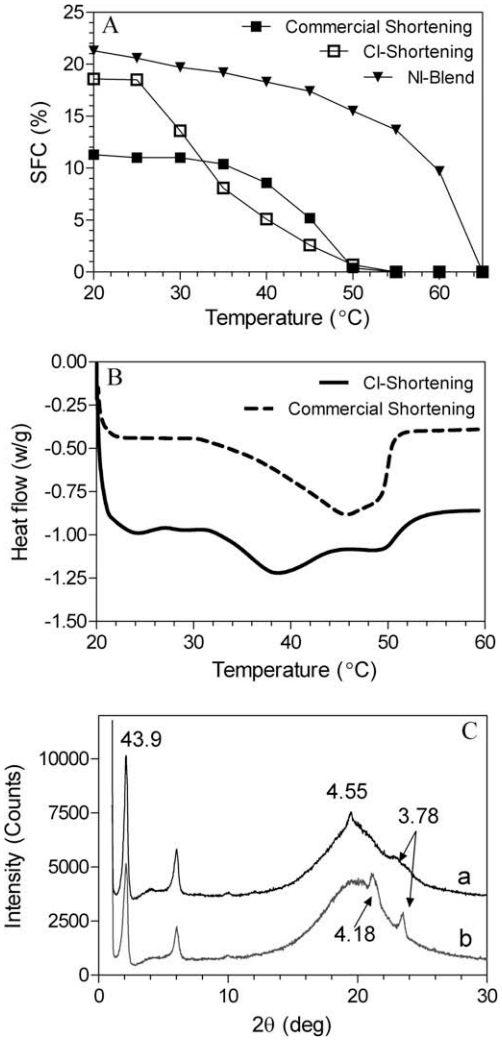

Fig. 1. (A): SFC as a function of temperature in 30%-NI-blend, 30% CI-shortening and commercial shortening, and (B) Differential scanning calorimetery melting thermograms of 30% CI-shortening and commercial shortening. C: Powder X-ray diffraction pattern for commercial and CI-shortening crystallized at room temperature for 24 h; (a) CI-shortening and (b) commercial shortening.

The total amount of unsaturated molecules in the commercial shortening was 63.9% and linoleic acid (41.5%) was in the highest amounts. Due to the high oxidative susceptibility of linoleic acid, replacing it with oleic acid should improve the oxidative stability of the product. The major saturated fatty acids in the CI-shortening and commercial shortening were stearic acid (29.9% and 12.9%, respectively) and palmitic acid (6.8% and 14.7%, respectively). Trans fatty acids were identified in the CI-samples (0.9%) and shortening (4.2%) (Table 2). The presence of trans fatty acid indicated that the commercial shortening contained partially hydrogenated fat, even though the product was labelled as trans-fat free. GLC analysis revealed that the majority of trans fatty acids were elaidic acid (18:1t).

Table 3 shows the TAG composition of the two shortenings. In the commercial shortening, TAGs contained linoleic acid such as LLL, LLO, LOS, and LLP which constituted 73.1% of the total TAGs. In the CI-shortening, TAGs containing oleic acid and stearic acid, such as OSO, SOS and POS were the main species present. Polymorphic behaviour is influenced by TAG and fatty acid composition (Takeuchi, Ueno, & Sato, 2002). In some cases the presence of a specific TAG has a beneficial effect on the polymorphic form and its stability (deMan & deMan, 2001). For example, 1-palmitoyl, 2-ste-

Table 2
Fatty acid composition of a mixture of 30% chemically interesterified of fully hydrogenated canola–soybean oil (FHCO/FHSO) with high oleic sunflower oil (HOSO) and a commercial shortening.

Fatty acid	CI-Shortening	Commercial shortening
	wt [%] of total fatty acids	
16:0	6.8 ± 0.2	14.7 ± 0.4
18:0	29.9 ± 0.1	12.9 ± 0.2
18:1	51.5 ± 0.3	17.0 ± 0.2
18:2	8.0 ± 0.1	41.5 ± 0.4
18:3	0.2 ± 0.0	5.4 ± 0.1
20:0	0.6 ± 0.0	0.3 ± 0.0
Total trans	0.9 ± 0.3	4.2 ± 0.3
SFAs	37.3 ± 0.2	27.9 ± o.4
MUFAs	51.5 ± 0.3	17.0 ± 0.2
PUFAs	8.2 ± 0.1	46.9 ± 0.3
U/S	1.6	2.3

SFAs, saturated fatty acids; UFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; U/S, unsaturated/saturated.
Mean value of two replicates with standard deviation.

Table 3
Triacylglycerol composition (% peak area) of a mixture of 30% chemically interesterified of fully hydrogenated canola–soybean oil (FHCO/FHSO) and high oleic sunflower oil (HOSO) (CI-shortening) and commercial shortening.

Compound	CI-shortening	Commercial shortening
DAG	2.9 ± 0.2	⩽1
LLLn	0.0 ± 0.3	7.9 ± 0.1
LLL	0.0 ± 0.0	29.3 ± 0.1
LLO	0.0 ± 0.0	20.3 ± 0.2
LLP	0.0 ± 0.0	15.6 ± 0.1
OOL	5.8 ± 0.0	5.9 ± 0.2
LLS + LOP	0.0 ± 0.0	10.0 ± 0.4
POL	0.7 ± 0.2	0.0 ± 0.0
OOO	20.1 ± 0.1	0.9 ± 0.3
LOS	0.0 ± 0.2	1.4 ± 0.2
OPO	7.7 ± 0.5	1.0 ± 0.1
PPP	4.6 ± 0.0	0.0 ± 0.0
OSO	39.1 ± 0.0	0.0 ± 0.0
POS	4.0 ± 0.2	0.0 ± 0.0
SOS	13.7 ± 0.3	0.0 ± 0.0
PPS	0.0 ± 0.0	2.0 ± 0.1
SSS	0.0 ± 0.0	0.7 ± 0.2
Total	98.6	95.5

O, oleic acid; L, linoleic acid; Ln, linolenic acid; P, palmitic acid; S, stearic acid.
TAG shown constitutes the major species present (i.e. >0.1%).
Values represent the mean value of two replicates with standard deviation.

aroyl, 3-palmitate (PSP) exists in a β′ form, whereas 1-stearoyl, 2,3-dipalmitate (SPP) and 1-palmitoyl, 2,3-distearate (PSS) crystallize in both β and β′ forms (Lutton, 1950). PSP in the tested commercial shortening was probably a factor affecting β′ polymorphism. In addition, a higher amount of palmitic acid in the commercial shortening can lead to increases in β′ polymorphism. D'Souza et al. (1991) concluded that the difference between β and β′ in margarines could be related to the C16 fatty acid. In the β-tending margarines the C16 fatty acid content was below 11%, but it was above 17% in the β′-tending margarines. deMan, deMan, and Blackman (1989) showed that the shortening containing partially hydrogenated fat was in β′ form compared to shortening without partially hydrogenated fat in the β form.

SFC: The SFC profiles of the experimental CI and commercial shortenings are shown in Fig. 1A. The SFC of the physical blend (NI-Blend, i.e. non-interesterified) was changed dramatically after chemical interesterification. The SFC of the interesterified blends was consistently lower than those of the NI blends. This is due to the randomization of fatty acids in the TAG molecules during the interesterification and creation of TAG species with melting points below that of trisaturated molecules (Chu et al., 2001). Lee, Akoh, and Lee (2008) found the same results for an interesterified sample produced with fully hydrogenated soybean oil, rapeseed oil and palm stearin in three different ratios. The observed changes in the slope of the CI-shortening curve were attributed to the presence of intermediate TAGs created after the interesterification process (Litwinenko, Rojas, Gerschenson, & Marangoni, 2002; Metzroth, 2005). The CI-sample showed an SFC profile close to that of the commercial shortening and the SFC of both samples decreased sharply with increases in temperature. Products with the type of profile are referred to as high stability shortenings (Metzroth, 2005). Overall, the CI-shortening had plasticity curves in the range of the commercial shortening.

Even though the CI-sample had a higher SFC at 20 °C than the commercial shortening, both samples melted completely at 50 °C. DSC results showed the same end point melting temperature for both samples (52 °C) (Fig. 1B). The physical characteristics of this shortening make it a suitable choice to use as a deep fat frying, as ingredient in cookies, crackers and some other confectionary products. Because these type of products need a fat with high SFC at low temperature and low SFC around 45 °C (Metzroth, 2005).

Crystal structure and crystal morphology: Polymorphism, crystal size and crystal shape were studied using polarised light microscopy and X-ray diffraction on samples stored for 24 h at room temperature. The polymorphic form of the crystals can affect the shape and size of the crystals and crystal aggregation (Hoerr & Waugh, 1955; Kellens, Meeussen, & Reynaers, 1992). Powder X-ray diffraction (Fig. 1C) indicated that the β′ polymorph was predominant in the commercial shortening, with short spacings observed at 4.2 and 3.8 Å. The CI-sample was mostly in the β form with a short spacing of 4.55 Å. Storage of the sample at room temperature gave enough time for crystals to convert to a stable polymorphic form. The level of softness or graininess of the shortening product may be associated, in part, with the polymorphic form. However the same polymorphic forms could have different crystal sizes and dif-

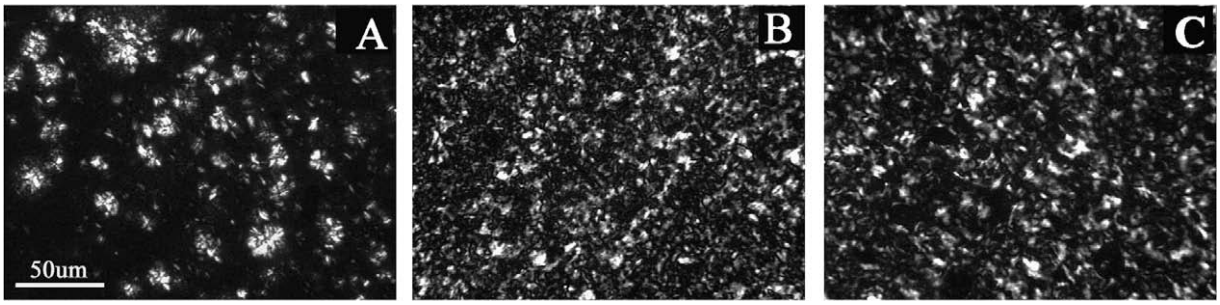

Fig. 2. Polarized light micrograph of (A) CI-shortening (static), (B) CI-shortening (under shear), and (C) commercial shortening stored at 25 °C/24 h.

ferent crystal shapes which in turn could lead to a greater or lesser graininess of the product (Kellens et al., 1992).

The β′ polymorph is usually believed to be the most functional polymorphic form in shortenings because of its small size and needle-like shape (Ghotra et al., 2002). Observing the crystal morphology of the CI-samples by PLM (Fig. 2) clearly demonstrates that even though the polymorphic form is different in the two samples, their morphology was the same. Crystal size and shape are the key factors influencing fat functionality and rheological properties of plastic fat (Tang & Marangoni, 2006). Large crystals impart a sandy mouth feel, whereas smaller crystals lead to firmer products (Rodriguez, Castro, Salinas, Lopez, & Miranda, 2001).

Polarized light images of the CI-sample before and after shearing were taken in order to understand how the appearance and morphology of crystals was affected by the thermal and mechanical treatment. Fig. 2A shows larger crystals (10.2 ± 3.42 μm), more aggregation and a more heterogeneous distribution of crystals for crystallization under static conditions compared to the sheared samples. After shearing and crystallization, images showed a smaller crystal size (3.5 ± 1.28 μm), less aggregation, and a more homogenous distribution of the crystals (less void space) (Fig. 2B). Shear induces more nucleation and less crystal growth and crystal breakage which resulted in a smaller crystals size. Therefore, products become more firm, more stable and possess a greater plastic range as the crystal size decreases. Decreasing crystal size induces a greater combined surface area and provides a strong network (Metzroth, 2005).

Rheological properties: At the same storage temperature (25 °C/24 h), the commercial sample of shortening had a higher storage elastic modulus than the CI-sample ($P < 0.05$) (Table 4), even though the CI-sample had a higher SFC. Small differences in the storage modulus value could be attributed to different chemical composition or different processing and tempering between the two samples. The measurement of G′ in the CI-sample before crystallization and shearing was not possible because the sample was too soft. Measuring the hardness of the shortenings by cone penetrometry revealed that the commercial shortening had a firmer texture relative to the CI-shortening. Penetration depth (0.1 mm) values of both of the shortenings were within the range of those reported for a soft shortening (50–300 g/cm^2) (Haighton, 1959).

Physical characteristics of cookies: The physical characteristics of cookies made using the different fats are shown in Table 5. Cookies made with the commercial shortening had a relatively lower spread ratio than cookies made with 30% FHCO and FHSO ($P < 0.05$). In contrast, the hardness of cookies was unaffected by the kind of shortening, and both samples had the same hardness index ($P > 0.05$). Jacob and Leelavathi (2007) reported the cookies made with sunflower oil had a higher spread value than cookies containing non-emulsified hydrogenated fat, but cookies made using sunflower oil were harder than cookies made using hydrogenated fat. There were no significant differences in the measured hardness between the two samples ($P > 0.05$).

Sensory evaluation test: A triangle test was carried out using 37 panellists. The results showed that 21 of the 37 tasters could not distinguish between cookies made using the CI-shortening and the commercial shortening ($P < 0.01$). Therefore, despite the differences in spread ratio of the cookies, they showed the same hardness and the same sensory attributes.

In conclusion, a chemically randomized blend of a 30% stearic acid-rich (FHCO and FHSO) and oleic acid-rich (HOSO) can be used as a zero trans shortening. Comparable plasticity was achieved by chemical interesterification of the mixture and then crystallization and shearing of the fat. This high stability shortening can find application in deep fat frying and many all-purpose bakery applications. This product had a comparable plasticity and melting characteristics as a commercial shortening, but with the added health benefits of high stearic and oleic acids.

Table 4
Rheological properties of CI-shortening and commercial shortening stored at 25 °C for 48 h.

Physical properties	CI-shortening	Commercial shortening
Storage modulus (G′) (Pa)	$1.25 \times 10^5 \pm 0.342 \times 10^5$	$2.02 \times 10^5 \pm 0.505 \times 10^5$
Penetration depth (0.1 mm)	212 ± 7.0	126 ± 8.2
Yield value (g/cm^2)	88.65	199.7

Values represent means and standard deviation of 10 replicates.

Table 5
Effect of shortening type on the physical specifications of cookies.

Shortening type	Width (mm)	Thickness (mm)	Spread ratio (mm)	Yield force (N)
30% CI-blend	70.55 ± 0.25^a	9.69 ± 0.09^a	7.38 ± 0.28^a	16.63 ± 3.97^a
Commercial	67.62 ± 0.11^b	10.38 ± 0.05^a	6.54 ± 0.15^b	17.99 ± 5.05^a

Values represent means and standard deviation of 10 replicates.
Different superscript letters indicate significant differences ($P < 0.05$) of values within each column.

Acknowledgments

The authors acknowledge the financial support of the Natural Sciences and Engineering Research Council of Canada and the Ontario Ministry of Agriculture, Food and Rural Affairs. The authors are thankful to Prof. John Kramer in Agriculture and Agri-Food Canada for his assistance in determining the fatty acids and triacylglycerols content of the fats. We are grateful to Dr. Gary List for providing the samples of fully hydrogenated soybean and canola oil.

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25, 50, and 75 mW/cm^3 and exposure times (PT) of 2, 5, and 8 min; and both treatments were incubated (IC) at 25 °C for 24, 36, and 48 h. Untreated raw peanuts served as controls. Two replications of the 27 UV and US treatments, and one control were prepared, a total of 110 samples analysed for *trans*-RES and *trans*-piceid in duplicate. When large coefficient of variation (COV) > 35% occurred between two replicates, a third duplicate analysis was conducted to decrease COV to <35%. Outlying results were dropped from the data set if COV remained >35%.

2.2. Sample preparation

Raw peanuts, (*Arachis hypogaea* cv Georgia green medium runners, 2005 crop year; Golden Peanut Co., Alpharetta, GA) stored for 2 months at 4 °C were used. All processing implements and surfaces were washed and sanitised with 200 ppm chlorine solution prior to use. About 1.8 kg peanuts were washed twice in 4 kg tap water, drained, surface sanitised in 2 kg of 150 ppm chlorine solution for 15 min, and rinsed with sterilised deionised water, prepared by passing through a 0.2 μm nylon filter (Millipore Corp., Bedford, MA). Peanuts were fully imbibed in 2 kg sterilised water for 16 h, drained, and manually sliced to about 7 mm.

UV treatment: Sliced peanuts, three batches of about 900 g each, were spread 1 cm layer deep on a plastic tray (56L × 30.5W × 60H cm, HDPE, Gage Industries Inc., Lake Oswego, OR). Three trays were positioned side by side along their length, a total of 91.5 cm, to allow equal exposure to 122 cm UV light (UPV XX-40S, 40 W, 254 nm, UltraViolet Products, Upland, CA) at a specific distance, and trays were removed after specific time according to experimental design. Peanuts were mixed after half of exposure time for equal exposure of surfaces to UV.

US treatment: Sliced peanuts, approximately 900 g/batch, were placed in a 2.5 L stainless steel cylindrical container (20 cm, i.d.) then filled with deionised water to 1.5 L. US at 20 kHz was applied through a 25 mm diameter probe of an ultrasonic processor with temperature controller (750W, 115 VAC, 50/60 Hz, Cole Parmer, Vernon Hills, IL) operating continuously at 25 °C and positioned at the centre of peanuts. The amplitude to achieve the desired power delivered to the probe was calculated using the formula, power (J or W-s) = PD (mW/cm^3) × volume of water (cm^3) × time (s) × 1 W/1000 mW, and empirically determined from preliminary sonication of peanuts immersed in water resulting in 21%, 48% and 68% amplitudes to achieve PD of 25, 50 and 75 mW/cm^3, respectively. Peanuts were sonicated and then drained, 15 min, to remove excess water.

Treated peanuts were packed in half gallon glass mason jars (Ball Corp., Muncie, IN), sealed with two-piece lids (Alltrista Corp., Muncie, IN), wrapped with foil and incubated at 25 °C (Environmental Growth Chamber, Chagrin Falls, OH) for 24, 36, and 48 h. Incubated samples were accumulated in a walk-in freezer at −18 °C, then thawed in a walk-in chiller, 5 °C for 24 h, poured to aluminium trays (40.6L × 38.1W × 1.2H, cm; 0.4 cm i.d. perforations) to one layer depth, and dried in a convection oven (645 Freas, Precision Scientific, Winchester, VA) at 45 °C for 24 h to about 10% moisture by weight. Peanuts were roasted (Lincoln Impingement Oven Model 1452, Fort Wayne, IN) at 158 °C for 4.5 min to an *L* value of 50 ± 1 (Chroma meter, Model CR-200, Minolta, Japan), cooled using an industrial fan, skins were manually removed then blown away by the fan. Roasted peanuts were packaged (10.16 × 45.72 cm vacuum plastic bags, Koch Packaging, Kansas, MO), flushed with nitrogen gas (medical grade, South Air Gas, Griffin, GA), and vacuum sealed (Ultravac, Koch Packaging, Kansas, MO). The entire process was conducted under yellow light to avoid isomerisation or degradation of stilbene compounds (Trela & Waterhouse, 1996).

Samples were stored at −18 °C prior to analysis, about 1 year.

2.3. Extraction of trans-RES and trans-piceid

Only *trans* isomers of RES and piceid were measured using the reverse HPLC method of Potrebko and Resurreccion (2009). About 30 g sample was ground (coffee mill, Model K9M2-4, BrAun, Mexico) for 1 min, then 10g were weighed into a 250 mL centrifuge bottle and added with 30 mL of 80% ethanol and 2 mL of 60 μg/mL freshly prepared phenolphthalein (Aldrich Chemical Company, Milwaukee, WI) solution as internal standard. Contents were homogenised (PowerGen 700, Fisher Scientific, Pittsburg, PA) for 2 min on ice and centrifuged (Model J2-21 M, Beckman, Palo Alto, CA) for 5 min at 1380G at 25 °C. A 2 mL supernatant was drained by gravity, about 20 min, into a 5 mL tube through a clean-up column, to remove interfering compounds that co-eluted with RES and piceid (Sanders, McMichael, & Hendrix, 2000). Clean-up column was a 3 mL disposable syringe fitted with about 5 mm diameter pre-filter (AP25, Millipore, Bedford, MA) to prevent loss of packing, consisting of 1 g mixture of aluminium oxide (neutral activity 1, particle size of 0.063–0.200 mm, EM Science, Gibbstown, NJ) and silica gel 60 C_{18} (EM Science, Gibbstown, NJ), 1/1, w/w. Column was washed with 0.5 mL ethanol and filtrate was collected in the same tube. Contents of six tubes placed parallel in a heating block (Thermolyne, Dubuque, IO) set at #5 to maintain 60 °C, were dried for about 1 h by blowing nitrogen directly over the samples through a hollow six-pin blowdown manifold, then tubes were capped, wrapped with foil and stored at −20 °C for about 1 week until analysed. Extraction was conducted under yellow light.

2.4. HPLC analysis of trans-RES and trans-piceid

Before HPLC analysis, 0.40 mL ethanol (15%) was added to dried peanut extract, vortexed for 30 s. Six tubes at a time were immersed into water in a round plastic container (13 cm i.d. × 6 cm depth) for 2 min indirect sonication at 50% amplitude using the ultrasonic processor described in Section 2.2 Sample was poured into a 3 mL glass syringe with attached inorganic membrane filter (Anotop 10, 0.2 μm, Whatman International Ltd., Maidstone, England), filtered into a 300 μL polypropylene insert placed in a 2 mL HPLC amber vial and sealed with a screw cap fitted with a Teflon/silicon septum (National Scientific Co., Lawrenceville, GA). HPLC analysis used a Varian ProStar HPLC system consisting of ProStar410 auto sampler, solvent delivery module 210, and ProStar 335 Diode Array Detector (DAD) to set UV spectrum from 240 to 400 nm, and controlled by LC Module Add-In Star version 6 and Star WS version 5.X, 6.41 software system (Varian Inc., Palo Alto, CA). The Eclipse Plus C18 reverse column, 250 × 4.6 mm, 5 μm particle size, preceded by an Eclipse Plus C18 guard column, 7.5 × 4.6 mm, 5 μm particle size (Agilent Technologies, Deerfield, IL) was maintained at ambient temperature, 25 °C. A 1.5 mL/min flow rate was used for solvent A, 100% double deionised water vacuum filtered through a 0.2 μm nylon filter and solvent B, 100% acetonitrile (HPLC Grade, Aldrich, Milwaukee, WI). Gradient elution included: 0 min, 5% B; 7 min, 22% B; 13 min, 23% B; 26 min, 63% B; 28 min, 80% B; then finally returned to 5% B in 1 min, and held at 5% B for additional 5 min. Samples were injected at 40 μL.

Peak areas of *trans*-RES and piceid, were quantified at 307 nm and of phenolphthalein, at 280 nm (Rudolf & Resurreccion, 2005) and concentrations were calculated as follows:

$$\mu g \text{ of } i \text{ in sample} = \frac{\left(\frac{\mu g \text{ of } i \text{ in standard}}{\text{PA of } i \text{ in standard}}\right) \times \text{PA of } i \text{ in sample}}{\left(\frac{\mu g \text{ of IS in standard}}{\text{PA of IS in standard}}\right) \times \text{PA of IS in sample}} \times \mu g \text{ of IS in sample}$$

where *i* = *trans*-RES or *trans*-piceid, IS (internal standard) = phenolphthalein, and PA = peak area. Six levels of standards, 10, 5, 3, 2, 1, and 0.5 μg/mL for *trans*-RES and piceid, and 20, 10, 6, 4, 2 and

1 μg/mL for phenolphthalein were analysed at the beginning of each HPLC sample set. *Trans*-RES and piceid were reported as μg/g peanut, dry weight basis, and their sum, as total stilbenes.

2.5. Consumer acceptance test

A consumer acceptance test (Resurreccion, 1998) was conducted recruiting 50 panelists using the criteria: 18–70 years old, non-smoker, not allergic to peanuts, consumes peanuts and peanut products at least twice/month, willing to evaluate peanuts, and available during test sessions. Twenty five panelists evaluated replication 1 and the other 25, replication 2 from 8:00 am to 7:50 pm. Each panelist evaluated a total of 28 samples in two sessions conducted 1 h apart, 14 samples/session presented in three blocks with 4–5 samples/ block; had 10 min compulsory breaks in-between blocks; and expectorated all samples to prevent fatigue during evaluation. Panelists were asked to fill-out demographical questionnaire, and read and sign two copies of consent to participate forms approved by the University of Georgia Institutional Review Board. Panelists were supplied with water and crackers for rinsing in-between samples. Samples were presented using a monadic sequential order controlled by *Compusense five* (version 4, Compusense Inc., Guelph, Ontario, Canada). Panelists evaluated overall acceptance (OA) and acceptance of appearance, colour, aroma, flavour and texture using a 9-point hedonic scale, with 1, dislike extremely; 5, neither like nor dislike; and 9, like extremely.

2.6. Statistical analysis

Data were analysed using SAS, 2001 statistical software, version 8 (SAS Institute Inc., Cary, NC). General Linear Model (PROC GLM) was used to detect significant differences amongst treatments for the response variables, RES, piceid, total stilbenes, and OA. The relative significance of independent variables, ID, IT and IC for UV treatment and PD, PT and IC for US treatment, was established by ANOVA with the variable contributing the largest percentage to the total sum of squares as the most significant. Mean separation test by Fisher's least significant difference at $P \leqslant 0.05$ was used to compare means of response variables.

Regression analysis (PROC REG) was conducted to develop prediction models for each response variable based on independent variables. A second order polynomial regression model with three linear terms was used as full model as follows: $Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_1 x_1^2 + \beta_2 x_2^2 + \beta_3 x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{123} x_1 x_2 x_3 + \varepsilon$, where Y, response variable; β_0, intercept when x_1, x_2, and x_3 are equal to zero; β_1, β_2, and β_3 = parameter estimates of the linear terms for UV treatment, ID (x_1), IT (x_2), and IC (x_3) and for US treatment, PD (x_1), PT (x_2), and IC (x_3); and their squared and cross product terms.

RSM determined the effects of independent variables on the levels of response variables. Significant regression models were identified ($P < 0.05$) and used to generate response surfaces and contour plots using PROC GCONTOUR with two independent variables at a time, holding the third fixed at one level. Regions of interest were shaded on the contour plots to cover the area with $\geqslant$2.64 μg/g *trans*-RES (McMurtrey, Minn, Pobanz, & Schultz, 1994), $\geqslant$1.85 μg/g *trans*-piceid (Lamuela-Raventos, Romero-Perez, Waterhouse, & de la Torre-Boronat, 1995), and $\geqslant$4.33 μg/g total stilbenes (Lamuela-Raventos et al., 1995), representing predicted $\geqslant$100% of these compounds in red wines, and an OA rating $\geqslant$5.0. When response variables could not meet the set criteria for the region of interest, maximum values achievable were shaded in lieu of pre-determined values. Contour plots for all response variables were superimposed to identify the region of overlap that determined the optimum UV or US process parameters.

3. Results and discussion

3.1. RES, piceid and total stilbenes of UV treated peanuts

The mean concentrations of RES, piceid and total stilbenes of UV treated peanuts are shown in Fig. 1. UV increased ($\alpha < 0.05$) RES, range = 0.54 ± 0.05 to 3.30 ± 0.58 μg/g, and total stilbenes, 0.66 ± 0.04 to 4.00 ± 0.07 μg/g, but not piceid, 0.35 ± 0.02 to 1.05 ± 0.78 μg/g of 27 treated peanuts compared to controls with 0.02 ± 0.002, 0.05 ± 0.004 and 0.03 ± 0.002 μg/g, respectively which correspond to 27–165, 13–80, and 12–35 fold increase, respectively. UV induces increase in enzymes responsible for the biosynthesis of secondary metabolites such as flavonoids and RES, which act as screens to prevent UV-induced damage to genetic material of plant cells (Cantos, Garcia-Viguera, de Pascual-Teresa, & Tomas-Barberan, 2000) causing increased RES in UV treated peanuts. RES in controls were less than 0.29–0.48 μg/g (Rudolf & Resurreccion, 2005, 2007) but within 0.02–0.31 μg/g analysed from 14 of 15 cultivars of three market types of raw peanuts (Sanders et al., 2000). Sanders et al. (2000) reported 1.79 μg/g RES only for small white Spanish cultivar and therefore, was not included as basis for comparison. Highest ($\alpha < 0.05$) RES of 3.30 μg/g and 4.00 μg/g total stilbenes were achieved after 30 min UV treatment at 40 cm ID and incubated for 36 h. Rudolf and Resurreccion (2005) obtained similar maximum RES of 3.42 μg/g in peanuts UV treated for 10 min at 40 cm ID and incubated for 48 h. UV increased ($\alpha < 0.05$) piceid in only 4 of 27 treatments including samples exposed to UV at 40 cm ID for 10 min and IC for 24 and 48 h; and for 30 min at the same ID then incubated for 24 and 36 h for which no trend was identified, whilst others did not significantly differ from controls suggesting that UV was not effective in increasing piceid in sliced peanuts.

ANOVA (Table 1) showed that ID was the most significant ($\alpha < 0.05$) factor affecting stilbenes concentrations of UV treated peanuts. ID contributed the largest sum of squares of 29.1, 19.5 and 34.4% to the total sum of squares for RES, piceid and total stilbenes, respectively, compared to IT and IC. Mean separation test (data not shown) indicated that all stilbenes were highest at ID of 40 cm and lowest at 20 and 60 cm. Cantos, Espin, and Tomas-Barberan (2001) likewise found that 40 cm was the optimum ID achieving the highest RES compared to 20 and 60 cm in UV-treated grapes. Lower RES resulting from UV treatment at 20 cm may have been

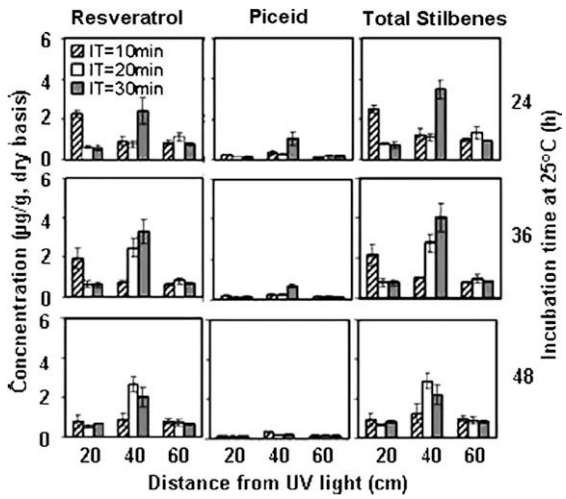

Fig. 1. Mean *trans*-resveratrol, *trans*-piceid and total stilbenes of peanuts exposed to varying doses of UV and incubated at 25 °C for varying times. IT means UV exposure time. Controls had 0.02 ± 0.002 μg/g *trans*-resveratrol, 0.03 ± 0.001 *trans*-piceid μg/g and 0.05 ± 0.002 μg/g total stilbenes.

Table 1
Summary of results of analysis of variance (ANOVA) showing the effects of distance from UV light (ID), UV exposure time (IT), incubation time (IC) on stilbenes concentrations and overall acceptance of UV treated peanuts.

Response	Source/factor	Degrees of freedom	Sum of squares	Percentage[a]	Mean square	*F* value[b]	*P*-value
Resveratrol	ID	2	20.88	29.1	10.44	21.68***	<0.0001
	IT	2	0.83	1.1	0.41	0.86	0.4261
	IC	2	1.29	1.8	0.64	1.34	0.2675
	Error/residual	101	48.64	68.0	0.48		
	Corrected Total	107	71.64	100.0			
Piceid	ID	2	1.44	19.5	0.72	13.81***	<0.0001
	IT	2	0.36	4.9	0.18	3.47*	0.0349
	IC	2	0.28	3.8	0.14	2.65	0.0755
	Error/residual	101	5.26	71.8	0.05		
	Corrected total	107	7.34	100.0			
Total stilbenes	ID	2	33.11	34.2	16.55	20.06***	<0.0001
	IT	2	2.11	2.2	1.05	1.78	0.1732
	IC	2	1.71	1.8	0.86	1.45	0.2387
	Error/residual	101	59.60	61.8	0.59		
	Corrected total	107	96.53	100.0			
Overall acceptance	ID	2	0.99	0.02	0.50	0.14	0.8655
	IT	2	1.00	0.02	0.50	0.15	0.8647
	IC	2	64.22	1.40	32.11	9.34***	<0.0001
	Error/residual	1343	4618.62	98.56	3.44		
	Corrected total	1349	4684.83	100.0			

[a] Percent contribution of the factor to response = (sum of squares of the factor/total sum of squares) × 100%.
[b] *Significant at $\alpha < 0.05$; **significant at $\alpha < 0.01$; ***significant at $\alpha < 0.001$.

"too strong" causing damage to the "biosynthetic system" of RES whilst 60 cm delayed the induction of RES biosynthesis (Cantos et al., 2001).

3.2. RES, piceid and total stilbenes of US treated peanuts

US increased ($\alpha < 0.05$) the mean concentrations of RES, 0.32 ± 0.16 to 6.39 ± 2.15 μg/g, piceid, 0.16 ± 0.05 to 6.39 ± 2.27 μg/g and total stilbenes, 0.48 ± 0.16 to 9.86 ± 0.82 μg/g, compared to controls (Fig. 2) corresponding to 16–319, 5–213, and 10–197 fold increase, respectively. US treatment at low intensities of ginseng cell cultures, stimulated growth and biosynthesis of secondary metabolites through mechanical stress and microstreaming induced by acoustic cavitations which disrupted the cell wall (Lin, Wu, Ho, & Qi, 2001). US caused rapid increase in levels of phenylammonia lyase (PAL), polyphenol oxidase and peroxidase in ginseng cell cultures with PAL enhanced most dramatically with

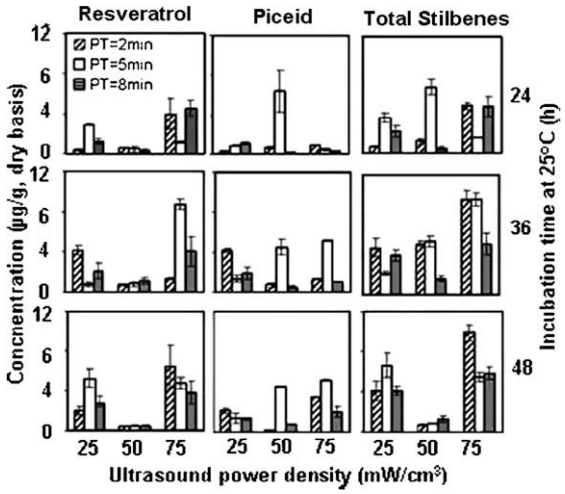

Fig. 2. Mean *trans*-resveratrol, *trans*-piceid and total stilbenes of peanuts exposed to varying doses of ultrasound and incubated at 25 °C for varying times. PT is ultrasound exposure time. Controls had 0.02 ± 0.002 μg/g *trans*-resveratrol, 0.03 ± 0.001 *trans*-piceid μg/g and 0.05 ± 0.002 μg/g total stilbenes.

5-fold higher at power level 4 after 4 days IC compared to controls (Wu & Lin, 2002). PAL is responsible for the deamination of phenylalanine, the initial step in the biosynthesis of coumaryl CoA, one of the precursors for RES synthesis (Soleas, Diamandis, & Goldberg, 1997). We believe that the release of PAL after US treatment could be responsible for increased concentrations of RES and piceid in US treated peanuts in this study and those of Rudolf and Resurreccion (2005, 2007).

ANOVA (Table 2) indicated that PD was the most significant ($\alpha < 0.05$) factor affecting RES and total stilbenes in US treated peanuts contributing the largest 35.6% and 18%, respectively, to the total sum of squares compared to PT and IC. Mean separation test (data not shown) indicated that exposing peanuts to 75 mW/cm^3 resulted in highest ($\alpha < 0.05$) increase in RES of 3.82 μg/g and total stilbenes of 5.41 μg/g, compared to 25 mW/cm^3 producing only 2.38 and 3.58 μg/g, and to 50 mW/cm^3 achieving 0.61 and 2.95 μg/g, respectively. PT ± was the most significant factor affecting piceid with the largest 6.5% contribution. Mean separation test (data not shown) indicated that the highest piceid of 2.34 μg/g was obtained at 5 min PT compared to 1.42 and 1.00 μg/g produced at 2 and 8 min, respectively.

Overall, US treatment resulted in higher ($\alpha < 0.05$) RES, piceid and total stilbenes compared to UV. US treatment had an overall mean concentrations of 2.27 ± 0.99 μg/g RES, 1.59 ± 1.09 μg/g piceid and 3.86 ± 0.92 μg/g total stilbenes, whilst UV resulted in 1.19 + 0.22, 0.24 + 0.03, and 1.43 + 0.23 μg/g, respectively. The average increase in concentrations in US compared to UV treated peanuts were 91% for RES, 562% for piceid, and 170% for total stilbenes. UV and US treated peanuts had higher RES, piceid and total stilbenes compared to blended peanut butters with 0.41 ± 0.02, 0.13 ± 0.01, and 0.54 ± 0.03 μg/g, respectively, and 100% natural peanut butters with 0.65 ± 0.02, 0.14 ± 0.01, and 0.814 ± 0.03 μg/g, respectively (Ibern-Gomez, Roig-Perez, Lamuela-Raventos, & de la Torre-Boronat, 2000). Compared to red wines with 1.85, 2.48 and 4.33 μg/mL RES, piceid, and total stilbenes, respectively (Lamuela-Raventos et al., 1995), US treated peanuts had comparable RES but lower piceid and total stilbenes whilst UV treated peanuts had lower mean values for all compounds. McMurtrey et al. (1994) reported even higher mean RES in red wines of 2.64 μg/mL, range: 0.99–5.01 μg/mL but did not report values for piceid.

Table 2
Summary of results of analysis of variance (ANOVA) showing the effects of ultrasound power density (PD) and exposure time (PT) and incubation time (IC) on stilbenes concentrations and overall acceptance of ultrasound treated peanuts.

Response	Source/factor	Degrees of freedom	Sum of squares	Percentage[a]	Mean square	*F* value[b]	*P*-value
Resveratrol	PD	2	187.11	35.6	93.56	303.00***	<0.0001
	PT	2	0.24	0.0	0.12	0.04	0.9618
	IC	2	26.07	5.0	13.04	4.22**	0.0173
	Error/residual	80	155.40	59.4	3.09		
	Corrected total	107	525.27	100			
Piceid	PD	2	10.94	2.1	5.47	1.21	0.3024
	PT	2	33.83	6.5	16.92	3.74*	0.0221
	IC	2	18.57	3.6	9.29	2.05*	0.1336
	Error/residual	80	201.89	87.8	4.51		
	Corrected total	107	520.02	100.0			
Total stilbenes	PD	2	147.83	18.0	73.91	12.58***	<0.0001
	PT	2	38.41	4.7	19.20	3.27*	0.0421
	IC	2	43.34	5.3	21.67	3.69*	0.0284
	Error/residual	80	125.52	72.0	5.88		
	Corrected total	107	822.97	100.0			
Overall acceptance	PD	2	6.74	0.11	2.25	0.75	0.4720
	PT	2	0.06	0.00	0.03	0.01	0.9935
	IC	2	20.74	0.35	10.37	2.36*	0.0505
	Error/residual	1321	5215.45	99.54	4.41		
	Corrected total	1349	5955.87	100.0			

[a] Percent contribution of the factor to response = (sum of squares of the factor/total sum of squares) × 100%.
[b] *Significant at $\alpha < 0.05$; **significant at $\alpha < 0.01$; ***significant at $\alpha < 0.001$.

3.3. Consumer sensory overall acceptance (OA) of UV and US treated peanuts

Results of *t*-tests showed that regardless of treatment, UV treated peanuts had higher ($\alpha < 0.05$) mean OA rating of 5.7 ± 1.7, range: 5.0 ± 1.7 to 6.3 ± 1.5, compared to US treated peanuts with 5.1 ± 1.6, range: 4.2 ± 2.5 to 6.0 ± 1.8. All UV or US treated peanuts, however, had lower ($\alpha < 0.05$) OA ratings than controls with 7.4 ± 1.4. The higher OA ratings obtained in UV compared to US treated samples were unexpected since UV exposure is known to enhance off-flavour development in food due to lipid peroxidation (Duh & Yen, 1995). Phenolics in food contribute to the off-flavours such as bitterness, astringency, colour, flavour, and odour (Naczk & Shahidi, 2006). Off-flavours contributed by phenolic compounds such as RES and other stilbenes may result in lower acceptance of the food. US produced higher concentrations of up to 9.86 μg/g total stilbenes compared to 3.85 μg/g by UV and this may explain the lower OA of US treated peanuts.

Tables 1 and 2 showed that OA of UV and US treated peanuts was significantly affected by IC rather than ID and IT or PD and PT. Mean separation test (data not shown) indicated that the shortest IC of 24 h had the highest OA of 5.9 for UV and 5.2 for US treated peanuts. The longest IC of 48 h produced the least acceptable products, OA of 5.6 for UV and 4.9 for US treated samples, which did not significantly differ from those at IC of 36 h, OA ratings of 5.6 and 5.1, respectively.

3.4. Modelling, mapping of contour plots and optimum region

Table 3 shows the regression coefficients for the significant prediction models for RES, piceid, total stilbenes, and OA of UV and US treated peanuts. The superimposed contour plots of these models showing the regions of overlap are presented in Fig. 3.

3.4.1. UV treatment

Resveratrol: The region of interest of 2.64 μg/g corresponding to 100% RES in red wines was not met. Only 2.0 μg/g or 75% was produced at IC of 36 h (Fig. 3) and shaded in the contour plot (not shown). The maximum RES of 2.06 μg/g was obtained after UV treatment for 29–30 min at 40–44 cm ID and IC for 36 h. RES was highest at IC of 36 h and then decreased with further increase or decrease in ID. With increasing IT at ID of about 30–50 cm, RES increased except when IC was 24 h wherein RES decreased with increasing IT from 10–20 min and at ID of 28–40 cm, and then increased when exposed from 20–30 min with increasing ID from 38–48 cm. These results indicate that ID of 30 to about 45 cm and IT for more than 30 min followed by IC for 36 h were necessary to allow maximum production of RES in UV treated peanuts.

Piceid: The region of interest of 1.85 μg/g or 100% in red wines was not met (contour plot not shown). Only 30% or 0.55 μg/g piceid was obtained at IC of 24 h (Fig. 3). Piceid increased with decreasing IC from 48 to 24 h and as IT increased from 20–30 min, regardless of IC. As IT increased from 10–20 min, piceid increased as 40 cm was approached from 20 or 60 cm ID. As IC increased from 24–48 h, high IT was needed to obtain equal increase in piceid content. Highest piceid of 0.60 μg/g or 32% in red wines was achieved when peanuts were exposed to UV for 30 min at 35.5–46 cm ID and IC for 24 h. Results indicate that ID approaching 40 cm and IT > 30 min followed by IC for 24 h would produce highest amounts of piceid in UV treated samples.

Total stilbenes: The region of interest of 4.33 μg/g total stilbenes or 100% in red wines was not met (contour plot not shown). Only 55% or 2.4 μg/g total stilbenes was achieved at 36 h IC (Fig. 3). Total stilbenes increased from 20 or 60 cm to 40 cm ID, and increased further with increasing IT from 17–30 min, or decreasing IT from 17–10 min achieving the maximum 2.55 μg/g after 30 min UV exposure at 40–42 cm ID and IC for 36 h. Results show that ID approaching 40 cm and IT > 30 in followed by 36 h IC will achieve highest total stilbenes.

Overall acceptance (OA): The region of interest of OA rating of 5 was met (plot not shown). However, a maximum OA rating > 5.5 could be achieved (Fig. 3). A rating of 5.5 assumes that half of the panel rated the sample a 6.0 or like slightly whilst the other half rated 5.0 or neither like nor dislike. OA increased with decreasing IC from 48 to 24 h. High OA ratings were met when IC, ID and IT decreased indicating that any UV treatment will diminish acceptance. Highest rating of 6.3 or like slightly was achieved when peanuts were exposed to UV for 10–14 min at 20 cm ID and IC for 24 h.

The optimum region: This region of overlap represents the optimum UV process that will meet at least 75% RES, 20% piceid, and 55% total stilbenes in red wines and mean OA $\geqslant$ 5.5. Only peanuts incubated for 36 h met these criteria (Fig. 3). The optimum UV

Table 3
Regression coefficients for the significant prediction models of UV and ultrasound treated peanuts[a].

Coefficient	Resveratrol	Piceid	Total stilbenes	Overall acceptance	Coefficient	Resveratrol	Total stilbenes	Overall acceptance
A. UV treatment					*B. Ultrasound treatment*			
Intercept	0.87601	−0.12828	0.74774	10.92222	Intercept	11.56110	−0.01809	6.89889
Linear					*Linear*			
ID	0.08297	0.04020	0.12317	−0.10111	PD	−0.45346	−0.33600	−0.00603
IT	−0.20424	−0.02291	−0.22715	−0.13133	PT	−0.51448	1.12147	−0.36148
IC	0.01339	−0.00985	0.00354	−0.14657	IC	−0.11287	0.34505	0.04417
Quadratic					*Quadratic*			
ID * ID	−0.00230	−0.00061	−0.00291	0.00014	PD * PD	0.00399	0.00305	−0.00004
IT * IT	0.00039	0.00088	0.00127	0.00050	PT * PT	−0.01025	−0.13594	−0.00370
IC * IC	−0.00155	0.00011	−0.00144	0.00035	IC * IC	0.00123	−0.00482	−0.00030
Interaction					*Interaction*			
ID * IT	0.00429	0.000322	0.00144	0.02650	PD * PT	0.01733	−0.01041	0.00186
ID * IC	0.00020	0.000209	0.00221	0.00239	PD * IC	0.00215	0.00211	0.00030
IT * IC	−0.00042	−0.000272	0.00389	0.00294	PT * IC	−0.15660	−0.00724	0.09440
ID * IT * IC	−0.00008	−0.000007	−0.00009	−0.00007	PD * PT * IC	−0.00045	−0.00034	−0.00004
Adjusted R^2	0.41	0.39	0.45	0.02	Adjusted R^2	0.42	0.29	0.01
P-value	0.0069	0.0111	0.0017	0.0005	*P*-value	<0.0001	<0.0001	0.0532

[a] ID, distance from UV light; IT, UV exposure time; IC, incubation time at 25 °C; PD, ultrasound power density; PT, ultrasound exposure time.

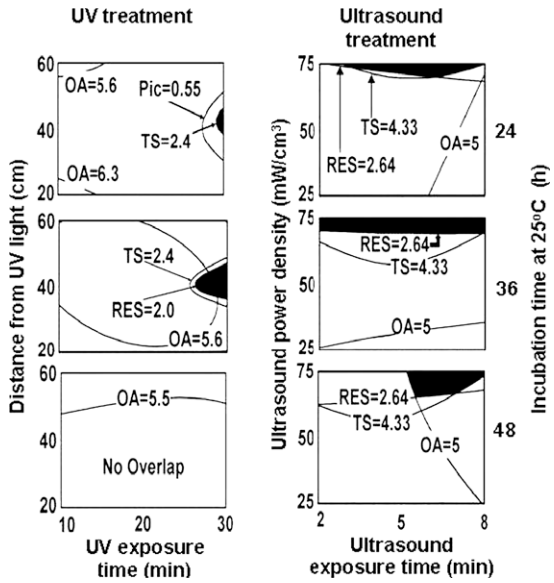

Fig. 3. Superimposed contour plots for the significant ($P < 0.05$) prediction models of resveratrol (RES, μg/g), piceid (Pic, μg/g), total stilbenes (TS, μg/g) and sensory overall acceptance (OA) of peanuts stressed by varying doses of UV and ultrasound. The regions of overlap (shaded areas) represent the optimum UV and ultrasound process at the specified incubation times.

process include all process combinations within the area of a triangle with bounded by the points, ID of 47, 41, and 33 cm for 30, 26.25, and 30 min IT, respectively and will achieve 2.00–2.06 μg/g RES, 0.40–0.47 μg/g piceid, and 2.42–.55 μg/g total stilbenes or 76–78%, 22–25%, and 56–59%, in red wines.

3.4.2. US treatment

Resveratrol: The region of interest of 2.64 μg/g or 100% of RES in red wines was met (plot not shown). RES increased as PD approached 75 and 25 mW/cm^3, with higher maximum at 75 mW/cm^3, regardless of PT from 2–8 min, with minimum RES at 44–48 mW/cm^3 PD. The areas of minimum RES concentrations decreased further as IC increased from 24–48 h. Highest RES of 4.4 μg/g was obtained when peanuts were incubated for 48 h and treated with 75 mW/cm^3 PD regardless of PT from 2–8 min. Results indicated that at the longest IC of 48 h, high RES > 3.96 μg/g or > 150% RES in red wines will be achieved by treatment with high PD of 71–75 mW/cm^3 for 2–6 min. At medium PD, e.g. 40–55 mW/cm^3, lower RES concentrations were observed.

Total stilbenes: The total stilbenes of 4.33 μg/g or 100% in red wines was met (plot not shown). At 45 mW/cm^3, amount of total stilbenes were minimum, and decreased as PT was either increased or decreased from 5 min. Highest total stilbenes, 6.5 μg/g, was reached at 75 mW/cm^3 for 2–6 min at IC of 48 and 36 h. Results indicated that both higher and lower PD of 65–75 and 25–35 mW/cm^3 will result in higher total stilbenes, reaching a maximum when using higher PD, e.g., 75 mW/cm^3.

Overall acceptance: The region of interest, OA rating of 5 was met (plot not shown). OA increased with decreasing IC from 48–24 h. At 24 h IC, OA decreased as exposure time to US increased from 2–8 min, remained at the same ratings at 36 h IC, and then increased at 48 h IC, regardless of PD. Highest OA rating of 5.4 was reached at the lowest IC of 24 h and lowest PT of 2 min at any PD.

The optimum region: This region represents the optimum US process parameters that will met at least 100% RES and total stilbenes in red wines, and OA $\geqslant$ 5. US process at all IC's met all these criteria (Fig. 3). However at 48 h IC, the highest 4.4 μg/g RES and 6.5 μg/g total stilbenes corresponding to 167% and 150% in red wines will be achieved, and determined as the optimum US process which include all US process combinations within the pentagon bounded by the points, PD of 75, 75, 72, 67 and 66 mW/cm^3 for 5.2, 8, 8, 7.1 and 5.5 min, respectively.

4. Conclusions

UV and US treatments significantly increased RES, piceid and total stilbenes in peanuts but decreased OA compared to controls. US, compared to UV, had higher stilbenes but lower OA. The optimum US process will produce 2.64–4.4 μg/g RES and 4.5–6.5 μg/g total stilbenes which are greater 2.00–2.06 and 2.64–3.8 μg/g, respectively, achieved by the optimum UV process. On a per serving, a peanut bar containing 30 g peanuts will require 3–5 bars of US- or 6 bars of UV-treated peanut bars to meet the same RES contents in a 140 mL glass serving of red wines. Further investigations on the additive or synergistic effects of combined UV and US process in increasing the amounts of stilbenes in sliced peanuts are recommended.

Acknowledgments

This project was supported in part by the Peanut Collaborative Research Project Grant No. LAG-G-00-96-00013-00 and the National Research Initiative Grant No. 20063550316989 from the USDA Cooperative State Research, Education, and Extension Service NRA 71.1 Improving Quality and Value.

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peroxide, sodium salicylate, pyrogallol, 2-thiobarbituric acid and other chemicals in the studies were of highest quality commercially available from local suppliers (Shanghai, China).

2.2. Sample extraction

The *P. koraiensis* seeds were collected from the Yichun Forestry Store (Heilongjiang Province, China). The voucher specimen was identified by Dr. Zhenyu Wang, College of Food Science and Engineering, Harbin Institute of Technology, China. The dried putamina of PKS (1500 g) was grinded by pulveriser and extracted with proportional ethanol (40%) by ultrasonic apparatus (KX-1740QT), at ultrasonic procedure of 80 °C, 200 W and 2 h. The extract was centrifuged at 3000 rpm for 5 min and the residue was extracted once again with new solvent under the same conditions. The supernatant was evaporated and the extract powder was obtained by freeze drying. The PKS extract powder (50 g) was stored at 4 °C until required. Before the assays, the extract was dissolved at required concentration.

2.3. Determination of total phenolic content

Total phenolic content was performed according to the Folin–Ciocalteau method (Slinkard & Singleton, 1977) with some modifications. Briefly, 0.1 ml of sample (0–5 mg/ml), 1.9 ml distilled water and 1.0 ml of Folin–Ciocalteau's reagent were seeded in a tube, and then 1.0 ml of 100 g/l Na_2CO_3 was added. The reaction mixture was incubated at 25 °C for 2 h and the absorbance of the mixture was read at 765 nm. The sample was tested in triplicate and a calibration curve with six data points for gallic acid was obtained. The results were compared to a gallic acid calibration curve and the total phenolic content of PKS extract was expressed as mg of gallic acid equivalents per gram of extract.

2.4. $OH^{\cdot}$ scavenging assay

The scavenging capacity of PKS extract on $OH^{\cdot}$ was evaluated according to the reaction of sodium salicylate and residual hydroxyl radical. $OH^{\cdot}$ scavenging assay was performed according to a literature procedure (Wang, Gao, Zhou, Cai, & Yao, 2008) with a few modifications. Hydroxyl radicals were generated by Fenton reaction in the system of $FeSO_4$ and H_2O_2. The reaction mixture was consisted of 0.5 ml $FeSO_4$ (8 mM), 0.8 ml H_2O_2 (6 mM), 0.5 ml distilled water, 1.0 ml of various concentrations PKS extract and 0.2 ml sodium salicylate (20 mM). The total mixture (3.0 ml) was incubated at 37 °C for 1 h and then the absorbance of the mixture was recorded at 562 nm. Ascorbic acid was used as the positive control. The scavenging activity was calculated using the following Eq. (1):

$$\text{Scavenging (or inhibition) rate } (\%) = [1 - (A_1 - A_2)/A_0] \times 100$$

where A_0 is the absorbance of the control (without extract), A_1 is the absorbance of the extract addition and A_2 is the absorbance without sodium salicylate. Matlab 7.0 analysis software was applied to analyse the regression of concentration and scavenging rate.

2.5. DPPH radical scavenging activity assay

The capacity of PKS extract to reduce the 2,2-diphenyl-picrylhydrazyl (DPPH) radical was assessed using the method in a literature (Blois, 2002). Two millilitres of a solution of DPPH in ethanol (8.62×10^{-2} mM) were mixed with 2 ml of various concentrations extract during 30 min at room temperature and the absorbance was recorded at 517 nm (Hsu, Coupar, & Ng, 2006). The normal purple colour of DPPH will turn into yellow when its single electron is paired with a hydrogen atom coming from a potential antioxidant. Ascorbic acid was used as the positive control. In this method, the percentage of DPPH scavenging activity was calculated according to absorbance difference, as has been depicted in Eq. (1), where A_0 is the absorbance of DPPH alone, A_1 is the absorbance of DPPH + extract and A_2 is the absorbance of the extract only.

2.6. O_2^- scavenging assay

In brief, 0.2 ml of different concentrations extract was added to 5.7 ml of 50 mM Tris–HCl buffer (pH 8.2). The mixture was incubated at 25 °C for 10 min and 0.1 ml of 6 mM pyrogallol (25 °C) was added. The absorbance of the reaction mixture was measured at 320 nm every 30 s until the reaction proceeded to 5 min (the same concentration extract was used as the blank to eliminate interference). O_2^- scavenging activity was expressed by the oxidation degree of a test group in comparison to that of the control. The percentage of inhibition effect was calculated according to Eq. (1), where A_0 is the absorbance of the Tris–HCl buffer with pyrogallol, A_1 is the absorbance of the extract addition and A_2 was the absorbance of extract blank.

2.7. Inhibition of lipid peroxidation in rat liver homogenate

The inhibition effect of PKS extract on lipid peroxidation was determined according to the thiobarbituric acid method. $FeCl_2$–H_2O_2 was used to induce the liver homogenate peroxidation (Yen & Hsieh, 1998). In this method, 0.2 ml of PKS extract (0–10 mg/ml) was mixed with 1.0 ml of 1% liver homogenate (each 100 ml homogenate solution contains 1.0 g rat liver), then 50 μl of $FeCl_2$ (0.5 mM) and H_2O_2 (0.5 mM) was added. The mixture was incubated at 37 °C for 60 min, then 1.0 ml of trichloroacetic acid (15%) and thiobarbituric acid (0.67%) was added and the mixture was heated up in boiled water for 15 min. The absorbance was recorded at 532 nm (Buege & Aust, 1978). Ascorbic acid was used as the positive control. The percentage of inhibition effect was calculated according to Eq. (1), where A_0 is the absorbance of the control (without extract), A_1 is the absorbance of the extract addition and A_2 is the absorbance without liver homogenate.

2.8. Inhibition of erythrocyte haemolysis in rat blood

The blood sample was obtained from mice eyepit and made to 0.5% erythrocyte suspension for the assay. The reaction mixture was consisted of 1.0 ml of erythrocyte suspension (0.5%), 1.0 ml extract (0.02–0.2 mg/ml) and 0.1 ml H_2O_2 (100 mM). The mixture was incubated at 37 °C for 60 min, then four times volume distilled water was added to the mixture and centrifuged at 1000 rpm for 10 min. The absorbance of the supernatant was read at 415 nm. The percentage of erythrocyte haemolysis inhibition effect was calculated according to Eq. (1), where A_0 is the absorbance of the supernatant without extract, A_1 is the absorbance of the extract addition and A_2 was the absorbance of extract solution.

2.9. Reducing ability assay

The reducing ability of PKS extract was assayed as described in a reference (Tsai, Huang, & Mau, 2006). Various concentrations extract (0–1.0 mg/ml) was added to 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferrocyanate $[K_3Fe(CN)_6]$ (1%). After incubation at 50 °C for 20 min, 2.5 ml of trichloroacetic acid (10%) was added to the mixture before centrifugation at 1000 rpm for 10 min. The supernatant (5 ml) was gathered and mixed with 5 ml of distilled water and 1 ml of ferric chloride (0.1%). The absorbance was measured at 700 nm. The reducing ability was weighed by the absorbance value of the reaction solution and a higher absorbance indicated increased reducing

power (Jayaprakasha & Bhimanagouda, 2007). Ascorbic acid was used as positive control.

2.10. Ageing model

Kunming male mice, weighing about 18–22 g were obtained from the Experimental Animal Center of Heilongjiang Tumour Hospital. Mice were kept in our animal house at an ambient temperature of 25 °C and 45–55% relative humidity with a 12 h each of dark and light cycle. Animals had free access to food and water. After 1 week of acclimatisation, the mice were randomly divided into five groups (12 mice per group) and i.p. injected with 0.3 ml of D-galactose (150 mg/kg) once daily for 60 days. Simultaneously, the ageing control group (group AC) mice were p.o. administered with 0.3 ml of physiological saline each; the PKS treatment group (group PKS) mice were p.o. administered with different dose of extract (250, 500 and 1000 mg/kg). The normal control group (group NC) mice were i.p. injected and p.o. administered with 0.3 ml of physiological saline.

2.11. Radiation model

γ-Ray was used to induce radiation oxidative damage (radiation dose is 5 grey, dose-rate 0.95 grey/min; radiation time 6.15 min, radiation radius 145 cm). The mice were divided into five groups (12 mice per group). The PKS groups were p.o. administered with 250, 500 and 1000 mg/kg of extract once a day. The radiation control (group RC) and normal group (group NC) were p.o. administered with 0.3 ml of physiological saline. After 21 days, the mice were irradiated by γ-ray according to foregoing dose expect the normal group. The determination was carried out at the fifth day after γ-ray irradiation.

2.12. Measurement of superoxide dismutase (SOD) activity and malondialdehyde (MDA) level

Mice were sacrificed by dislocating; the brains and livers were removed rapidly and homogenised in physiological saline (ice-bath). Brain and liver were used for ageing model and radiation model assays, respectively. The homogenate was centrifuged at 4000 rpm (4 °C) for 15 min and the supernatant was gathered for assays. Test of total SOD activity was carried out by using commercial kits. SOD activity was expressed as units per microgram of brain or liver protein. MDA content was determined by thiobarbituric acid reaction (TBAR) method and expressed as nanomoles per milligram of brain or liver protein.

2.13. Determination of glutathione peroxidase (GSH-px) activity and glutathione (GSH) content

Mice were sacrificed by dislocating; the brains and livers were removed rapidly and homogenised in cold physiological saline (ice-bath). Brain and liver were used for ageing model and radiation model assays, respectively. GSH content and GSH-px activity were measured by commercial kits.

2.14. Protein assay

Protein concentration was measured by Bradford method (Lu, Li, Huang, & Zhang, 2007). Bovine serum albumin was used as the standard.

2.15. Statistical analysis

Data was expressed as mean ± SD. The Duncan test and a one-way analysis of variance were used for multiple comparisons by analysis of SPSS 14.0. The results were considered statistically significant if the *P*-values were 0.05 or less.

3. Results and discussion

3.1. Total phenolic content

Proportional ethanol (40%, v/v) was used for extraction and the extract yield was 3.5 g/100 g. This solvent system destroys the cell membranes, simultaneously dissolving the phenolic compounds. Phenolic content of PKS extract was measured by Folin–Ciocalteau method and the total phenolic content in the extract was 264 ± 10.52 mg/g. The Folin–Ciocalteau method is simple and can be used in characterising botanical samples, for example sorghum (Awika, Rooney, & Waniska, 2004), soybean (Takahashi et al., 2005), white and black sesame seed (Shahidi, Liyana-Pathirana, & Wall, 2006). The result showed PKS extract contained high phenolic content compared with some other plants, such as black soybean seed (Astadi, Astuti, Santoso, & Nugraheni, 2009), bayberry (Zhou et al., 2009) and Vietnam bitter tea (Thuong, Nguyen, Ngo, et al., 2009). PKS was a nice potential antioxidant plant.

3.2. $OH^{\cdot}$ scavenging activity

As the positive control, ascorbic acid showed an excellent scavenging activity on $OH^{\cdot}$, the half inhibition concentration (EC_{50}) = 0.103 ± 0.031 mg/ml. PKS extract was also observed to have strong scavenging activity, with an EC_{50} value of 0.391 ± 0.055 mg/ml (Fig. 1a).

3.3. DPPH radical scavenging activity

Fig. 1b showed the scavenging capacity of PKS extract on DPPH (ascorbic acid as the positive control). A dose–response relationship is found in DPPH scavenging activity of PKS extract and an increase in concentration is synonymous of an increase in scavenging capacity. Ascorbic acid showed high scavenging activity (EC_{50} = 0.002 ± 0.0001 mg/ml). The PKS extract also had strong DPPH scavenging effect, with an EC_{50} value of 0.023 ± 0.004 mg/ml. Among the levels used in the experiment, 0.09 mg/ml variety was the strongest one with a scavenging rate of 91.64%, ascorbic acid as standard was 96.46% at the same concentration. At 0.09 mg/ml, the scavenging ability of PKS extract on DPPH was not significantly different from ascorbic acid. The strong scavenging capacity of PKS extract on DPPH was possibly due to the phenolic compounds which could act as a hydrogen donor antioxidant.

3.4. O_2^- inhibition activity

Spontaneity oxidation of pyrogallol will proceed in slightly alkaline condition and the O_2^- was produced with absorption at 320 nm. Antioxidant can inhibit the spontaneity oxidation of pyrogallol but it has been found that the reaction is instable in 1–3 min. Repetitious tests showed the reaction was stable in 4–5 min and consequently the data at 5 min was adopted to evaluate the inhibition activity. Matlab 7.0 analysis software was applied to analyse the regression of concentration and inhibition rate. PKS extract was found to have inhibition activity and the EC_{50} was 4.37 ± 0.19 mg/ml (Fig. 2a), as the positive control, EC_{50} of ascorbic acid was 0.069 ± 0.013 mg/ml. The result showed PKS extract had a certain extent inhibition on O_2^- but was not as strong as ascorbic acid. PKS extract effectively inhibited O_2^- at concentrations of 4–8 mg/ml. The inhibition rate was above 80% at 8 mg/ml.

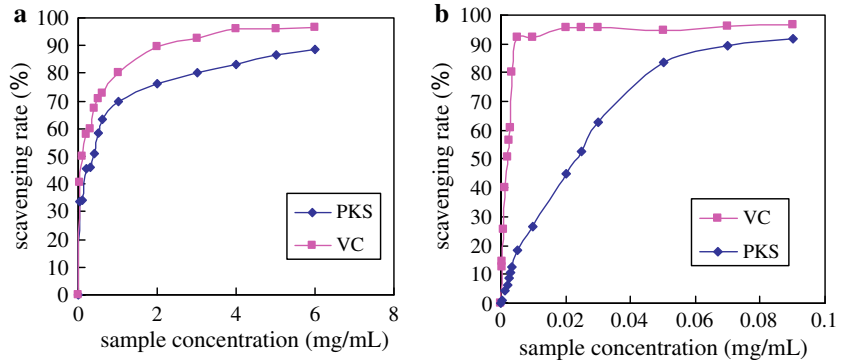

Fig. 1. (a) The OH· scavenging activity of PKS extract. (b) The DPPH radical scavenging activity of PKS extract.

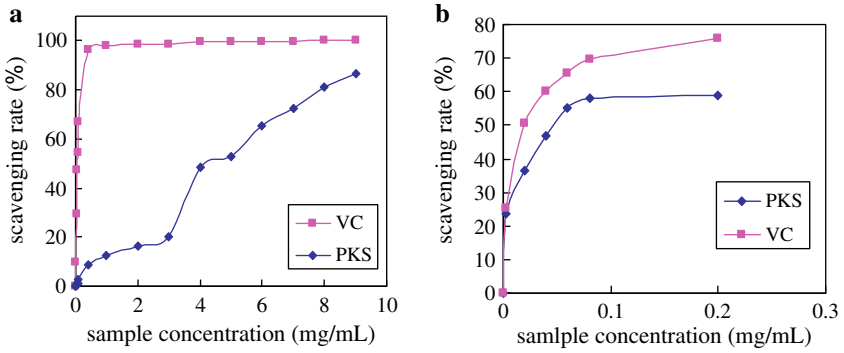

Fig. 2. (a) The O_2^- inhibition activity of PKS extract. (b) The inhibition of PKS extract for erythrocyte haemolysis in rat blood.

3.5. Inhibition of lipid peroxidation in rat liver homogenate

Lipid peroxidation was an oxidative deterioration process of polyunsaturated fatty acids which induced by radical. Lipid structure of cell velum was damaged by radical and accordingly lipid peroxidation of viscera homogenate was increased. MDA content can indicate the degree of lipid peroxidation. $FeCl_2$–H_2O_2 system was used to induce lipid peroxidation in rat liver homogenate. In the assay, PKS extract was confirmed to have strong inhibition for lipid peroxidation. 88.47% of the peroxidation was inhibited by PKS extract at concentration of 4 mg/ml (Fig. 3a). At 7 mg/ml, PKS extract showed an inhibition rate of 90.21%. Ascorbic acid, as the positive control, at concentrations of 4 and 7 mg/ml, 51.93% and 83.13% of the peroxidation was inhibited, respectively. So PKS extract has highly strong resistance on peroxidation. The phenolic compounds in PKS extract can provide phenolic hydroxyl to accept electrons and scavenge OH· which induced by $FeCl_2$–H_2O_2. The results are considered to be noteworthy when compared to the findings of other studies concerning medicinal plants (Sultana, Anwar, & Przybylski, 2007; Tepe, Sokmen, Akpulat, Yumrutas, & Sokmen, 2006).

3.6. Inhibition of erythrocyte haemolysis in rat blood

Oxidant damage of cell film which induced by H_2O_2 can result in erythrocyte haemolysis. The result showed erythrocyte haemolysis was effectively inhibited by PKS extract at concentrations of 0.02–0.2 mg/ml. At concentration of 0.04 mg/ml, the inhibition rate was nearly 50%. At concentrations of 0.06, 0.08 and 0.2 mg/ml, the inhibition rate was 55.3%, 57.9% and 59%, respectively. Inhibition rate of ascorbic acid (the positive control) was 60% at 0.04 mg/ml. At 0.06, 0.08 and 0.2 mg/ml, the inhibition rate was 65.1%, 69.4% and 75.8%, respectively (Fig. 2b). In this assay, PKS extract was found to have inhibition activity for erythrocyte haemolysis; this

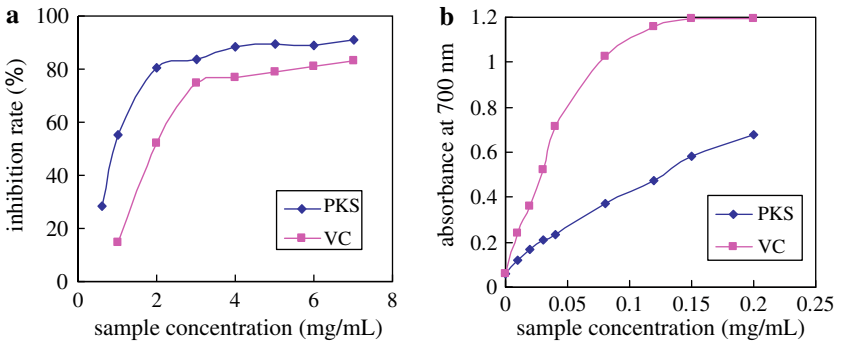

Fig. 3. (a) The lipid peroxidation inhibition activity of PKS extract. (b) The reducing ability assay of PKS extract.

Table 1
Effect of PKS extract on SOD, GSH-px activity and MDA, GSH content for ageing mice brain.

Group	SOD (U/mg protein)	GSH-px (U/mg protein)	MDA (nmol/mg protein)	GSH (mg/g protein)
NC	391 ± 32.8	688 ± 47.3	2.88 ± 0.77	3.85 ± 0.84
AC	316 ± 21.9[b]	540 ± 43.7[b]	3.64 ± 0.61[a]	3.09 ± 0.60[a]
PKS (250 mg/kg)	321 ± 36.0	550 ± 34.6	3.40 ± 0.54	3.35 ± 0.55
PKS (500 mg/kg)	345 ± 15.9[c]	559 ± 39.7	2.98 ± 0.48[c]	3.71 ± 0.58[c]
PKS (1000 mg/kg)	374 ± 36.3[d]	561 ± 30.0	2.87 ± 0.53[c]	3.86 ± 0.54[c]

$N = 12$.
[a] $P < 0.05$ vs. NC group.
[b] $P < 0.01$ vs. NC group.
[c] $P < 0.05$ vs. AC group.
[d] $P < 0.01$ vs. AC group.

effect can prevent cell films from lipid oxidation and protect erythrocyte.

3.7. Reducing ability assay

The reducing power of PKS extract was presented in Fig. 3b. As described in Fig. 3b, PKS extract was found to have strong and increased reducing ability at concentrations of 0.12–0.2 mg/ml. In this assay, the yellow colour of the test solution changes to various shades of green and blue (Barros, Baptista, & Ferreira, 2007). The presence of reducers causes the conversion of the Fe^{3+}/ferricyanide complex to the ferrous form. The reducing power of PKS extract increased with a higher concentration. All spectrophotometric measurements were repeated twice with at least three replicates. At concentrations of 0.02, 0.04 and 0.08 mg/ml, the reducing power of PKS extract was around 0.17, 0.23 and 0.38, respectively, while a solution of ascorbic acid at the same concentration, the positive control used in this test, had a reducing power value of 0.358, 0.712 and 1.713, respectively.

3.8. Antioxidative effects for ageing mice

Subacute mice ageing were induced by injection of D-galactose. The biological redox substance in mice can be disturbed by long-term injection of D-galactose, such as decreasing of GSH level, SOD and GSH-px activity. The antioxidant effect of PKS extract for ageing mice was presented in Table 1. The results indicated that PKS extract could strongly inhibit mice ageing. Mice were p.o. administered with PKS extract and the SOD activity was enhanced 9% and 18% at dose of 500 and 1000 mg/kg, respectively. The MDA content was decreased 18% and 21% at 500 and 1000 mg/kg, respectively, the GSH content was also effectively increased at dose of 500 and 1000 mg/kg. At dose of 1000 mg/kg, the PKS extract showed the greatest antioxidant effect for ageing mice. The results also indicated that PKS extract might be used as natural antioxidants and alternatives to synthetic anti-ageing reagent. The potent anti-ageing effect of PKS extract *in vivo* maybe owe to its antioxidative activity. The antioxidant principle was to reduce organism harm and prevent radical formation and histiocytic period was prolonged.

3.9. Antioxidative effects for radiation mice

Radiation can result in oxidant damage and disturb the balance of enzyme activity in mice livers. The important enzyme activity and substance content related to radiation injury were detected in this assay. The results of radiation model test were showed in Table 2. It was found that PKS extract could potently inhibit oxidant damage induced by γ-ray. PKS extract was ingested by p.o. administration and the radiation damage on mice was highly decreased. The results showed ingestion of PKS extract *in vivo* could increase SOD and GSH level of radiation mice to normal level. Ingestion of PKS extract can maintain the balance of enzyme action and release the radiation damage. At dose of 1000 mg/kg, PKS extract can remarkably enhance SOD activity. Also the MDA content was highly decreased at the same dose (decreasing of 11% and 16%, respectively). At dose of 500 and 1000 mg/kg, the GSH content in mice liver was much higher than the RC group.

PKS (homologous from *Pinus* plant) was reported to have many bioactivities, such as anti-cancer, antioxidant, anti-virus and antibacterial. Free radicals, oxidant-related enzyme and lipid peroxidation are important factors which highly impact oxidation and ageing. In this study, antioxidant properties of PKS extract (40% ethanol) were demonstrated by various assay systems. The results suggested that PKS extract had potent antioxidant activities in multiple mechanisms. Especially, the scavenging activity on $OH^{\cdot}$ was very strong and nearly as effective as ascorbic acid (Fig. 1a). It was worth mentioning that inhibition effect of PKS extract for lipid peroxidation was even higher than ascorbic acid (Fig. 3a). *In vivo* assays, the PKS extract was found to increase the levels of antioxidant enzymes (SOD, GSH) and decrease MDA content in brain and liver. It was confirmed that PKS extract could protect the tissues against oxidative damage. Enhancing of SOD activity and GSH content in mice viscera indicated the *in vivo* antioxidant activity of PKS extract and the related mechanism. The present study can be considered a forward step in this direction.

Table 2
Effect of PKS extract on SOD, GSH-px activity and MDA, GSH content for radiation mice liver.

Group	SOD (U/mg protein)	GSH-px (U/mg protein)	MDA (nmol/mg protein)	GSH (mg/g protein)
NC	501 ± 38.6	890 ± 48.2	3.98 ± 0.48	4.42 ± 0.65
RC	417 ± 22.2[b]	740 ± 43.7[b]	4.62 ± 0.48[a]	3.26 ± 0.78[b]
PKS (250 mg/kg)	423 ± 36.5	750 ± 34.6	4.40 ± 0.63	3.44 ± 0.51
PKS (500 mg/kg)	434 ± 23.4	759 ± 39.7	4.08 ± 0.42	3.83 ± 0.56[d]
PKS (1000 mg/kg)	475 ± 34.4[d]	761 ± 27.6	3.86 ± 0.52[c]	4.20 ± 0.49[d]

$N = 12$.
[a] $P < 0.05$ vs. NC group.
[b] $P < 0.01$ vs. NC group.
[c] $P < 0.05$ vs. RC group.
[d] $P < 0.01$ vs. RC group.

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the biochemical and anticoagulant properties of *M. edulis* anticoagulant peptide (MEAP) isolated from blue mussel were identified. These prolonged blood coagulation times by multiple interactions with blood coagulation factors.

2. Materials and methods

2.1. Materials

Fresh live mussel (*M. edulis*) was obtained from an aquafarm in Tongyoung, S. Korea. The approximately 1 year old mussels had been cultured on a hanging line. The average width of the shell was about 7–8 cm and the average shell on weight was ~15 g. A HiPrep™ 16/10 DEAE FF anion exchange column and a Superdex™ 200 10/300 GL gel filtration column were purchased from Amersham Biosciences Co. (Uppsala, Sweden). An ACL® Coagulation analyzer (ACL® 7000), a blood coagulation assay kit (HemosIL™ APTT Lyophilized silica for APTT, HemosIL™ TT, and HemosIL™), and a Factors-Deficient Plasma were produced by the Instrumentation Laboratory Co. (Lexington, MA, USA). Human blood coagulant factors (zymogens of FII, FV, FVII, FIX, FX, FXI, FXII, and their serine proteinase type enzymes of the activated FII, FV, FVII, FIX, FX, FXI, FXII) and 6-amino-1-naphthalenesulphonamide (ANSN)-based fluorogenic substrates (SN-7; Mes-D-LGR-ANSN$(C_2H_5)_2$ and SN-59; D-VPR-ANSN$HC_4H_9 \cdot 2HCl$) were obtained from Haematologic Technologies, Inc. (Essex Junction, VT, USA). Fibrinogen, plasmin, and molecular markers for electrophoresis were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The BIAcore® 2000 system for surface plasmon resonance (SPR) analysis, a sensor chip CM5, surfactant P20, HBS buffer (10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA, 0.05% surfactant P20, pH 7.4), an amine-coupling kit containing *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-diethylaminopropyl) carbodiimide (EDS) and ethanolamine hydrochloride were obtained from Biacore AB Co. (Uppsala, Sweden). A NISSEI ACE homogenizer AM-6 with cup, cutter, vessel, and motor was a product of Nihonseiki Kaisa Ltd. (Tokyo, Japan). A Micro Acilyzer Model G3electrodialyzer was purchased from Asahi Chemical Industry Co. (Tokyo, Japan). A fast performance liquid chromatographic system, FPLC ÄKTA was produced by Amersham Bioscience Co. (Uppsala, Sweden). To perform an enzyme-linked immunosorbent assay (ELISA), an Ultramark microplate imaging system plate reader was purchased from Bio-Rad Co. (Hercules, CA, USA). The BIAcore 2000® system was produced by Biacore AB Co. (Uppsala, Sweden). The ESI-QTOF tandem mass spectrometry was produced by Micromass Co. (Manchester, UK). All other chemicals used in this study were of analytical grade.

2.2. Isolation of an exogenous anticoagulant peptide from M. edulis

All operations were done at 4 °C unless stated otherwise. Fresh *M. edulis* was washed three times with tap water to remove salts and other contaminants. After removing the shells, all of the edible parts (500 g) were minced and homogenised at 10,000 rpm for 15 min with 10 mM Tris–HCl buffer (pH 7.7), and the insoluble homogenates were removed by centrifugation at 9500*g* for 15 min with a Micro 17R centrifuge (Hanil Co., Seoul, Korea) and the supernatant was treated with 150 ml of 20% (v/v) CCl_4 and cooled to −20 °C to remove lipid. Soluble extracts in the water layer were desalted using an electrodialyzer with a 1 kDa molecular weight cut-off (MWCO) membrane (Asahi Chemical Industry Co.) until conductivity was under 0.1 mA. The dialysate was separated using a HiPrep™ 16/10 DEAE FF anion exchange column (16 × 100 mm) preliminarily equilibrated with 10 mM Tris–HCl buffer (pH 7.7) in a fast performance liquid chromatographic

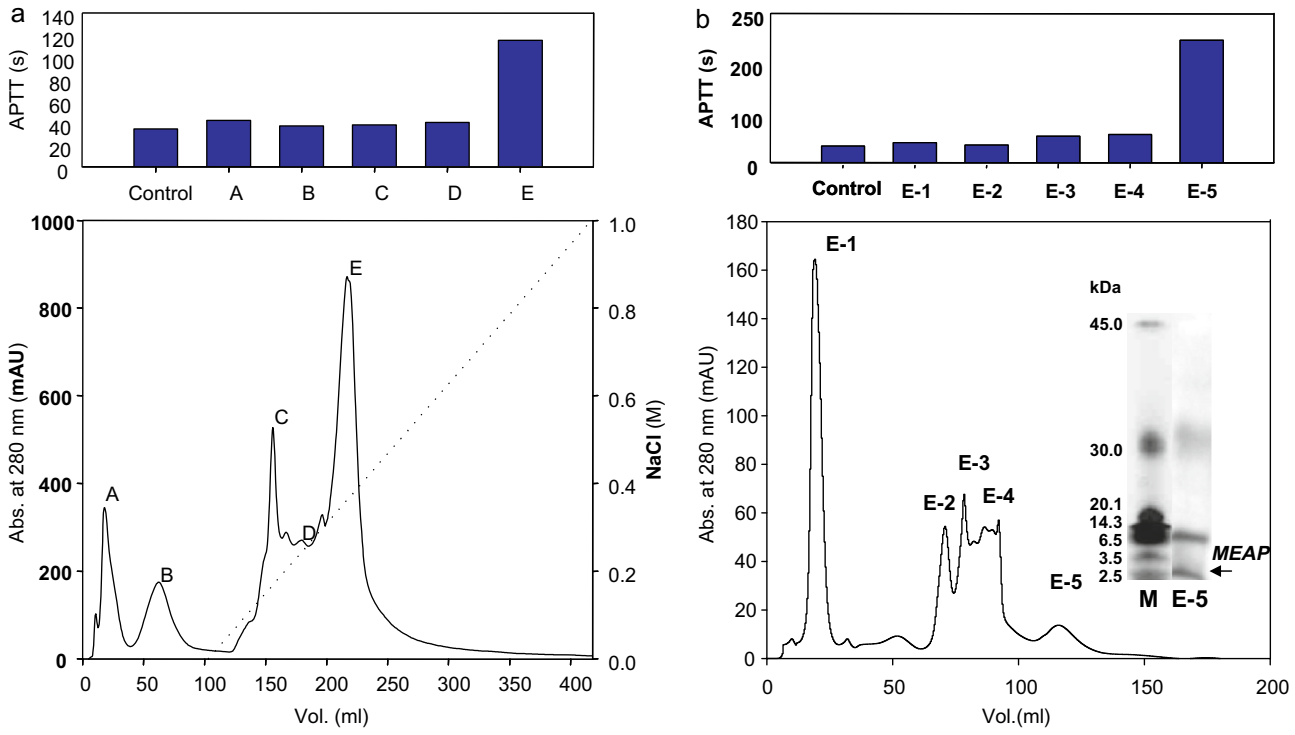

Fig. 1. Purification profiles and anticoagulant activity of MEAP. (a) Anion exchange chromatography on a HiPrep™ 16/10 DEAE FF anion exchange column (16 × 100 mm) preliminarily equilibrated with 10 mM Tris–HCl buffer (pH 7.4) in a FPLC system. A linear gradient of NaCl (0–1 M) in the same buffer was maintained at a flow rate of 1.5 ml min^{-1} and 5.0 ml fractions were collected. The upper panel shows anticoagulant activity for the APTT (s) and (b) gel filtration chromatography on a Superdex™ 200 10/300 GL gel filtration column (10 × 300 mm) preliminarily equilibrated with distilled water. Three millilitres fractions were collected at a flow rate of 0.7 ml min^{-1}. The upper panel shows the anticoagulant activity for the APTT. An inner-panel is the SDS–PAGE profile of the active fraction (E-5) under denaturing conditions.

system. A linear gradient of NaCl (0–1.0 M) in the same buffer was maintained at a flow rate of 1.5 ml min^{-1} and 5.0 ml per a fraction was collected. Absorbance was monitored at 280 nm (Fig. 1a) and anticoagulant activity was evaluated using the blood coagulation assay for the activated partial thromboplastin time (APTT) as described herein. The pooled fraction E, exhibiting the highest activity, was subsequently loaded onto a Superdex™ 200 10/300 GL gel filtration column (10 × 300 mm) with distilled water at a flow rate of 0.7 ml min^{-1} (Fig. 1b). After being separated through consecutive FPLC columns, the purity of the active fraction (E-5) was analysed by electrophoresis on a 15% SDS–PAGE using a Bio-Rad Mini-PROTEAN II electrophoresis cell (Hercules, CA, USA) according to the method of Laemmli (1970). Samples (5 μg) incubated with 2% 2-mercaptoethanol for 5 min at 100 °C were loaded on a 15.0% slab gel and stained with Coomassie Brilliant Blue R-250. Molecular markers (lane M) are ovalbumin (45 kDa), carbonic anhydrase (30 kDa), lysozyme (14.3 kDa), aprotinin (6.5 kDa), insulin β chain (3.5 kDa), and insulin α chain (2.5 kDa). For further purification, the fraction E-5 containing two peptides (Fig. 1b) was re-separated with Superdex™ 200 10/300 GL gel filtration column (10 × 300 mm) at a flow rate of 0.5 ml min^{-1}. The arrow (Fig. 1b) indicated the final active fraction (MEAP) re-separated with the Superdex™ 200 10/300 GL gel filtration column. The purified *M. edulis* anticoagulant peptide (MEAP) was analysed using ESI-QTOF tandem mass spectrometry after gel extraction. To extract the peptide from the gel, the stained peptide was excised from the slab gel and digested with trypsin (Promega Co., Madison, WI, USA). After washing with 10 mM NH_4HCO_3 and 50% CH_3CN, the gel piece was swollen in a digestion buffer containing 50 mM NH_4HCO_3, 5 mM $CaCl_2$ and 12.5 ng μl^{-1} trypsin and incubated at 37 °C for 12–16 h. The peptide was recovered by two extraction steps with 50 mM NH_4HCO_3 and 100% CH_3CN. During the chromatographic process, protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin (Thermo Fisher Scientific Inc., Rockford, IL, USA) as a standard material.

2.3. Peptide sequencing and identification by mass analysis and database searching

All MS/MS experiments were performed on a QTOF tandem mass spectrometer equipped with a nano-ESI source. The molecular mass and amino acid sequence of the purified peptide were determined. The peptide solution was desalted using C18 Zip-Tip® pipette tips (Millipore, Billerica, MA). Approximately 5 μl of the sample was placed into a metal-coated glass capillary (Protana Co., Ødense, Denmark), and a 1500 eV voltage was applied to produce an electrospray; cone voltage was 30 eV. The MS/MS spectra were acquired in the data-dependent MS/MS mode, in which collision energy was increased stepwise to 25, 30, and 35 eV from 10 eV. Argon was introduced as a collision gas at a pressure of 10 psi. The instrument was calibrated externally, and no post-acquisition recalibration of the MS/MS spectra was performed. Sequencing information for the active peptide was acquired over the *m*/*z* range 50–2500 and sequenced by using the PepSeq *de novo* sequencing algorithm (Micromass Co.). For peptide identification, MS/MS spectra were searched by MASCOT (Matrix science, http://www.matrixscience.com, London, UK) using the NCBInr database. Mass tolerance was set at ±1.0 Da for the masses of peptide precursors and at ±0.8 Da for the masses of fragment ions. Proteins containing at least one significant peptide (⩾individual score) were selected from the database search results.

2.4. Blood coagulation time assay

Nine parts of blood was drawn by venipuncture into one part of 3.2% sodium citrate from three healthy human volunteers and pooled. The blood was separately centrifuged at 1600*g* for 10 min to obtain platelet poor plasma (PPP). All coagulation assays were performed with three individual replicates using the ACL® 7000 coagulation analyzer and diagnostic kit, and mean values were used. Briefly, normal citrated PPP (80 μl) was incubated with sample solution (20 μl) for 3 min at 37 °C. For the activated partial thromboplastin time (APTT) assay, 100 μl of APTT reagent was added to the mixture (PPP and sample) and incubated at 37 °C for 3 min, and clotting time (time for the change of light scatter associated with the formation of a fibrin clot) was immediately recorded after the addition of 100 μl of 20 mM $CaCl_2$. In the prothrombin time (PT) assay, PT reagent was added to the incubated mixture of PPP (80 μl) and sample (20 μl), and clotting time was recorded. For thrombin time (TT) measurements, 100 μl of anticoagulant was incubated with human thrombin (100 μl) for 3 min at 37 °C, and clotting time was determined after the addition of 200 μl PPP. For the specific factor inhibitory assays, HemosIL™ Factors-Deficient Plasma kits were used according to the manufacturer's manual, and the effects of pH, temperature, and divalent cation on MEAP activity were tested on the basis of the TT assay according to our previous method (Jung et al., 2001).

2.5. Surface plasmon resonance (SPR) analysis

To identify the binding affinity of MEAP to blood coagulation factors, surface plasmon resonance (SPR) sensorgrams were determined using a BIAcore 2000® system. A ligand peptide was directly immobilised on a CM5 sensor chip by coupling through the free amino group to a carboxylated dextran matrix, activated with a mixture of *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*′-(3-diethylaminopropyl) carbodiimide (EDS) after pH scouting. The sensor chip surface was coated with a ligand peptide in 10 mM sodium acetate buffer at optimal pH, and unreacted groups were blocked with 1 M ethanolamine, pH 8.5. Among the four flow cells in the sensor chips, the first flow cell in each was similarly activated and blocked without immobilisation of the peptide to serve as a control surface. After immobilisation of coagulation factors on the sensor chip, an analyte (50 μg ml^{-1}) was injected onto the surface of the sensor chip in HBS buffer (Hepes-buffered saline containing 1 mM 3-[(3-cholamidopropy-l) dimethylammonio]-1-propanesulfonate, CHAPS, 0.005% surfactant P20, 5 mM $CaCl_2$, 10 nM tissue factor, pH 7.4) at 25 °C at a flow rate of 30 μl min^{-1} for 3 min followed by 2 min of dissociation. Resonance was monitored as a function of time and binding affinity and is represented as a resonance or response unit (RU) in real time. The Langmuir equation was applied for evaluation of equilibrium kinetics with a normal definition of the constants for the association rate and dissociation rate, k_a and k_d. The constants were calculated by the manufacturer's BIA Evaluation software for kinetic experiments.

2.6. Preparation of phospholipid complexes

To investigate an inhibitory activity of the exogenous anticoagulant on amidolytic functions of the major factors in the blood coagulation system, the following assays were performed according to the previous methods (Butenas, van't Veer, & Mann, 1997; Shi & Gilbert, 2003). Phospholipid (PL) vesicles were prepared by evaporating chloroform from the desired phospholipids (PS–PE–PC percentage ratio, 15:20:65), resuspending in methylene chloride, and re-evaporating twice under argon. Phospholipids were then suspended by gently swirling Tris (tris-(hydroxymethyl) aminomethane)-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4) over the dried lipid suspension until all the lipid was suspended. Vesicles were filtered through 2 stacked polycarbonate membranes (100 nm pore size, Millipore, Bedford, MA, USA) and were used as multilamellar vesicles. Some of the resuspended vesicles were then

sonicated using Branson Sonicator (Model 5510, Branson Ultrasonics, Danbury, CT, USA), and some were made by extruding these phospholipid suspensions 20× through 2 stacked polycarbonate membranes under argon in a High Pressure Extrusion Device (Sciema Technical Services, Vancouver, BC, Canada). Vesicles were used fresh; or 1.0 ml aliquots were quick-frozen in liquid nitrogen, stored at −80 °C, and thawed at 37 °C. Storage at 4 °C before incubation with microspheres or blood clotting factors did not exceed 24 h.

2.7. Intrinsic factor tenase (FXase) assay

The activation of FX by FIXa in the presence of exogenous anticoagulants was measured with an amidolytic substrate assay. FIXa (0.1 nM) was incubated with the specified concentrations of FX and varied concentrations of sample in 150 mM NaCl, 50 mM Tris (pH 7.4) containing PL vesicles, 1.5 mM $CaCl_2$, 1 nM FVIII, and 0.1 nM thrombin for 5 min at 25 °C; the final reaction volume was 40 μl. The reaction was then stopped by the addition of 0.5 M ethylenediaminetetraacetic acid (EDTA) solution to 7 mM final concentration. The amount of FXa generated was determined immediately with the use of the 6-amino-1-naphthalene-sulphonamide (ANSN) based fluorogenic substrate SN-7, Mes-D-LGR-ANSN$(C_2H_5)_2$ on an enzyme-linked immunosorbent assay (ELISA) plate reader in kinetic mode.

2.8. Prothrombinase assay

Cleavage of prothrombin (FII) to thrombin (FIIa) was measured in an amidolytic substrate assay analogous to that for FX activation. First, 1 nM FVa, 6.2 pM FXa, and the sample at specified concentrations were incubated for 5 min at 25 °C in a solution containing 150 mM NaCl, 50 mM Tris containing PL vesicles, 1.5 mM $CaCl_2$, and 0.05% w/v ovalbumin (Sigma Chemical Co., St. Louis, MO, USA), pH 7.8, prior to the addition of 1 μM prothrombin. After 5 min at 25 °C, the reaction was stopped by the addition of EDTA to a final concentration of 7 mM. Thrombin formation was assessed in a kinetic microplate reader immediately after addition of 0.1 mM fluorogenic substrate SN-59, D-VPR-ANSNH-$C_4H_9 \cdot 2HCl$.

2.9. Cytotoxicity assay

A venous endothelial cell line (ECV-304) was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% (v/v) heat inactivated foetal bovine serum (FBS) supplemented with penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37 °C under 5% CO_2 (Daesung Industrial Gases Co. Ltd., Seoul, Korea) in air. The cytotoxic effect of exogenous MEAP on endothelial cells (ECV-304) was measured by using an MTT assay using heparin as the basis of comparison. The cells were seeded in a 96-well plate at a concentration of 2×10^4 cells ml^{-1} using the RPMI medium. After incubation in a humidified 37 °C, 5% CO_2 incubator (MCO-18AIC, Sanyo Co., Tokyo, Japan) for 16 h, MEAP was added to the wells at a concentration range from 0 to 500 μg ml^{-1}. The cells were then further incubated for an additional 72 h at 37 °C. MTT stock solution (50 μl of 2 mg ml^{-1} in PBS) was then added to each well for a total reaction volume of 250 μl. After incubating for 4 h in a humidified atmosphere of 5% CO_2 at 37 °C, the plate was centrifuged at 800*g* for 5 min and the supernatants were aspirated to remove untransformed MTT. The formazan crystals in each well were dissolved in 150 μl of DMSO (Merck, Darmstadt, Germany). The amount of purple formazan was determined by measuring the absorbance at 540 nm using the enzyme-linked immunosorbent assay (ELISA) plate reader. For treated cells, viability was expressed as a percentage of control cells.

2.10. Statistics

Data was analysed using Microsoft Excel 2003 (Microsoft Co., Redmond, WA, USA), and statistical analysis was performed with SPSS 8.0 (SPSS Inc., Chicago, IL, USA). All determinations were carried out in triplicate. Results are presented as mean ± SD of the mean ($n = 3$). The Student's *t*-test was used to determine the level of significance unless otherwise stated.

3. Results

3.1. Anticoagulant activity and structural conformation of M. edulis anticoagulant peptide (MEAP)

During screening for anticoagulant activity from marine bivalves, blood coagulation inhibitory activity was detected in the soluble extracts of blue mussel (*M. edulis*). In the preliminary test, the extracts prolonged blood clotting time on TT and APTT but not PT. A control clotting time on TT (11.6 ± 0.4 s) was prolonged to 42.1 ± 0.9 s by the addition of 20 μl soluble *M. edulis* extracts (3.8 mg of total soluble extract ml^{-1}). In the APTT assay, the addition of extracts with the same concentration could prolong the control (35.3 ± 0.5 s) up to 75.8 ± 0.9 s. After purification, the purified *M. edulis* anticoagulant peptide (MEAP) prolonged the normal clotting time to 321 ± 2.1 s on APTT and to 81.3 ± 0.8 s on TT, respectively, in a dose-dependant manner (Fig. 2). The results of ESI-QTOF tandem mass analysis showed that MEAP has a molecular mass of 2468 Da similar to the results with SDS–PAGE (Fig. 1b), and consistent with the amino acid sequence of MEAP which is EADIDGDGQVNYEEFVAMMTSK.

3.2. Identification and characterisation of M. edulis anticoagulant peptide (MEAP)

Specific factor inhibitory assays with normal human plasma showed that MEAP could inhibit FIX, FX, and FII containing γ-carboxyglutamic acid (gla) domains (Fig. 3). In the direct-binding assay using SPR sensorgraphy (Fig. 4), real-time resonance units (RUs) were measured for identifying molecular interactions between MEAP (ligand) and FII, FIX, FX (analytes). The binding affinity between MEAP and the factors was in the order: FIX > FX > FII; kinetic analysis determined K_D values of MEAP with FIX, FX, and FII of 11.3 ± 0.69, 21.4 ± 1.28, and 65.6 ± 1.97 nM, respectively, in the presence of Ca^{2+}. The present data suggests that MEAP has a higher

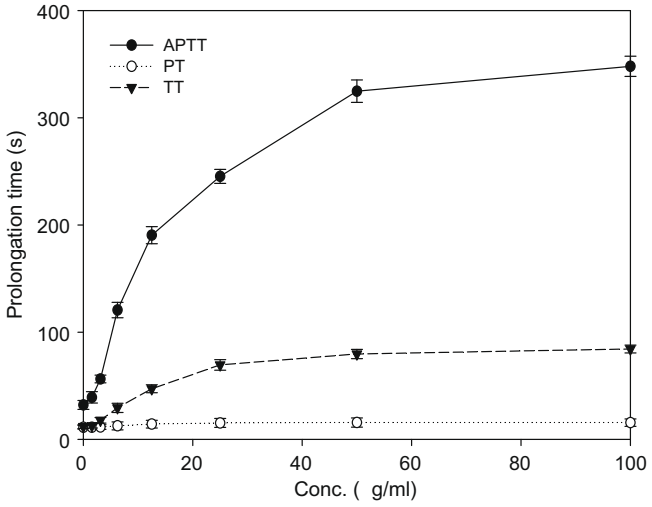

Fig. 2. Dose-dependant prolongation of MEAP. PT, TT, and APTT assays were performed with three individual replicates as described herein.

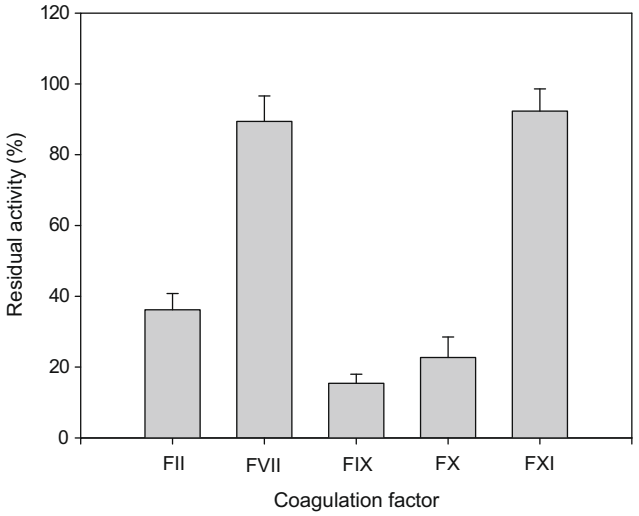

Fig. 3. Specific inhibitory assays for blood coagulation factors. The assays were performed with three individual replicates. Values are expressed as replicate means (±SD expressed as error bars).

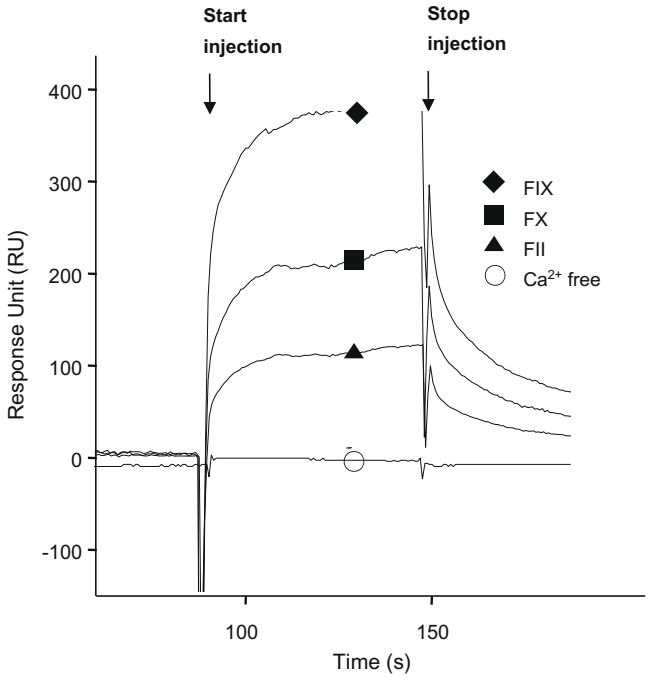

Fig. 4. The binding affinity assays with MEAP. The resonance units (RUs) of SPR real-time sensorgrams were measured to identify molecular interaction between MEAP (ligand) and FII, FIX, FX (analytes), in the presence of Ca^{2+}.

affinity for FIX and FX, which are included in the intrinsic pathway, and could potently prolong APTT as shown in Fig. 2. From the results of the amidolytic assay using fluorogenic substrates, it was shown that MEAP could inhibit the proteolytic conversion of FII by the prothrombinase complex and activation of FX by the intrinsic FXase complex. As shown in Fig. 5, the exogenous anticoagulant peptide (MEAP) inhibited in a dose-dependent manner the amidolytic activation of FX (IC_{50}; 13.6 μg protein ml^{-1}). In the prothrombinase complex, MEAP could inhibit the conversion of FII to FIIa in the prothrombinase complex (IC_{50}; 42.9 μg protein ml^{-1}). The effects of pH, temperature, and cation on MEAP activity were measured using the APTT assay (data not shown). MEAP was stable in the range of pH 6.5 and 8.0, but the activity below pH 6.0 and above pH 9.0 was destroyed. MEAP activity could be maintained at temperatures from 45 to 55 °C. After removing cations by

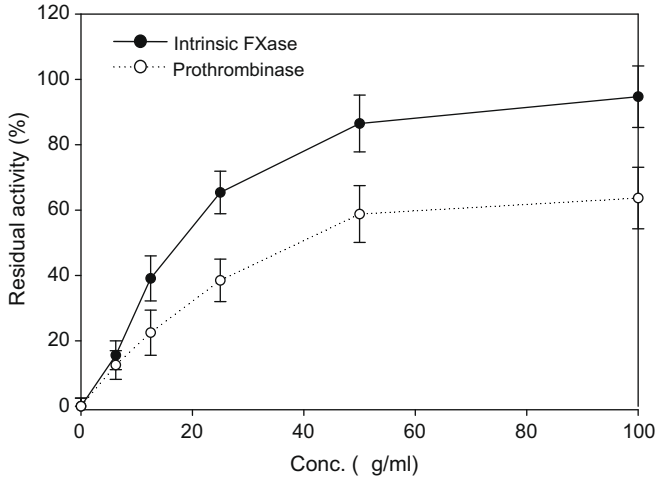

Fig. 5. Inhibitory activity of MEAP against amidolytic functions of the intrinsic FXase and the prothrombinase complex. The amount of FXa and FIIa (thrombin) generated in the blood coagulation complexes were determined immediately with the use of the fluorogenic substrates SN-7, Mes-D-LGR-ANSN(C_2H_5)$_2$, and SN-59, D-VPR-ANSNH-$C_4H_9 \cdot 2HCl$, respectively. The assay was performed with three individual replicates. Values are expressed as replicate means (±SD expressed as error bars).

EDTA-treatment and dialysis, MEAP activity could be recovered by addition of Zn^{2+}, Mg^{2+}, and Mn^{2+}, but not Cu^{2+} and Fe^{2+}. To investigate cytotoxicity of MEAP, the MTT assay was run on a venous endothelial cell line (ECV 304). MEAP-treated cell viability is slightly lower than that of heparin-treated cells; however, the result of the MTT assay showed that MEAP is not significantly cytotoxic ($P < 0.05$) to the venous endothelial cells.

4. Discussion

M. edulis anticoagulant peptide (MEAP) prolonged the normal clotting time to 321 ± 2.1 s on APTT and to 81.3 ± 0.8 s on TT in a dose-dependant manner, respectively. As reported in a previous study (Jung et al., 2001), *Scapharca broughtonii* anticoagulant protein only prolonged the activated partial thromboplastin time (APTT), corresponding to inhibition of the major coagulation factors (FVII, FIX, FX, FIX, PLs, etc.) in the intrinsic blood coagulation pathway. In that study, the addition of 100 μg ml^{-1} of *S. broughtonii* anticoagulant prolonged a 32 s control clotting time to 325 s. According to Davie et al. (1991), prolongation of thrombin time (TT) implies an inhibition of the conversion of prothrombin (FII) to thrombin (FIIa) by negative interactions with the major factors (FII, FV, FX, PLs, etc.) in the blood coagulation pathway. MEAP could specifically inhibit FII, IX and FX, all of which have a gla domain, in a calcium (Ca^{2+}) dependant manner. Among blood coagulation factors, especially, FII, FVII, FIX, and FX with a gla domain play a key role in the Ca^{2+}-mediated formation of phospholipid-factors complexes such as the intrinsic factor tenase, the extrinsic factor tenase, and the prothrombinase complex (Davie et al., 1991). The FII, FIX, and FX are considered as essential components in the intrinsic FXase and prothrombinase complex to complete human blood clotting (Mann, 1976). Specially, FII (prothrombin) with 10 gla residues converts specific glutamic acid residues to gla, which contribute to the ability of FII to bind to the negatively charged PL membranes. FIX has 12 gla residues which also facilitates the calcium dependent binding of FIX to the negatively charged PL surfaces and the epidermal growth factor (EGF) homology domains. The activated FIX contains the catalytic component of the intrinsic FXase complex (FIXa/FVIIIa/Ca^{2+}/PL) which proteolytically activates FX to FXa. Factor X also has 11 gla residues and 2 EGF homology domains. The activation of FX is catalysed by the

intrinsic FXase complex. Factor Xa serves as the enzyme component of the prothrombinase complex (FXa/FVa/Ca^{2+}/PL), which is responsible for the rapid conversion of FII to FIIa (Mann, Jenny, & Krishnaswamy, 1988). As summarised above, the gla residues enable blood coagulation factors with a gla domain to bind PL membranes (i.e., cell surfaces) in a Ca^{2+}-dependent manner for assembly of the intrinsic FXase and prothrombinase complex. It means that exogenous molecules with a high affinity for the Ca^{2+}/PL membrane may interrupt normal activation of blood coagulation factors by the PL complexes. From the NCBInr database search, it was determined that the amino acid sequence of MEAP (-EADI DGDGQVNYEE FV**A**MMTSK-) has a high identity (95%) with that of the EF-hand domain (127-EADI DGDGQVNYEE FV**T**MMTSK-148) of the calcium-binding protein, calmodulin (CaM) from scallop (Patinopecten) adductor muscle on the C-terminal end (Accession No. 0711223A) (Toda et al., 1981). These results showed that the MEAP peptide could be a fragment of the mussel calmodulin.

It is supposed that the presence of MEAP in human plasma might interrupt regular activation of FX in the intrinsic FXase complex (FIXa/VIIIa/PLs) and conversion of FII to FIIa in the prothrombinase complex (FXa/FVa/PLs) by negative interactions with gla domains in FII, FIX, and FX, due to the high affinity of MEAP for the Ca^{2+}/PL membrane. The results of the amidolytic assay showed that MEAP could potently inhibit the activation of FX and FII by prothrombinase and intrinsic FXase complex. These results suggest that the prolongation of blood clotting time in the presence of MEAP may be caused by interrupting the calcium-dependent molecular interactions between major coagulation factors with gla domains and PL membranes in the formation of the intrinsic FXase complex which activates FX and the prothrombinase complex to convert FII to FIIa (Fig. 6). In the previous study (Jung et al., 2001), the blood coagulation assay with human plasma showed that the anticoagulant protein isolated from marine arkshell (*S. broughtonii*) prolonged the activated partial thromboplastin time (APTT) and inhibited factor IX in the intrinsic pathway of the blood coagulation cascade. As reported by Koyama, Noguchi, Aniya, and Sakanashi (1998), plancinin, a potent anticoagulant peptide isolated from starfish (*Acanthaster planci*) could inhibit the prothrombinase complex by the anticoagulant action of placinin on the activation step of FII and FXa. After removing cations by EDTA-treatment and dialysis, MEAP activity could be recovered by addition of Zn^{2+}, Mg^{2+}, and Mn^{2+} in addition to Ca^{2+}. The results of the blood coagulation assay in the presence of various divalent cations showed that MEAP may easily interact with FII, FIX, and FX by stabilisation and coordination of divalent cations; moreover, Zn^{2+}, Mg^{2+}, and Mn^{2+} may play a role as a substitute for Ca^{2+} in the anticoagulant activity of MEAP in the formation of FXase and prothrombinase complex. From the result of the MTT assay, MEAP exhibits low cell cytotoxicity towards the endothelial ECV-304 cells. Most anticoagulants injected into veins encounter endothelial cells. The primary process of the haemostatic mechanism (platelet aggregation) is to be set in motion when the endothelial lining of blood vessels is denuded following vascular injury (Davie et al., 1991).

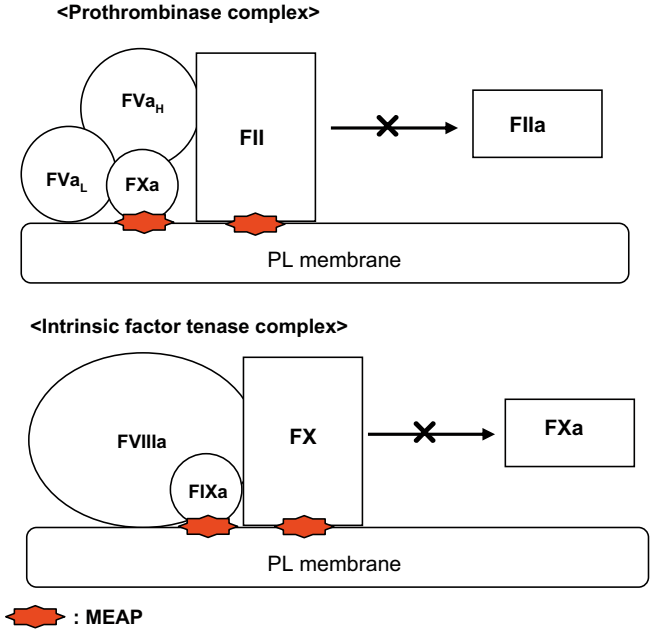

Fig. 6. The anticoagulant mechanism of MEAP in the prothrombin and intrinsic factor tenase complex. MEAP in human plasma might interrupt the conversion of FII to FIIa in the prothrombinase complex (FXa/FVa/PLs) and the regular activation of FX in the intrinsic FXase complex (FIXa/VIIIa/PLs) by negative interactions with gla domains in FII, FIX, and FX, due to the high affinity of MEAP for the Ca^{2+}/PL membrane.

5. Conclusion

This study showed that MEAP can prolong blood clotting time by inhibiting activation of FX in the intrinsic tenase complex (FIXa/VIIIa/PLs) and conversion of FII to FIIa in the prothrombinase complex (FXa/FVa/PLs).

Acknowledgement

This research was supported by a grant from the Marine Bioprocess Research Center of the Marine Bio 21 Center funded by the Ministry of Land, Transport and Maritime, Republic of Korea. This study was also supported by research funds from Chosun University 2008.

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In this paper we report on quantifying EC in commercial brands of pot still cachaças from Paraíba State, Northeastern Brazil; and discuss the results in light of the brands' characteristics and distillation profile.

2. Materials and methods

2.1. Sampling of cachaças and selecting distilleries

Duplicate samplings of 25 brands of cachaças, produced in all 19 approved pot still distilleries in Paraíba, the third major Brazilian State in pot still production of cachaça (Cachaça Official Guide, 2005), were conducted from retail outlets in Paraíba's capital, between September and October 2007. In order to obtain a valid representation of each brand's EC level, samples of different batch codes were purchased. General characteristics of the brands, including the distillation method, were obtained from local inspecting authorities and from label information. Detailed information on distillation was collected in visits to 11 distilleries, which were selected on the basis of their interest in participating in the project. According to label information, the samples contained no added sugar.

2.2. Chemicals

Ethyl carbamate (99.0%), for calibration, and propyl carbamate (98.0%), internal standard, were purchased from Chem Service (West Chester, USA) and Aldrich (Milwaukee, USA), respectively. The analytical solutions were dissolved in LC grade ethanol (Merck, Darmstadt, Germany) at 40% (v/v).

2.3. Analysis of ethyl carbamate

Preparation of calibration curves and EC analysis were based on Andrade-Sobrinho et al. (2002) and Reche et al. (2007), respectively. An Agilent 6873 autosampler was used to introduce, in splitless mode, 2 μl aliquots of each cachaça brand ($n = 2$) onto a capillary column (FFAP, 50 m × 0.2 mm i.d. × 0.3 μm film thickness; HP) installed in an Agilent 6890 gas chromatograph (GC) coupled to an Agilent 5973 mass selective detector (MS). The MS was operated in the electron impact mode with an ionisation energy of 70 eV and helium at 1.5 ml/min was used as carrier gas. The GC oven was initially kept at 90 °C (2 min), followed by an increase to 150 °C (0 min) at 10 °C/min, then up to 230 °C at 40 °C/min. The injector temperature was 250 °C, and the GC/MS interface was maintained at 230 °C. Quantification was performed in terms of single ion monitoring mode for m/z 62 mass fragment and was based on an internal standard procedure. The limits of detection and quantitation were 10 and 40 μg/l of EC, respectively.

2.4. Analysis of alcoholic strength

The alcoholic strenghts (% volume at 20 °C) of the spirits were determined using a Densimat hydrostatic balance coupled to an Alcomat converter (Gibertini Elettronica, Milano, Italy).

3. Results and discussion

3.1. EC levels in commercial brands of pot still cachaças

Table 1 shows the EC concentrations, in increasing order, of 25 brands of pot still cachaças produced in Paraíba State, Brazil. EC was detected in all samples. The brands' EC concentration range and average value were 55–700 μg/l and 221 μg/l, respectively. Ethyl carbamate levels in 70% of the brands exceeded the international limit for spirits (150 μg/l).

The average EC levels found in pot still cachaças from Paraíba State are considerably lower than the mean values reported previously for 34 samples of pot still cachaças from different parts of Brazil (630 μg/l, Andrade-Sobrinho et al., 2002) and for several

Table 1
Ethyl carbamate (EC) levels in brands of pot still cachaças from Paraíba State, Brazil, and corresponding product descriptions, according to visual and label information.

Cachaça brand	EC[a] ± SD[b] (μg/l)	Alcoholic strength (% vol)	Cachaça colour[c]	Distillation[d]	Bottle colour[e]	Distillery
01	55 ± 5	44.3	W	S	A	A
02	55 ± 5	39.9	W	S	C	B
03	60 ± 10	38.0	W	S	C	C
04	75 ± 5	37.2	W	S	C	D
05	90 ± 0	43.5	W	S	C	E
06	100 ± 0	39.0	W	S	C	F
07	125 ± 15	44.4	Y	D	C	F
08	140 ± 30	42.0	Y	S	C	H
09	165 ± 15	42.4	Y	S	C	I
10	165 ± 5	42.0	W	S	A	J
11	180 ± 10	42.0	W	S	A	K
12	195 ± 5	38.3	Y	S	C	L
13	195 ± 25	42.3	Y	S	C	A
14	200 ± 50	43.6	W	S	C	M
15	220 ± 20	41.3	W	S	A	N
16	225 ± 45	39.2	W	S	C	O
17	230 ± 30	38.4	Y	S	C	D
18	235 ± 15	40.4	W	S	A	G
19	240 ± 0	37.9	Y	S	C	P
20	295 ± 15	36.7	Y	S	C	B
21	360 ± 60	38.9	Y	D	C	Q
22	385 ± 85	38.5	Y	S	C	N
23	410 ± 10	40.1	W	D	C	Q
24	420 ± 10	43.2	W	S	C	R
25	700 ± 100	46.9	W	S	A	S
Mean	221					

[a] Ethyl carbamate levels (in increasing order) of cachaça brands are the mean of duplicate sampling of different batch codes.
[b] SD, standard deviation.
[c] Cachaça colour, according to visual inspection, varied from white (colourless, W) to yellowish (pale to dark yellow, Y).
[d] Distillation type (single – S or double – D) of product, according to information obtained from label and local inspecting authorities.
[e] Bottle colour, according to visual inspection, varied from clear (colourless, C) to amber (brown, A).

Table 2
Ethyl carbamate (EC) levels in brands of white single-distiled cachaças and their corresponding distillation profile.

Distillery	Brand[a]	EC[b] (μg/l)	Profile of copper pot still distillation[c]			
			Distillation scale[d]	Heating system[e]	Kettle shape[f]	Cooling system[g] (pot still column)
B	02	55	M	SS	O	TD
C	03	60	S	SS	O	TD
D	04	75	M	SS	O	TD
E	05	90	S	DF	O	HC
F	06	100	S	SS	O	HC
M	14	200	S	DF	C	HH
N	15	220	S	DF	C	HH
O	16	225	S	DF	C	HH
G	18	235	S	SS	O	HH
R	24	430	L	SS	B	HH
S	25	700	M	DF	O	HH

[a] Brands of white single-distiled cachaças, according to Table 1.
[b] Ethyl carbamate levels (in increasing order) of cachaça brands.
[c] Distillation profile was obtained via data collection during visits to distilleries.
[d] Distillation scale, according to total kettle volume of distillery: small (S), 1.000–3.000 l; medium (M), 3.000–9.000 l; large (L), >9.000 l.
[e] Kettle heating system *via* internal steam serpentine (SS) or external fire with direct combustion of dried cane bagasse (DF).
[f] Kettle shape, according to Fig. 1: onion (O), conic (C), and boiler (B).
[g] Cooling system of pot still column (ascending parts), according to Fig. 2: tubular dephlegmator (TD), head cooler (HC), and hot head (HH).

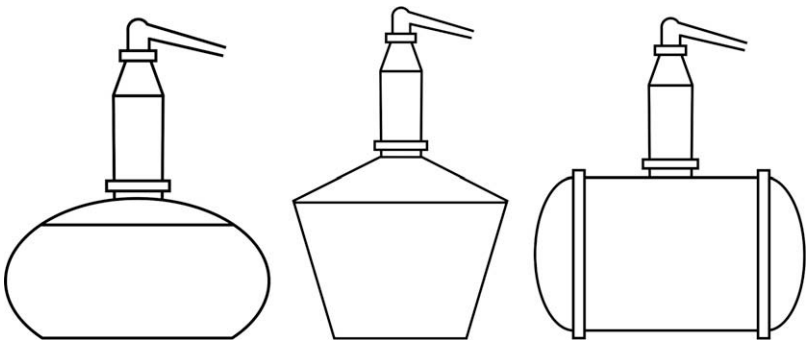

Fig. 1. Schematic cachaça alembics (ascending parts) showing different kettle shapes: onion (left), conic (centre), and boiler (right).

commercial cachaças produced in Minas Gerais State (1205 μg/l, Baffa Júnior et al., 2007; 893 μg/l, Labanca et al., 2008). Although not stated, the samples investigated by Baffa Júnior et al. (2007) and Labanca et al. (2008) are likely to be pot still types, because Minas Gerais State, in Southeastern Brazil, is the national leader in pot still production of cachaça (Cachaça Official Guide, 2005). However, the average level reported here are higher than the values reported in 13 commercial samples of pot still cachaças produced in Rio de Janeiro State (123 μg/l, Bruno, Vaitsman, Kunigami, & Brasil, 2007).

No clear connections between the brands' characteristics (cachaça's colour, distillation [single or double], and bottle colouration) and EC were observed, as shown by these characteristics' random distribution along the EC concentration range (Table 1).

The relationship between the bottle's colour and the EC level was researched because EC can form in spirits not only during distillation, but also during storage, especially by the light-induced formation mechanism from hydrogen cyanide (Mildau, Preuß, Frank, & Heering, 1987). However, this mechanism appears to be negligible in cachaça, because our finding regarding bottle colour is in line with Andrade-Sobrinho et al. (2002), who also found no connections between EC levels and bottle colour in 103 samples of various types of cachaças.

Ethyl carbamate has a low volatility (bp 182–184 °C); and therefore, double distillation has been proposed as a measure to reduce the amount of EC in pot still cachaças, which are typically single-distiled. In fact, an early study by Nagato, Silva, Yonamine, and Penteado (2000) on 13 samples of commercial cachaças reported an average EC level of approximately 317 μg/l; but the authors observed that samples with the lowest concentrations (~38–48 μg/l) were double-distiled. Our study, in which three brands were double-distiled (Table 1), found no such low levels, suggesting that double distillation alone is an insufficient mitigating measure. Other factors might also play important roles in reducing the EC level, such as using low distillation rates and adequately separating the first ("heads") and last ("tails") distiling fractions.

No apparent association was seen between the cachaça's colour (which reflects cask maturation) and their EC levels, as shown by the random distribution of white and yellowish cachaças along the EC concentration range (Table 1). When we look at the white and yellowish single-distiled cachaças produced by distilleries A (brands 01 and 13), B (brands 02 and 20), and D (brands 04 and 17), however, we see that the EC concentration in yellowish cachaças is four times higher (on average) than in the corresponding white cachaças. This effect was less evident for distillery N (brands 15 and 22), which showed a 1.7-fold increase for the yellowish cachaça. Interestingly, no such effect was observed for white and yellowish double-distiled cachaças produced by distilleries F (brands 06 and 07) or Q (brands 21 and 24).

According to information collected from the distilleries in Paraíba State, freshly single-distiled cachaças, with "heads" and "tails" already separated to some extent, are typically matured in large (~20,000–30,000 l) freijó wood (*Cordia goeldiana*) containers or small (~250 l) reconditioned oak casks, if white or yellowish/matured cachaças are desired, respectively. The study observed that the cachaças' maturation time was typically 6–12 months.

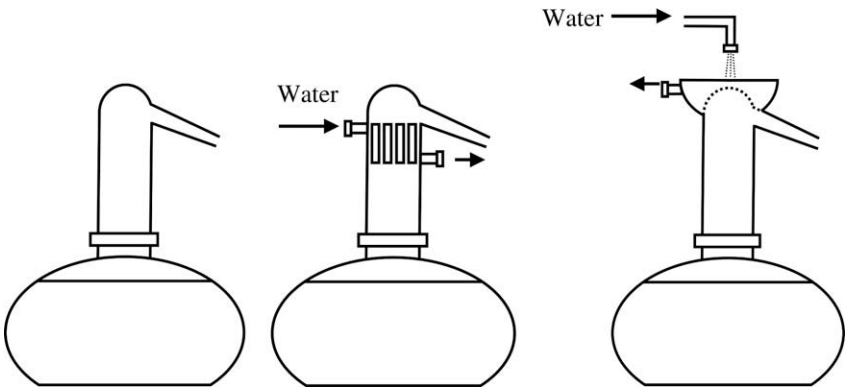

Fig. 2. Schematic cachaça alembics (ascending parts) showing different cooling systems: hot head (left), tubular dephlegmator (centre), and head cooler (right).

According to Aylott et al. (1990), the final amount of EC present in mature grain spirits includes the initial EC measured immediately after distillation, plus that formed from active precursors such as hydrogen cyanide, cyanate, and copper cyanide complexes. Taking into account the pot still cachaças' maturation periods, and the fact that most EC forms ~24–48 h after distillation (Aylott et al., 1990; Riffkin, Wilson, Howie, & Müller, 1989), it appears that factors other than maturation time are playing important roles to cause the high EC levels in yellowish single-distiled cachaças (Table 1). The relatively similar EC levels in white and yellowish double-distiled cachaças (Table 1) can be explained by the depletion of EC precursors between the first and second distillations (prior to maturation), combined with inadequate second distillation procedures (e.g., high distillation rates and poor separation of "heads" and "tails").

3.2. Distillation profile of distilleries and EC levels

Table 2 shows the EC levels, in increasing order, of 11 brands of pot still cachaças and the distillation profiles obtained during visits to corresponding distilleries. To maintain homogeneity in the comparisons, the distillation profiles are associated with only one type of product, white, single-distiled cachaças.

No clear connections were observed between EC levels and distillation scale, heating system, or kettle shape (Fig. 1), as shown by these characteristics' random distribution along the EC concentration range (Table 2).

With regard to the cooling system, however, a clear concentration of hot head systems (Fig. 2) in the most heavily contaminated range (200–700 μg/l) was observed. Contrarily, a clear prevalence of dephlegmator and head cooler systems, particularly the former, in the less contaminated range (55–100 μg/l) was observed (Table 2). These observations are in line with those of Bruno et al. (2007), who also found a close connection between low levels of EC in cachaças and distillation in alembics using high reflux rates.

With respect to brand 25, reasons were sought in distillery S for its exceedingly high EC level (Table 2). Contrary to the other distilleries visited, distillery S did not separate the "heads" at all. Interestingly, brand 25 has the highest ethanol concentration (Table 1); thus strengthening the information collected at distillery S. This observation is in line with results of Andrade-Sobrinho et al. (2009), who found higher EC levels in "heads" than in other distiling fractions of pot still cachaças. Although Andrade-Sobrinho et al. (2009) explained their finding solely on the basis of EC's solubility (higher in ethanol than in water), the fact that "heads" contain the abundance of cyanides (Christoph, Schmitt, & Hildenbrand, 1987; Guerain & Leblond, 1992), which may react with ethanol forming EC, is a more plausible explanation than solubility. The exceedingly high EC levels observed in brand 25 might thus be explained by the combined effects of full incorporation of "heads" into the final product with the use of a "hot head" alembic.

4. Conclusions

This work has shown that most brands of pot still cachaças produced in Paraíba State, Brazil, do not comply with the maximum permissible EC level for spirits (150 μg/l) tolerated in several countries, including Brazil. No clear connection between the characteristics of cachaça brands (colour, distillation [single or double], and bottle colour) and their corresponding EC levels was observed. However, focusing on the white and yellowish single-distiled cachaças from the same distilleries provided some evidence suggesting that the yellowish cachaças tend to be more contaminated than the white. An on-site investigation of 11 pot still distilleries shows that the brands with low (55–100 μg/l) and high (200–700 μg/l) contamination levels in white single-distiled cachaças are associated with pot stills equipped with cooled and non-cooled columns, respectively. A study larger than the present one is currently in progress to strengthen these observations.

Acknowledgments

The authors thank the Brazilian Government through BNB/FUNDECI (Project No. 2004-1-276; Agreement No. 103/2006) for financial support.

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are unrecognizable, being of great utility in the questions previously described.

In this field, the DNA analyses are the best option, since provide more information than protein due to the degeneracy of the genetic code and the presence of many non-coding regions (Lockley & Bardsley, 2000). By other way, mitochondrial DNA has several advantages over nuclear DNA, including its high mutation rate, multi-copy nature and maternal inheritance (Mackie et al., 1999). Particularly, the mitochondrial *cytochrome b* gene has been widely used by many authors to carry out the genetic identification of very different taxa (Infante et al., 2006; Perez & Garcia-Vazquez, 2004; Santaclara, Cabado, & Vieites, 2006; Santaclara, Espiñeira, & Vieites, 2007). The main advantage of this gene is that accepts mutations quickly enough, so that closely related organisms can show nucleotide differences, but not so rapidly that the variation within a species is significant (Bartlett & Davidson, 1992). In this manner, even closely related species as tuna and bonito or frigate tuna can be identified (Bartlett & Davidson, 1991; Infante, Catanese, Ponce, & Manchado, 2004; Pardo & Perez-Villareal, 2004; Ram, Ram, & Baidoun, 1996; Terol, Mascarell, Fernandez-Pedrosa, & Perez-Alonso, 2002; Unseld, Beyermann, Brandt, & Hiesel, 1995). The methodological approaches used to date are varied, amongst them are the *PCR–RFLP* (Polymerase Chain Reaction–Restriction Fragment Length Polymorphism) (Aranishi, 2005; Quinteiro et al., 1998), the phylogenetic analysis (Terol et al., 2002), Taqman RT–PCR technology (Lopez & Pardo, 2005) and the Multiplex primer-extension PCR assay (Lin & Hwang, 2008). Many of these works only take into account one or few genera of the family Scombridae and do not study all species included in each genus, so that, incorrect identifications can take place.

In the present work, a method of authentication of scombroid products based on DNA amplification by PCR and phylogenetic analysis of DNA sequences was designed. This methodology allows discriminating successfully scombroid species of important commercial value using the mitochondrial *cytochrome b* as molecular marker.

2. Materials and methods

2.1. Sample collection, storage and DNA extraction

Samples of different scombroid species were collected from several locations around the world (Table 1). The individuals were

Table 1
Samples included in this work and location of collection. Locations abbreviations: AR, Argentina; AU, Australia; BR, Brazil; BS, Bahamas; CN, China; CU, Cuba; CV, Cape Verde; EC, Ecuador; ES, Spain; GR, Greece; IL, Israel; IN, India; IT, Italy; JP, Japan; KR, Korea; MA, Morocco; MX, Mexico; NZ, New Zealand; PA, Panama; PE, Peru; PH, Philippines; PT, Portugal; SC, Seychelles; SN, Senegal; UK, United Kingdom; US, United States; ZA, South Africa.

Genus	Species	Common name[a]	Sample location	Samples[b]	Seq.[c]	Accession number[d]
Allothunnus	*A. fallai*	Slender tuna	ZA, AU, PE	3	3	EU935743–EU935745
Auxis	*A. rochei*	Bullet tuna	CN, PE, MA, US, BR	9	10	EU349320–EU349329
	A. thazard	Frigate tuna	PH, AU, EC, CN, PA, SC	7	9	EU349330–EU349338
	E. affinis	Easter little tuna	CN	1	4	EU349371–EU349374
Euthynnus	*E. alletteratus*	Little tunny	Unknown	1	3	EU349375–EU349377
	E. lineatus	Black skipjack	MX	1	3	EU349378–EU349380
Katsuwonus	*K. pelamis*	Skipjack tuna	ES, UK		3	EU349416–EU349418
Rastrelliger	*R. kanagurta*	Indian mackerel	JP	1	3	EU349419–EU349421
	S. chiliensis	Pacific bonito			3	EU349339–EU349341
Sarda	*S. orientalis*	Striped bonito	MX		3	EU349345–EU349347
	S. sarda	Atlantic bonito	IT, US, GR		3	EU349342–EU349344
	S. australasicus	Spotted mackarel	MX, AU	2	3	EU349317–EU349319
Scomber	*S. colias*	Atlantic chub mackerel	ES, MX, US, IL, ZA, AR, PT	11	14	EU349291–EU349304
	S. japonicus	Pacific mackerel	KR, US, MX, EC	6	8	EU349309–EU349316
	S. scombrus	Atlantic mackerel	ES, UK	4	4	EU349305–EU349308
	S. brasiliensis	Serra spanish mackerel	UK, CU		3	EU349348–EU349350
	S. cavalla	King mackerel	US, unknown	2	3	EU349351–EU349353
Scomberomorus	*S. commerson*	Narrow barred mackerel	AU, US	2	5	EU349354–EU349358
	S. maculatus	Atlantic spanish mackerel	US	2	4	EU349359–EU349362
	S. niphonius	Japanese spanish mackerel	JP	3	5	EU349363–EU349367
	S. regalis	Cero	Bahamas	1	3	EU349368–EU349370
	S. tritor	West African mackerel	*NCBI*			AF231666
Thunnus	*T. alalunga*	Albacore	IN, ES, IT, US, EC	5	10	EU349381–EU349385 EU935746–EU935750
	T. albacares	Yellowfin tuna	IN, ES, GR, IL, US	5	10	EU349386–EU349388 EU935751–EU935757
	T. atlanticus	Blackfin tuna	US, BR, EC	5	10	EU349389–EU349393 EU935758–EU935762
	T. maccoyii	Southern bluefin tuna	AU, NZ	5	10	EU349394–EU349399 EU935763–EU935766
	T. obesus	Bigey e tuna	ES, PT, MX, US, EC	5	10	EU349400–EU349402 EU935767–EU935773
	T. orientalis	Pacificbluefin tuna	JP, CN, AU, US	5	10	EU349403–EU349407 EU935774–EU935778
	T. thynnus	Bluefin tuna	ES, IT, GR	5	10	EU349408–EU349411 EU935779–EU935784
	T. tonggol	Longtail tuna	JP, AU	5	10	EU349412–EU349415 EU935785–EU935790
Decapterus	*D. macarellus*	Mackerel scad	SN	1	3	EU349422–EU349424
	D. maruadsi	Japanese scad	*NCBI*			EF512291

[a] Only it is shown one of the possible common names for each species.
[b] Each sample included between 3 and 10 individuals.
[c] Number of DNA sequences obtained in this work.
[d] Accession numbers to sequences deposited in the *GenBank* database.

identified attending to morphological characteristics (Collette & Nauen, 1983; Collette, Reeb, & Block, 2001).

DNA was extracted from 30 mg of muscle in fresh and frozen samples, according to the method described by Roger and Bendich with slight modifications (Rogers & Bendich, 1985). In processed products, DNA was extracted from 150 mg in processed products, using the *NucleoSpin Tissue kit* (*Macherey-Nagel*). The purity and concentration of the extracted DNA was measured using a UV–vis spectrophotometer (*Biophotometer*, *Eppendorf*). Scombroid samples and DNA extractions were appropriately labelled and stored at −80 °C for subsequent studies.

2.2. PCR amplification and DNA sequencing

A fragment of the *cytochrome b* gene was amplified by PCR using the forward primer L14735 (Burgener, 1997) and the reverse BRmod (primer BR described by Terol (Terol et al., 2002) slightly modified). This primer set amplified the herein called *Fragment B*569.

In all cases, PCR reactions were carried out in a total volume of 50 μL with the following composition: 100–300 ng of DNA template were added to PCR mix consisting of 0.8 mM of dNTP mix (*Bioline*), 5 μL of 10X buffer, 2 mM of $MgCl_2$, 0.75 unit of BioTaq™ DNA pol (*Bioline*), 0.8 μM of each primer (*Sigma Genosys*) and molecular biology grade water (*Eppendorf*) to adjust to the final volume.

PCR were carried out in a *MyCycler*™ thermocycler (*BIO-RAD*). Conditions for the primer set L14735/BRmod were as follows: a preheating step at 95 °C for 3 min, 35 cycles of amplification (95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s) and a final extension step of 72 °C for 3 min.

PCR amplicons were visualised on 2% agarose gels (*Sigma*) in 1X TBE buffer (*Sigma*) with 0.3 μg/mL of ethidium bromide (*Sigma*). DNA fragments were visualised using the *Molecular Imager Gel Doc XR System* transilluminator and the software *Quantity One® v* 4.5.2 (*BIO-RAD*). The *pGEM* (*Promega*) and 50 *Base Pair Ladder* (*GE Healthcare*) DNA markers were used to estimate the size of the amplicons.

The PCR products were purified using the *Nucleospin Extract II kit* (*Macherey-Nagel*) according to the manufacturer's instructions. The concentration and purity were estimated by means of a UV–vis spectrophotometer *Biophotometer* (*Eppendorf*). Subsequently were sequenced on an *ABI Prism* 310 *Genetic Analyzer* (*Applied Biosystems*) using the primers of the PCR amplification and the *BigDye Terminator Cycle Sequencing Ready Reaction Kit v* 1.1 (*Applied Biosystems*) following the supplier's recommendations.

The electropherograms obtained were analysed using the *DNA Sequencing Analysis Software v* 3.4 (*Applied Biosystems*) and corrected at hand with *Chromas v* 1.45 (Mc Carthy, 1996). The nucleotide sequences obtained were aligned with *BioEdit v* 7.0 software (Hall, 1999) and used for estimating intraspecific and interspecific distances with *DnaSP v* 4.0 (Rozas, Sanchez-DelBarrio, Messeguer, & Rozas, 2003).

2.3. Primers design for species identification in canned products

From the sequences of the *fragment B569* obtained two new internal primers were designed: BD1-R, BD2-D. These primers were used together BD described by Terol et al. (2002) and BRmod. The PCR products obtained from BD/BD1-R and BD2-D/BRmod primer sets were herein called *Fragment* 1 and *Fragment* 2, respectively. These fragments are overlapped, generating the *Fragment* B239 (Table 2 and Fig. 1).

Table 2
Primers used in this work.

Name	Sequence 5′–3′	Described
L14735	AAA AAC CAC CGT TGT TAT TCA ACT A	Burgener (1997)
BRmod	AAT CGG GTG AGG GTG GCR TTG T	In this work
BD	GGC CGA GGC CTT TAC TAC GGC	Terol et al. (2002)
BD2-D	ACG TMC TTC CCT GAG GRC A	In this work
BD1-R	RGT AAT GAC GGT AGC TCC T	In this work

The PCR amplifications of the *Fragment* 1, *Fragment* 2 and *B*239 were carried out with the following conditions: a preheating step of 94 °C for 5 min was followed by 35 cycles of amplification (94 °C for 20 s 52 °C for 20 s and 72 °C for 20 s) and a final extension step of 7 min at 72 °C.

Next, PCR products obtained were visualised, cleaned, and sequenced as reported previously.

2.4. Development of FINS methodology using the cytochrome b gene

The sequences herein obtained and those ones downloaded from *GenBank* database were used to carry out the phylogenetic analyses. The following DNA sequences belonging to scombroid species were downloaded from the *NCBI* database: NC005313, AB105165 (*A. rochei*), NC005318, AB105447 (*A. thazard*), AB098092 (*E. affinis*), AB099716 (*E. alleteratus*), NC005316 (*K. pelamis*), DQ497857–DQ497859 (*R. kanagurta*), AB098098, EF141178, DQ497888, DQ497890 (*S. orientalis*), EF439573, EF427593, EF392613 (*S. sarda*), AB032519 (*S. australasicus*), AB032517 (*S. japonicus*), NC006398 (*S. scombrus*), AB032518, AB098098, EF141178, NC008109 (*S. cavalla*), DQ497885–DQ497887 (*S. niphonius*), AF231666 (*S. tritor*), AB098103 (*T. albacares*), AB098106 (*T. maccoyii*), AB098110 (*T. obesus*), AB185022 (*T. orientalis*), X81563 (*T. thynnus*), AB098115, AF239964 (*T. tonggol*), EF512291 (*D. maruadsi*).

The phylogenetic analyses were carried out with *Mega 3.0* (Kumar, Tamura, & Nei, 2004) using the Tamura–Nei model (Tamura & Nei, 1993) to calculate the genetic distances between sequences. The inference of the phylogenetic tree was carried out with the Neighbor-Joining method (Saitou & Nei, 1987). The three species of the genus *Decapterus* were used as outgroup. The reliability of the groups was evaluated by means of bootstrap test with 2000 replications.

2.5. Methodological validation

Individuals of the different species were authenticated on the basis of their morphological traits. These reference individuals were used to manufacture different products (salted, smoked and canned) in the pilot plant of CECOPESCA (*Spanish National Centre of Fish Processing Technology*). The most extreme treatment applied

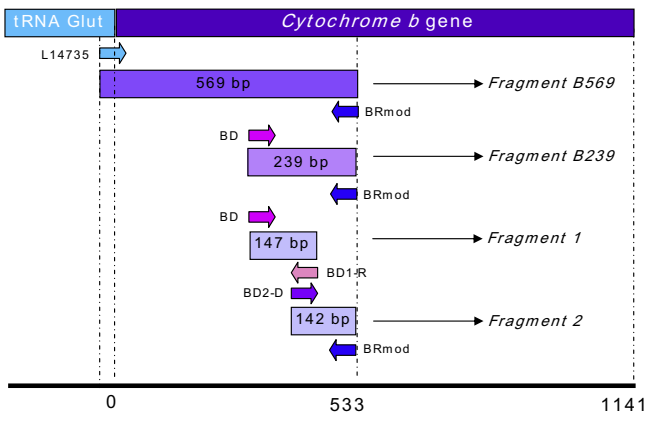

Fig. 1. Location and size of the DNA fragments of *cytochrome b* amplified in this work and position of primer sets used.

to the samples was the sterilization in a horizontal retort steel–air, at 115 °C for 50 min, with 1.2 bars of overpressure (cans of 125 mL).

Next the samples were analysed with the methods developed in the present work. Results of the species assignment on the basis of morphology and genetic probes were compared. The coincidence percentage between the species identified on the basis of morphological traits and the genetic methodology developed was calculated to establish the specificity of the method.

2.6. Application to commercial samples

Once the methods were validated, these ones were applied to 20 cans labelled as tuna, bonito, mackerel and frigate tuna. These products were purchased in supermarkets and shops from European countries, amongst them Spain, Germany, Finland, Portugal and Poland, with the aim of evaluating their correct labelling.

3. Results and discussion

3.1. PCR amplification and DNA sequencing

A high source of genetic markers which can be used for species identification has been created associated to the development of molecular techniques (Taggart, Hynes, Prodohl, & Ferguson, 1992; Young, Wheeler, Coryell, Keim, & Thorgaard, 1998). Specifically, in the present study the mitochondrial *cytochrome b* gene was used as target to develop a DNA-based method for the identification of Scombridae species.

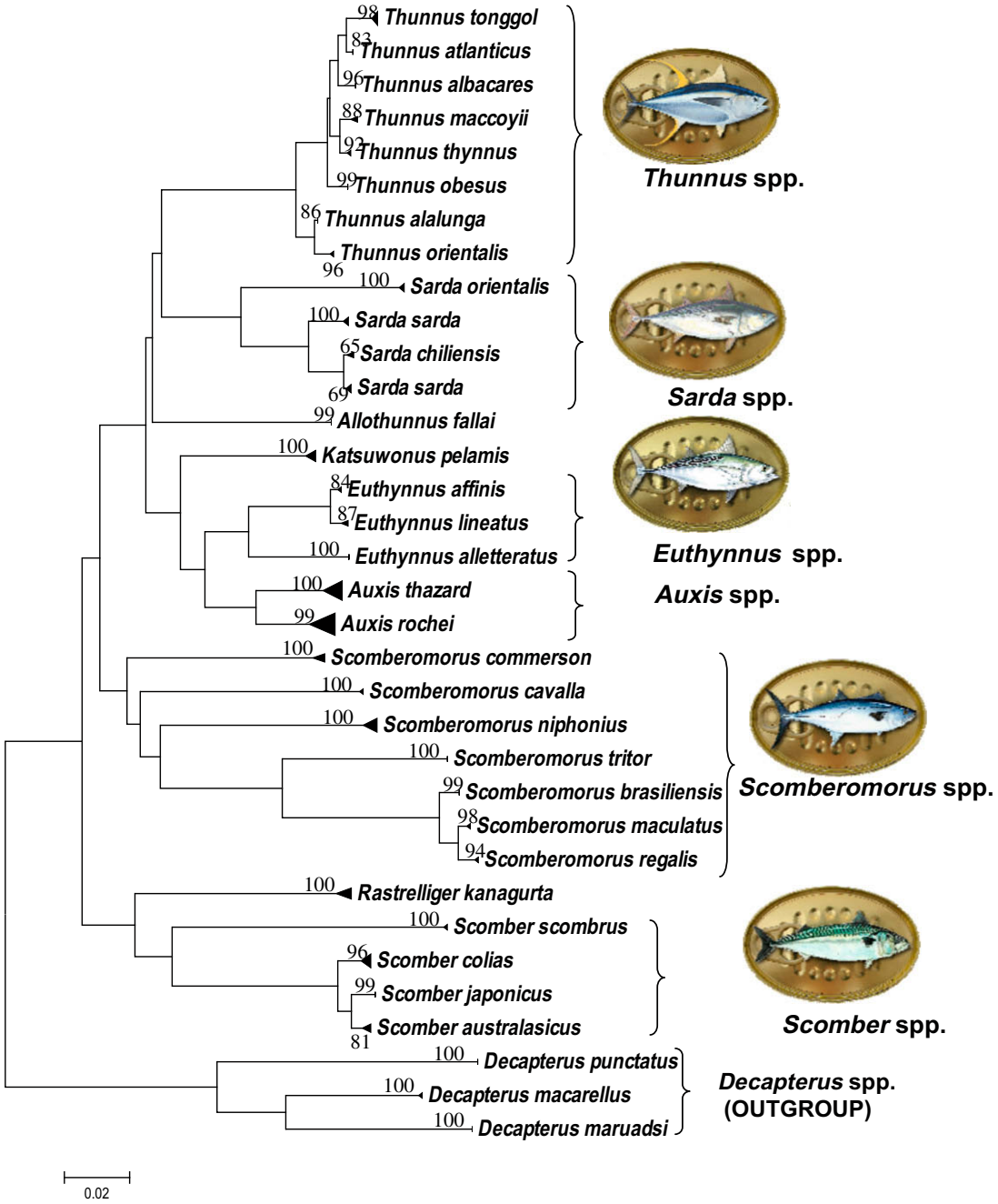

Fig. 2. Neighbor-Joining tree of genetic relationships amongst studied species, carried out from the alignment of 522 bp sequences.

The sequences of *Fragment B*569 were obtained from different species of *Thunnus*, *Scomber*, *Auxis*, *Sarda*, *Scomberomorus*, *Euthynnus*, *Allothunnus*, *Katsuwonus*, *Rastrelliger* and *Decapterus* genera (Accession numbers in Table 1). In all the species analysed the length of this PCR product was 569 bp, except for *Rastrelliger kanagurta* that was 570 bp. This PCR product is located from position 37 of the glutamic acid tRNA to position 533 of the *cytochrome b* (Fig. 1). The additional base in *R. kanagurta* is situated on tRNA of the glutamic acid, so that, there is not reading frame shift of the *cytochrome b* protein.

3.2. Development of the genetic method: PCR product amplification

In order to allow the authentication of scombroid species in canned and other seafood products, several methodological strategies were developed. In fresh or frozen fish, it is possible to amplify the *Fragment B*569. Subsequently, in order to carry out the genetic identification easily. In the case of fishes undergoing different treatments, it is not possible to amplify the mentioned PCR product because the thermal treatment generates DNA fragmentation. For this reason, in products only subjected to moderate temperature in the processing (such as smoked or salted fish), the *Fragment B*239 was amplified (Fig. 1).

In the case of cans, fragments of little size were amplified because the thermal treatment and pressure applied generated high levels of DNA fragmentation. Quinteiro et al. (1998) established a maximum fragment size of 176 bp to ensure the amplification in canned products. Pardo & Perez-Villareal, 2004 under certain conditions, amplified fragments higher than 200 bp from canned products. However, we consider that it is important to have at one's disposal a method that can be used routinely, allowing the amplification of DNA easily and that provides a reliable species identification. Because of this, PCR amplifications of *Fragment* 1 and *Fragment* 2 were carried out. The length of these amplicons was 147 and 142 bp, respectively. These fragments are overlapped and generate the *Fragment B*239 (Fig. 1).

3.3. Phylogenetic analysis: FINS methodology

The *FINS* technique was evaluated because it has been extensively used in the past for the genetic identification of fish and shellfish as for instance cephalopods (Santaclara et al., 2007), bivalves (Espiñeira, González-Lavín, Vieites, & Santaclara, 2009; Santaclara et al., 2006), flatfishes (Espiñeira, González-Lavín, Vieites, & Santaclara, 2008a), anglerfishes (Espiñeira, González-Lavín, Vieites, & Santaclara, 2008b) or anchovies (Santaclara, Cabado, & Vieites, 2006). The main advantage of this technique is that uses the information of all nucleotide positions of the amplified DNA, compared to *RFLP* or other techniques which only assess a low number of nucleotides. However, the *FINS* technique allows the genetic identification of species by means of phylogenetic analyses using a DNA sequence database. Like this, reference sequences belonging to morphologically identified individuals and the sequence of an unknown individual are compared. From this analysis, a distance matrix is constructed and this allows the assignment of the unknown species to a group according to the genetic distance. The genetic distances between the obtained *cytochrome b* gene sequences reveal that the intraspecific distance mean was 0.03 ± 0.00 and interspecific ones were one order of magnitude higher than the intraspecific ones (the mean value for these ones was 0.141 ± 0.012). The mean interspecific distance between species of the same genus was 0.099 ± 0.009.

From the distance matrix, the phylogenetic tree was constructed allowing the genetic identification of species because samples belonging to the same species were grouped into the same clade. The bootstrap method can be used to obtain the support of the different groups obtained in the phylogenetic tree. It has been calculated that bootstrap values higher or equal to 70% usually correspond to a probability higher or equal to 95% that the corresponding cluster is real (Hillis & Bull, 1993), giving a quantitative measurement of the certainty of the assignment of a sample to a particular species. The phylogenetic tree constructed from 522 bp sequences (*Fragment B*569 without primers) shows that all the sequences belonging to individuals of the same species are grouped in the same cluster (Fig. 2). All clusters are strongly supported, with bootstrap values higher than 80% (except for *Sarda chiliensis*), allowing the reliable assignation of each individual to a particular species. Also, bootstrap values higher than 80% were obtained in the phylogenetic reconstruction carried out with the 196 bp fragment (*Fragment B*239 without primers) (Fig. 3), showing that *Fragments B*569 and *B*239 allows the reliable assignment of an unknown sample to its species.

Therefore, the proposed strategies of amplification allow the amplification of a DNA fragment sufficiently long to discriminate successfully all the scombroid species of commercial importance, even in canned products and others formats in which the DNA is highly degraded.

3.4. Inclusion of Decapterus species in the methodology

The species of *Decapterus* genus were included in this work because canned products elaborated with the species *Decapterus*

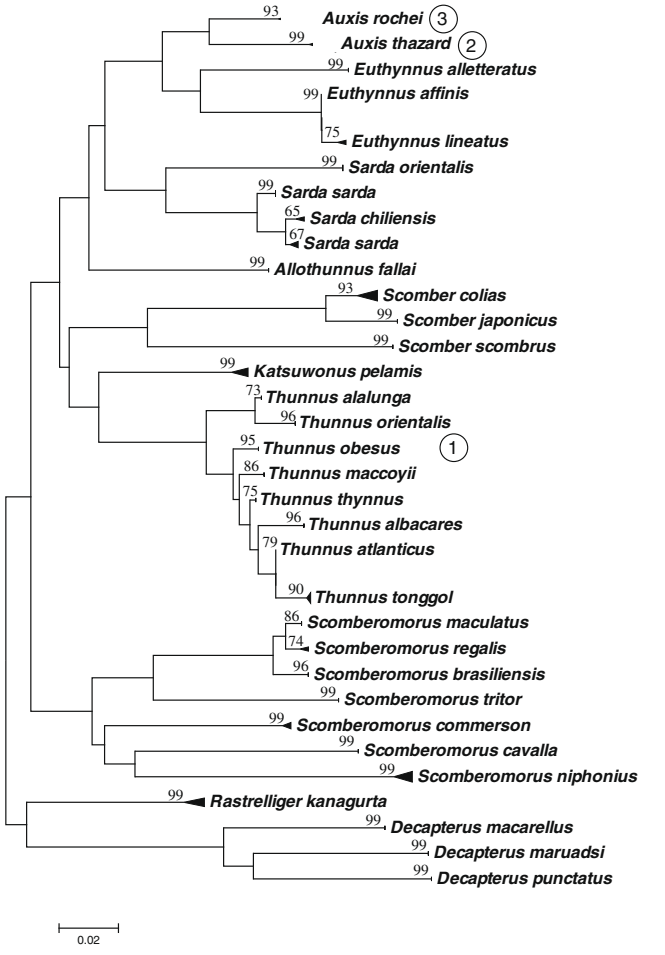

Fig. 3. Neighbor-Joining tree of genetic relationships amongst studied species, carried out from the alignment of 196 bp sequences. Circled numbers belong to the commercial samples analysed where a mislabelling was detected.

macarellus are labelled using the commercial denomination "Mackerel scad". This fact can produce confusion with several scombroid species, which include "Mackerel" in their denomination. Moreover, the organoleptic properties of some canned *Decapterus* species are very similar to the *Scomber* ones. The phylogenetic analysis shows that all the *Decapterus* species form a taxon clearly differentiated from the family Scombridae. Therefore, the three *Decapterus* species included in this study can be identified with total reliability with the proposed methodology.

3.5. Considerations derived from the phylogenetic analysis

The phylogenetic analysis clarified and confirmed some questions about several taxonomic groups. First of all, the results observed in the phylogenetic reconstruction performed in this work are in accordance with the recognition by fish taxonomy of the species *Scomber colias*, distributed in the Atlantic Ocean as a separate species from *S. japonicus* that inhabits the Pacific Ocean (Fig. 2). These species are included in two separated clades strongly supported. Infante, Blanco, Zuasti, Crespo, and Manchado (2007) carried out a phylogenetic differentiation between *S. colias* and *S. japonicus* based on nuclear 5S rDNA sequences. The results of this work are in agreement with the ones obtained in the present study based on mitochondrial sequences.

Also, it is worth mentioning that there are two groups for the species *Sarda sarda*. There are several genetic studies of *S. sarda* that suggest the existence of two subpopulations in the Mediterranean Sea (Pujolar, Roldán, & Pla, 2001; Viñas, Alvarado Bremer, & Pla, 2004). This is in accordance with the results obtained in the phylogenetic reconstruction made in the present work.

Regarding the clade of *Thunnus* genus, although the internal structure was weak, the bootstrap values for each species were higher than 83%, allowing the assignment of an unknown sample to a species belonging to this genus. Moreover, a high number of individuals belonging to all the geographical distribution range for each species were considered to carry out the phylogenetic reconstruction.

3.6. Methodological validation

The results of the genetic analyses of the samples manufactured in the pilot plant of CECOPESCA were in accordance with the expected based on the morphology characterisation. Therefore, the developed method showed a specificity of 100%. Also it was checked whether the canning process or any other kind of processing, such as smoking salting, or canning do not affect to the correct operation of the developed methods.

3.7. Application to commercial samples

The aim of this study was to assess the fulfilment of labelling requirements for the commercial canned samples. The *FINS* method herein developed was applied to all commercial canned samples analysed. Three analysed samples contained a different species from those indicated in the label, meaning 15% of the samples were incorrectly labelled.

Except for one sample of tuna, and two samples of bullet and frigate tuna, the labelling and the species identified by the proposed technique were in agreement. The exceptions were canned products labelled as *T. albacares*, *A. rochei* and *A. thazard* that were respectively identified as *T. obesus*, *A. thazard* and *A. rochei*.

In conclusion, the technique described in this paper is a useful tool to authenticate scombroid species in all kind of products, from fresh fish to canned products. It can be useful to verify the correct labelling of fishing products elaborated with these species. Also, this methodology could be useful to check the import and export of these species and therefore their traceability or origin and to control the catch of protected species, for example the "bluefin tuna" *Thunnus thynnus*, which is nowadays overexploited.

Moreover, the analysis can be carried out in less than two work days, and it allows a high throughput screening of all kind of samples. Also, different methodological strategies are proposed, allowing analysis of fresh, frozen or processed products with total reliability.

Amongst the advantages of this method it is worth mentioning its rapidness and reliability to authenticate processed commercial products. Also, it includes 33 species and all of which are used as substitutes of others of higher value in processed food, meaning this is the most complete method developed up to now. Moreover, it can be applied to any product, independently of the degree and type of processing to which it has been subjected (fresh, frozen, salted, smoked, and canned, amongst other products).

Acknowledgements

We thank Jorge L. García, Fátima C. Lago and Arrate Aldasoro for their helful assistance. We also thank Douglas H. Adams (Florida Marine Research Institute, USA); Stefania Ancora (Università de Siena, Italy); H. Banford (University of West Georgia, USA); Jeffrey Buchanan (Lincoln Marine Science Centre, Australia); K. Carpenter (Old Dominion University, USA); Naritoshi Cho (National Research Institute of Fisheries Science, Japan); B. Collette (Virginia Institute of Marine Science, USA); Elaine Espino (Programa Pesca Ribereña–Instituto Nacional de la Pesca, Mexico); Javier Fuertes (Comercial Coper, S.L., Spain); Jose Antonio González (Instituto Canario de Ciencias Marinas, Spain); John Graves (Virginia Institute of Marine Science, USA); Alistair Hobday (CSIRO, Australia); Keitaro Kato (Fisheries Laboratory of Kinki University, Japan); Masaaki Kodama (School of Fisheries Sciences, Kitasato University, Japan); Masashi Maita (Tokyo University of Marine Science and Technology, Japan); Elba Mora (Instituto Nacional de Pesca del Ecuador, Ecuador); Melissa Paine (Virginia Institute of Marine Science, USA); David Richardson (University of Miami, USA); Enrique Rodriguez-Marin (Instituto Español de Oceanografía, Spain); Daniel R. Scoles (Virginia Institute of Marine Science, USA); Chugey Sepúlveda (Pileger Institute of Environmental Research, USA); J.M. de la Serna (Instituto Español de Oceanografía, Málaga); Raewyn Street (University of Queensland, Australia); Hiroshi Yokoyama (University of Tokyo, Japan); H.J. Walker (University of California, USA); Wan-Duen Chiou (National Kaohsiung Marine University, Taiwan); Bob Ward (CSIRO, Australia), Chris Whipps (Oregon State University, USA); and Nikolaos Zampoukas (National and Kapodistrian University of Athens, Greece) for providing some of the samples included in this work.

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et al., 2009; Spanjer, Rensen, & Scholten, 2008; Sulyok, Berthiller, Krska, & Schuhmacher, 2006), are more frequently applied. Most of these chromatographic methods are based on the determination of a single mycotoxin or multiple mycotoxins belonging to the same group (Berthiller et al., 2005; Cervino et al., 2008; D'Arco et al., 2008; Faberi et al., 2005; Silva et al., 2009), whereas the number of methods for the simultaneous determination of more than one group of mycotoxin is still limited (Cavalieri et al., 2005; Lattanzio et al., 2007; Sulyok et al., 2006). In general, these methods require considerable analysis time (higher than 20 min) (Cavalieri et al., 2005; Lattanzio et al. 2007; Spanjer et al., 2008; Tanaka et al., 2006), and in some cases, two injections are required (Sulyok et al., 2006), as well as the application of a clean-up step (Cavalieri et al., 2005; Lattanzio et al., 2007; Tanaka et al., 2006). On the other hand, mycotoxins are compounds with different physicochemical properties, and multimycotoxin determination requires the development of appropriate optimisation methods for the simultaneous extractions of these compounds.

Ultra-high performance liquid chromatography (UHPLC) is a relatively new technique that shows improved sensitivity, resolution and speed compared to LC (Pastor-Montoro, Romero-González, Garrido-Frenich, Hernández-Torres, & Martínez-Vidal, 2007). These advantages result from the use of columns filled with sub-2 μm particle size and instruments with high pressure fluidic modules. There are few published studies in which mycotoxins have been analysed in food using UHPLC (Mol et al., 2008; Ren et al., 2007). For instance, the second method requires a clean-up step, and two consecutive chromatographic runs of 7.5 min each.

The aim of this study was to develop and validate a simple and rapid UHPLC–MS/MS multimycotoxin method for the simultaneous determination of aflatoxins B1, B2, G1, G2, M1, fumonisins B1 and B2, ochratoxin A, deoxynivalenol, HT-2 and T-2 toxin and zearalenone in maize, walnuts, biscuits and breakfast cereals at trace levels. The method is based on a single extraction step using acetonitrile/water mixture (80/20 v/v), without the need of any clean-up procedure. The chromatographic analysis time was reduced to 8.5 min, which meets the requirement for a high throughput determination; in addition the method shows better detection limits than previously published methods for the target compounds (Cavalieri et al., 2005; Lattanzio et al., 2007; Sulyok et al., 2006; Tanaka et al., 2006).

2. Materials and methods

2.1. Reagents and chemicals

Ochratoxin A, deoxynivalenol, T-2 toxin and stock solution of aflatoxin M1 (in acetonitrile) were obtained from Biopure (Tulln, Austria). Aflatoxins B1, B2, G1, G2, zearalenone and stock standard solution of fumonisin B1 and HT-2 toxin (in acetonitrile) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Stock solution of fumonisin B2 (in acetonitrile) was supplied by Fluka (Steinheim, Germany). First, stock standard solutions (200 mg/L) were prepared by exact weighing of those mycotoxins obtained in powder and dissolved in 50 mL of HPLC-grade acetonitrile (Sigma). Then, a multicompound working solution (2 mg/L except for aflatoxin M1 which was 0.2 mg/L) was prepared by combining suitable aliquots of each individual standard stock solution and diluting them with appropriate amounts of acetonitrile. These solutions were kept at 4 °C and renewed weekly.

Acetic acid (purity > 97%), formic acid (purity > 98%), ammonium formate and ammonium acetate were obtained from Panreac (Barcelona, Spain). HPLC-grade methanol was supplied by Sigma. Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA).

2.2. Equipment

Chromatographic analyses were performed in an ACQUITY UPLC™ system (Waters, Milford, MA, USA), using an Acquity UPLC BEH C_{18} column (100 mm × 2.1 mm), with 1.7 μm particle size, from Waters. MS/MS detection was performed using an Acquity TQD tandem quadrupole mass spectrometer (Waters, Manchester, UK). The instrument was operated using an electrospray (ESI) source in positive mode. Data acquisition was performed using MassLynx 4.0 software with QuanLynx software (Waters). Centrifugations were performed in a high-volume centrifuge from Centronic (Barcelona, Spain). A Vortex mixer Heidolph, model Reax 2000 and an analytical AB204-S balance (Mettler Toledo, Greinfesee, Switzerland) were also used. A Braun MX 32 kitchen blender (Barcelona, Spain) was used to process samples. A Reax-2 rotary agitator from Heidolph (Schwabach, Germany) was used for sample extraction.

2.3. UHPLC–MS/MS analysis

Chromatographic analyses were carried out using a gradient elution with eluent A being methanol and eluent B consisting of an aqueous solution of ammonium formate (5 mM). The analysis started with 25% of eluent A, which was increased linearly up to 75% in 3 min, and then, to 100% in 2 min. This composition was held for further 1.5 min before being returned to 25% of eluent A in 1 min, followed by a re-equilibration time of 1 min, to give a total run time of 8.5 min. The flow rate was set at 0. 35 mL/min and column temperature was maintained at 30 °C. Aliquots of 5 μL of sample extract were injected into the chromatographic system.

For MS/MS detection, the ionisation source parameters were: capillary voltage 3.5 kV, extractor voltage 3 V, source temperature 120 °C, desolvation temperature 350 °C, cone gas flow 50 L/h and desolvation gas flow 650 L/h (both gases were nitrogen). Collision-induced dissociation was performed using argon as collision gas at a pressure of 4×10^{-3} mbar in the collision cell. The multiple reaction monitoring (MRM) transitions and the applied cone voltages and collision energies are summarised in Table 1.

2.4. Sample preparation

Mycotoxins were extracted from samples using a solid–liquid extraction procedure. Briefly the procedure was as follows: 5 g of ground samples (maize, walnut, biscuit and breakfast cereal) were weighed in a polypropylene centrifuge tube (40 mL) and 10 mL of an acetonitrile/water solution (80/20, v/v) was added and the mixture was vortexed for 2 min. However for biscuit samples, 20 mL of the mixture acetonitrile/water were needed. After that, the tube was put into a rack in the rotary agitator for 10 min at 60 rpm. After centrifugation at 4500*g* for 5 min, 2 mL of the supernatant layer were taken and filtered through a Millex-GN nylon filter (0.20 μm, Millipore, Carrightwohill, Ireland), before the sample was injected into the UHPLC–MS/MS system.

2.5. Samples

The following food items were collected: three samples of maize and walnuts were obtained from farms located in the Southeast of Spain. Five breakfast cereals and five different brands of biscuits were purchased from local supermarkets (Almeria, Spain) and were analysed before the expiration date. The maize, walnut and the content of each package were grounded before mycotoxin extraction. All samples were analysed following the procedure described above and those samples showing the absence of the target compounds were used as blank samples in the preparation of standards and recovery studies.

Table 1
Retention time windows (RTWs) and UHPLC–MS/MS parameters.

Compound	RTW (min)	Channel	Dwell time (ms)	Cone voltage (V)	Quantitation transition[a]	Confirmation transition[a]
Deoxynivalenol	1.41–1.59	1	0.200	25	297.4 > 249.4 (10)	297.4 > 231.3 (15)
Aflatoxin G2	2.61–2.75	2	0.015	60	331.4 > 313.5 (25)	331.4 > 245.3 (30)
Aflatoxin M1	2.72–2.94	2	0.015	50	329.4 > 273.4 (20)	329.4 > 259.3 (25)
Aflatoxin G1	2.77–2.91	2	0.015	45	329.2 > 243.1 (25)	329.2 > 311.4 (25)
Aflatoxin B2	2.93–3.07	2	0.015	50	315.2 > 259.2 (30)	315.2 > 243.3 (35)
Aflatoxin B1	3.07–3.21	2	0.015	30	313.3 > 285.5 (25)	313.3 > 241.3 (30)
Fumonisin B1	3.43–3.59	3	0.025	45	723.1 > 334.7 (40)	723.1 > 352.8 (35)
Ochratoxin A	3.59–3.71	3	0.025	25	404.2 > 239.2 (20)	404.2 > 358.2 (15)
HT-2 Toxin	3.67–3.77	4	0.015	25	442.6 > 263.4 (15)	442.6 > 215.3 (15)
T-2 Toxin	3.89–4.05	4	0.015	25	484.7 > 215.3 (20)	484.7 > 245.4 (15)
Fumonisin B2	4.01–4.12	4	0.015	55	707.1 > 336.7 (30)	707.1 > 354.7 (30)
Zearalenone	4.14–4.32	4	0.015	30	319.5 > 301.6 (10)	319.5 > 283.6 (12)

[a] Collision energies (eV) are given in brackets.

3. Results and discussion

The aim of this work was to develop a simple and fast method for high throughput determination of mycotoxins in cereals and related foods by UHPLC–MS/MS.

3.1. Optimisation of the UHPLC–MS/MS determination

First, the experiments to select the optimum MS conditions and the appropriate ions were performed by column injection of individual standards at 500 μg/L. ESI positive and negative mode were evaluated, observing that whereas all toxins were detectable in the positive mode, not all of them were efficiently ionised in the negative mode, and ESI in positive mode was selected for all the mycotoxins. Full-scan mass spectra and product ion scan were acquired in order to obtain at least one precursor and two product ions for each compound for both identification and quantification purposes, selecting the most abundant product ion for quantification and the second one for confirmation. Table 1 shows the MS/MS transitions as well as the cone voltages and collision energies optimised for each compound. In all the cases, $[M+H]^+$was selected as parent compound, except for T-2 and HT-2 toxins, which formed the ammonium adduct $[M+NH_4]^+$. For ochratoxin A, the most intensive product ion was at *m/z* 358 which correspond with the loss of water and carbon monoxide whereas the other ion selected corresponded with the loss of phenylalanine at *m/z* 239 (Sforza, Dall'Asta, & Marchelli, 2006). On the other hand, non-specific transitions such as loss of water were avoided although for some compounds such as aflatoxin G2 and G1 was selected due to its higher sensitivity.

Finally, it must be indicated that although some published papers use ESI in negative mode to determine some mycotoxins such as deoxynivalenol and zearalenone (Sorensen & Elbaek, 2005) good sensitivity was obtained when ESI in positive mode was applied.

Secondly, the influence of the several variables involved in the chromatographic process was evaluated in order to reduce analysis time, increase sensitivity and provide good peak shape. Due to the chemical diversity of the analysed toxins, a suitable composition of the mobile phase must be selected. The organic solvent used in the mobile phase was evaluated, observing that methanol provided better sensitivities than acetonitrile for all analytes, especially for trichothecenes such as deoxynivalenol, T-2 and HT-2 toxins.

Ammonium acetate or formate can be added to avoid the formation of stable sodium adducts, mainly for trichothecenes and aflatoxins. On the other hand, fumonisins have four carboxylic groups in the molecular structure and acidic chromatographic conditions can be used in order to obtain good peak shape (Sulyok et al., 2006). Formic acid (0.1%), ammonium acetate (5 mM) and ammonium formate (5 mM) were added to the mobile phase and better results were obtained for aflatoxins, deoxynivalenol, T-2 and HT-2 toxin when ammonium formate was used, whereas for fumonisin B1 and B2, slightly better results were obtained when formic acid was used. Furthermore, better responses were always obtained when formate was selected instead of acetate. Based on these data, 5 mM of formate ammonium was used in the mobile phase, bearing in mind that is still acidic for the suitable separation of fumonisins.

Several initial percentages of the mobile phase were studied, ranging from 5% to 40% of methanol, observing that good peak shape and high sensitivity were achieved with the increase of methanol content in the initial composition, and analysis time can decreased. However, it can be noted that when the gradient started at 40% of methanol, bad peak shapes were obtained for fumonisin B1. Considering the combined factors between separation efficiency and sensitivity, 25% of methanol was selected as the initial composition of the mobile phase.

Other parameters such as flow rate, injection volume, and column temperature were optimised selecting as optimum conditions: 0.35 mL/min as flow rate, 30 °C as column temperature and 5 μL as injection volume.

With the optimised conditions, the analysis time was lower than 9 min, including cleaning and re-equilibration steps, which allows high sample throughput, and retention time ranges from 1.5 (deoxynivalenol) to 4.3 (zearalenone). The analytes were distributed in four overlapping functions, using a maximum of five mycotoxins (10 transitions) per function. Different dwell times were evaluated (0.005–0.200 s) to find the best detection parameters, observing that better results were obtained when 0.015 s was selected except for function 1 and 3 (Table 1), where 0.200 and 0.025 s were used as the dwell time, respectively, due to the different compounds analysed simultaneously in the selected overlapping functions. Using the selected dwell time, a sufficient number of data points per peak can be acquired in order to have enough sensitivity and allow reproducible integration for quantitative results.

Furthermore, the use of UHPLC–MS/MS combines the separation capability of UHPLC and the selective detection power of MS/MS, which facilitates the identification of unresolved peaks even if coeluted interfering peaks are present. For instance, aflatoxin G1 and M1 coeluted and they have the same parent ion (*m/z* 329). Bearing in mind that retention time and precursor ion were very similar for both analytes, the product ions were selected taking into account that they must be different in order to avoid this interference. In this sense, both aflatoxins produce an intensive product ion at *m/z* 229, and this can be rejected because both compounds can interfere each other during their determination, so specific transitions for each aflatoxin were selected to monitor both compounds (see Table 1).

3.2. Optimisation of the extraction procedure

In multiresidue mycotoxin methods, the critical step is the extraction and clean-up procedure. To reduce sample handling and increase throughput, a simple solid–liquid extraction was used before chromatographic determination. For the optimisation, maize was used as representative matrix.

First, the optimum extraction solvent was evaluated, checking different solvents such as methanol, acetonitrile, a mixture of acetonitrile/methanol (50/50, v/v), water and several ratios of methanol and acetonitrile aqueous solution, obtaining better results when acetonitrile was used. Only, T-2 and HT-2 toxins shown slightly better responses when methanol was used. Most of the published paper used a mixture of acetonitrile/water (Zöllner & Mayer-Helm, 2006), and different ratios of acetonitrile/water was checked. For all the mycotoxins, the highest peak areas were obtained when a acetonitrile/water composition of 80/20 (v/v) was used as extracting solvent, except for fumonisin B1 and B2 that slightly better signals were achieved when the ratio was 60/40 (acetonitrile/water), as it was indicated by Sulyok et al. (2006). Furthermore, it can be indicated that when the percentage of water increased, the signal decreased considerably.

Finally, the extraction time was evaluated, testing times from 0 to 60 min. It can be highlighted that mycotoxins can be extracted just with vortex and no further agitation steps are needed except for T-2 and HT-2 toxins. For these compounds, peak area increased with extraction time up to 10 min, and then kept constant, so 10 min was selected as extraction time.

The proposed extraction method was evaluated in other type of matrices such as walnuts, breakfast cereals and maize based food such as some biscuits. It can be highlighted that mycotoxins can be extracted using the proposed method in these matrices, but fumonisin B1 and B2 were not extracted from walnuts and biscuits, as it was observed by other authors in other matrices, (Spanjer et al., 2008; Sulyok et al., 2006), indicating that the method should be modified to extract these critical compounds. Furthermore, for the extraction of mycotoxins from biscuits, 20 mL of extractant agent must be used instead of 10 mL, in order to obtain a suitable volume to be injected into the chromatographic system and reproducible results, because when 10 mL were used a doughy mixture was obtained, and irreproducible results were achieved.

Fig. 1 shows a typical chromatogram of a blank maize sample spiked with 50 μg/kg of the target mycotoxins (5 μg/kg for aflatoxin M1). It can be observed that the optimised extraction pro-

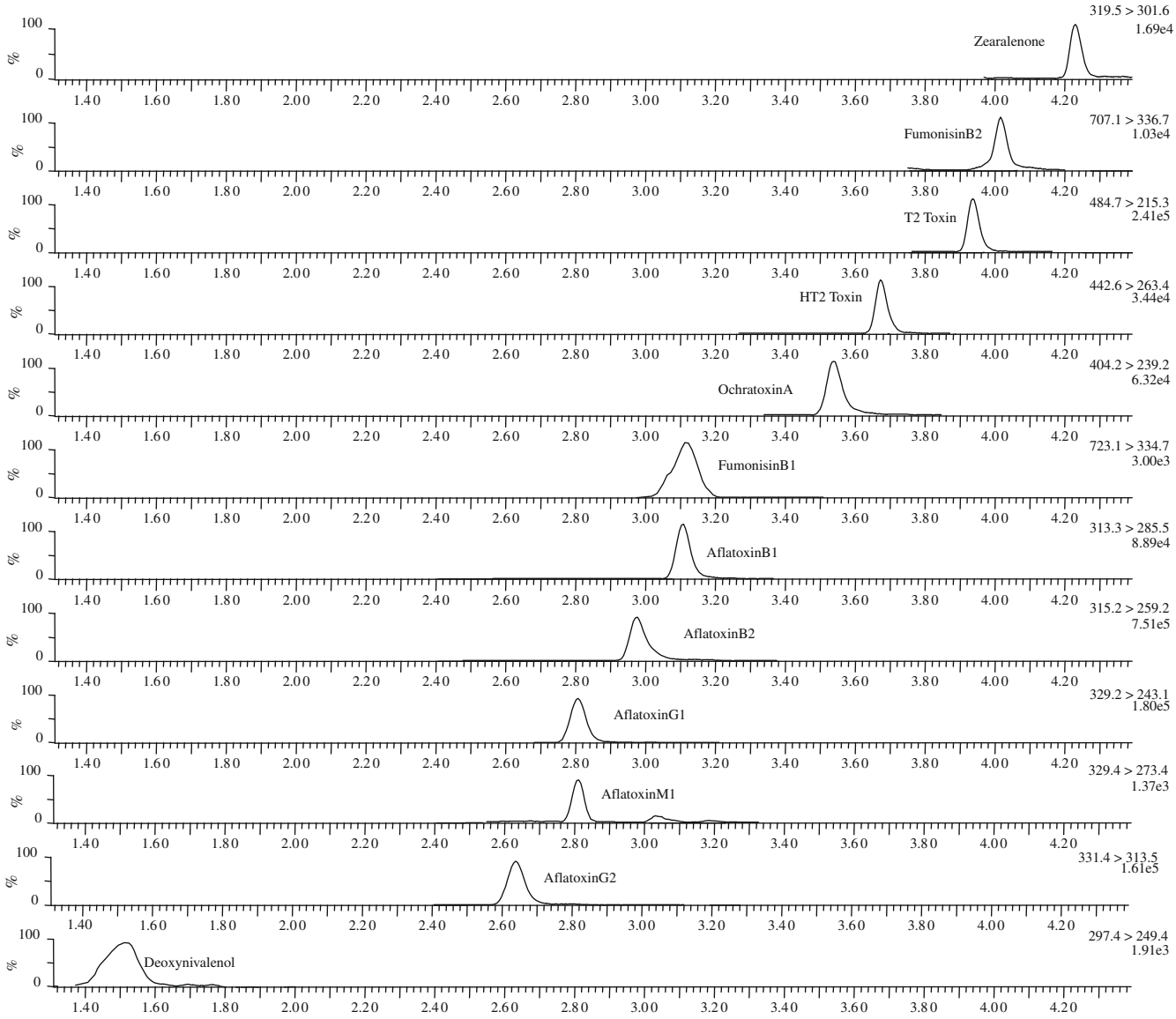

Fig. 1. UHPLC–MS/MS chromatograms obtained from a blank maize sample spiked at 25 μg/kg (2.5 μg/kg for aflatoxin M1).

cedure coupled to MS/MS provides a clean chromatogram without interferences, so further clean-up procedures were avoided in order to increase sample throughput, when the proposed extraction method is combined with UHPLC separation. Furthermore, it can be observed that complete resolution was not obtained but MS/MS detection allows the selective analysis of all the compounds.

3.3. Validation of the proposed method

Method validation was performed in terms of selectivity, linearity, accuracy, repeatability, interday precision, limits of detection (LODs) and quantification (LOQs).

One of the main problems of LC–MS/MS is that that the presence of matrix components can affect the ionisation of the target compounds, reducing or enhancing the response compared with standards in solvents, and the influence of the matrix effect on the response must be studied. In this work, several matrices were selected for evaluation of matrix effect, including maize, walnuts, breakfast cereals and biscuits. Several concentrations (from 5 to 100 μg/kg, except for aflatoxin M1 which was 10× lower) were analysed in pure solvent and in blank samples, and the slopes of the calibration curves were compared by an analysis of covariance (García-Campaña, Cuadros-Rodríguez, Aybar-Muñoz, & Alés-Barrero, 1997). Table 2 shows the obtained results. It can be noted that except for ochratoxin A and HT-2 toxin, the slopes are statistically different (*p*-value was lower than 5% for all the mycotoxins), indicating that a matrix effect was detected and this effect must be corrected in order to obtain reliable results. It can be observed an extensive matrix effect mainly due to the solvent:sample ratio, which were lower than other ratios published by Sulyok et al. (2006) and Spanjer et al. (2008), which used a ratio of 8:1, and minor matrix effects were obtained.

Because the diversity of the matrices and compounds evaluated, a representative matrix can not be used for this type of matrices, because depending on the matrix and the mycotoxin the matrix effect is different. For instance, for aflatoxin G2, the same matrix matched calibration can be used if maize and walnuts are analysed, whereas different calibrations must be prepared if zearalenone is determined in these matrices. For routine analysis, if several matrices must be evaluated, one matrix-matched calibration should be prepared for each matrix in order to obtain accurate results. Bearing in mind the short extraction time and chromatographic analysis, the preparation and injection of several matrix-matched calibrations does not increase the analysis time per batch considerably.

Although representative matrices can be selected in order to correct matrix effect, it could be detected some differences in the matrix effect within a given matrix (Sulyok, Krska, & Schuhmacher, 2007) and other approaches can be applied such as the use of isotopically labelled internal standard. However standard addition methodology can be used when mycotoxin concentrations were close to the limits indicated in international regulations in order to assure the reliability of the obtained results.

The linearity of the method was tested by spiking blank extract samples at five concentration levels between 1 and 100 μg/kg (except for aflatoxin M1 which was 10× lower). Peak area was selected as peak response and good linearity was found for all the mycotoxins within the tested interval, with determination coefficients higher than 0.98.

The recovery of the extraction step of each mycotoxin at two fortification levels (5 and 50 μg/kg, and 0.5 and 10 μg/kg for aflatoxin M1) was studied, showing the obtained results in Table 3. It can be noted that recovery of the extraction step ranged from 70.0% to 108.4% that fulfils the requirements established by European Union (Commission Regulation 401/2006, 2006). Furthermore, recoveries do not depend on the matrix evaluated and no significant differences were observed.

Repeatability (intraday precision) was evaluated at the two concentration levels of the recovery studies, performing six replicates for each level (Table 3), whereas interday precision was studied analysing one spiked sample at 10 μg/kg (1 μg/kg for aflatoxin M1) during six consecutive days (Table 4). For all the cases, repeatability was lower than 20%, except for fumonisin B1 and B2, which show some values slightly higher than 20%. In relation to interday precision, RSDs values lower than 20% were obtained for all the mxycotoxins and matrices evaluated, except for aflatoxin M1 and zearalenone in biscuit and fumonisin B2 in breakfast cereal, with RSDs values of 21.9, 21.8 and 24.5, respectively.

LODs and LOQs were calculated analysing blank spiked samples, and they were determined as the lowest concentration of the analytes that produce chromatographic peak at S/N of 3 and 10, respectively (Table 4). For instance, the lowest LODs and LOQs were obtained for aflatoxins (0.01 and 0.03 μg/kg for aflatoxin M1 in maize), whereas the highest values were achieved for deoxynivalenol and zearalenone, which LOQs values ranging from 3.70–5.90 and 5.10–6.30 μg/kg, respectively. In all the cases, LOQs were always lower than the maximum residue limits established by European Union (Commission Regulation 2006/1881/EC, 2006), indicating the suitability of the proposed method for the determination of trace concentration of these compounds, as well as they are lower than other obtained by related methods (Spanjer et al., 2008), which can be mainly attributed to the reduction of chromatographic dispersion when UHPLC is used, that provokes an increase in the analytical sensitivity.

Identification of the mycotoxins was carried out by searching in the appropriate retention time windows (RTWs), defined as the retention time ± three standard deviations of the retention time

Table 2
Evaluation of matrix effect, using maize, walnut, biscuits and breakfast cereal matrix-matched calibration and solvent-based standards.

Mycotoxin	Solvent	Maize	Walnut	Biscuit	Breakfast cereal	*p* (%)[a]
Deoxynivalenol	11.0	1.6	1.6	3.2	1.5	0.01
Aflatoxin G2	170.1	102.6	106.9	154.7	170	0.5
Aflatoxin M1	37.9	28.4	32.3	43.9	43.0	1.1
Aflatoxin G1	134.8	62.8	103.9	118.3	126.8	0.1
Aflatoxin B2	378.9	59.6	209.6	251.5	333.3	0.1
Aflatoxin B1	329.4	159.2	250.4	287.5	301.6	0.4
Fumonisin B1	19.2	9.3	16.5	12.6	13.4	4.2
Ochratoxin A	45.0	37.4	61.2	41.1	39.9	6.6
HT-2 toxin	21.7	19.4	17.3	19.2	19.9	28.9
T-2 toxin	103.3	100.8	116.3	133.5	146.4	2.2
Fumonisin B2	11.6	18.1	10.1	3.6	13.7	0.2
Zearalenone	12.2	10.9	19.1	17.7	17.6	1.2

[a] *p*-Value (in %) obtained when the selected matrices were evaluated.

Table 3
Recovery values (%) and relative standard deviations (%) given in brackets[a].

Mycotoxin	Maize		Walnut		Biscuit		Breakfast cereal	
	5 μg/kg	50 μg/kg	5 μg/kg	50 μg/kg	5 μg/kg	50 μg/kg	5 μg/kg	50 μg/kg
Deoxynivalenol	74.8 (17.4)	90.2 (4.0)	88.9 (18.0)	92.0 (4.0)	98.5 (9.2)	77.5 (5.8)	77.4 (19.6)	82.6 (11.3)
Aflatoxin G2	80.4 (10.7)	72.5 (3.6)	103.1 (7.8)	85.4 (4.4)	74.2 (14.0)	92.5 (8.1)	73.2 (8.5)	80.2 (5.6)
Aflatoxin M1[b]	73.2 (10.5)	74.0 (10.8)	71.3 (20.0)	79.0 (14.1)	87.2 (11.4)	71.3 (8.6)	104.7 (19.7)	79.6 (14.6)
Aflatoxin G1	77.1 (11.9)	79.6 (5.0)	80.6 (4.6)	74.1 (1.7)	108.4 (8.8)	71.9 (3.9)	81.9 (12.3)	89.9 (9.7)
Aflatoxin B2	90.2 (6.2)	80.5 (2.3)	73.7 (10.9)	77.9 (7.6)	100.5 (6.7)	85.0 (4.5)	87.1 (5.3)	84.3 (5.1)
Aflatoxin B1	85.7 (6.3)	87.8 (2.9)	73.2 (13.4)	75.4 (3.2)	102.0 (20.0)	104.8 (13.4)	93.9 (18.9)	73.8 (5.7)
Fumonisin B1	83.6 (12.0)	73.7 (8.8)	NE[c]	NE	NE	NE	70.2 (20.3)	74.5 (14.3)
Ochratoxin A	83.4 (15.2)	85.1 (4.7)	76.6 (15.3)	82.3 (14.0)	102.4 (12.8)	93.4 (3.8)	98.7 (11.6)	103.5 (4.3)
HT-2 toxin	71.1 (4.4)	90.5 (2.1)	73.0 (20.0)	74.9 (15.3)	105.4 (13.7)	97.7 (3.1)	102.3 (15.3)	70.0 (13.2)
T-2 toxin	75.5 (3.8)	92.1 (1.9)	96.7 (13.6)	75.5 (7.1)	82.4 (11.4)	95.6 (6.7)	86.9 (10.6)	100.5 (6.1)
Fumonisin B2	75.1 (12.5)	87.2 (9.2)	NE	NE	NE	NE	72.0 (20.4)	73.7 (17.3)
Zearalenone	91.2 (15.1)	78.9 (5.4)	77.9 (11.5)	102.9 (8.9)	100.8 (13.4)	74.6 (4.8)	89.4 (13.4)	74.3 (9.6)

[a] Number of replicates: 6.
[b] 0.5 and 5 μg/kg for aflatoxin M1.
[c] Not extracted.

Table 4
Interday precision, (n = 6 days) at 10 μg/kg[a] and LODs and LOQs (μg/kg) obtained for the four matrices evaluated.

Mycotoxin	Maize			Walnut			Biscuit			Breakfast cereal		
	RSD[b]	LOD	LOQ	RSD	LOD	LOQ	RSD	LOD	LOQ	RSD	LOD	LOQ
Deoxynivalenol	10.0	1.10	3.70	7.6	1.30	4.30	9.2	2.00	5.90	9.2	1.60	4.80
Aflatoxin G2	11.3	0.20	0.80	4.4	0.90	2.90	8.3	0.40	1.30	3.9	0.40	1.40
Aflatoxin M1	15.0	0.01	0.03	12.4	0.07	0.23	21.9	0.10	0.30	13.5	0.10	0.30
Aflatoxin G1	11.9	0.20	0.50	7.3	0.30	1.00	8.0	0.20	0.50	12.3	1.10	3.50
Aflatoxin B2	5.8	0.10	0.30	10.9	0.60	2.10	5.6	0.10	0.30	5.8	0.40	1.50
Aflatoxin B1	12.0	0.02	0.10	15.9	0.30	0.90	7.5	0.05	0.17	5.9	0.03	0.10
Fumonisin B1	13.1	0.10	0.50	NE[c]	NE	NE	NE	NE	NE	16.9	2.10	6.20
Ochratoxin A	9.0	0.30	0.90	15.3	0.40	1.40	6.6	1.30	4.30	12.1	0.60	1.90
HT-2 toxin	15.5	1.00	3.30	16.2	0.40	1.50	10.9	0.30	0.80	15.5	0.50	1.60
T-2 toxin	4.8	0.10	0.50	7.9	0.10	0.30	13.5	0.40	1.30	6.1	0.60	2.00
Fumonisin B2	9.2	0.20	0.60	NE	NE	NE	NE	NE	NE	24.5	0.70	2.50
Zearalenone	18.9	1.50	5.10	15.4	1.70	5.70	21.8	2.00	6.00	14.8	2.10	6.30

[a] 1 μg/kg for aflatoxin M1.
[b] Intermediate precision expressed as relative standard deviation (RSD).
[c] Not extracted.

Table 5
Comparison of the matrix effects and detection limits obtained in the proposed method and bibliographic data.

Compound	Proposed method		Spanjer method[a]		Sulyok method[b]	
	Matrix effect (%)	LOD (μg/kg)	Matrix effect (%)	LOD (μg/kg)	Matrix effect (%)	LOD (μg/kg)
Deoxynivalenol	−85	1.10	15	50	8	16
Aflatoxin G2	−39	0.20	15	1.0	−38	4
Aflatoxin M1	−25	0.01	NS	NS[c]	NS	NS
Aflatoxin G1	−53	0.20	−1	2.0	−44	4
Aflatoxin B2	−84	0.10	21	1.0	−52	4
Aflatoxin B1	−52	0.02	17	0.5	−82	80
Fumonisin B1	−52	0.10	−17	100	1	16
Ochratoxin A	−17	0.30	11	1.0	0	3.5
HT-2 toxin	−11	1.00	20	25	−26	16
T-2 toxin	−2	0.10	13	25	−8	4
Fumonisin B2	36	0.20	−2	100	4	8
Zearalenone	−11	1.50	12	10	8	0.5

[a] Data obtained from Spanjer et al., 2008.
[b] Data obtained from Sulyok et al., 2006.
[c] Not studied.

of 10 blank maize samples spiked at 25 μg/kg for each mycotoxin, except for aflatoxin M1 which was 2.5 μg/kg (Table 1), and confirmation was carried out by comparison of the signal intensity ratios of the two transitions (quantification and confirmation), with those obtained using fortified blank samples. Furthermore, the selectivity of the proposed method was evaluated by the analysis of blank samples. The absence of any chromatographic signal at the same retention time as the target compounds indicated the absence of chemical or matrix interferences.

In order to show the feasibility of the proposed method, matrix effects and LODs obtained with the proposed method were compared with those published in bibliography (Table 5). It can be ob-

served that despite of higher matrix effects were obtained when the proposed method is used, LODs were considerably lower than previous results, indicating that the sensitivity of the presented method has been improved at the cost of increased matrix effect, due to the solvent:sample ratio used. The observed matrix effects can be compensated by the use of matrix-matched calibration, standard addition methodology or by the use of isotopically labelled internal standards.

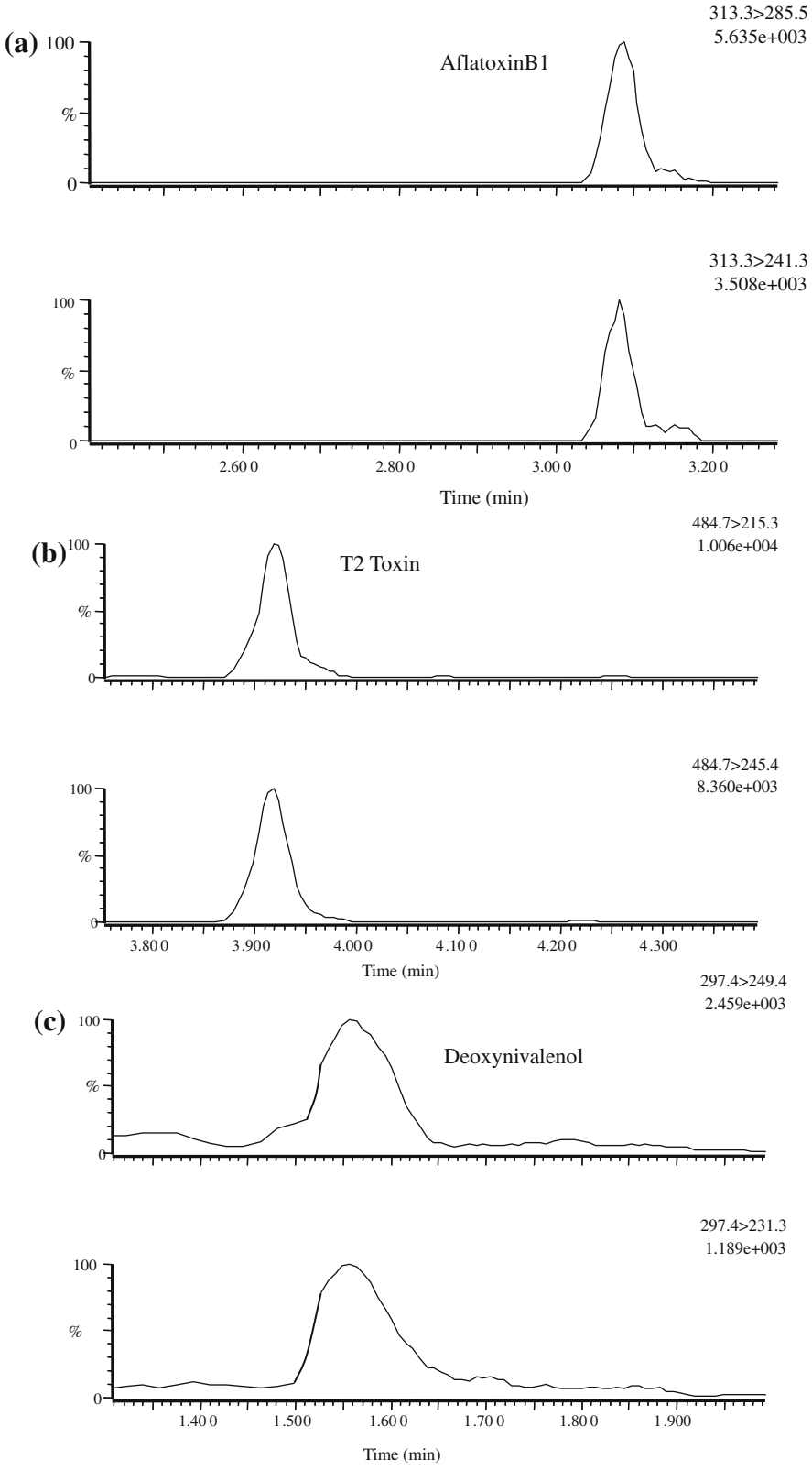

Fig. 2. UHPLC–MS/MS chromatograms for: (a) maize containing aflatoxin B1 at 2.7 μg/kg; (b) the same sample containing T2 at 5.1 μg/kg, and (c) breakfast cereal containing deoxynivalenol at 42.1 μg/kg. Quantification and confirmation transitions were shown for the three compounds.

3.4. Sample analysis

The developed method was applied to several samples (maize, walnuts, biscuits and breakfast cereals). An internal quality control was carried out for every batch of samples to check if the system is under control, and it implies a matrix-matched calibration, a reagent blank, a matrix blank and a spiked blank sample at 10 μg/kg in order to check the reliability of the proposed method.

Three sets of maize samples were collected from two farms located in two different areas. Two sets were collected from the same farm located in Granada, but in two different years (2007 and 2008), whereas the third set was collected from another farm. Mycotoxins were only detected in the maize collected in 2007, which was stored at room temperature in the farm. Aflatoxin G2 was detected at 3.4 μg/kg, aflatoxin G1 at 3.3 μg/kg, aflatoxin B2 at 2.2 μg/kg and aflatoxin B1 at 2.7 μg/kg. Furthermore, T2 toxin was found at 5.1 μg/kg, and traces of HT-2 toxin were detected. Although these levels were below the maximum level established by European Union (Commission Regulation 2006/1881/EC, 2006), they can indicate that more attention should be paid to storage conditions, in order to minimise the content of these analytes.

Three different sets of walnuts were collected from two different farms from the South of Spain, and only traces of deoxynivalenol were detected in one set.

Finally, five types of biscuits and breakfast cereals were collected from stores located in Almeria, and only deoxynivalenol was detected in one breakfast cereal sample at 42.1 μg/kg, which was far below the maximum residue limit established by the European Union (200 μg/kg).

Fig. 2 shows representative chromatograms of aflatoxin B1 and T2 Toxin in maize and deoxynivalenol in breakfast cereal. It can be observed that despite of the relatively high complexity of the analysed matrices, good selectivity and sensitivity were obtained.

4. Conclusions

A rapid and quantitative method has been developed for the simultaneous determination of mycotoxins in several types of matrices, with minimal sample preparation and clean-up. The use of a triple quadrupole mass spectrometer allows the unambiguous identification of 12 mycotoxins, allowing the determination of these compounds in the low μg/kg, fulfilling the requirements established by the European Union. Although significant matrix effect was observed, it was successfully compensated using matrix matched calibration. This quantitative method has many advantages including simple pre-treatment, avoid time-consuming and error-prone clean-up steps, rapid determination by the use of UHPLC, and high sensitivity due to the low LODs and LOQs obtained, which could be applied to the determination and quantification of multicomponent mycotoxins in routine analysis or in monitoring food programs, in which a large number of samples must be analysed.

Acknowledgements

The authors gratefully acknowledge Spanish Ministry of Education and Science (MEC-FEDER) (Project Ref. AGL2006-12127-C02-01) for financial support. RRG is also grateful for personal funding through the Ramon y Cajal program (Spanish Ministry of Education and Science-EFS).

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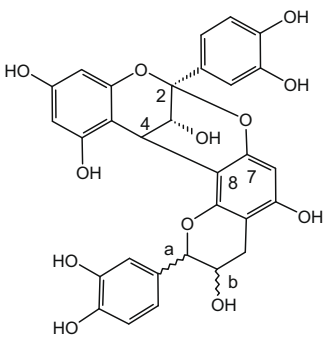
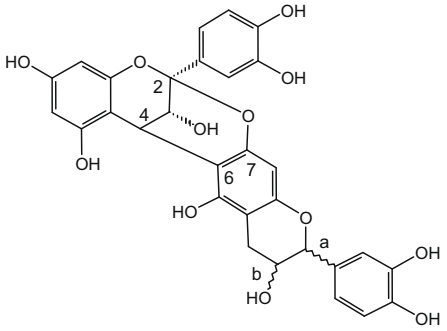


A-type dimer (2-*O*-7, 4-8) linkage



A-type dimer (2-*O*-7, 4-6) linkage

1) EC - C (a = ▌ , b = ⋮)

2) EC - EC (a = ▌ , b = ▌)

3) EC - entC (a = ⋮ , b = ▌)

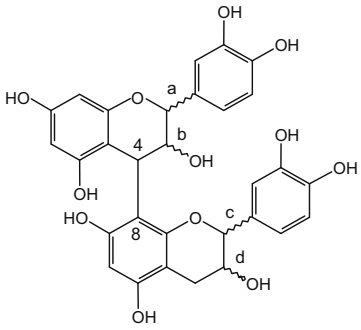
4) EC - entEC (a = ⋮ , b = ⋮)

5) EC - C (a = ⋮ , b = ▌)

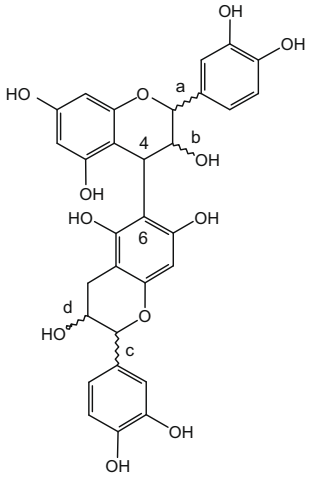
6) EC - EC (a = ⋮ , b = ⋮)

7) EC - entC (a = ▌ , b = ⋮)

8) EC - entEC (a = ▌ , b = ▌)



B-type dimer (4-8) linkage



B-type dimer (4-6) linkage

9) EC - C (a = ⋮ , b = ⋮, c = ▌ , d = ⋮)

10) EC - EC (a = ⋮ , b = ⋮, c = ⋮, d = ⋮)

11) C - C (a = ▌ , b = ⋮, c = ▌, d = ⋮)

12) C -EC (a = ▌, b = ⋮, c = ⋮, d = ⋮)

13) EC - C (a = ⋮, b = ⋮, c = ▌, d = ⋮)

14) EC - EC (a = ⋮, b = ⋮, c = ⋮, d = ⋮)

15) C - C (a = ▌, b = ⋮, c = ▌, d = ⋮)

16) C -EC (a = ▌, b = ⋮, c = ⋮, d = ⋮)

Fig. 1. The structures of A-type and B-type dimers present in grape seeds and peanut skins, respectively. EC = epicatechin, C = catechin and *ent* = enantiomer.

degree of polymerisation (DP) > 2 are believed to be unabsorbable, data on the absorption of dimers are not consistent (Baba, Osakabe, Natsume, & Terao 2002; Donovan et al., 2002; Holt et al., 2002; Sano et al., 2003). Furthermore, A-type PAs have not been included in these bioavailability studies. In order to fully explore the biofunctional potential of PAs, research should focus on the relation between their structure, bioavailability and bioactivity. Isolated and well-characterised PA dimers are, therefore, of great importance.

Efficient isolation of different types of PA dimers requires sources containing high amounts and a large diversity of dimers. Commonly used sources for the isolation of B-type PCs are apple and cocoa, which primarily consist of epicatechin units (Guyot, Marnet, Laraba, Sanoner, & Drilleau, 1998; Rigaud, Escribanobailon, Prieur, Souquet, & Cheynier, 1993) that are mainly 4-8 linked (Foo & Lu, 1999; Rigaud et al., 1993). Grape seeds are also rich in B-type PCs (Gu et al., 2004) with relatively more catechin units (Saucier, Mirabel, Daviaud, Longieras, & Glories, 2001) compared to apple and cocoa. Additionally, 4-6 linkages and galloylated derivatives are present (Ricardo da Silva, Rigaud, Cheyner, Cheminat, & Moutounet, 1991; Rigaud et al., 1993; Santos-Buelga, Francia-Aricha, & Escribano-Bailon, 1995). Therefore, grape seed is a promising source to isolate a large diversity of B-type dimers. A-type PCs can be found in peanuts, plums, cranberries and cinnamon (Gu et al., 2004). Peanut skins contains up to 17% (w/w) PCs (Karchesy & Hemingway, 1986) and both C4–C8 and C4–C6 linked A- (with an additional C2–*O*–C7 linkage) and B-types have been identified (Karchesy & Hemingway, 1986; Lou et al., 1999).

The methods reported to obtain pure A- and B-type dimers are laborious as they include multiple chromatographic steps. The first

step in isolation usually consists of a separation based on DP. Previously, size exclusion materials like Sephadex LH-20 (McMurrough, Madigan, & Smyth 1996; Foo & Lu, 1999) and Toyopearl (Ricardo da Silva et al., 1991; Yanagida, Shoji, & Shibusawa, 2003) were used for this purpose. A combination of five different columns, including four size exclusion columns, was required to isolate several A-type dimers from peanut skins (Lou et al., 1999). The high diversity of B-type PC dimers in grape seeds makes it a good but also challenging source for the current chromatographic methods to isolate the dimers. Recently, a high-speed counter-current chromatographic (HSCCC) technique was used to isolate PC dimers from grape seeds (Kohler, Wray, & Winterhalter, 2008). However, an elution of many hours was needed and additional reversed-phase (RP) HPLC was a prerequisite to obtain pure dimer fractions. Nowadays, more specific silica-based stationary phases are used (Gu et al., 2002; Kelm, Johnson, Robbins, Hammerstone, & Schmitz, 2006; Natsume et al., 2000; Rigaud et al., 1993; Yanagida et al., 2000) and separations of PAs from several sources up to DP 10 have been described (Gu et al., 2002). Additionally, fractions up to DP 8 have been purified from apple (Shoji, Masumoto, Moriichi, Kanda, & Ohtake, 2006). Nevertheless, when pure dimer fractions are to be isolated, often size exclusion chromatography, instead of normal phase (NP) HPLC, in combination with RP-HPLC has been used. Advantages to use NP-HPLC followed by RP-HPLC are: (1) only one HPLC system is required instead of the multiple systems needed for HSCCC, (2) peak shape on NP and thereby purity of the different DP fractions is better than that on size exclusion or HSCCC, (3) a baseline separation of dimers on NP-HPLC facilitates isolation of a fraction containing only all dimers while size exclusion or HSCCC often results in inefficient isolation of dimers to prevent contamination with monomers or trimers due to overlap of peaks.

The combination of NP- and RP-HPLC to obtain pure dimers has rarely been used and little is known about the behaviour of A-type PCs on such columns. In this study an efficient method for the isolation of both A- and B-type dimers is described by combining NP- and RP-HPLC chromatography, yielding sufficient amounts of material for bioavailability studies.

2. Materials and methods

2.1. Materials

Sources to isolate PCs were peanut skins, kindly provided by Imko-The Nut Company BV (Doetinchem, The Netherlands) and a commercially available procyanidin extract from grape seeds (Vitaflavan, DRT, Levita Chemical International NV, Antwerpen, Belgium). PC dimer standards B1, B2, B3 and B4 were obtained at Apin Chemicals (Abingdon, UK). Organic solvents used for HPLC analysis were all HPLC-grade. Milli-Q water from a Millipore system was used.

2.2. Extraction of peanut skins

Peanut skins (~75 g) were defatted with hexane using soxhlet extraction. The residue was air dried. The defatted skins (~60 g) were extracted three times with 1 L of 20% (v/v) aqueous methanol. After each extraction the suspension was filtered over a 595 ½ filter (Schleicher & Schuell, Dassel, Germany), after which the pellet was subjected to the next extraction. The three 20% (v/v) methanol fractions were combined, concentrated by evaporation under vacuum and lyophilised. During the extractions and evaporation, light was excluded as much as possible by the use of marquees and tinfoil. Solvent partitioning was used for further purification. The lyophilised extract was put into a separation funnel to which 500 mL H_2O and 500 mL ethyl acetate was added. This was placed in an ultrasonic bath for 10–15 min at room temperature. After vigorous mixing, the H_2O phase and ethyl acetate phase were collected separately. The H_2O phase was put back into the funnel and the solvent partitioning procedure was repeated twice resulting in three ethyl acetate phases and one H_2O phase. The ethyl acetate phases were combined and subsequently extracted with H_2O (1:1, v/v) twice. The ethyl acetate phase was vacuum evaporated, dissolved in H_2O and lyophilised, resulting in the final extract referred to as: peanut skin extract.

2.3. Removal of gallic acid from grape seed procyanidins

Gallic acid substituents were enzymatically removed from grape seed PCs as described elsewhere (Appeldoorn, Vincken, Aura, Hollman, & Gruppen, 2009) to improve resolution on NP-HPLC. In short, grape seed PCs were dissolved in a sodium acetate buffer (0.1 M, pH 5.0), and incubated with tannase (EC 3.1.1.20, Gamma Chemie, Darmstadt, Germany). PCs were separated from free gallic acid by solid phase extraction. PCs were eluted with methanol, vacuum evaporated, dissolved in H_2O and lyophilised.

2.4. Preparative isolation of A- and B-type dimers

2.4.1. Normal phase HPLC

Fractionation by DP was performed on an Inertsil PREP-SIL 30 mm ID × 250 mm column with a 10 μm particle size (GL sciences Inc., Tokyo, Japan). A Waters system equipped with a 2767 sample manager, a 2525 binary gradient module, a 2996 photodiode array detector (PDA) and an UV fraction manager was used. The binary mobile phase consisted of (A) hexane and (B) acetone. The elution was as follows: 0–30 min, 40–60% B; 30–50 min, isocratic at 60% B; 50–70 min, 60–75% B, followed by a washing step at 98% B for 3 min, and reconditioning of the column. The flow rate was 27.22 mL/min and PDA spectra from 210–300 nm were recorded. In a run, 10 mL (~70 mg/mL in acetone/hexane/ethanol (7:3:2)) peanut skin extract or the tannase-treated grape seed extract was applied. The fraction containing dimers was collected from 22.3 to 28.2 min for the peanut skin extract and from 28.2 to 38.1 min for the grape seed extract. The fractions were evaporated under vacuum and lyophilised.

2.4.2. Reversed phase HPLC

An XterraRP 50 mm ID × 100 mm column with a 5 μm particle size (Waters) was used with a flow rate of 82.7 mL/min. The fractions containing dimers (~70 mg), which were obtained with preparative NP-HPLC, were dissolved in methanol using ultrasonic treatment if needed and filtered over a Spartan® 0.45 μm filter (Schleicher & Schuell, Dassel, Germany) before injection. The binary mobile phase consisted of (A) H_2O + 0.1% (v/v) acetic acid and (B) acetonitril + 0.1% (v/v) acetic acid. The elution for the separation and isolation of the individual peanut skin dimers was as follows: the first 3 min isocratic on 0% B; 3–23 min, B: linearly 0–22%; 23–28 min, B: linearly 22–89% followed by reconditioning of the column. The elution for the grape seed dimers was as follows: the first 10 min isocratic on 10% B; 10–23 min, B: linearly 10–50%; 23–27 min, B: linearly 50–95% followed by reconditioning of the column. PDA spectra were recorded from 210 to 300 nm. The fractions that were collected after injecting peanut skin dimers were; I_P (15.7–16.6 min), II_P (17.6–18.7 min), III_P (18.7–19.6 min), IV_P (19.6–20.5 min), V_P (21.2–22.0 min) and VI_P (22.0–22.8 min). The fractions that were collected after injecting grape seed dimers were; I_G (10.0–12.2 min), II_G (13.0–13.7 min), III_G (13.9–14.2 min), IV_G (14.8–15.5 min), V_G (15.7–15.9 min), VI_G (16.1–16.3 min), VII_G (16.5–17.0 min) and $VIII_G$ (17.4–17.8 min). Subsequently, the fractions were evaporated under vacuum and either lyophilised or

solubilised in methanol prior to further analysis. Fraction IV_G consisted of two PC dimers, which were further purified on an Atlantis dC18 (19 mm ID × 100 mm, 5 μm particle size; Waters). The fraction (~16 mg) was dissolved in 10 mL 50% (v/v) aqueous methanol, filtered over a Spartan® 0.45 μm and injected. The flow rate was 17.1 mL/min. The binary mobile phase consisted of (A) H_2O + 0.1% (v/v) acetic acid and (B) methanol + 0.1% (v/v) acetic acid combined in the following elution pattern: 0–16.7 min, B: linearly 5–45%; 16.7–20 min, B: linearly 45–60%; 20–23.3 min, isocratic at 60% B, followed by reconditioning of the column. Fractions were collected from 9.5 to 10.5 min (IV_{G1}) and from 10.8 to 12.0 min (IV_{G2}).

2.5. Analytical HPLC

2.5.1. Normal phase HPLC

A Thermo Spectra system was used containing a P 4000 pump, an AS 300 autosampler and an UV 3000 detector (Thermo Separation products, Fremont, CA, USA). Analysis was performed on a Luna Silica (2) 4.6 mm ID × 250 mm column with a 5 μm particle size (Phenomenex, Torrance, CA) operated at room temperature. Samples were dissolved in methanol. The mobile phase consisted of (A) dichloromethane, (B) methanol and (C) acetic acid/H_2O (50% v/v). The flow rate was 1 mL/min and detection was performed at 280 nm. The elution was as follows; C was kept constant at 4% throughout the elution; 0–20 min, B: linearly 14–23.5%; 20–50 min, B: linearly 23.5–35% followed by a washing step at 96% B for 10 min and reconditioning of the column.

2.5.2. Reversed phase HPLC

The same Thermo Spectra system was used and analysis was performed on a XterraRP dC18, 4.6 mm ID × 150 mm column with a 3.5 μm particle size (Waters). Samples were dissolved in methanol. The mobile phase was composed of (A) H_2O + 0.1% (v/v) acetic acid and (B) acetonitril + 0.1% (v/v) acetic acid. The flow rate was 0.7 mL/min and detection was performed at 280 nm. The elution for peanut skin dimers was as follows; the first 5 min isocratic on 10% B; 5–35 min, B: linearly 10–30%; 35–40 min, B: linearly 30–90% and reconditioning of the column. The elution for grape seed dimers was as follows; the first 15 min isocratic on 10% B; 15–35 min, B: linearly 10–50%; 35–40 min, B: linearly 50–95% followed by reconditioning of the column. Samples were compared to retention times of B-type dimer standards B1, B2, B3 and B4.

2.6. Characterisation of B-type dimers by phloroglucinolysis

Fractions I_P and VI_P, obtained after preparative HPLC, were analysed on RP-HPLC before and after acid-catalysed degradation as described by Fournand and coworkers (Fournand et al., 2006). Briefly, intact dimers were analysed on RP-HPLC-UV. After acid-catalysed degradation of the dimers in the presence of excess phloroglucinol, released terminal units and extension unit-phloroglucinol adducts were analysed by RP-HPLC-UV. Dimers were identified based on the retention time of the intact dimers and the retention time of (epi)catechin and their phloroglucinol adducts, released after acid-catalysed degradation.

2.7. Spectroscopic-analysis

2.7.1. ESI-MS detection

An LCQ Classic equipped with an ESI source was used and controlled by the software Xcalibur (Thermo Finnigan, San Jose, CA). The detector was coupled to an HPLC system by means of a splitter (Acurate, LC Packings, Amsterdam, The Netherlands), which reduced the flow rate 20 times. Measurements were performed in the negative mode with an ion spray voltage of 4.5 kV, a capillary voltage of −5.0 V and a capillary temperature of 270 °C. The scan range was set from *m/z* 100–2000. The MS/MS function was performed in the data dependant mode. The collision energy value was 27%.

2.7.2. Maldi-TOF-MS

Samples were analysed on an Ultraflex instrument equipped with a nitrogen laser of 337 nm and controlled by the software Flexanalysis (Bruker Daltonics B.V., Wormer, The Netherlands). A laser intensity of 29% was applied and data were collected within a mass range of 400–2000 Da. The apparatus was operated in both the positive and the negative mode with a delayed extraction time of 100 ns and an acceleration voltage of 20 kV. Samples were mixed with the matrix 2,5-dihydroxybenzoic acid (Bruker Daltonics) (10 mg/mL H_2O) (1:1) and 2 μL was put on a stainless steel metal plate, crystallised under a blow drier and analysed.

2.7.3. NMR spectroscopy

NMR experiments were performed on a Varian UNITY INOVA 500 MHz spectrometer (Varian NMR instruments, Palo Alto, CA, USA) equipped with a 3 mm indirect detection probe operating at 500 MHz for ^1H and 125.7 MHz for ^{13}C. One-dimensional (1D) ^1H, two dimensional (2D) ^1H TOCSY and ROESY, and 2D (^1H–13C) HSQC and HMBC spectra of samples dissolved in methanol-d_4 (fractions I_P, II_P and IV_P) or dimethyl sulphoxide-d_6 (fractions III_P, V_P and VI_P) were recorded at 298 K. Chemical shifts (δ) are given in ppm and coupling constant (J) values are given in Hertz. Spectra signals were referenced to the solvent signals, either DMSO (^1H signal at 2.5 ppm and ^{13}C signal at 39.5 ppm) or methanol (^1H signal at 3.3 ppm and ^{13}C signal at 49.0 ppm). Spectra were processed and analysed with V NMR software (Varian, CA, USA).

3. Results and discussion

3.1. Preparative isolation of A- and B-type dimers

3.1.1. Use of NP-HPLC for the isolation of a dimeric fraction

To be able to isolate the grape seed dimers, gallic acid substituents were enzymatically (tannase) removed, which improved the resolution especially between the dimers and trimers, as described previously (Rigaud et al., 1993). The dimers from the tannase-treated grape seed extract, as well as from the peanut skin extract, were isolated with preparative NP-HPLC (Fig. 2), which separates by DP. The total recovery of all material after NP-HPLC was 85% (w/w) (n = 1) for the peanut skin extract and 78% (w/w) ± 4.8 (n = 4) for the grape seed extract. This is comparable to recoveries obtained for apple extracts obtained with the same method (Shoji et al., 2006). The dimers represented 16.8% (w/w) (n = 1) of the peanut skin extract and 21.2% (w/w) ± 2.1 (n = 5) of the grape seed extract, similar to previously reported amounts in grape seeds (Nunez, Gomez-Cordoves, Bartolome, Hong, & Mitchell, 2006). Both dimer fractions were free of monomers and trimers as their masses, *m/z* 289 $[M-H]^-$ and *m/z* 865, 863 (1 A-interflavanic linkage (IFL)), and 861 $[M-H]^-$ (2 A-IFL), respectively, were not detected by NP-HPLC–UV–MS. The A- and B-type dimers could be distinguished by their parent ions (*m/z* 575 $[M-H]^-$ and *m/z* 577 $[M-H]^-$, respectively) and accompanying MS/MS spectra. PCs have three characteristic fragmentation routes, which have been described as: quinone methide (QM), retro-Diels–Alder (RDA) and heterocyclic ring fission (HRF) cleavage (Gu et al., 2003). A lower retention of the peanut skin dimers compared to grape seeds dimers on the silica stationary phase (Fig. 2) could hint at the presence of A-type dimers, which are less polar than the B-type dimers due to the additional bond. This was confirmed by the MS/MS spectra, which showed parent ions of *m/z* 575 for the peanut skin dimers with *m/z*

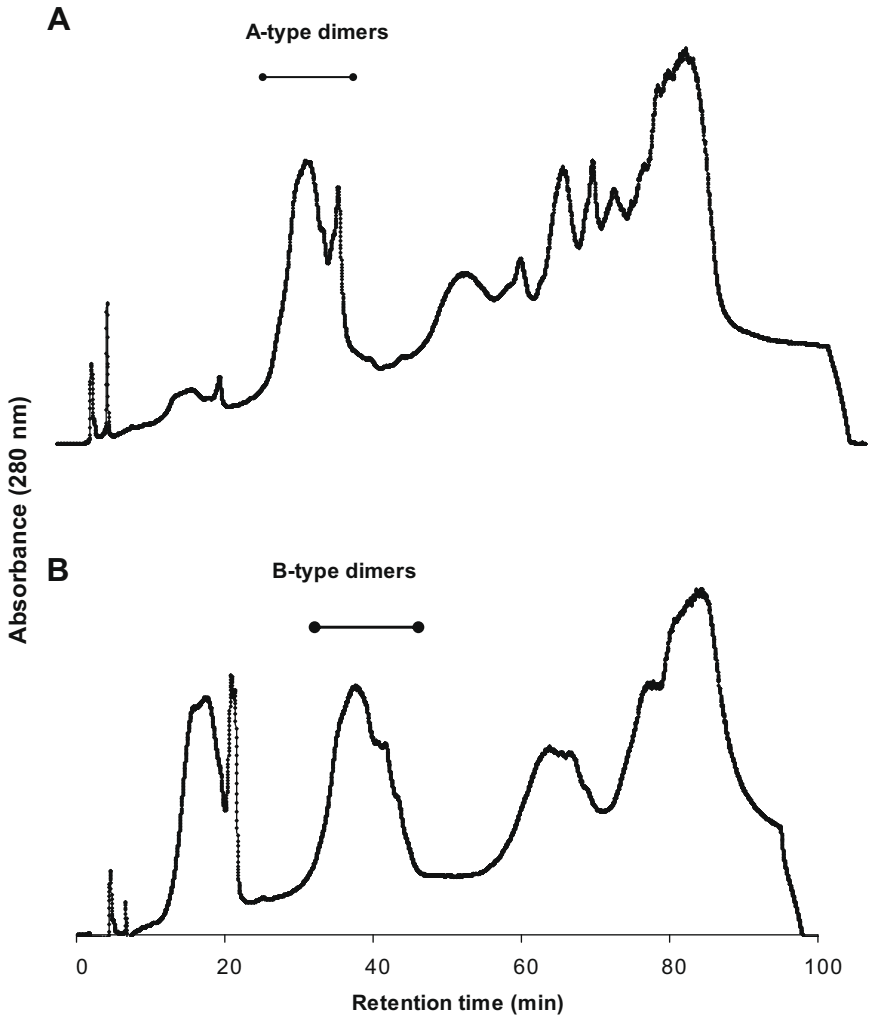


Fig. 2. Isolation of A-type dimers from peanut skins (panel A) and B-type dimers from grape seeds (Panel B) by preparative NP-HPLC. The fractions containing the A- or B-type dimers were collected, evaporated under vacuum and lyophilised.

289 + 285 (QM), *m/z* 423 + 407 (RDA) and *m/z* 449 (HRF) as fragment ions. The grape seed dimers had parent ions of *m/z* 577, and *m/z* 289 + 287 (QM), *m/z* 425 + 407 (RDA) and *m/z* 451 (HRF) as fragment ions. The observation that the fragmentation of A-type dimers, just like the B-type, yields a fragment ion of 407 rather than 405 has been reported earlier (Zhu, Hammerstone, Lazarus, Schmitz, & Keen, 2003).

3.1.2. Use of RP-HPLC for purification of individual dimers

The fractions containing the dimers were further separated on an XterraRP dC18 column. The total recovery of all material after RP-HPLC was 65.1% (w/w) ($n = 1$) for the peanut skin dimers and 68.7% (w/w) ± 5.3 ($n = 3$) for the grape seed dimers. The peanut skin dimers contained 6 peaks in total indicated by I_P till VI_P (Fig. 3, panel A). Separation of the grape seed dimers resulted in 8 peaks, I_G till $VIII_G$ (Fig. 3, panel B). Peak IV_G consisted of 2 components based on reinjection on an Atlantis dC18 column. Preparative separation on this column resulted in two pure fractions: IV_{G1} and IV_{G2} (Fig. 4). The total recovery was 66.4% (w/w) ($n = 1$).

3.2. Structure elucidation of isolated dimers

3.2.1. A-type dimers

Four peaks (II_P to V_P) were characterised as A-type dimers based on their parent ions (*m/z* 575 $[M-H]^-$) and MS/MS fragments. Because no commercial standards were available for A-type dimers, NMR spectroscopy was performed for structural identification (Tables 1 and 2 in Supplementary material).

3.2.2. Determination of interflavanic linkage (IFL)

The NMR spectra of the fractions II_P, III_P, IV_P and V_P were very similar and typical for A-type dimers as described previously (Lou et al., 1999). In short, an isolated AB system in the heterocyclic proton region (δ 4.0–4.5 ppm, J = 2.8–3.5 Hz) was attributed to protons at carbon 3 and 4 of the C-ring (C3-C and C4-C). Two meta-coupled doublets and a singlet in the aromatic region (δ ± 6 ppm) were assigned to the protons of the A-ring and the residual proton of the D-ring, respectively. Two AMX systems in the aromatic downfield region (δ 6.5–7.5) were detected as B- and E-ring protons. The other signals were unambiguously attributed to the F-ring protons. Therefore, the A-IFL involved both the C4 and C2 of the upper unit. The junctions at the lower unit were determined through the analysis of both ^{1}H–13C long-range HMBC and ^{1}H through space ROESY correlations.

The bond positions of fractions II_P, III_P and IV_P were determined in a similar way as illustrated in Fig. 5, panel A. First, C8a-D was attributed through its correlation with the proton at C2-F, whereas C5-D was identified through its correlations with both protons at C4-F. The residual proton of the D-ring showed correlations with two deshielded carbons (δ > 150 ppm). One could easily be

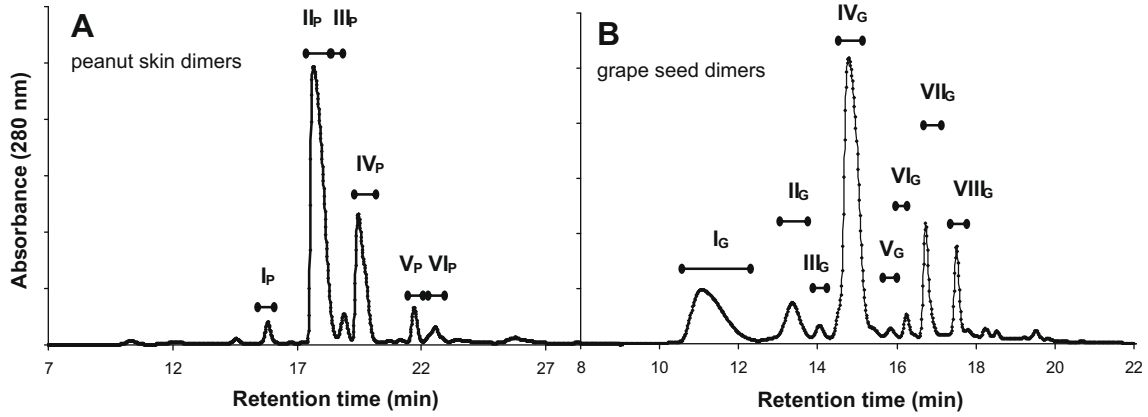


Fig. 3. Isolation of dimers from peanut skins (A) and grape seeds (B) on a preparative XterraRP C18 column. Fractions were collected as indicated by I through VIII. The origin is indicated by P (peanut skins) and G (grape seeds).

determined to be the C5-D, while the other one was deduced to be C7-D, since its chemical shift was different from that of C8a-D. Therefore, the residual proton was determined to be at C6-D. Consequently, the IFL implied attachment to C8 of the D-ring. Also the long range correlation between the proton at C4-C and C8a-D and ROESY spectra, which showed correlations between the proton at C4-C and E-ring protons, were in agreement with a C4–C8 linkage. The ether bond was deduced to be C2–*O*–C7, as it is the only possibility in case of a C4–C8 linkage. Therefore, the components of fractions II_P, III_P and IV_P were identified to be (C2–*O*–C7, C4–C8) double linked dimers.

In the case of fraction V_P, no conclusive evidences of the IFL positions could be found based on long-range HMBC correlations. The residual D-ring proton could not be determined, because C8a-D and C5-D overlapped. However, ROESY correlations between the proton at C4-C and protons of E-ring were clearly lacking, which indicated a C4–C6 linkage (Lou et al., 1999) (Fig. 5, Panel B). In this case, the ether linkage can be either C2–*O*–C7 or C2–*O*–C5. Since a weak, but clear, correlation occurred between the proton at C8-D and the proton at C2-F, as well as one between the proton at C8-D and protons at C2 and C6 of the B-ring, the linkage can be deduced to be C2–*O*–C7. The C2–*O*–C7 linkage was also in accordance with the ^{13}C chemical shift of C7-D, which was upfield (δ 150) compared to free hydroxylated C7-A (δ 156.3). Based on all the data the component of fraction V_P was identified as a (C2–*O*–C7, C4–C6) double linked dimer.

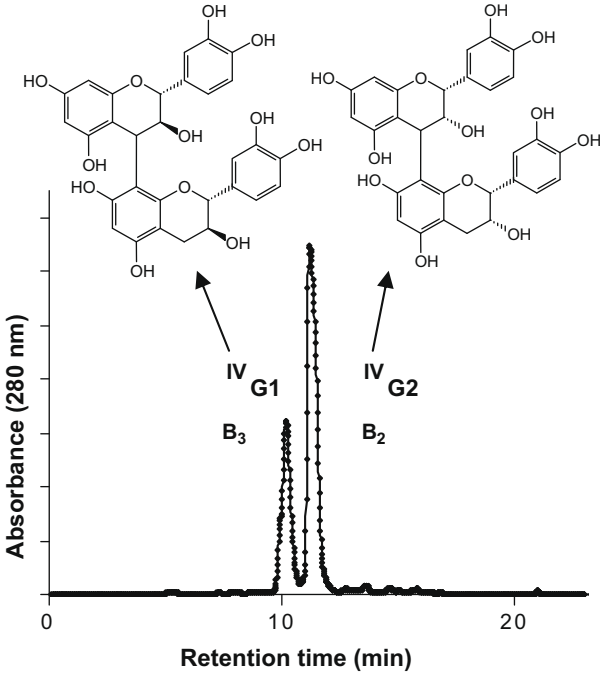


Fig. 4. Isolation of dimers B2 (IV_{G2}) and B3 (IV_{G1}) on a preparative Atlantis dC18 column from fraction IV_G, previously isolated on XterraRP (Fig. 3, Panel B).

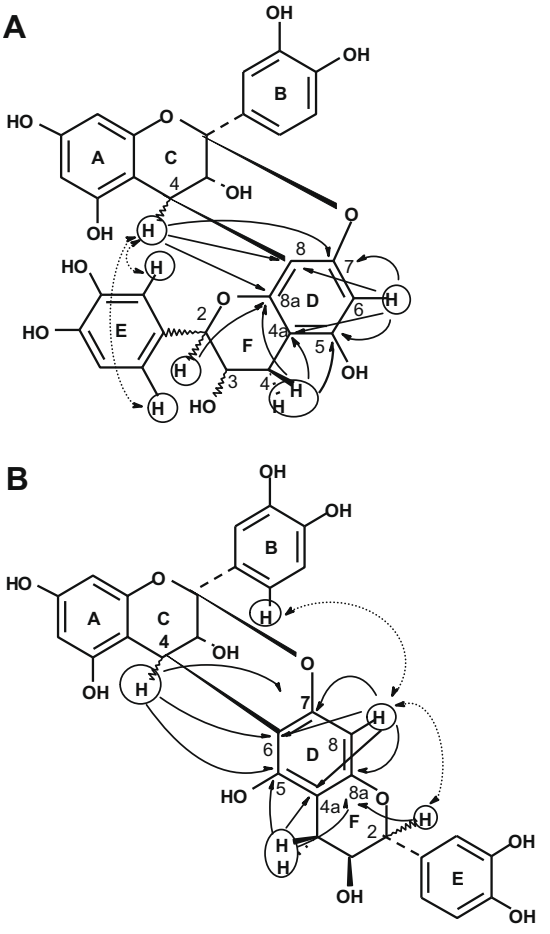


Fig. 5. Main HMBC (→) and ROESY (→) correlations allowing linkage positions to be established, in either (C2–*O*–C7, C4–C8) (A) or (C2–*O*–C7, C4–C6) (B) dimers.

NMR spectra of fraction VI_P were not conclusive because of its low purity. The major compound, which, based on NMR signals, represented about 35% of fraction VI_P, exhibited some typical NMR signals (an AB C-ring system) of an A-type dimer. However, structural identification was difficult due to numerous overlaps and poor signal intensities. Phloroglucinolysis of fraction VI_P did not result in depolymerisation, which supported the hypothesis of the presence of an A-type dimer in this fraction.

3.2.3. Monomeric composition

In the case of fractions II_P, III_P, and V_P, the large values observed for the J_{23} F-ring coupling constants ($J \sim 7$ Hz) were characteristic for catechin. In contrast, for fraction IV_P this value was relatively low (~1 Hz), which is characteristic for an epicatechin unit. The upper units of fractions II_P–V_P were supposed to be epicatechin, as Lou and coworkers (Lou et al. 1999) found that A-type dimers from peanut skins always contained epicatechin as upper units.

Therefore, the two major peaks, II_P and IV_P, were identified as epicatechin-(2-*O*-7, 4-8)-catechin (**1**), also referred to as A1, and epicatechin-(2-*O*-7, 4-8)-epicatechin (**2**), referred to as A2. Fraction III_P showed similar NMR spectra compared to fraction II_P (A1). Dimer A1 has been identified in peanut skins before and seems to be abundantly present (Karchesy & Hemingway, 1986; Lou et al., 1999). Therefore, the major fraction II_P was annotated to be A1, while the less abundant fraction III_P, containing a similar NMR spectra, was tentatively identified as the enantiomer of A1 (**3**). The presence of enantiomers in peanut skins was also found by Lou and coworkers (Lou et al., 1999), who identified the enantiomer of dimer A2. However, the enantiomer of A1 has not been identified in peanut skins before. Fraction V_P was characterised as epicatechin-(2-*O*-7, 4-6)-catechin (**5**).

3.2.4. B-type dimers

The fractions obtained from grape seeds all represent B-type dimers (*m/z* 577 $[M-H]^-$) based on RP-HPLC-UV-MS and their fragment products with MS/MS. Comparison with standards resulted in the identification of I_G as B1 (**9**), II_G as B4 (**12**) and IV_G, which consisted of both B2 (**10**) and B3 (**11**). The remaining peaks could not be identified further. Peak IV_G was further separated on an Atlantis dC18 column, resulting in pure fractions of B2 (**10**) (IV_{G2}) and B3 (**11**) (IV_{G1}) (Fig. 4).

One peak isolated from peanut skins (I_P, Fig. 3, panel A) was identified as a B-type dimer, which was B7 (epicatechin-(4-6)-catechin) (**13**) based on its retention time before phloroglucinolysis and its products after phloroglucinolysis. This was confirmed with NMR (Tables 1 and 2 in Supplementary material). Both 1D and 2D homonuclear ^{1}H and 2D heteronuclear ^{1}H–13C experiments were performed. The main difference compared to the spectra of an A-type dimer is the presence of a proton at C2-C. The structure of this dimer was determined as follows: C8a-D (δ 154.7) was first identified through its long range correlation with the proton at C2-F. The bond location was determined from the long range correlations of the proton at C4-C. This proton had no correlation with C8a-D at δ 154.7 but exhibited correlations with several deshielded carbons at δ 155.6, 159.2, 159.2 and 157.8, which were attributed to C5-D, C7-D, C8a-A and C5-A. A long range correlation of the proton at C4-C with C5-D and the lack of a correlation with C8a-D indicated that C4-C is linked to C6-D (De Bruyne et al., 1996). The J_{23} F-ring coupling constant values allowed to discriminate between epicatechin ($J \sim 1$ Hz) or catechin ($J \sim 7$ Hz) (2,3 *cis* or *trans*) configuration of the lower unit (Davis, Cai, Davies, & Lewis, 1996) and was determined to be catechin since the J_{23} coupling constant value was 7.7 Hz.

4. Conclusion

The main A-type dimers, A1 (**1**) and A2 (**2**), were efficiently isolated. In comparison to a previously published method (Lou et al., 1999), the required chromatographic methods were reduced from five to two, while simultaneously the yields of A1 and A2 increased 20–400 times. To compare both methods, the yields of A1 and A2 were expressed as weight percentage of the total amount of peanut skins that was used. Lou and coworkers (Lou et al., 1999) used skins of 279 kg peanuts. The amount of skins they used was calculated to be ~11 kg, assuming that the skins represents 4% (w/w) of the total peanut (Pominski, Daquin, Molaison, McCourtney, & Vix, 1952). Differences in extraction procedure and the origin of peanut skins might also have contributed to the pronounced yield differences found. Next to A1 and A2, four other peanut skin dimers were isolated including the tentatively identified enantiomer of A1 (**3**), not previously detected in peanut skins, epicatechin-(2-*O*-7, 4-6)-catechin (**5**), B7 (**13**) and one unidentified dimer.

Although grape seeds have been used before as a source to isolate PC dimers, data on the efficiency of those isolations are not well documented. Dimers isolated with NP-HPLC resulted in about 21% (w/w) of the PC extract, which is in accordance with compositional LC–MS analysis of PC dimers in grape seed extracts performed by others (Nunez et al., 2006; Santos-Buelga et al., 1995). The yield for B1–4 from the grape seed extract using NP/RP-HPLC was about 10 times higher compared to a recently published HSCCC method (Kohler et al., 2008). Recoveries for the additional

Table 1
Characteristics of the isolated A- and B-type dimers obtained after successive preparative normal and reversed phase HPLC.

Source	Fraction[a]	Composition/structure[b]	Purity[c]	% (w/w)[d]
Peanut skin extract	I_p	EC-(4-6)-C (13)	93	0.2
	II_p	EC-(2-O-7, 4-8)-C (1)	99	6.9
	III_p	EC-(2–0 -7, 4-8)-ent C (3)	81	0.3
	IV_p	EC-(2–0 -7, 4-8)-EC (2)	98	2.1
	V_p	EC-(2–0 -7, 4-6)-C (5)	99	0.3
	VI_p	–	<75	0.2
Grape seed extract	I_g	EC-(4-8)-C (9)	95	3.2
	II_g	C-(4-8)-EC (12)	81	1.2
	III_g	–	<75	0.2
	IV_{g1}	C-(4-8)-C (11)	88	1.5
	IV_{g2}	EC-(4-8)-EC (10)	99	7.1
	V_g	–	80	0.1
	VI_g	–	65	0.2
	VII_g	–	93	1.2
	$VIII_g$	–	96	0.9

[a] The fractions represent the peaks as shown in Figs. 4 and 5.
[b] The numbers represent the structures shown in Fig. 1.
[c] Based on the peak area (280 nm) assuming equal molar response factors, analysed on analytical RP-HPLC.
[d] Calculated as a weight % of the peanut skin extract and grape seed extract.

RP-HPLC that was performed after HSCCC were not given, but would likely lower their total yield. Furthermore, pre-purification steps like precipitation or SPE to remove polymers and improve resolution, prior to HSCCC, were not needed.

The combination of NP/RP-HPLC is suitable to efficiently isolate a large variety of both A- and B-type dimers with a limited number of chromatographic steps. Table 1 summarises the purity, yield and structure of each peak that was isolated. Sufficient amounts of material were obtained to study the bioavailability of both A- and B-type dimers, which is subject of further research in our laboratory.

Acknowledgements

The authors thank Jean-Marc Souquet for performing phloroglucinolysis. This work was financially supported by the Dutch Ministry of Agriculture, Nature Management and Food Quality and the Graduate School VLAG.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2009.04.047.

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The overall sampling scheme for each pork cut was

$$3\,\text{States} \times 3\,\text{SEC} \times 2\,\text{replicate suburbs} \times 2\,\text{retailer types} \times 2\,\text{conditions (raw, cooked)} = 72\,\text{samples (purchases)}$$

where State = Australian State; retailer type = butcher/supermarket; SEC = socioeconomic status of suburb (high, medium, low).

The 13 cuts which were advised to be the more popular consumer cuts and therefore selected were: loin chop, loin steak, fillet, rump steak, round steak, topside steak, silverside steak, diced pork, pork strips, pork mince, loin roast, round (mini) roast, scotch (neck) roast. One cut, the loin chop, was studied in more detail across all three States, in order to provide additional data on variability.

Sample collection and handling generally followed Food and Agriculture Organization/International Network of Food Data Systems (FAO/INFOODS) international guidelines (Greenfield & Southgate, 2003). Pork loin chop samples were collected in December 2005, and the other cuts progressively from February to May 2006. For the 13 cuts the number of actual purchases was 911 instead of the planned 936 because of a small number of cuts that were unavailable on the day of collection. Sample collection was organised by APL representatives in each of the three States and shoppers were commissioned to purchase duplicate samples (e.g. two pork roasts or ~2 kg of smaller cuts) anonymously from each nominated retail outlet. Shoppers were instructed not to specify whether trimmed or untrimmed cuts were desired. The meat samples were packed and freighted chilled to the Food Science Australia (FSA) laboratory in Queensland by air from Melbourne, Victoria and Perth, Western Australia and by road from Brisbane. Meat temperatures were checked on arrival at FSA and all samples were stored at 1–2 °C until required for processing, either immediately or later on the same day. Where incorrect cuts had been supplied attempts were made to obtain replacements, or were later designated as missing values along with those that were not available.

The duplicate samples purchased for each cut were randomly allocated to either raw or cooked treatment. Samples were held at 1–2 °C before and during handling, and kept covered in closed polyethylene bags to minimise evaporation of moisture.

2.2. Cooking methods

All cuts for analysis were analysed in the raw state and in the cooked state. Cooking methods were as specified by APL home economists to reflect household practice then adapted for the available equipment. Details of cooking methods are given in Müller et al. (2009).

2.3. Preparation of analytical homogenates

Preparation of the analytical homogenates was carried out following FAO/INFOODS international guidelines for food composition studies (Greenfield & Southgate, 2003, Fig. 5.1, Table 5.4). For each cut, except loin chop, duplicates of four national analytical homogenates (namely, raw lean, cooked lean, raw fat and cooked fat) were formed as homogenised composites from the State duplicate purchases. For loin chop, 24 analytical homogenates were formed so that for each of the three States, duplicate homogenates were formed for each of the following: raw lean, cooked lean, raw fat, cooked fat. The State duplicates were obtained by combining the two retailer types from one randomly selected set of a high, a medium and a low SEC area as one composite, and the remaining high, medium and low SEC areas as a second composite. The dissected meat was weighed and aliquot samples were allocated for aggregation according to the experimental design for the particular cut. The aggregate meat sample was coarsely cut, hand mixed and minced through a meat mincer (IRCEM Srl Tritacarne Type I-E 22, 240 V, Italy) using a single blade and a 4 mm mincing plate. The samples were then recombined and minced again to attain adequate homogeneity. Contact with metal was minimised wherever possible. The mincer was dismantled and washed after each sample was minced to eliminate cross contamination between samples. Temperatures were maintained at 1–2 °C throughout preparation of analytical homogenates.

Initially, a number of individual samples were assessed by measuring homogeneity by the reproducibility of their moisture contents to establish the degree of mixing required. Samples within a 2% moisture range were assessed as sufficiently homogenised, e.g., 75% ± 2%. The samples were then packed in plastic jars and stored chilled or frozen until required for further evaluation. The four (4) composite samples that were obtained for each of the cuts were raw lean, cooked lean, raw fat and cooked fat. For some cuts, only raw lean and cooked lean data were required due to the absence of separable fat on cuts such as fillet.

2.4. Transport of analytical homogenates

Depending on the nutritional analyses that were required, samples were coded and sent chilled (for folate analysis) to the University of NSW, and either chilled (for water, thiamin and riboflavin analyses) or frozen (for the rest of the analyses) to National Measurement Institute (NMI) in Melbourne. All samples were freighted in insulated containers by overnight courier. All chilled samples were dispatched on the same day as dissection and aggregation, and all frozen samples on the day following. A small amount (100 g) of each sample was vacuum-packed and retained by FSA in a −80 °C freezer as back-up samples for re-analysis if required.

2.5. Variability study of pork loin chop

All 24 composites of loin chop (2 duplicate composites for each of 4 portions, raw lean, raw fat, cooked lean, cooked fat from each of three States) were analysed. The cut collection and gross composition work was carried out in December 2005. The nutrient analytical work was completed in February 2007.

2.6. Representative study, all cuts

The remaining 12 cuts were analysed as 34 national composites (for 5 cuts: national duplicates × 4 portions, lean/fat x raw/cooked, total = 20; for 7 cuts: national duplicates × 2 portions, lean only (e.g. fillet) or lean + fat mixed (e.g. mince), raw/cooked, total = 14). The cut collection and gross composition work was done from February to May 2006. The nutrient analytical work was completed in April 2007.

2.7. Methods of nutrient analysis

Methods of analysis were agreed in terms of availability and appropriateness for pork, and in terms of the expected ranges of nutrient levels in this commodity.

Methods for proximate components were: moisture, air-drying at 102 °C, AOAC (1995) method nos., 934.06, 950.46B; protein, nitrogen by Kjeldahl method, AOAC (1995) method nos. 981.1, 928.08, protein conversion factor 6.25; fat, Soxhlet extraction, gravimetric determination, AOAC (1995) method nos. 920.39, 960.39; ash, incineration and gravimetry, AOAC (1995) method nos. 923.03, 920.153. Energy calculation was: (g protein × 17 kJ) + (g fat × 37 kJ) (Greenfield & Southgate, 2003).

Methods for vitamins were: retinol, HPLC (Brubacher, Müller-Mulot, & Southgate, 1985); α- and β-carotene, HPLC (Brubacher et al., 1985); α-tocopherol, HPLC (De Leenheer, Lambert, & de

Ruyter, 1985); thiamin and riboflavin, HPLC (Wehling & Wetzel, 1984); niacin, capillary zone electrophoresis and HPLC (Ward & Trenerry, 1997); vitamin B_6, HPLC (Bergaentzlé, Arella, Bourguignon, & Hasselmann, 1995); pantothenic acid, gas–liquid chromatography (Davidek, Velisek, Cerna, & Davidek, 1985); vitamin B_{12}, microbiological assay using *Euglena gracilis* (Anderson & Sourial, 1983); total folate, microbiological assay with *Lactobacillus casei (subsp. Rhamnosus ATCC 7469).* The method followed was the tri-enzyme treatment according to Tamura, Mizuno, Johnson, and Jacob (1997) with a modification using a cryoprotected frozen culture and preparation of the extract as suggested by Shrestha, Arcot, and Paterson (2000). Extraction was carried out on the chilled samples with an additional lipase treatment (Vishnumohan & Arcot, 2006). Separately, comparisons of the tri-enzyme treatment and the tri-enzyme plus lipase treatment were carried out on fresh convenience samples of pork purchased from local retail outlets.

Methods for minerals were: ICP/MS (Inductively Coupled Plasma Mass Spectrometry) (AOAC (1995), method nos. 984.27, 993.14) for sodium, potassium, iron, calcium, magnesium, zinc, manganese, selenium, iodine; ion selective electrode (Dabeka & McKenzie, 1981; Dabeka, McKenzie, & Conacher, 1979) for fluoride.

2.8. Apparent retention of nutrients

Apparent nutrient retention (%) was calculated as: nutrient content per g of cooked food (dry basis)/nutrient content per g of raw food (dry basis) × 100 (Murphy, Criner, & Gray, 1975).

2.9. Quality control

All analyses were performed in duplicate apart from folate (in triplicate) and vitamin E (single analyses).

Quality control procedures for all nutrient analyses included the use of National Institute of Standards and Technology (NIST) Standard Reference Material 1546 Meat Homogenate, in-house reference materials, precision of replicates, recoveries of standards, use of blanks and other routine quality control checks. For folate analyses NIST Certified Reference Material Pig Liver CRM 487 was used and additional quality control included triplicate analyses and performing recovery studies on samples. Quality control procedures are shown in Table 1.

2.10. Data scrutiny

All authors scrutinised the analytical data on receipt. Further scrutiny, including integrity checks, of the complete data sets was performed by three of the authors (JB, HG and JC). All scrutineers compiled their own independent lists of queries. After agreement between the scrutineers reached by teleconference, a consolidated list of queries and, in some cases, requests for reanalyses was sent to NMI, Melbourne. Further scrutiny was done, including of repeated analytical results, until all scrutineers were satisfied with the reliability of the analytical data.

2.11. Statistical analyses

For each loin chop variable a three-way analysis of variance was performed; 3 States × 2 types (lean/fat) × 2 cooking categories (cooked/raw). A split plot-type analysis was performed as each of the six States × replicates was regarded as a main plot and was split for the four types × cooking categories. Variation between duplicate (or triplicate) readings was obtained as a final split within the analysis, which enabled the reliability of the analytical methods to be assessed. For moisture, fat, protein, ash, total N, thiamin, niacin, vitamin B_6, vitamin B_{12}, Se, Zn, Na, K and Mg, measurements on lean and fat samples were analysed separately by two-way analyses of variance, as the values for lean and fat were distinctly different with the type with lower values having lower variability, making it inappropriate to use the single analysis. In all cases the key assumptions of analysis of variance, namely homogeneity of variation across the range of values and normally distributed error terms, were checked and found to hold, so no variables required transformation before analysis. The package GenStat for Windows (2007) was used for these analyses.

As composites for all the other cuts were formed over all replicate samples, no estimates of variability could be obtained and results are expressed as means over duplicate (or triplicate) determinations.

3. Results and discussion

3.1. Performance of analytical methods

Results of quality assurance procedures as shown in Table 1, were rated as satisfactory overall.

The analytical variability for individual methods is demonstrated in Table 2 which shows that confidence can be placed in the reliability of most of the analytical methods; high variance ratios and low *P*-values indicate that intra-sample variance is much lower than inter-sample variance. *Fat:* It had been decided to use the Soxhlet method as this is a standard method for fat in meat. It is comparable with the Mojonnier acid hydrolysis method for meat (Mills, van den Voort, & Usborne, 1983) therefore comparing results with those obtained in the 1980s (Hutchison et al., 1987, acid hydrolysis) and the 1990s (Barnes et al., 1996, Soxhlet) is valid. *Retinol*: Table 2 shows poor results for analytical variability for retinol analysis (CVs 27–29%). *Vitamin E:* Initial analyses on loin chop produced data that were below the level of reporting for this nutrient, and it was therefore decided not to do further analyses on the other cuts. Subsequently the analyses were repeated on stored samples (where available) by NMI using newly acquired improved instrumentation. Single (not duplicate analyses) were performed and indicative results were provided. While method performance cannot be assessed from these vitamin E analytical data, the results have been included in Tables 3 and 5. *Folate:* Total folate is notoriously difficult to measure in foods (Hyun & Tamura, 2005). The analytical method used in this study for total folate performed well in terms of precision with a low coefficient of variation at 0.3%. Recoveries from the pig liver reference material were moderately high at about 68% and also of folic acid (measured as total folate) from pork lean tissue at 66%. Results reported are those from the internationally accepted tri-enzyme technique (Tamura et al., 1997) with an additional lipase treatment (Vishnumohan & Arcot, 2006). *Vitamin B_{12}:* As this is a method developed for analysis of serum not foods (Anderson & Sourial, 1983), poor precision for these food samples was not unexpected. *Pantothenic acid*: The variance ratio was disappointingly and unaccountably low.

3.2. Variability study of pork loin chop

The analysis of two composites per State for loin chop enabled testing for differences between States, between cooked and raw, between lean and fat, and the interactions between these factors. For all nutrients, the majority of significant differences were between cooked and raw, and between lean and fat. There were some significant differences between the States and a few significant interactions, however these were not readily interpretable and the differences were generally much less than those for cooked vs. raw and lean vs. fat. Accordingly, means and standard deviations were calculated for nutrients in raw lean, raw fat, cooked lean and cooked fat, for this cut, across the six composite samples (3 Australian States × 2 replicates) (Table 3). It must be borne in mind

Table 1
Quality assurance results for nutrient analyses of pork.

Nutrient	Units	Limit of reporting	Difference between test sample duplicates (%)	Recovery of pure standard (%)	Reference/control materials	Certificate value/control value for reference/control materials	Analysed values for reference/control materials
Moisture	g/100 g	0.2 g/100 g	<1	NA	Flour check sample, batch 0706 RACI	12.0 g	11.1–12.8
Total solids	g/100 g	0.2 g/100 g	NA	NA	NIST 1546	40.5 ± 2.6	38.3–41.1
Protein	g/100 g	0.2 g/100 g	<1–1.6	Tyrosine 99.3–99.9	(ii) NIST1546 (N × 6.25)	14.9 ± 1.0	14.0–14.5
					(i) Flour check sample, batch 0706 RACI (N × 5.7)	11.9	11.9–12.0
Fat	g/100 g	0.2 g/100 g	<1–8	NA	(i) NIST1546 (solvent extraction)	20.7 ± 2.1	21.5 – 21.6
			NA	NA	(ii) NIST 1546 (sum of fatty acids)	19.7 ± 2.1	19.9 – 20.6
Ash	g/100 g	0.2 g/100 g	0–7	NA	(i) NIST 1546	3.21 ± 0.21	3.1–3.2
					(ii) Flour Check Sample, batch 0706 RACI	0.53	0.47–0.58
Thiamin	mg/kg	0.25	0–7	99–117	(i) NIST 1546	2.16 ± 0.44 (B1.HCl)	2.7–3.5
					(ii) Homogenised wheat breakfast cereal (in-house)	3.2	2.3–4.1
Riboflavin	mg/kg	0.5	0–7	96–108	(i) NIST 1546	2.0 ± 0.59	1.9–2.4
					(ii) Homogenised wheat breakfast cereal (in-house)	2.0	1.9–2.4
Niacin	mg/kg	50	0–8	82–105	(i) NIST 1546	36.3 ± 3.8 mg/kg	31–38
					(ii) Homogenised wheat breakfast cereal (in-house)	20	18.3–24.0
Vitamin B6	mg/kg	0.2	0–9	82–118	(i) NIST 1546	1.3 ± 0.61	0.8
					(ii) Homogenised infant formula powder (in-house)	0.54	0.42–0.71
Folate	μg/100 g	1	0–12.5	66–67 (Tri-enzyme) 85–89 (Tetra-enzyme)	SRM 487, Pig liver (dry matter)	1335 ± 198 (without lipase)	811 ± 110 (without lipase) 1122 ± 98.7(with lipase)
Vitamin B_{12}	mg/kg	0.34	<20	95–105	Meat extract (in-house control)	6.74 ± 1.56 (2SD)	5.96–7.5
Pantothenic Acid	mg/kg	10	<20	NA	Milk powder (in-house control)	37.0 + 5.0	Within stated limits of acceptability
Retinol	mg/kg	0.05	10	80–94	(i) NIST 1846	5.84 ± 0.68	5.4
					(ii) Homogenised "Milo" (in-house)	590	520–640
Carotenoids	mg/kg	0.05		71–86	NA	NA	NA
Vitamin E	mg/kg	1			NIST 1846	271 ± 25	260
Sodium	mg/kg	10	0–9	85–112	(i) NIST 1546	9990 ± 716	9890
					(ii) AGAL-4 Bovine liver	2236 ± 161	1856–2079
					(iii) AGAL-7 Wheat flour	61 ± 2.4	62
Potassium	mg/kg	10	0–17	80–111	(i) NIST 1546	2460 + 160	2410
					(ii) AGAL-4 Bovine liver	9616 ± 1277	8174–9039
					(iii) AGAL-7 Wheat flour	1602 ± 86	1618–1666
Magnesium	mg/kg	1	0–18	99–109	(i) NIST 1546	165 + 12	173
					(ii) AGAL-4 Bovine liver	609 ± 52	536–591
					(iii) AGAL-7 Wheat flour	425 ± 10	400–417
Manganese	mg/kg	0.01	0–18	99–110	(i) NIST 1546	0.292 + 0.044	0.26
					(ii) AGAL-4 Bovine liver	9.88 ± 0.86	8.50–9.39
					(iii) AGAL-7 Wheat flour	14.9 ± 0.6	13.3–14.5
Copper	mg/kg	0.2	0–24	83–109	(i) NIST 1546	0.93 + 0.18	0.7
					(ii) AGAL-4 Bovine liver	149 ± 10	131–155
					(iii) AGAL-7 Wheat flour	4.00 ± 0.37	3.60–4.32
Zinc	mg/kg	0.5	0–11	84–118	(i) NIST 1546	18.5 + 1.6	18.3
					(ii) AGAL-4 Bovine liver	157 ± 11	146–152
					(iii) AGAL-7 Wheat flour	10.0 ± 0.4	9.9–10.2
Selenium	mg/kg	0.01	0–27	89–118	(i) AGAL-4 Bovine liver	1.55 ± 0.23	1.16–1.47
					(ii) AGAL-7 Wheat flour	0.56 ± 0.093	0.45–0.46
Calcium	mg/kg	1	0–30	101–110	(i) NIST 1546	323 ± 28	349
					(ii) AGAL-4 Bovine liver	164 ± 21	138–166
					(iii) AGAL-7 Wheat flour	188 ± 7	203–209
Iron	mg/kg	1	0–27	84–115	(i) NIST 1546	11.4 ± 1.0	9.7
					(ii) AGAL-4 Bovine liver	428 ± 32	389–458
					(iii) AGAL-7 Wheat flour	40 ± 3	32–35
Fluoride	mg/kg	0.2	0	86–97	GBW 07602 Bush branches & leaves	24 ± 3	20–22
Iodine	mg/kg	0.01	0–34	96–111	(i) BCR 186 Pig kidney	0.145 (non certified)	0.155–0.171
					(ii) AGAL-3 Prawn tissue	1.62 (non certified)	1.57

Key: NA = not applicable/not available.
NIST 1546, NIST SRM 1546, meat homogenate. National Institutes of Standards and Technology, Gaithersberg, MD, USA.
NIST 1846, NIST SRM 1846, infant formula (milk-based). National Institutes of Standards and Technology, Gaithersberg, MD, USA.

Table 1 (*continued*)

AGAL-4, Bovine liver. The elemental composition was certified from results obtained in interlaboratory round-robin comparison organised by The Australian Government Analytical Laboratories (now National Measurement Institute).
AGAL-7, wheat flour. The elemental composition was certified from results obtained in interlaboratory round-robin comparison organised by The Australian Government Analytical Laboratories (now National Measurement Institute).
AGAL-3, prawn tissue. The elemental composition was certified from results obtained in interlaboratory round-robin comparison organised by The Australian Government Analytical Laboratories (now National Measurement Institute) 0706 RACI, Flour Check Sample, batch 0706. Royal Australian Chemical Institute, Australia.
GBW 07602, bush branches and leaves. Institute of Geophysical and Geochemical Exploration Office of Certified Reference Materials, China.
BCR 186, trace elements in lyophilised Pig kidney. European Commission Joint Research Centre, Institute for Reference Materials and Measurements, Belgium.
BCR 487, lyophilised pig liver. European Commission Joint Research Centre, Institute for Reference Materials and Measurements, Belgium.

Table 2
Analytical variability of measured variables for pork loin chop.

Variable	Variance ratio[a]	*p*	Between duplicate variation significantly less than between sample variation
Moisture	25.08	<0.001	✓
Fat	37.84	<0.001	✓
Protein	17.99	<0.001	✓
Ash	19.76	<0.001	✓
Total N	18.12	<0.001	✓
Retinol	1.54	0.225	
Thiamin	55.67	<0.001	✓
Riboflavin	9.83	<0.001	✓
Niacin	7.40	<0.001	✓
Vitamin B_6	13.02	<0.001	✓
Pantothenic acid	1.31	0.283	
Vitamin B_{12}	1.42	0.235	
Folate[b]	2811.98	<0.001	✓
Se	14.49	<0.001	✓
Zn	3.78	0.004	✓
Na	3.81	0.004	✓
K	6.80	<0.001	✓
Mg	4.75	0.001	✓
Mn	10.01	<0.001	✓
Fe	8.65	<0.001	✓
Cu	3.63	0.006	✓
Ca	7.09	<0.001	✓
Iodine	3.10	0.014	✓
F-Total	93.67	<0.001	✓

[a] Variance ratio = variation between samples after removing effects of state, cooking and fat vs. lean, divided by variability between analytical duplicates. Higher values indicate more reliable analytical techniques.
[b] Triplicate analyses were performed for folate.

that these data reflect in part variability in method performance (particularly for fat tissue, which is difficult to handle in the laboratory) as well as any variability across States and replicate composites. Inspection of the standard deviations shows that those for fat were much greater than for lean, reflecting the difficulty of obtaining good homogenates for fat tissue (this is supported by calculations for the proximate analyses alone which showed that the CVs for lean samples ranged from 0.7 to 16.1, while those for fat samples ranged from 4.2 to 28.1). The standard deviations in Table 3 must be interpreted with caution as they reflect analysis of six composite samples, not analysis of individual pork chop samples, so do not represent true variation in nutrient composition of loin chop between States. This compromise was an understood condition of the sampling plan adopted (Müller et al., 2009).

3.2.1. Differences in nutrient composition within States for pork loin chop

Due to the fact that samples within each State were bulked to two composite samples to reduce the number of analyses for cost reasons, it is not possible to carry out statistical analyses which would indicate whether there were significant variation within States. Inspection of the data, however, indicated sufficient variability in some cases to demonstrate that it was wise to collect and analyse State duplicates, and that this feature should be part of future projects of this nature.

Table 3
Nutrient composition of loin chop (means ± SD for 6 national composites)/100 g.

Analyte	Raw lean Mean ± SD	Cooked lean Mean ± SD	Raw fat Mean ± SD	Cooked fat Mean ± SD
Moisture (g)	74.1 ± 0.7	63.3 ± 0.5	23.8 ± 4.9	21.3 ± 2.4
Fat (g)	1.75 ± 0.21	4.47 ± 0.38	69.2 ± 4.6	70.7 ± 3.1
Protein (g)	23.2 ± 0.8	30.6 ± 0.3	7.9 ± 1.6	8.7 ± 1.1
Ash (g)	1.22 ± 0.04	1.38 ± 0.24	0.36 ± 0.07	0.57 ± 0.14
Total N (g)	3.71 ± 0.12	4.89 ± 0.04	1.25 ± 0.25	1.39 ± 0.17
Energy (kJ)	459 ± 12	685 ± 14	2693 ± 153	2766 ± 111
α-Carotene (μg)	<5 [a]	<5	[b]	<5
β-Carotene (μg)	<5	<5	[b]	<5
Retinol (μg)	<2.5	<2.5	18 ± 4	17 ± 5
Vitamin E (mg)	0.06 ± 0.03	0.07 ± 0.04	0.34 ± 0.13	0.20 ± 0.05
Thiamin (mg)	0.76 ± 0.18	0.86 ± 0.21	0.17 ± 0.06	0.21 ± 0.06
Riboflavin (mg)	0.143 ± 0.032	0.147 ± 0.037	0.107 ± 0.043	0.114 ± 0.033
Niacin (mg)	6.83 ± 1.75	9.10 ± 1.89	1.60 ± 0.85	3.18 ± 0.88
Vitamin B_6 (mg)	0.34 ± 0.06	0.30 ± 0.04	0.06 [c]	0.07 ± 0.01
Pantothenic acid (mg)	0.79 ± 0.17	0.88 ± 0.16	0.34 ± 0.19	0.37 ± 0.06
Vitamin B_{12} (μg)	0.20 ± 0.05	0.24 ± 0.07	1.06 ± 0.18	1.07 ± 0.09
Folate[d] (μg)	42 ± 34	24 ± 23	14 ± 12	27 ± 30
Se (μg)	27.4 ± 5.7	35.2 ± 7.9	6.0 ± 1.4	7.3 ± 1.8
Zn (mg)	1.28 ± 0.07	1.78 ± 0.13	0.37 ± 0.10	0.43 ± 0.06
Na (mg)	52.4 ± 2.0	58.2 ± 1.8	24.4 ± 1.5	33.3 ± 3.6
K (mg)	426 ± 31	400 ± 20	104 ± 21	159 ± 19
Mg (mg)	25.8 ± 1.0	27.8 ± 0.8	6.0 ± 1.3	9.5 ± 1.2
Mn (μg)	7.6 ± 1.3	8.6 ± 1.6	4.3 ± 0.7	5.3 ± 0.6
Fe (mg)	0.46 ± 0.07	0.63 ± 0.04	0.29 ± 0.07	0.36 ± 0.12
Cu (μg)	48.2 ± 5.6	64.9 ± 9.6	31.8 ± 5.6	39.0 ± 5.6
Ca (mg)	10.6 ± 1.3	15.3 ± 2.6	15.0 ± 6.3	17.3 ± 5.2
I (μg)	0.74 ± 0.05	0.96 ± 0.08	0.71 ± 0.06	0.84 ± 0.24
F[e] (mg)	0.04 ± 0.02[e]	0.05 ± 0.03[e]	0.05 ± 0.03[e]	0.04 ± 0.02[e]

[a] Contains values below level of reporting.
[b] No data, limited sample.
[c] No SD calculated because $n = 2$.
[d] Calculated from means for tetra-enzyme treatment using individual replicates.
[e] Approximate only; includes samples (5 of 24) with levels below detection level (<0.02 mg/100 g) where 0.01 was used.

3.3. Summary of nutrient composition of pork loin chop

Inspection of Table 3 allows some comment to be made regarding the occurrence of nutrients in the lean and the fat portions of pork loin chop and their behaviour on cooking.

3.3.1. Proximate composition

Overall recoveries (sum of proximate components) ranged from 99.8 to 101.4 g/100 g, which can be regarded as satisfactory. Moisture was lost on cooking and other proximate components increased as a result of this loss, causing their concentration. Calculation of apparent retention of fat during cooking showed that there was a slight tendency for fat to move into the lean tissue during cooking. True retention of fat (Murphy et al., 1975) cannot be calculated as each individual cut was not weighed before and after cooking.

3.3.2. Fat-soluble vitamins

Carotenes: Neither α- nor β-carotene were detectable in fat or lean of loin chop. Therefore, analyses for these nutrients were not carried out in the other pork cuts studied. *Retinol*: This vitamin

Table 4
Proximate composition and energy content of 12 other pork cuts, raw and cooked (means for 2 national composites)/100 g.

Cut	Cooking method	Moisture (g)	Fat (g)	Protein (g)	Ash (g)	Total N (g)	Energy (kJ)
Loin steak or medallion	Dry-fried						
Lean, raw		75.5	1.6	24.1	1.2	3.86	469
Lean, cooked		67.7	3.5	29.5	1.3	4.70	631
Fat, raw		26.4	68.5	6.3	0.4	1.01	2642
Fat, cooked		30.8	55.5	14.9	0.8	2.38	2307
Fillet	Roast						
Lean, raw		75.2	1.1	23.1	1.4	3.70	433
Lean, cooked		68.9	2.2	28.5	1.5	4.56	566
Rump steak	Dry-fried						
Lean, raw		73.7	2.1	25.3	1.2	4.04	508
Lean, cooked		68.3	3.8	28.6	1.4	4.58	627
Fat, raw		27.1	66.4	8.6	0.5	1.38	2603
Fat, cooked		34.9	53.7	12.0	0.8	1.91	2190
Round steak	Dry-fried						
Lean, raw		75.6	1.1	22.1	1.4	3.54	416
Lean, cooked		69.3	1.7	29.4	1.5	4.70	563
Topside steak	Dry-fried						
Lean, raw		75.1	2.0	24.0	1.5	3.83	482
Lean, cooked		69.3	2.3	29.2	1.4	4.66	582
Silverside steak	Dry-fried						
Lean, raw		75.4	2.0	22.5	1.2	3.60	457
Lean, cooked		68.8	3.0	27.9	1.3	4.44	585
Diced pork	Dry-fried						
Raw		75.2	3.1	22.8	1.1	3.64	502
Cooked		65.7	3.1	31.3	1.5	5.01	647
Pork strips	Dry-fried						
Raw		75.4	2.3	22.4	1.2	3.58	466
Cooked		66.4	2.5	31.2	1.3	4.98	623
Pork mince	Dry-fried						
Raw		70.6	9.4	20.9	1.0	3.32	703
Cooked		64.8	10.2	25.9	1.1	4.14	818
Loin roast	Roast						
Lean, raw		74.3	2.2	23.3	1.2	3.72	478
Lean, cooked		61.4	8.6	31.9	1.2	5.10	861
Fat, raw		19.3	77.1	5.2	0.3	0.82	2941
Fat, cooked		26.1	67.0	8.8	0.4	1.40	2629
Round (mini) roast	Roast						
Lean, raw		75.5	1.1	23.7	1.3	3.78	444
Lean, cooked		64.5	2.5	34.7	1.4	5.55	682
Fat, raw		45.3	44.6	11.3	0.7	1.81	1842
Fat, cooked		41.4	45.5	16.3	0.9	2.60	1961
Scotch roast	Roast						
Lean, raw		72.6	7.6	20.6	1.0	3.29	631
Lean, cooked		58.9	12.0	30.6	1.2	4.88	964
Fat, raw		38.1	52.5	10.7	0.5	1.70	2124
Fat, cooked		39.8	50.5	12.2	0.6	1.94	2076

was only measured above the limit of detection in the fat portions of loin chop. Due to the indications of presence in the lean portions of loin chop it was decided that both lean and fat portions of the other cuts should be analysed for retinol. *Vitamin E*: Levels were higher in fat tissue than lean tissue, as expected.

3.3.3. Water-soluble vitamins

All water-soluble vitamins were at higher levels in lean tissue than fat tissue; an exception was vitamin B_{12} which was, unexpectedly, higher in fat tissue than in lean tissue. Water-soluble vitamins were well-retained in the loin chop by the cooking method employed. The exceptions were vitamin B_6 and folate, and to a lesser extent, riboflavin. *Folate*: the total folate data derived from the tri-enzyme method plus lipase treatment are very unusual. Animal tissues (apart from liver) are not usually considered to be significant sources of folate. There was a very high level of variation between samples and the four means presented in Table 3 were not significantly different; further the results are high in terms of existing recorded knowledge about total folate levels in pork. Noting the high reliability of the technique for measuring folate (Tables 1 and 2) it is possible that folate was distributed non-uniformly in the composite samples. These values in Table 3 for loin chop, adjusted for comparisons made with the tri-enzyme method alone on different samples as described in Section 2, would become in μg/100 g: raw lean, 35.5 ± 11.8; cooked lean, 44.3 ± 1.5; raw fat, 20.0 ± 8.0; cooked fat, 7.9 ± 3.4.

3.3.4. Minerals

Minerals tended to be at higher levels in lean tissue than fat tissue, except in the case of calcium. Minerals were reasonably well retained by cooking except for potassium. *Iron*: Iron levels were low when compared with earlier studies, and with pork loin values from other countries. Special attention was paid in data scrutiny and re-analyses for iron, together with inspection of the analytical quality control reports for this nutrient. Even though the method for iron used in this study, ICP/MS, was different from that used

in the earlier studies in the 1980s and 1990s (atomic absorption spectrophotometry (AAS)) no reason was found to reject the iron data reported in this study. Feeds are fortified to the level of 100 g iron/tonne (Gannon, pers. commun.) *Iodine:* Iodine levels were low and not detectable in some samples. Geographical location might have had some influence on iodine levels. Feeds are fortified to the level of 1 g per tonne (Gannon, pers. commun.) *Fluoride:* In some samples of loin chop, fluoride was not detectable. Levels would, to some extent, indicate water fluoridation practices in the different parts of the country. It should be noted, however, that due to industry distribution practices, pork may be retailed in a different State from where it was produced.

Table 5
Vitamin composition of 12 other pork cuts (means for 2 national composites)/100 g.

Cut	Retinol (μg)	Vitamin E (mg)[a]	Thiamin (mg)	Riboflavin (mg)	Niacin (mg)	Vitamin B_6 (mg)	Pantothenic acid (mg)	Vitamin B_{12} (μg)	Folate (adj)[b] (μg)
Loin steak/medallion									
Lean, raw	<5	0.21	0.84	<0.05	9.4	0.57	1.0	0.25	68 (57)
Lean, cooked	<5	0.26	0.82	<0.05	6.3	0.46	1.2	0.30	10 (8)
Fat, raw	30	ND[c]	0.21	<0.05	3.7	0.15	0.5	0.88	36 (11)
Fat, cooked	24	ND	0.39	<0.05	7.3	0.15	1.0	1.00	36 (11)
Fillet									
Lean, raw	<5	0.11	1.50	<0.05	9.7	0.73	1.2	0.26	38 (32)
Lean, cooked	<5	0.05	1.50	<0.05	10.7	0.63	1.0	0.25	52 (44)
Rump steak									
Lean, raw	<5	0.12	1.10	0.07	11.5	0.71	1.3	0.19	42 (35)
Lean, cooked	<5	0.18	1.10	0.07	5.3	0.60	1.4	0.35	32 (27)
Fat, raw	26	0.60	1.10	0.07	4.1	0.30	0.9	0.87	14 (4)
Fat, cooked	31	ND	0.52	<0.05	3.6	0.29	0.8	1.35	28 (8)
Round steak									
Lean, raw	<5	0.07	1.20	<0.05	9.7	0.68	0.8	0.17	49 (41)
Lean, cooked	<5	0.15	1.20	<0.05	10.4	0.59	1.4	0.40	30 (25)
Topside steak									
Lean, raw	<5	0.12	1.20	0.05	10.0	0.62	1.1	0.14	61 (51)
Lean, cooked	<5	0.14	1.25	0.07	4.4	0.57	1.2	0.59	51 (43)
Silverside steak									
Lean, raw	<5	0.20	0.96	0.07	11.0	0.51	1.1	0.46	61 (52)
Lean, cooked	<5	0.23	0.92	0.06	9.0	0.45	0.5	0.49	90 (76)
Diced pork									
Raw	<5	0.07	0.74	0.27	9.2	0.44	1.3	0.53	5 (4)
Cooked	<5	0.15	0.94	0.26	5.5	0.35	1.6	0.68	26 (22)
Pork strips									
Raw	<5	0.23	0.87	0.24	9.8	0.51	1.1	0.44	24 (20)
Cooked	<5	0.09	1.05	0.22	11.0	0.26	1.4	0.56	35 (30)
Pork mince									
Raw	5	0.16	0.72	0.16	7.0	0.41	0.8	0.54	11 (9)
Cooked	<5	0.05	0.70	0.10	10.7	0.42	1.2	0.64	12 (10)
Loin roast									
Lean, raw	<5	0.18	0.73	0.13	8.8	0.12	0.9	0.47	43 (36)
Lean, cooked	<5	0.10	0.72	0.15	10.0	0.17	1.0	0.41	18 (15)
Fat, raw	28	0.53	0.19	0.10	0.8	0.05	0.3	1.11	36 (11)
Fat, cooked	22	0.52	0.24	0.12	2.0	0.08	0.6	1.14	26 (8)
Round (mini) roast									
Lean, raw	<5	0.12	0.85	0.22	6.0	0.34	1.2	0.69	45 (38)
Lean, cooked	<5	0.06	0.86	0.31	4.5	0.30	1.4	0.73	2 (1.5)
Fat, raw	24	ND	0.33	0.17	4.1	0.18	ND	ND	10 (3)
Fat, cooked	19	ND	0.64	0.25	2.3	0.22	ND	ND	24 (7)
Scotch roast									
Lean, raw	<5	0.21	0.65	0.19	9.6	0.35	1.1	0.68	15 (12)
Lean, cooked	<5	0.08	0.52	0.28	8.1	0.24	1.8	0.87	13 (11)
Fat, raw	17	0.43	0.33	0.08	3.8	0.22	0.4	0.96	13 (4)
Fat, cooked	<5	0.15	0.37	0.14	4.2	0.14	1.1	0.95	11 (3)

[a] Single analytical samples only.
[b] Adjusted for comparisons made with the tri-enzyme method alone shown in parentheses.
[c] ND, not determined.

Table 6
Mineral composition of 12 other pork cuts (means for 2 national composites)/100 g.

Cut	Se	Zn	Na	K	Mg	Mn	Fe	Cu	Ca	F
	(μg)	(mg)	(mg)	(mg)	(mg)	(μg)	(mg)	(μg)	(mg)	(mg)
Loin steak/medallion										
Lean, raw	28	1.55	46	420	27	5.3	0.54	39	5.6	<0.02
Lean, cooked	34	1.85	52	455	29	7.2	0.63	51	6.1	<0.02
Fat, raw	6	0.49	23	150	8	3.9	0.48	23	4.9	<0.02
Fat, cooked	9	0.91	47	340	18	9.8	0.70	80	15.5	<0.02
Fillet										
Lean, raw	18	1.75	43	390	26	10.5	0.86	82	3.8	<0.02
Lean, cooked	24	2.40	44	390	27	12.5	0.99	100	4.2	<0.02
Rump steak										
Lean, raw	17	1.70	50	365	25	10.5	0.84	69	5.0	<0.02
Lean, cooked	24	2.40	52	380	26	10.5	0.94	93	5.5	<0.02
Fat, raw	5	0.47	23	140	8	5.1	0.60	33	3.3	<0.02
Fat, cooked	14	0.58	36	230	14	7.0	0.67	47	4.4	ND
Round steak										
Lean, raw	11	2.00	49	385	25	10.5	0.88	78	4.0	<0.02
Lean, cooked	13	2.90	51	390	25	13.0	1.10	105	4.7	<0.02
Topside steak										
Lean, raw	17	1.50	48	370	25	8.6	0.73	61	4.3	0.03
Lean, cooked	24	2.20	55	440	30	11.0	0.87	87	5.5	0.03
Silverside steak										
Lean, raw	25	1.85	53	410	26	8.0	0.79	63	4.5	<0.02
Lean, cooked	32	2.55	59	425	28	10.0	0.97	90	5.1	0.02
Diced pork										
Raw	19	2.40	59	465	27	8.9	0.84	76	5.9	<0.02
Cooked	29	3.75	90	465	30	12.0	1.15	115	7.3	<0.02
Pork strips										
Raw	27	2.05	47	385	26	7.8	0.75	72	4.6	<0.02
Cooked	38	2.65	58	470	33	9.8	0.90	97	5.1	<0.02
Pork mince										
Raw	14	2.30	58	360	21	9.3	1.00	67	7.8	0.22
Cooked	19	2.95	62	400	24	10.5	1.15	81	8.1	0.02
Loin roast										
Lean, raw	25	1.60	52	370	25	5.4	0.41	40	6.4	<0.02
Lean, cooked	44	2.55	57	340	25	6.3	0.52	64	7.2	<0.02
Fat, raw	4	0.22	27	70	4	2.7	0.17	23	3.8	<0.02
Fat, cooked	7	0.45	41	145	8	3.1	0.27	36	2.8	<0.02
Round (mini) roast										
Lean, raw	19	2.60	50	390	24	8.9	0.83	85	4.3	<0.02
Lean, cooked	29	3.70	55	435	27	10.0	1.35	110	5.5	<0.02
Fat, raw	7	0.94	38	200	12	4.9	0.64	37	4.9	<0.02
Fat, cooked	12	1.55	54	345	23	10.5	1.45	76	5.7	<0.02
Scotch roast										
Lean, raw	13	2.70	49	355	20	10.5	0.79	65	3.9	0.04
Lean, cooked	24	4.60	57	430	25	12.0	1.35	110	7.8	0.06
Fat, raw	6	1.05	30	190	10	4.9	0.56	36	8.6	0.10
Fat, cooked	8	1.45	41	280	15	7.6	0.88	54	9.0	0.08

Table 7
Comparison of nutrient values for pork, lean only, raw, from this study with those of the 1980s and 1990s/100 g.

Study		Protein g	Fat g	Energy (kJ)	Na mg	K mg	Fe mg	Mg mg	Zn mg	Thiamin mg	Riboflavin mg	Niacin mg
This study	All cuts	20.6–25.3	1.1–7.6	416–631	43–53	355–426	0.41–0.88	20–27	1.28–2.7	0.65–1.5	<0.05–0.22	6.0–11.5
	Loin chop	23.2	1.75	459	52.4	426	0.46	25.8	1.28	0.76	0.143	6.83
Barnes et al., (1996)	All cuts	19.5–23.4	1.1–3.1	417–476	45–73	370–430	0.6–1.3	21–27	1.5–3.6	0.66–1.2	0.09–0.3	4.1–8.0
	Loin chop	22.2	1.7	440	66	410	0.8	24	1.9	0.71	0.15	5.2
Hutchison et al. (1987)	All cuts	20.4–24.4	0.8–2.2	393–478	52–72	340–360	0.7–1.1	16–27	1.6–3.4	0.56–0.91	0.19–0.3	3.3–5.7
	Loin chop	23.1	1.7	393	72	350	0.7	16	1.7	0.85	0.2	5.7

3.4. Summary of representative nutrient study of 12 other pork cuts

3.4.1. Proximate composition

Results for proximate and energy composition are shown in Table 4. Total recoveries as% ranged from 100.2 to 104.0, reflecting a tendency towards over-extraction. Data examination revealed no consistent trend to over-extract particular proximate components, however, the higher recoveries were for fat tissue homogenates, which tend to be difficult to handle in the laboratory. Fat content of the lean section of all cuts was low (range 1.0–2.2 g/100 g) except for Scotch (neck) roast at 7.6 g/100 g, probably reflecting the difficulty in totally separating fat from lean tissue in this shoulder cut

which is comprised of several muscles with some intermuscular fat (Australian Pork Limited (APL), 2002). Mince, strips and diced, all being mixed lean and fat tissue, had slightly overall higher fat levels, ranging from 2.3 to 9.4 g/100 g. The method used for fat analysis in this study was Soxhlet extraction, as used in the study in the 1990s, but different from the acid hydrolysis method used in the 1980s. Since both methods are standard methods for meat, any variation in fat content is unlikely to be due to method changes. Since the separable fat section of pork has been reduced and the lean tissue increased, the energy content of pork cuts has been reduced because fat provides more kJ per gram than protein. Fat content of cooked lean ranged from 1.7 g/100 g for dry-fried round steak to 12.0 g/100 g for scotch roast. Lean-only cuts (fillet, round steak, topside steak, and silverside steak) appeared to lose little fat during cooking (from apparent nutrient retention data, not shown, calculated according to Murphy et al., 1975) while cuts with separable fat according to similar calculations tended to retain lipid in the lean tissue from the fat tissue during the cooking process.

3.4.2. Vitamin composition

Results for vitamin composition of 12 other pork cuts are given in Table 5. All water-soluble vitamins were at higher levels in the lean tissue than the fat tissue, as expected, but the reverse was true for vitamin B_{12} as was found for loin chop. The USDA food tables SR21 also give values for vitamin B_{12} of a similar distribution and order, i.e. 0.92 μg/100 g for vitamin B_{12} in pork fresh, separable fat, raw (NDB No: 10006); 0.67 μg/100 g for vitamin B_{12} in pork, fresh, composite of trimmed retail cuts (leg, loin, shoulder), separable lean only, raw (NDB No: 10002); and, 0.53 μg vitamin B_{12} in pork, fresh, loin, centre rib (chops or roasts), boneless, separable lean only, raw (NDB No: 10199) (USDA., 2009).

Thiamin levels appear to be high and well-retained on cooking. Riboflavin levels were low or undetectable in some cuts, notably those from some thinly sliced cuts. Retinol and vitamin E levels were higher in the fat tissue than the lean tissue of cuts, as anticipated. Vitamin E levels must be interpreted with caution as values are from single unreplicated analytical samples.

As for loin chop, total folate levels were highly variable. Results reported in Table 5 are those from the lipase treatment in addition to the tri-enzyme extraction with an adjacent column showing, in parentheses, the results adjusted according to comparative studies with the tri-enzyme method alone. According to Gannon (1991), folate levels could be expected to fluctuate widely in pig tissue depending on conditions. Folic acid is added to pig grower/finisher feeds at the level of 0.25 g per tonne of feed (Gannon, pers. commun.) The findings of folate in pork may reflect this fortification. However, this is a question that can only be resolved by controlled feeding studies and the application of a validated and reliable method for measuring folate in pork tissue. The reported results should be accepted as a guide to the presence of moderately high levels of total folate in pork lean tissue, but it would be inadvisable to promote lean pork as a good source of folate without further confirmatory research. Folate was not detectable in current Australian red meats (Williams, Droulez, Levy, & Stobaus, 2007) but those analytical samples were frozen not chilled so a true comparison cannot be made. The most recent release of the US nutrient database, SR21 (USDA, 2009) lists folate levels for fresh pork, derived from 2007 tri-enzyme method analyses, in the range 0–12 μg/100 g, while UK pork has been reported to contain folate at levels of 1–11 ug/100 g with data from 1995 (Food Standards Agency, 2008).

3.4.3. Mineral composition

Results for mineral composition of the 12 cuts are given in Table 6. The low iron levels of pork loin chop (Table 3) were confirmed by the data from the other cuts. Cuts from the loin area were the lowest in iron. The iron level of loin chop has fallen by about 50% since the 1980s and 1990s. It is suspected that this is due to current feeding regimes geared to producing a faster-growing, leaner carcase without any adjustment to the iron levels in vitamin and mineral premixes. Research is needed to identify feeding practices that could rectify these lower levels of iron in present-day Australian pork. They are lower than levels reported for UK pork (Food Standards Agency, 2008) and US pork (USDA, 2009). Other minerals appear to be unchanged since the 1980s. These results provide comprehensive new information for additional minerals, notably selenium, manganese, copper and fluoride, not previously analysed in Australian pork. These 12 cuts were not analysed for iodine, as levels in loin chops were very low so no further analyses were warranted.

3.5. Summary of comparisons with previous studies and current data from other countries

Previous Australian studies: Table 7 summarises the changes over time for the lean only, raw, of all cuts analysed in each study, and for loin chop only. As composition varies from muscle to muscle, the most appropriate comparison is for loin chop. The decline in iron over 20 years can be noted, with a possible trend to an increase in niacin. These trends are unlikely to be due to the method change for minerals from AAS to ICP/MS. For niacin, the method in this study was CZE/HPLC, not the colorimetric method utilising cyanogen bromide used in the 1980s and 1990s, and the observed increase may therefore be due to a more efficient analytical method.

Current data from other countries: Comparisons with data for fresh pork cuts from other countries are not explored in detail since such comparisons are hampered by the different cut names and butchering criteria, and by the lack of source information for the data given in national food composition tables. Experimental pig production studies are available but tend to focus more on lipid composition outcomes than the nutrients of interest in this study.

4. Conclusions

Present-day pork separable lean tissue continues to be low in fat, probably due to the stated objectives of the improved breeding, feeding and rearing practices implemented by the industry in Australia, and remains a good source of protein, and of thiamin and other vitamins. It would appear that previous studies underestimated the levels of water-soluble vitamins in edible pork since only the lean tissues were analysed. Research is needed to improve the estimates of folate levels in pork. Mineral levels have been maintained apart from those of iron, which appear to have declined. It is concluded that pork can no longer be promoted as a source of iron and that research is needed to optimise iron and other nutrient levels which may be affected by feeds in Australian pig production.

Acknowledgements

The authors thank Janet Stark, Sharon Kennedy, Keith Pitts and Steve Humphries of FSA for technical assistance, dissection and processing of meat samples and Mendi Sulentic for data entry and data verification. We are grateful to Zuraidah Nasution for technical assistance with folate analyses, AgriQuality North Melbourne for assistance with the pantothenic acid analyses, and PathWest, Perth, for assistance with the vitamin B_{12} analyses. This study was funded by Australian Pork Limited.

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acetate were obtained from Sigma–Aldrich (St. Louis, MO, USA). Toluene-α-thiol was from Fluka (St. Louis, MO, USA). Polyphenol standards were supplied as follows: gallic acid, protocatechuic acid, (+)-catechin, (−)-epicatechin, phloridzin, and hydroxycinnamic acids (*p*-coumaric acid, caffeic acid and chlorogenic acid), by Sigma (St. Louis, MO, USA); procyanidin B2 and quercetin glycosides (hyperin, avicularin and quercitrin) by Extrasynthèse (Genay, France), and reynoutrin was from Apin Chemicals (Abingdon, UK). Phloretin-2′-xyloglucoside, trimer C1 and tetramer were kindly furnished by Dr. Lea (Reading, UK). Water was purified with a Milli-Q system from Millipore (Bedford, MA, USA). Solvents were purchased from Panreac (Barcelona, Spain) and were of analytical or HPLC grade.

2.2. Samples

Five apple pomaces (1 kg each one) were obtained from local cider-making industries and six single-cultivar pomaces (0.5–0.6 kg each one) from experimental pressings, which characteristics are summarised in Table 1. To obtain the experimental pomaces, between 1.5 and 2.0 kg of healthy and ripe apples were milled (SPEC RC2000) and quickly pressed by means of a small hydraulic press (Hafico). The resulting pomaces were immediately packed in plastic bags and frozen at −20 °C. The whole process (milling, pressing and packing) took about 15 min. Mean moisture content of apple pomaces were between 70% and 76%. The samples were dried (oven-dried at 60 °C for 48 h) finely milled (sieve 0.5 mm) with a Cyclotek 1093 (Foss-Tekator) and kept protected from light and humidity until analysis.

2.3. Polyphenol extraction

Between 2 and 2.5 g of apple pomace powder were extracted with acetone:water (70:30) (3 × 15 mL) for 5 min in an ultrasonic bath at 20 °C. The extracts were pooled, centrifuged (10 min at 10 °C and 17,000*g*) and evaporated to dryness at 30 °C with a rotary evaporator. The residue was reconstituted in 10 ml of methanol, filtered through 0.22 μm PVDF membrane (Teknokroma, Barcelona, Spain) and analysed.

2.4. Analytical procedures

HPLC analysis was performed according to a previously validated method (Suárez, Palacios, Fraga, & Rodríguez, 2005), by means of a Waters system, equipped with a 717 automatic injector, a column oven (30 °C), two pumps (model 510), a diode array detector (model 2996), and Empower v.2.0 software data module. The interval for absorbance measurements was between 190 and 450 nm. Separation of polyphenols was carried out on a reversed-phase Nucleosil 120 C_{18} (250 × 4.6 mm I.D, 3 μm) column from Teknokroma (Barcelona, Spain). The elution solvents were aqueous 2% acetic acid (solvent A) and 100% methanol (solvent B). The samples were eluted according to the following gradient: a linear step from 0% to 45% of solvent B in 55 min, an isocratic step for 15 min and a final linear increase of solvent B to 55% in 10 min. Flow rate was 0.8 ml/min and the injection volume was 10 μl.

Quantification was done by the external standard method, using as standards (−)-epicatechin for flavanols, phloridzin for dihydrochalcones (λ = 280.0 nm), quercitrin for flavonol glycosides (λ = 350.0 nm), and *p*-coumaric acid (λ = 313.0 nm) and chlorogenic acid (λ_{max} = 326.3; sh. at 297.7 nm) for hydroxycinnamic acid derivatives.

Total phenol analyses were done by spectrophotometry by the Folin–Ciocalteu method, according to that described by the European Union Official Methods of Analysis (1998), by means of a Perkin–Elmer Lambda 35 equipped with an autosampler and a 1 cm optical quartz flow cell. The reaction is developed in 100 ml volumetric flasks, where the different reactants are added in this order: 1 ml of sample, 50 ml of water, 5 ml of Folin–Ciocalteu reagent, 20 ml of 20% sodium carbonate and water, to reach the final volume. The absorbance was measured at 750 nm, after 30 min at room temperature. The results are expressed as g gallic acid/kg of dry matter (DM).

2.5. Antioxidant capacity

2.5.1. DPPH assay

The antiradical activity was determined by spectrophotometry in 1 cm disposable plastic cells. 40 μL of sample or standard (diluted 1/10 with methanol, reaching a concentration range of 100–300 mg gallic acid/L) were added to 1.460 mL of DPPH$^{\bullet}$ solution (40 mg/L) in methanol, and left to stand in the dark (Brand-Williams, Cuvelier, & Berset, 1995). The absorbance at 515 nm was measured at the beginning ($A_{t=0}$) and after 240 min ($A_{t=240\ min}$), when the reaction reached the steady state. The inhibition percentage was calculated as follows:

$$\%IP = \frac{(A_{control} - A_{t=240\,min})}{A_{control}} \times 100$$

The antioxidant capacity was referred to as ascorbic acid (AA) equivalents (g AA/kg DM).

2.5.2. FRAP assay

The working FRAP reagent was prepared freshly every day by mixing 2.5 mL of TPTZ (10 mM in 40 mM hydrochloric acid), 2.5 mL of ferric chloride (20 mM) and 25 mL of sodium acetate buffer (300 mM, pH 3.6). The FRAP assay was carried out at 37 °C, in 1-cm disposable plastic cells. 900 μL of the FRAP reagent were mixed with 90 μL of water and 30 μL of apple pomace extract (diluted 1:25 with methanol). After 120 min, the absorbance at 595 nm was measured (Pulido, Bravo, & Saura-Calixto, 2000).

The antioxidant capacity of the apple pomaces were expressed as AA equivalents.

2.6. Thiolysis of the crude apple pomace and extracts

In order to confirm the structure of the unknown flavanols, the thiolysis reaction was applied both to the acetone extract and directly to the dried apple pomace, according to the procedure described elsewhere (Guyot, Marnet, Sanoner, & Drilleau, 2001). Briefly, between 50 and 100 mg of apple pomace or 500 μL of the pomace acetone:water extract were treated with acidic methanol (400 μL, HCl 3.3% v/v) and toluene-α-thiol (800 μL 5% in methanol), incubated at 40 °C for 30 min, cooled, filtered through a PVDF

Table 1
Technological descriptions of the apple pomaces.

Origin	Characteristics	References
Industrial pomaces	Mixture of Asturian apples, hydraulic press, and different degrees of exposure to air during the pressing process	M1, M3: 48 h M2: 10 h M4: 36 h
	Mixture of Asturian and foreign apples Pneumatic, Bucher–Guyer type press	G: 1.5 h
Single-cultivar pomaces	Limón Montés (acidic)	LM
	Meana (bittersharp)	M
	Durona de tresali (acidic, mild bitter)	DT
	De la Riega (mild sharp)	DR
	Perezosa (Sweet)	P
	Carrió (Mild sharp)	C

membrane and injected. The mean polymerisation degree (DPn) was calculated by the molar ratio between all the flavan-3-ols (benzylthioether adducts + terminal units) to the sum of terminal units ((+)-catechin and (−)-epicatechin)).

2.7. Statistical analyses

ANOVA, non-linear regression and Principal Component Analysis, taking a 95% confidence interval were performed by means of the SPSS v.11.0 statistical package for Windows. Multivariate partial least squares regression (PLSR) analyses were done with the PARVUS v.1.0 statistical package (Forina, Leardi, Armanino, & Lanteri, 1988).

3. Results and discussion

3.1. Phenolic profiles of cider apple pomaces

In Fig. 1 are shown two characteristic profiles of apple pomaces. As seen in Fig. 1A, major compounds correspond to dihydrochalcones (phloridzin and phloretin-2′-xyloglucoside, and others, probably 3-hydroxyphloretin), acids (protocatechuic, chlorogenic, caffeic and others), flavanols ((−)-epicatechin, procyanidin B2, trimer, tetramer, and others), and flavonols (hyperin, isoquercitrin, rutin, reynoutrin, avicularin, and quercitrin). At the beginning of both chromatograms (Fig. 1A and B), a series of four unknown compounds were observed. These components were not assigned

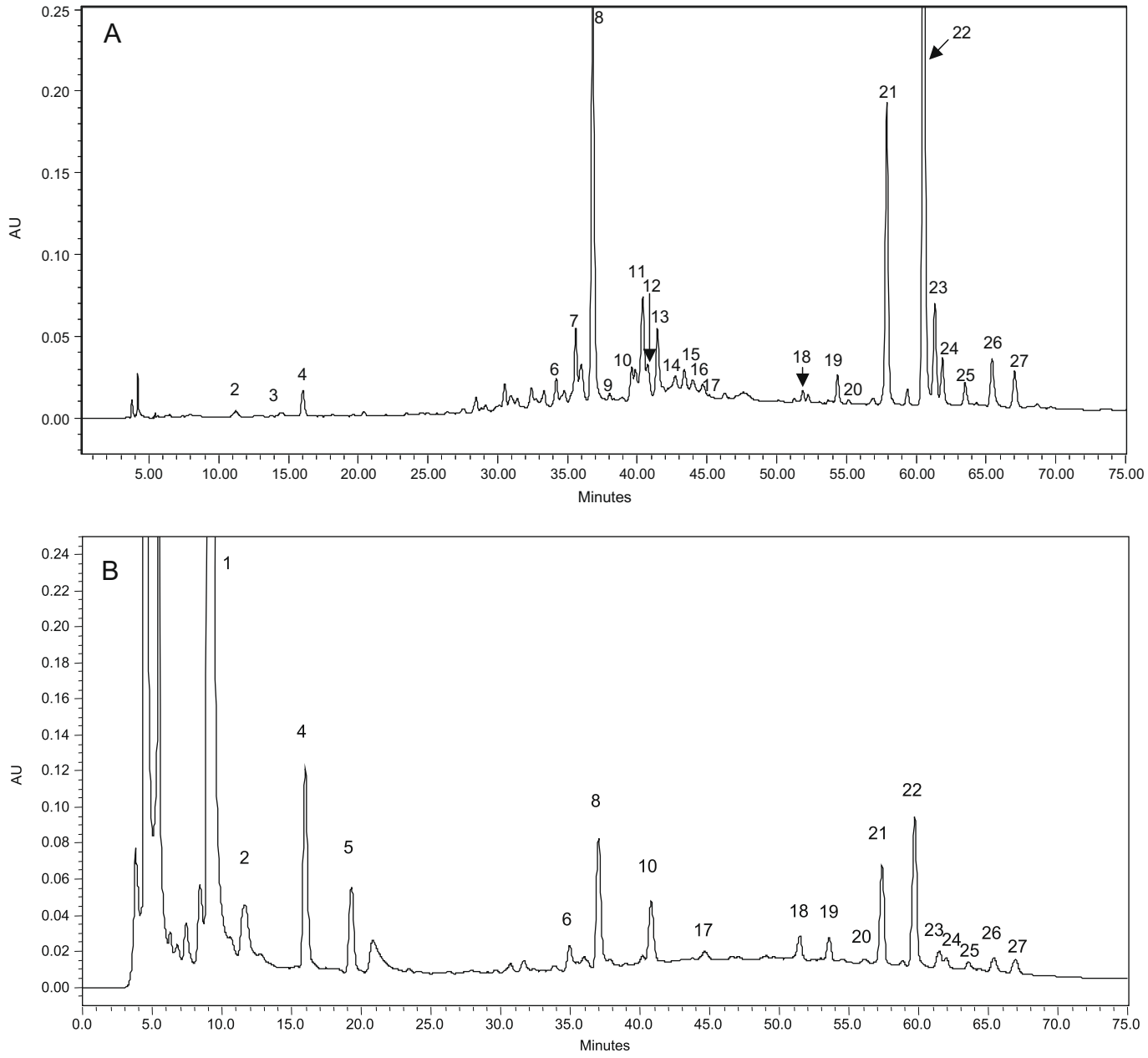

Fig. 1. Typical chromatogram at 280 nm of apple pomace (A: single-cultivar; B: industrial). Analytical conditions, see Section 2. Peaks: 1, Unk-1 (λ_{max} 268 nm); 2, Unk-2 (λ_{max} 270 nm); 3, Unk-3 (protocatechuic acid-type; λ_{max} 256 nm); 4, Unk-4 (λ_{max} 284 nm); 5, protocatechuic acid; 6, cinnamic acid-1 (λ_{max} 315 nm; sh. 283 nm); 7, procyanidin B2; 8, chlorogenic acid; 9, caffeic acid; 10, cinnamic acid-2 (λ_{max} 312 nm); 11, trimer + tetramer; 12, (−)-epicatechin; 13, cinnamic acid-3 (λ_{max} 312); 14–16, unknown flavanols; 17, cinnamic acid-4 (λ_{max} 312 nm); 18–20, unknown dihydrochalcones; 21, phloretin-2′-xyloglucoside; 22, phloridzin; 23, hyperin; 24, rutin + isoquercitrin; 25, reynoutrin; 26, avicularin; 27, quercitrin.

to any group because their UV-spectra did not correspond to any of the phenolic families analysed. The absorption maxima ranged between 256 and 284 nm, and their amounts were expressed in area $\times 10^{-6}$/kg DM.

The compounds tentatively identified as flavanols by spectra and by co-elution with the corresponding standards (procyanidin B2, trimer and tetramer, (−)-epicatechin) were confirmed as flavanols. Thus, one single-cultivar apple pomace, both the direct powder and the extract, were submitted to thiolysis, according to the method reported elsewhere (Guyot et al., 2001). As expected, the reaction with the thiol reactant gave rise to the disappearance of the peaks assigned to procyanidin B2, trimer + tetramer and other flavanols, by cleavage of the interflavanyl linkages, leading to the formation of the benzylthioether adducts at the end of the chromatogram, and the release of the terminal monomeric units ((+)-catechin and (−)-epicatechin), thus confirming these components as flavanols. No differences were observed between the treatments with toluene thiol of the dried apple pomace powder and the corresponding extract. The resulting mean polymerisation degree was close to 4 in both samples. The (−)-epicatechin monomer content was 4.5-fold higher than that of (+)-catechin.

3.1.1. Single-cultivar samples

Six single-cultivar pomaces from different technological groups were chosen among the cider apple varieties included in the Protected Designation of Origin "Cider from Asturias".

Significant differences were observed among varieties for all of the parameters analysed ($p < 0.05$). Folin index varied in this order: C > DR = M > DT = P > LM. The flavanol contents ranged between 1.7 and 2.5 g/kg. As shown in Table 2, Meana was the cultivar with the lowest amount of trimer + tetramer, and the highest in other flavanols, followed by De la Riega and Carrió. Phloridzin was always the main dihydrochalcone present in the apple pomaces, with contents ranging between 0.6 and 1.5 g/kg. Phloretin-2′-xyloglucoside was the second one, with contents in the range of 0.08 and 1.0 g/kg. Considering phenolic acids, their contents were between 0.5 and 1.6 g/kg, being chlorogenic acid the major one in all the cases. Chlorogenic acid contents varied in the order DR > C > DT > P = LM > M. Maximum content of caffeic acid was 25 mg/kg, and corresponded to DT cultivar. Protocatechuic acid was always a minor component, excepting in LM cultivar, with 144.7 mg/kg. Six quercetin glucosides were identified in the apple pomaces: hyperin, isoquercitrin, rutin, reynoutrin, avicularin and quercitrin, as described by others (Lu & Yeap Foo, 1997; Schieber, Hill, Conrad, Beifuss, & Carie, 2002; Sánchez Rabaneda et al, 2004). These components were present in high levels in apple pomaces, ranging between 0.4 and 1.4 g/kg, hyperin being the major one.

Comparing with previous results on single-cultivar apple pomaces (Ćetković et al., 2008), the samples reported here exhibited higher levels of polyphenols. Phenolic profiles are closely related to cultivar (Alonso-Salces et al., 2004; Mangas, Rodríguez, Suárez, Picinelli, & Dapena, 1999; Marks, Mullen, & Crozier, 2007), the cultural conditions of the apple trees (Chinnici et al., 2004; Lea & Beech, 1978; Veberic et al., 2005) and season (Lata, Przeradzka, & Bińkowska, 2005).

On a dry weight basis, it is possible to compare the phenolic contents of the cider apple varieties analysed in this paper with others. Thus, these Asturian cultivars present higher mean contents of (−)-epicatechin (232.4 mg/kg), chlorogenic acid (824.6 mg/kg) and phloridzin (913.1 mg/kg) than those from the Basque region, (respectively, 162.1, 409.0 and 21.0 mg/kg, as calculated from Alonso-Salces et al. (2004)). The concentrations of (−)-epicatechin in the Spanish cider varieties were remarkably lower

Table 2
Mean polyphenolic contents (mg/kg) and antioxidant capacity of single cultivar cider apple pomaces.

	P	M	DR	LM	C	DT
Folin (g gallic acid/kg)	6.2b	7.2c	7.7c	5.5a	10.9d	6.3b
TP_{HPLC} (mg/l)	4818.3	5910.0	5230.4	4672.5	5862.6	5198.5
DPPH (g AA/kg)	11.4a	11.1a	13.5c	12.4b	15.9d	11.1a
FRAP (g AA/kg)	9.5a	10.8a	10.7a	9.8a	13.8b	9.7a
Phenolic acids						
Protocatechuic	20.8c	nd	nd	144.7d	4.1b	1.7a
Chlorogenic	693.2b	393.2a	1415.5e	681.5b	927.2d	836.9c
Caffeic	nd	20.7 cd	16.8c	10.5b	17.3c	25.1 cd
Other acids	230.6c	109.7a	110.6ab	118.1b	278.3d	409.5e
Sum of phenolic acids	944.7	523.7	1542.9	954.7	1226.9	1273.3
Flavanols						
(−)-Epicatechin	394.9d	222.8c	314.6d	161.1b	163.0b	136.0a
Procyanidin B2	477.0b	590.2c	437.3b	329.1a	348.7a	553.9c
Trimer C1 + tetramer	525.3b	372.0a	576.0b	572.9b	585.0b	569.6b
Other flavanols	664.1b	1228.6e	944.3d	652.1b	772.0c	619.6a
Sum of flavanols	2061.2	2413,7	2272.1	1715.8	1868.7	1881.2
Dihydrochalcones						
Phloretin-2′-xyloglucoside	332.8d	996.2f	136.9b	82.9a	457.2e	228.2c
Phloridzin	797.3c	1435.4f	730.2b	587.2a	1053.0e	875.7d
Other dihydrochalcones	38.7c	104.0f	22.7b	18.0a	61.7e	51.0d
Sum of dihydrochalcones	1168.8	2535.7	889.7	688.2	1571.9	1154.9
Flavonols						
Hyperin	223.4b	186.7a	175.3a	377.1d	462.9e	252.5c
Isoquercitrin + rutin	136.0c	44.6a	63.3b	159.6d	208.9e	218.7f
Reynoutrin	61.7c	37.5b	16.8a	144.3f	104.4e	84.2d
Avicularin	129.5b	99.2a	168.1c	364.0f	242.9e	223.5d
Quercitrin	93.0b	69.0a	96.0b	252.0e	168.7d	103.8c
Sum of flavonols	643.6	436.9	525.6	1313.7	1195.0	889.2

Different letters in the same line indicate significant differences at $p < 0.05$.
TP_{HPLC}: Sum of HPLC polyphenols.
nd: Not detected.
For cultivar references, see Table 1.

Table 3
Mean polyphenolic contents (mg/kg) and antioxidant capacity of industrial cider apple pomaces.

	G	M1	M2	M3	M4
Folin (g gallic acid/Kg)	4.7a	4.0a	7.4b	3.9a	13.9c
TP_{HPLC} (mg/l)	2467.5	3652.4	4418.9	1045.9	2059.5
DPPH (g AA/kg)	8.2c	7.6b	12.5e	4.5a	10.3d
FRAP (g AA/kg)	8.2b	8.1b	11.9c	4.3a	12.1c
Phenolic acids					
Protocatechuic	108.9b	36.8a	93.6b	29.6a	263.9c
Chlorogenic	259.8b	586.7d	602.4d	96.1a	375.3c
Caffeic	10.6b	18.3d	21.4e	8.2a	17.2c
Other acids	84.3a	229.4b	609.0d	63.3a	328.8c
Sum of phenolic acids	463,5	871,2	1326,4	197,2	985,2
Flavanols					
(−)-Epicatechin	167.5b	nd	nd	nd	nd
Procyanidin B2	144.6c	92.1b	287.1d	nd	nd
Trimer C1 + tetramer	96.0c	1316.2e	748.7d	nd	49.7b
Other flavanols	153.1d	105.4c	305.5e	43.5a	64.5b
Sum of flavanols	561.3	1513.6	1341.3	43.5	114.2
Dihydrochalcones					
Phloretin-2′-xyloglucoside	172.8c	150.4b	351.4d	19.7a	174.3c
Phloridzin	451.6c	302.5b	594.7d	159.4a	292.5b
Other dihydrochalcones	58.5b	59.5b	115.8d	11.4a	101.0c
Sum of dihydrochalcones	682.8	512.3	1061.8	190.5	567.8
Flavonols					
Hyperin	189.2b	232.0d	207.5c	191.6b	112.6a
Isoquercitrin + rutin	102.8c	119.6d	137.3c	71.1b	57.3a
Reynoutrin	17.6a	76.3d	70.4c	67.6c	41.7b
Avicularin	255.2d	180.6c	162.8b	160.8b	90.5a
Quercitrin	186.3e	144.2d	109.9b	118.3c	87.1a
Sum of flavonols	751.1	752.7	687.9	609.4	389.2
Unknown compounds[1]					
Unk-1 (λ_{max} 268 nm)	250.5a	548.2a	505.6a	397.7a	19777.0b
Unk-2 (λ_{max} 260 nm)	nd	25.9b	53.4c	46.9bc	397.4d
Unk-3 (λ_{max} 256 nm)	8.4c	6.8b	10.3d	6.5b	nd
Unk-4 (λ_{max} 284 nm)	160.5c	130.1b	72.6a	189.1d	1061.0e

Different letters in the same line indicate significant differences at $p < 0.05$.
TP_{HPLC}: Sum of HPLC polyphenols.
nd: Not detected.
For sample references and characteristics, see Table 1.
[1] Expressed in Area $\times 10^{-6}$/kg DM.

than those found in the English cider cultivars (632.2 mg/kg, calculated from Marks et al. (2007)).

Regarding the not identified compounds, that referred to as Unk-4 was the major one, followed by Unk-3 and Unk-1 (Table 4). The order observed for compound Unk-4 was M ≈ P > DR > DT > C > LM. Likewise, cultivars M and P exhibited the highest amounts of compounds Unk-3 and Unk-1, whereas the compound referred to as Unk-2 was present in DT cultivar only (data not shown).

3.1.2. Industrial samples

In Table 3 are presented the results for the industrial apple pomaces. As seen, the sample referred to as M4 had the highest Folin index, followed by M2. There were significant differences among the samples for all the parameters analysed. Unlike the single-cultivar samples, in the industrial pomaces protocatechuic acid was present in all the cases; among the flavanols, (−)-epicatechin was absent in all the samples, except that referred to as G. Chlorogenic acid was predominant among the phenolic acids, and phloridzin was the main dihydrochalcone, as observed for single-cultivar samples. It is worth to note the unknown compound 1, which proportion in the sample referred to as M4 is remarkable (Table 3). This component was found to be the major one among the non identified phenols, followed by Unk-4 and Unk-2.

Comparing with the single-cultivar pomaces, significant differences were found for many of the phenolic compounds analysed. No differences were observed in the cases of Folin index, caffeic and other acids, trimer + tetramer, other dihydrochalcones, most of the flavonols and the compound referred to as Unk-4 (Table 4).

Among the phenolic acids, chlorogenic acid content was significantly higher in the group of the single cultivars, whereas the industrial pomaces had higher concentrations of protocatechuic acid. Another difference between the two groups of pomaces were the contents in (−)-epicatechin procyanidin B2 and other flavanols, significantly lower in the industrial ones, or absent. Although not significantly different, the maximum concentration of trimer + tetramer observed in the industrial residues was nearly twice that of the single-cultivar pomaces (Table 4). Finally, the contents of dihydrochalcones were also lower in the industrial samples than in the experimental single cultivar ones.

The areas of the unknown compounds referred to as 1, 2 and 4 were significantly higher in the industrial pomaces than in the single cultivar samples, whereas the last ones had higher contents of compound referred to as 3 (Table 4). Even though chlorogenic acid, and in a lesser extent, flavanols and phloridzin are good substrates for enzymatic oxidation catalysed by polyphenol oxidases (Bernillon, Guyot, & Renard, 2004), and that the apple pomaces were exposed to different degrees of oxidation, we did not observed any trace of those compounds associated with phloridzin oxidation (λ_{max} = 430 nm), reported elsewhere (Le Guernevé, Sanoner, Drilleau, & Guyot, 2004). Instead, the unknown compounds analysed in the apple pomaces, which identification is still in progress, could be related to the colourless compounds resulting from the enzymatic oxidation of phloridzin by apple polyphenoloxidase at low pH values (Le Guernevé et al., 2004).

A data matrix was constructed with all the samples analysed and nine variables (protocatechuic acid, chlorogenic acid, (−)-epicatechin, procyanidin B2, other flavanols, phloretin-2′-xyloglucoside, phloridzin, hyperin and rutin + isoquercitrin), and a PCA was performed. Three significant components explaining the 84.17% of the variance were obtained. In Fig. 2 is shown the projection of samples and variables onto the plane formed by the first two principal components. From this figure, a data structurisation can be visualised, since the single-cultivar pomaces (referred to as 1) were placed on the right side of the first axe and the industrial ones (referred to as 2) were on the left side. Procyanidin B2, other flavanols and phloridzin had the highest score on the positive side of this axe, whereas hyperin and rutin + isoquercitrin had the highest score on the second axe.

3.2. Phenolic profiles and antioxidant activity of apple pomace

DPPH and FRAP assays are two of the most employed for antioxidant quantitation, due to their operating simplicity. The first one is usually performed by monitoring at 515 nm the changes of a mixture of the reactant and the antioxidant, until the absorbance is stable. The percentage of the DPPH remaining is proportional to the antioxidant concentration. The second one is typically run at 595 nm, taking 4 min as reaction time (Benzie & Strain, 1996).

One extract of apple pomace, properly diluted with methanol to reach a final range of total polyphenols between 30 and 300 mg gallic acid/L, was used to optimise the analytical methods for antioxidant activity.

For DPPH assay, as reaction time is a critical point of control of antioxidant activity assessment, the percentage of inhibition was monitored against time until not significant changes were observed. The steady stage of the inhibition reaction was obtained at 220 min, thus, a final time of 4 h was selected for further analyses. Subsequently, the antioxidant activity of three apple pomaces were determined five times within 1 day for repeatability, while reproducibility was examined in triplicate for three different days.

Table 4
Mean polyphenolic contents (mg/kg) and antioxidant capacity of the cider apple pomaces.

		Industrial				Single-cultivar			
		Mean	sd	Max	Min	Mean	sd	Max	Min
Folin (g gallic acid/kg)	ns	6.8	4.0	14.1	2.3	7.3	1.8	11.3	5.0
DPPH (g AA/kg)	**	6.6	2.8	12.8	4.4	12.6	1.8	16.0	10.7
FRAP (g AA/kg)	*	8.9	3.0	12.5	4.1	10.7	1.6	14.5	8.0
Phenolic acids									
Protocatechuic	**	106.6	88.2	268.5	28.2	28.6	54.0	147.4	nd
Chlorogenic	**	384.1	200.5	607.2	94.5	824.6	322.2	1463.2	383.4
Caffeic	ns	15.1	5.1	22.0	7.6	15.1	8.6	25.3	nd
Other acids	ns	262.9	205.8	633.3	62.2	209.5	113.8	412.8	106.5
Flavanols									
(−)-Epicatechin	**	33.5	69.4	169.6	nd	232.4	104.6	410.6	127.3
Procyanidin B2	**	104.8	110.6	295.6	nd	456.0	102.3	619.3	311.9
Trimer C1 + tetramer	ns	442.1	533.5	1352.3	nd	533.5	83.3	677.8	367.8
Other flavanols	**	134.4	96.8	310.0	42.2	813.6	221.6	1255.2	589.5
Dihydrochalcones									
Phloretin-2′-xyloglucoside	*	173.7	109.4	357.9	19.4	372.4	313.9	1020.7	79.4
Phloridzin	**	360.1	155.5	641.8	149.8	913.1	281.6	1476.6	560.2
Other dihydrochalcones	ns	69.2	38.0	117.2	10.9	49.4	29.6	108.4	16.8
Flavonols									
Hyperin	**	186.6	41.5	233.9	110.9	280.5	108.3	466.9	175.3
Isoquercitrin + rutin	*	97.6	30.8	139.7	57.0	139.5	67.1	227.6	43.6
Reynoutrin	ns	54.7	22.9	77.3	14.9	74.8	43.6	147.3	15.9
Avicularin	ns	169.9	54.6	258.0	80.2	204.5	89.6	375.7	97.5
Quercitrin	ns	129.2	35.2	189.7	86.3	130.4	64.2	256.4	67.6
Unknown compounds[1]									
Unk-1 (λ_{max} 268 nm)	*	4295.8	8014.1	20008.0	246.1	10.0	10.5	31.9	nd
Unk-2 (λ_{max} 260 nm)	*	104.7	153.2	404.7	nd	6.5	15.0	40.4	nd
Unk-3 (λ_{max} 256 nm)	**	6.4	3.6	11.0	nd	23.8	8.9	42.3	15.3
Unk-4 (λ_{max} 284 nm)	ns	322.7	384.2	1068.0	69.9	194.3	90.8	419.9	108.8

ns: Not significant.
nd: Not detected.
[1] Expressed in Area $\times 10^{-6}$/kg DM.
* Significant at the 5% level.
** Significant at the 1% level.

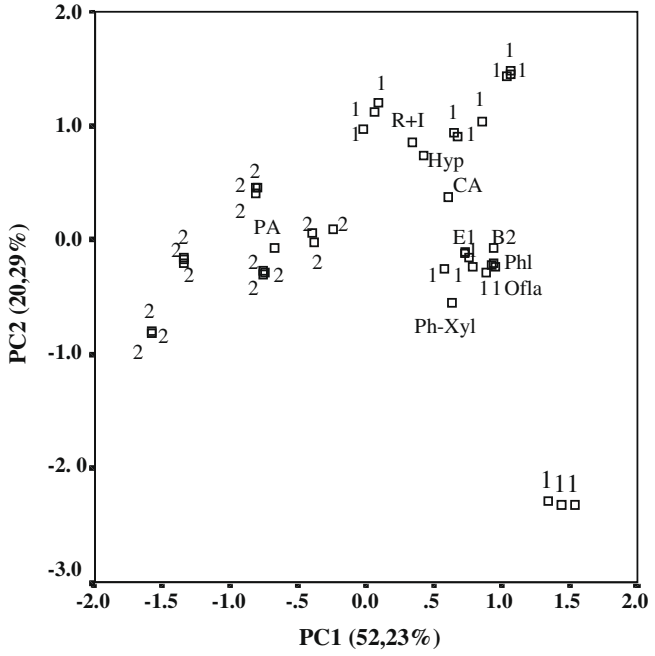

Fig. 2. Projection of samples and variables on the factorial plane formed by the first two principal components. Sample references: 1, single cultivars; 2, industrial apple pomaces. Abbreviations: CA, chlorogenic acid; B2, procyanidin B2; E, (−)-epicatechin; R + I, rutin + isoquercitrin; Ofla, other flavanols; Hyp, hyperin; PA, protocatechuic acid; Phl, phloridzin; Ph-Xyl, phloretin-2′-xyloglucoside.

The relative standard deviations in terms of ascorbic acid equivalents were, respectively, 2.6% and 5.4%.

Regarding the FRAP assay, most of the previous reports on the application of this assay for antioxidant measurements used a reaction time of 4 min, however, different time-dependent kinetic behaviours are observed when reaction times longer than 4 min are taken (Pulido et al., 2000). In the case of the apple pomace, the steady stage was not reached but the absorbance at 595 nm continuously increased. A final time of 120 min was chosen. Within and intra-day precision were calculated as described before. Mean values obtained for relative standard deviation were, respectively, 1.8% and 5.7%.

The antioxidant activities of cider apple pomaces ranged between 4.1 and 14.5 (FRAP assay), or 4.4–16.0 (DPPH assay) g ascorbic acid/kg DM (Table 4). Considering that this material is constituted by a 25% of dry matter, the presented values are equivalent to 1.0–4.0 g AA/kg of fresh weight (FW), Thus, comparing with previous studies, the apple pomace exhibited similar antioxidant activity than that of fresh apples (1.36 g AA/kg FW, reported by Kim et al. (2002), for Gala apples) or extracts of peels and pulps (1.25 g AA/kg FW for pulps, 3.3 g AA/kg FW for peels, as reported by Chinnici et al. (2004)), which confirms the suitability of apple pomace as a valuable material.

Among the single-cultivar pomaces, the antioxidant capacity determined by the DPPH assay showed this order: C > DR > LM > P = M = DT, whereas the results from the FRAP assay did not show significant differences among varieties (Table 2). Regarding the industrial samples, that referred to as M3 exhibited the lower antioxidant value. M2, exposed to air for 10 h, presented

the maximum value, followed by M4, aerated for 36 h (Table 3). The samples referred to as G (1.5 h pressing) and M1 (48 h pressing) had intermediate values for antioxidant capacity. Thus, no apparent relationship could be inferred from the present data between the pressing time, and therefore, the exposure of the pomaces to oxygen, and their antioxidant capacity.

Removing from the sample set those referred to as M, among the single-cultivars, and M4, among the industrial ones, highly significant correlations were observed between the antioxidant activity and several of the parameters analysed. Phloridzin (0.89; $p < 0.01$) and total phenols (0.85; $p < 0.01$) exhibited the highest values, followed by those of other flavanols (0.84; $p < 0.01$), chlorogenic acid (0.78; $p < 0.01$), procyanidin B2 (0.73; $p < 0.01$) and phloretin 2′-xyloglucoside (0.69; $p < 0.01$). Lower but still significant values for correlation were found for (−)-epicatechin, and for hyperin and rutin + isoquercitrin when using the DPPH assay. In general, the correlation values were higher when the antioxidant capacity was measured by the DPPH method, except for chlorogenic acid.

A multivariate partial least squares regression analysis was performed taking the antioxidant activity of the apple pomaces as dependent variables (A) and their phenolic profiles (X_n) as predictor ones. The linear models were constructed as:

$$A = b_0 + b_1X_1 + b_2X_2 + \cdots + b_nX_n$$

A final data matrix containing fourteen phenolic compounds (protocatechuic acid, chlorogenic acid, procyanidin B2, other flavanols, phloretin 2′-xyloglucoside, phloridzin, other chalcones, and flavonols) and all the samples analysed gave two models with three latent variables and good prediction ability. Values obtained for explained and cross-validated variances for prediction of the antioxidant activity as measured by the DPPH were 91.4% and 80.3%, respectively, and 84.4% and 72.2% in the FRAP assay. The variables with the higher modelling power were phloridzin > procyanidin B2 > rutin + isoquercitrin > protocatechuic acid > hyperin. All these compounds have been previously reported for their antioxidant activity (Chinnici et al., 2004; Nakamura, Watanabe, Miyake, Kohno, & Osawa, 2003; Natella, Nardini, Di Felice, & Scaccini, 1999; Tsao et al., 2005). It is interesting to note that phloridzin was the most relevant variable (even though its antioxidant activity is much lower than that of flavanols and flavonols), whereas chlorogenic acid, another major compound in cider apple pomace, with recognised antioxidant capacity (Moreira, Monteiro, Ribeiro-Alves, Donangelo, & Trugo, 2005) was not included in these models. In this sense, as quite long reaction times have been used in these assays, the influence on the antioxidant activity of less active compounds, as phloridzin, or more complex structures, as highly polymerised procyanidins may become important.

4. Conclusions

Apple pomace is a valuable source of polyphenols relevant for their antioxidant activity, as flavanols and flavonols. The antioxidant activity of apple pomace should be mainly related to apple cultivars, and it resulted to be equivalent to that previously reported for fresh apples. The apple pomaces coming from the cider-making industry exhibited lower, but still appreciable antioxidant activity than the single-cultivar samples considered in this study, which can be attributed to differences in their phenolic profiles. Both DPPH and FRAP assays, with optimised reaction times, allowed to perform reliable routine determinations of antioxidant capacity of apple pomace, and provided equivalent results. The antioxidant activity of apple pomace can be predicted by the contents of phloridzin, procyanidin B2, rutin + isoquercitrin, protocatechuic acid and hyperin.

Acknowledgements

Financial support for this work was provided by the National Institute of Agricultural of Research and Agro-Food Technology (INIA) and co-financed with ERDF and ESF funds (Project RTA2006-00-00069), the Regional Government of Asturias and the Town Hall of Villaviciosa for a grant (YDG).

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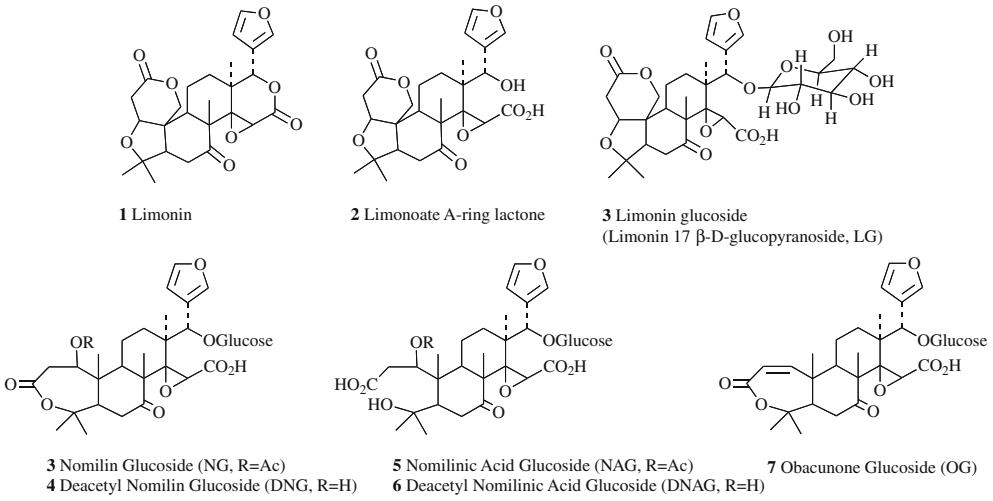

Fig. 1. Chemical structures of *Citrus* limonoids.

practice that chromatographic results from this method were less than satisfactory and ultimately traced the root cause of the deficiency to the lot-to-lot variability found among guard columns. Desiring to exploit the advantages that LC–MS affords in reduced sample preparation and sample analysis time while avoiding the potential pitfalls simultaneously, we have succeeded in generating an alternative chromatographic method based on a standard micro-bore HPLC column. Described within this report is the development of this new chromatographic method and its application as a screening method to rapidly determine the content and character of limonoid glucosides found in a variety of citrus derived samples.

2. Materials and methods

2.1. Materials

Solvents, HPLC grade acetonitrile and methanol, and formic acid (88%, ACS reagent grade) were purchased from Fisher Scientific Ltd. (Waltham, MA). Water was deionized to $\geqslant$18.1 MΩ/cm resistance using a Barnstead NANOpure Deionisation System (Dubuque, IA) and filtered through a 0.45 μm type HA membrane filter (Millipore, Billerica, MA) before use. Pure crystalline limonin glucoside was available in our laboratory from previous studies. Other limonoid glucosides used to establish chromatographic retention times were available as mixtures or obtained from extracts. Samples for this study were taken from our in-house limonin glucoside isolation efforts, with additional samples obtained from the USDA's A.H. Whitmore Foundation Farm located in Groveland, FL and from fruit purchased from local grocery stores.

2.2. Sample preparation

Juice samples or samples containing limonoid glucosides were clarified by centrifugation (14,000*g*, 5 min, 8 °C) and the resulting supernatant collected and filtered (0.45 μm, 25 mm GD/X filter, Whatman Inc., Clifton, NJ). Syringe filtration is not required for colorimetric determination, but is done as a precaution to prevent fouling of the HPLC column.

2.3. Estimation of total limonoid glucoside content

Before LC–MS analysis, the limonoid glucoside content of each sample was estimated by the colorimetric method of Breksa and Ibarra (2007) with the following modification: In place of the solid phase extraction step, samples (200 μL) were simply diluted with water (500 μL) and acetonitrile (300 μL) and analysed directly without further treatment. Samples having concentrations in excess of the calibration range were further diluted using 30% acetonitrile (aq) and reassessed.

2.4. HPLC apparatus for method development

A Waters HPLC system equipped with a Model 2695 Separations Module coupled to a Waters model 996 diode array detector (190–250 nm) (Milford, MA) and Sedex 55 ELS detector (50 °C, 2.5 bar N_2, S.E.D.E.R.E., Alfortville, France) was used for initial method development experiments. Instrument control and data acquisition were accomplished using Masslynx (Version 4.0) with a SAT/IN module used to capture the signal from the ELS detector. Initial experiments determined the effects of mobile phase acidity (formic acid 1–50 mM) on the chromatographic properties of limonin glucoside. Subsequent experiments used a sample containing all six limonoid glucosides to examine the effects of solvent polarity (acetonitrile 10–30%) and flow rate (0.2–0.5 mL/min) and were conducted using a Waters 2695 system controlled by Xcalibur (Version 1.4) and coupled to a TSP UV 2000 detector (l = 210 nm) and Thermo Finnigan LCQ Advantage Mass Spectrometer (San Jose, CA). Tuning of the mass spectrometer was accomplished in negative ion mode through optimisation on the limonin glucoside signal at *m/z* 649.3 generated by introduction of a limonin glucoside solution (5 ppm) into the mass spectrometer in the LC mobile phase at the flow rate used for analysis. Following tuning, the mass spectrometer was operated in the negative ion mode using a capillary temperature of 380 °C, spray voltage of 4.50 kV, and capillary voltage of 9.0 V. The mass spectrometer was set to acquire data over a mass range from 475–750 *m/z*. All method development experiments were conducted at 30 °C under isocratic conditions using a 50 × 2.0 mm Phenomenex Phenosphere-Next-5μ Phenyl column (Torrance, CA) equipped with a guard column of the same stationary phase. Injection volumes varied from 3 to 20 μL.

2.5. Screening method for evaluating limonoid glucoside content and character of samples

The screening method consists of two steps. First, the total limonoid glucoside content of each sample was estimated using the modified colorimetric method described above. Based on these results, samples were diluted with water to concentrations between 75 and 125 mg L^{-1} limonin glucoside equivalents. Next, the samples were analysed by LC–MS using the system and MS set-

tings described above and conducted as follows: Samples (10 μL) were injected into a 50 × 2.0 mm Phenosphere-Next-5μ Phenyl column equipped with a guard column of the same stationary phase maintained at 30 °C and flowing isocratically at a rate of 1.0 mL per min. The solvent composition was 15:85 acetonitrile: 4 mM formic acid, and the total run time was 4.0 min. The flow from the TSP UV 2000 detector was directed through an LC Packing's Acurate flow splitter (Sunnyvale, CA), so that only 1/5 of the flow was introduced into the mass spectrometer. Using the Xcalibur software package, mass traces for individual limonoid glucosides were extracted from the total ion chromatograms. Subsequent data analysis including identification of the limonoid glucosides present and integration of individual peak areas was accomplished using Xcalibur. For quantification of the limonoid glucosides, an approach commonly applied in metabolic profiling of arabidopsis plants was used (Nikiforova et al., 2005). On a per sample basis, raw peak areas of individual limonoid glucosides were normalised to the sum of the areas of the detected limonoid glucosides for each chromatogram and reported as a relative percent. Spike recovery experiments were conducted on some samples. In those experiments, samples were first diluted within the linear range of the LC–MS method, and then spikes of limonin glucoside were added at two concentrations (10 and 25 mg L^{-1}). To calculate the percent recovery, the observed concentration was divided by the expected concentration and resulting product multiplied by 100%. Spike recovery experiments were conducted in triplicate.

2.6. Evaluation of run-to-run and day-to-day variability

A set of eleven randomly chosen samples, including juice, extracts, and liquid samples of partially purified limonoid glucosides, were evaluated in triplicate on three consecutive days to evaluate inter-run and inter-day variability of the method.

3. Results and discussion

Initial experiments on the application of the phenyl stationary phase to the analysis of citrus limonoid glucosides were evaluated using an HPLC system coupled to an evaporative light scattering (ELS) detector. The choice of ELS detection during the method development stage was based upon recommendations found in the literature (Charlesworth, 1978; Webster & Diaz, 2002) and made by instrument manufacturers suggesting that ELS would be a less expensive alternative to a mass spectrometer during the development of LC–MS methods. A second reason for choosing ELS was to determine if ELS detection afforded any improvement over UV detection of limonoid glucosides since the ELS is mass dependent and not dependent on the presence of an appropriate chromophore. Limonin glucoside (LG) was evaluated first because it is the predominant limonoid glucoside found in most *Citrus* and was available in our laboratory from previous studies. Before starting these experiments, the ELS operating parameters of temperature and nebulizer pressure were optimised. Operating temperature (30–55 °C) and nebulizer pressure (1.9–2.5 bar N_2) were varied as 20 μL injections of LG (5 mg L^{-1}) were introduced into an aqueous solvent stream flowing into the detector at a rate of 500 μL per min. The best response (data not shown) was observed using an operating temperature of 50 °C in conjunction with a nitrogen pressure of 2.5 bar. Comparison of the ELS response to that of a UV (λ = 210 nm) detector over a range of LG concentrations (1–50 mg L^{-1}) revealed that ELS detection afforded at least a twofold increase in signal when the instrument's gain was set to its maximum level; however, in contrast to the linear response of the UV detector ($y = 8.896x + 37.373$, $R^2 = 0.987$), response of the ELS detector was parabolic and was fit using a polynomial equation ($y = 1.9032x^2 + 21.318x + 57.437$, $R^2 = 0.999$). From these experiments the instrument limit of quantitation for LG by ELS was estimated to be approximately 5 ng.

Limonoid glucosides contain one or two carboxylic acid groups and we anticipated that the pH of the mobile phase would affect retention time. Fig. 2 shows the effects of mobile phase acidity (formic acid 1–50 mM) on the retention of LG on the phenyl stationary phase. Retention time increased from 1.09 min to 2.14 min with increasing acidity of the mobile phase. Formic acid concentrations above 50 mM had no further effect on retention time, but did result in an increase in baseline noise from the ELS detector. Based upon the observed results, a formic acid concentration of 4 mM was selected for subsequent experiments examining the effects of solvent polarity and flow rate (0.2–0.5 mL min^{-1}).

For the next phase of the method development, the LC–MS system was used to analyse a sample containing all six limonoid glucosides. We wished to evaluate both methanol and acetonitrile as organic components of the mobile phase. A side-to-side comparison of chromatograms revealed that methanol runs were plagued with significant peak tailing (data not shown). We thus focused our evaluation exclusively on acetonitrile, varying its concentration stepwise from 10% to 30%. At the lowest acetonitrile concentration, the analytes were eluted as broad peaks barely discernable over the baseline and required a run time in excess of 10 min to complete their elution. Whereas when 30% acetonitrile was used, the analytes were not retained, but were immediately co-eluted. Ultimately, a mobile phase composition of 85:15, 4 mM formic acid:acetonitrile was chosen as the optimal compromise between analysis time and resolution. Changes in flow rate under the conditions examined affected neither peak shape nor resolution of the analytes.

Fig. 3 illustrates by mass spectrometry data the resolution and order of elution, such that quantitation of each of the six glucosides in a mixture may be accomplished without worry of isotope contribution. Of the limonoid glucosides present in the standard solution, DNAG was the first of the analytes to be eluted. Examining the structures of the six glucosides (Fig. 1), it is not surprising that DNAG was the first to be eluted considering that DNAG is the most polar of the six compounds. DNAG was followed by LG, DNG, NAG, NG, and finally OG. In contrast, the reported order of elution for the same glucosides from a C-18 stationary phase was LG followed by DNAG, DNG, NG, NAG, and finally OG (Herman et al., 1990; Ohta et al., 1993; Schoch et al., 2001). Thus, shifting from C-18 to a phenyl stationary phase, the elution order of two sets of compounds, LG and DNAG, and NG and NAG, are reversed.

The differences observed in elution order between the two stationary phases are the result of the modes in which each phase can interact with the analytes in solution. The long carbon chains of the C-18 phase are like long fingers that can interact with analytes with a significant degree of freedom in a number conformations, whereas the phenyl phase in contrast is much shorter, ending with a planar phenyl ring, which is likely constrained to interact directly with the furan ring of limonoid glucosides and perhaps the double bond in the A-ring of obacunone. The apparent contrast in the ability of individual phases to interact with analytes suggests that limonoid glucosides with open A-rings (NAG and DNAG) can have conformations in solution that allow additional interactions with a C-18 phase. These subtle differences in selectivity between the two phases may be useful in the isolation of limonoid glucosides.

Having established chromatographic conditions suitable to resolve limonoid glucosides we further optimised the method by reducing analysis time to maximize sample throughput. To reduce analysis time, we doubled the flow rate from 0.5 to 1.0 mL min^{-1} and found chromatographic resolution of the analytes unaffected by increased flow. Concurrent with the increase in flow rate, col-

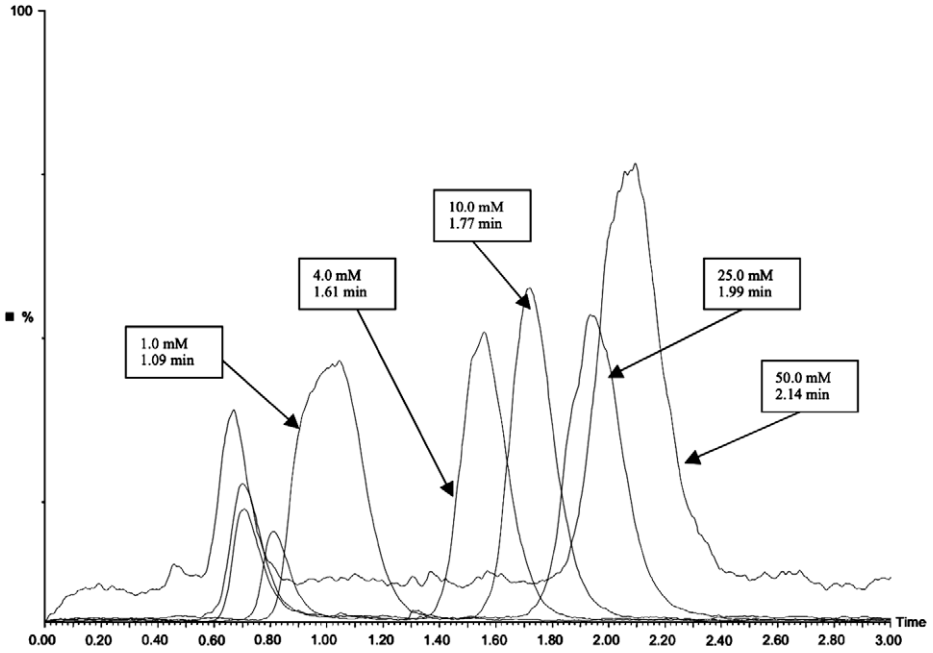

Fig. 2. Retention time of limonin glucoside on the phenyl stationary phase increases with increasing formic acid concentrations in the mobile phase. Experiments were conducted using a 25 mg L^{-1} solution of limonin glucoside and chromatograms monitored with an evaporating light scattering detector. Additional details are found in Section 2.

umn backpressure doubled and reached a value around 2200 psi. Although the LCQ Advantage mass spectrometer could handle a flow rate of 0.5 mL min^{-1}, it was overwhelmed by a flow of 1.0 mL min^{-1} so that it was necessary to introduce a flow splitter (1:5) prior to the mass spectrometer to accommodate the increased flow rate.

Utilizing the LC–MS conditions described above, response was confirmed to be linear over three orders of magnitude (1–150 mg L^{-1}; $R^2 > 0.98$) and the limit of quantitation was estimated to be near 2 ng. Both results are consistent with a previous report on the detection of limonoid glucosides by LC–MS (Schoch et al., 2001). Considering that limonoid glucoside concentrations in orange juice and citrus molasses typically range from 250 to 396 mg L^{-1} (Fong, Hasegawa, Herman, & Ou, 1990; Herman et al., 1990) and 470–7960 mg L^{-1} (Schoch et al., 2001), respectively, we were concerned that analyte concentrations in neat samples would be out of the linear range of the detector unless diluted. Therefore, before LC–MS analysis, the total limonoid glucoside content of each sample was estimated by the modified colorimetric method described in Section 2. After the concentration was determined, the sample was diluted with water to a total limonoid glucoside concentration between 50 and 100 mg L^{-1}. Since a single limonoid glucoside usually accounts for more than 50% of the total glucoside content of a given sample, this concentration range was targeted to ensure that the concentrations of the predominate glucosides would be within the linear range of the MS detector. A 5:1 dilution was sufficient for most juice samples, whereas for more concentrated samples (e.g., molasses or concentrated extracts) 50 to 1000-fold dilutions were required.

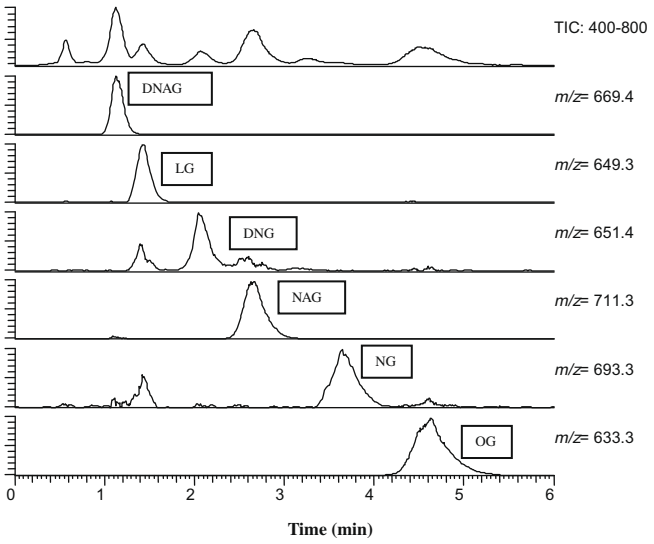

Fig. 3. Total ion and selective ion chromatograms resulting from the LC–MS analysis of a standard solution containing the six limonin glucosides using a Phenosphere-Next-5μ Phenyl column, 50 × 2.0 mm. Injection volume = 3.0 μl, column temperature of 30°, isocratic mobile phase of ACN:4.0 mM formic acid in water (15:85), flow rate = 0.5 mL min^{-1}.

Washington Navel, Hamlin and Valencia sweet oranges purchased from a local grocery store were hand squeezed, diluted 5:1 with water and analysed for their limonoid glucoside content. Results for the analysis of each of these samples along with the results previously reported for orange juices are listed in Table 1. Amongst the juices tested, Washington Navel and Valencia juices were the most similar and exhibited LG compositions close to those previously reported for orange juices. We have observed that NG is slowly converted to DNAG under the acidic conditions found in juice it is probable that its conversion into DNAG prior to the analysis may have been the cause for the reduced NG composition reported for the orange juices. The compositional differences between these samples and those for the Hamlin, Pera Rio and Natal samples suggest that in addition to harvest time (Fong et al., 1992), the limonoid glucoside contents found in *Citrus* fruits are also greatly influenced by variety.

Because we wished to apply this method on an ongoing basis and results were to be reported as relative percentages, we evaluated the inter-run and inter-day variability associated with the

Table 1
Limonoid glucoside contents found in common commercial varieties and previously reported values as relative percentage.

Limonoid glucoside ([M−H]$^-$ m/z)	LG (649)	NG (693)	DNG (651)	NAG (711)	DNAG (669)	OG (633)
Variety						
Washington Navel	53.6	26.2	0.8	10.6	8.1	0.7
Valencia	57.1	23.1	1.8	5.2	11.6	1.2
Hamlin	33.2	31.4	0.9	9.7	21.3	3.5
Previously reported						
Orange juice (Ave)[a]	56.3	nd	nd	nd	nd	nd
Orange juice (Ave)[b]	53.2	14.2	6.3	nd	25.4	0.9
Pera Rio orange[c]	82.8	5.7	0.0	1.1	9.2	1.1
Natal orange[c]	80.9	6.4	0.0	2.1	10.6	0.0

nd = not determined.
[a] Fong et al. (1990).
[b] Herman et al. (1990).
[c] Schoch et al. (2001).

method in order to determine if there were any limitations with this strategy. For this evaluation, a set of eleven randomly chosen samples, including juice, extracts and liquid samples of partially purified limonoid glucosides were evaluated in triplicate on three consecutive days. Each sample was diluted to within the linear range of the MS detector before analysis. Results from the intra-run evaluation are shown in Fig. 4. Variability was more heavily influenced by concentration rather than by limonoid identity. The more concentrated the analytes the smaller the variability. For limonoid glucosides that accounted for 10% or more of the normalised total, the observed %CV was typically less than 5%. As the percent composition decreased further, the %CV continued to increase. Results from the second two days of testing were similar (data not shown). Evaluating the results obtained across the three days, we found that the %CV increased by a factor of two or less for each of the samples tested and this result provided us with the confidence to move forward with our strategy to utilise relative concentrations as an alternative to reporting absolute concentrations and the need to run calibration curves on a daily basis. However, considering the variability observed, we adopted specific uncertainty levels (%CV = 5%, 10% and 20%) and recommend that these guidelines be used when evaluating results. For relative concentrations that were equal to or greater than 50%, a %CV of 5% was utilised (i.e., assigned uncertainty = relative concentration (%) × 0.05). A %CV of 10% was used for relative concentrations between 5 and 50% and for relative concentrations below 5% a %CV of 20% was applied.

As part of an ongoing project to characterise chemical phenotypes of genetic resources found within the USDA's A.H. Whitmore Foundation Farm, juice obtained from fruits from trees located within the collection were analysed. The UV (λ = 220 nm), total ion and selective ion monitoring (SIM) chromatograms obtained for two representative samples are shown in Fig. 5A–F. Detection of analytes by mass spectrometry can be hindered by matrix components, in particular by salts or other species in significant concentrations (e.g., sugars, organic acids) that are often not bound by reverse phase stationary phases. Thus with abbreviated chromatographic methods, such as this one and typical of many LC–MS methods, there is a need to resolve the majority of sample matrix components sufficiently from the analytes of interest. Comparison of the UV and SIM traces reveals that the limonoid glucosides are well-resolved from the unbound matrix components eluting in the void volume of the column. Spike recoveries observed after the addition of limonin glucoside (10 and 25 mg L^{-1}) to juice and other samples were observed to range from 95% to 103% and provide further support that, for at least limonin glucoside, the analytes are resolved from interfering matrix components.

The relative percentage concentration for each limonoid glucoside in these two representative samples was calculated as described above and in Section 2. Limonoid glucosides detected at greater than 1% for the first sample included (Fig. 5C) LG (14.2 ± 1.4%), NAG (74.0 ± 3.7%), and NG (10.2 ± 1.0%), whereas for the second sample (Fig. 5F) DNAG (19.6 ± 2.0%), LG (4.9 ± 1.0%), DNG (54.5 ± 2.7%), NAG (17.1 ± 1.7%), and NG (3.4 ± 0.7%) were detected. Although, the fruit samples came from two different trees that are members of a F1 population generated from a cross between *C. Grandis* and *Poncirus trifoliata* (Nakon × Flying Dragon) × *C. Sinensis* (Succari), the relative concentrations of the limonoid glucosides found in the samples is distinctly different. We are currently continuing our analysis of this population in

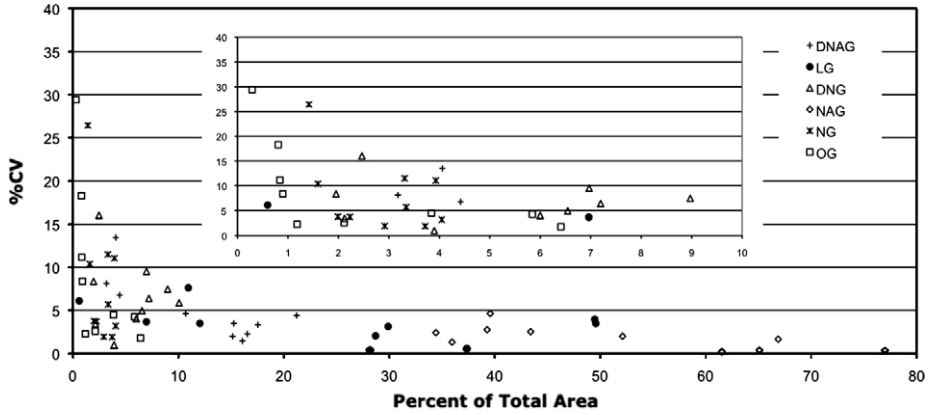

Fig. 4. Evaluation of intra-run variability: %CV and its relationship to percent total composition. Inset is a magnification of the same graph from 0% to 10%.

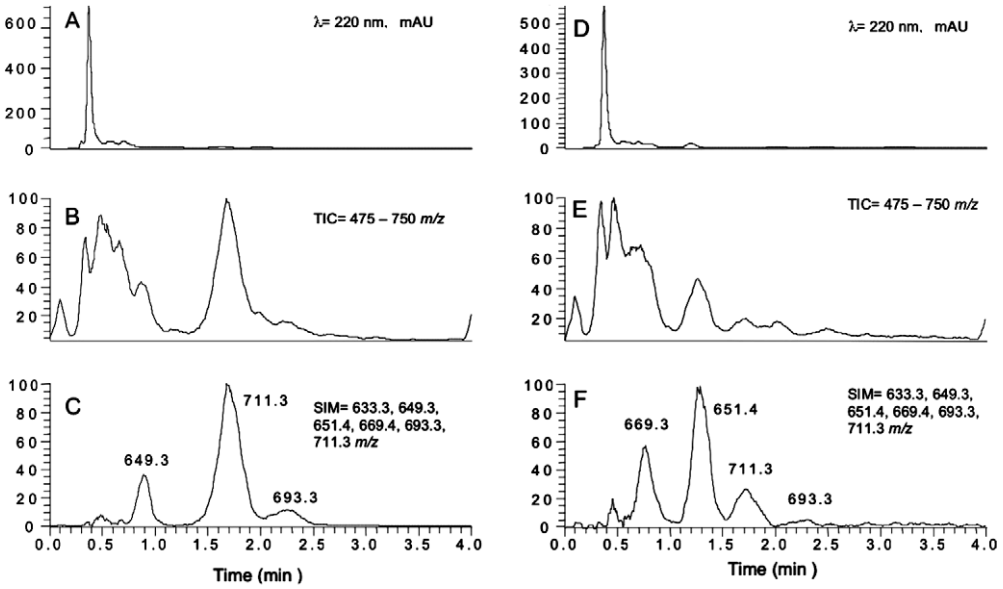

Fig. 5. UV (λ = 220 nm), total ion and selective ion monitoring (SIM) chromatograms obtained for two representative juice samples. Juice samples were obtained from two different trees that are members of a F1 population generated from a cross between *C. grandis* and *Poncirus trifoliata* (Nakon × Flying Dragon) × *C. sinensis* (Succari).

the hope that results from the analysis of their limonoid glucosides content and character will yield segregating populations that may be further examined by genomic tools in order to establish the genetic basis of the observed phenotypes.

Our goal was to develop a rapid and robust LC–MS method to characterise limonoid glucosides found in citrus juices, extracts, and fractions obtained from our isolation efforts. The majority of methods described thus far for the HPLC and LC–MS analysis of limonoid glucosides have relied upon C-18 stationary phases. In this report, we describe our evaluation of a phenyl stationary phase as an alternative to C-18 and show that, in addition to resolving the analytes of interest, the phenyl stationary phase, when compared to C-18 phases, also exhibits differences in selectivity that might further aid in isolating limonoid glucosides. Additionally, we demonstrate that the chromatography is rapid and robust, and, when paired with mass spectral detection, provides a method that is applicable to the analysis of samples having complex matrices. Furthermore, we believe that this method and its use, in conjunction with the colorimetric estimation of total limonoid glucoside concentration, will be of value to those with experience in the analysis of citrus limonoid glucosides and equally valuable to other researchers who do not have the means or resources to obtain analytical standards, but wish to evaluate citrus samples for their limonoid glucoside content and character.

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the prevention of cardiovascular diseases and hormone dependent cancers (Harris, Besselink, & Seeram, 2006; McCutcheon, Udani, & Brown, 2008; Seeram, Zhang, Reed, Krueger, & Vaya, 2006; Syed, Malik, Hadi, Schmitt, & Afaq, 2006). Although pomegranate and its juice are highly esteemed for their putative weak phytoestrogenic properties, the presence of substances responsible for such activities has not been confirmed and literature is mainly focused upon the efficacy of the flavonoidic fraction (Kim, Mehta, Yu, Neeman, & Livney, et al., 2002); moreover, the previously suggested presence of sexual hormones estrone or estradiol in *P. granatum* has been recently challenged (Choi, Kim, Choi, Jung & Kim et al., 2006; Heftaman & Bennett, 1966; Moneam, El Sharasky, & Badreldin 1988; van Elswijk, Schobel, Lansky, Irth, & van der Greef, 2004).

In this paper we report for the first time a complete description of lignan distribution in *P. granatum* juice, fruit parts (seeds, pulp, endocarp) and wood knots from different cultivars.

In this paper we report for the first time a complete description of lignan distribution in *P. granatum* juice, fruit parts (seeds, pulp, endocarp) and wood knots from different cultivars.

2. Materials and methods

2.1. Standards and reagents

Betulinol was purchased from Sigma–Aldrich; (+)-Pinoresinol was obtained from ArboNova (Turku, Finland); BSTFA (N,O-Bis trimethylsilyl-trifluoroacetamide) with 1% trimethylchlorosilane was purchased from Fluka. All solvents (analytical grade) have been obtained from Carlo Erba Reagenti (Milano, Italy).

2.2. Plant material

Pomegranate plant material was collected in September 2008. *P. granatum* L. var. Wonderful concentrated juice, its by-products (pulp, seeds, endocarp) and a concentrated juice from Tunisian pomegranates obtained from an experimental industrial process were kindly provided by Dr. Massimiliano Rinaldi, Dipartimento di Ingegneria Industriale, Università di Parma, Italy. Seeds and endocarp of the Tunisian variety were obtained by manual removing of the pericarp from the rest of the fruit, isolating the endocarp and subsequently carefully splitting the tegumental pulp surrounding the seeds. Wood knots were obtained from young branches of Italian pomegranate trees, following the manual procedure previously described for *Picea abies* (Piispanen, Willfor, & Saranpaa, 2008). Three different commercial pure pomegranate juices of different brands were purchased in local groceries and herbal shops in June 2008 and anonymised by means of numbering. Flax and sesame seeds utilised to confirm lignan identification were purchased in local herbal shops. Flax, sesame and pomegranate seeds, endocarp and wood knots were ground in a blade grinder and both the powders obtained and the juices were stored in the dark at −20 °C, until analysis.

2.3. Hydrolysis and extraction

Lignan glycosides were extracted as previously reported (Smeds, Eklund, Sjoholm, Willfor, & Nishibe et al., 2007); briefly, the plant materials (5 g, dry weight) or the juices (15 ml) were first delipidised under reflux with 100 ml *n*-hexane (1 h), then extracted with 100 ml acetone (1 h) and finally with 100 ml acetone–water (70:30, v/v, 1 h). The acetone and acetone–water fractions were then pooled and evaporated under vacuum to remove acetone. The aqueous residue (30 ml) was hydrolysed according to a protocol available in literature (Sicilia, Niemeyer, Honig, & Metzler, 2003), by adding 4 ml of HCl 6 N and heating for 1 h at 95 °C, in order to recover the lignan aglycones. After the addition of 0.1 ml betulinol (520 ppm, in acetone) as internal standard, the hydrolysed mixture was extracted three times with 10 ml of an ethyl acetate/*n*-hexane mixture (1:1, v/v). The organic phases were pooled, taken to dryness and then sylanized with 100 μl of BSTFA reagent (1 h, 60 °C) for the GC–MS analysis. The recovery of the ethyl acetate/n-hexane extraction was determined on pinoresinol, by directly injecting in GC–MS silylated pinoresinol and betulinol standards and the same standards subjected to the complete extraction procedure. The recovery resulted 90 ± 3%.

2.4. GC–MS

Gas chromatography–mass spectrometry (GC–MS) analysis was performed with an Agilent Technologies 6890 N gas chromatograph coupled to an 5973 N mass selective detector (Agilent Technologies, Santa Clara, CA, USA), under the following instrumental conditions: SLB-5MS capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA) with oven temperature programmed from 60 °C for 1 min, increased to 250 °C at 30 °C/min, kept at 250 °C for 10 min, increased to 280 °C at 1 °C/min, then kept at 280 °C for 13 min. Initial head pressure was 8.13 psi; injector temperature: 280 °C; injection mode: splitless 0.2 min; volume injected 1 μl; detector temperature: 280 °C; carrier gas: helium 5.0. MS conditions: ion source temperature: 230 °C; electron ionisation: 70 eV; acquisition mode: scan (*m/z* 50–750). The quantification of the lignans identified was performed utilising betulinol as internal standard, as previously reported (Willför, Ahotupa, Hemming, Reunanen, &, Eklund, 2003), integrating the total chromatografic area. The GC retention times of lignans were as follow: isolariciresinol 24.16 min; anhydrosecoisolariciresinol 24.74 min; secoisolariciresinol 25.44 min; pinoresinol 39.17 min; medioresinol 45.09 min; syringaresinol 51.62 min. Retention time of betulinol was 48.38 min. The GC–MS response factor, calculated in respect to pinoresinol, was 1.03 ± 0.02. The quantitative results are the mean value of three different extractions and three injections performed for each sample. It is important to highlight that the quantitative data reported represent an estimate, as the response factor was calculated only for pinoresinol and applied to all the other lignans.

2.5. Lignan identification

Because no mass spectra of silylated lignans were available in common commercial databases, lignans were identified by comparison with authentic reference compounds or utilising mass spectra previously reported in literature, and confirmed by analysing known sources of the specific lignans (Meagher, Beecher, Flanagan, & Li, 1999; Penalvo, Haajanen, Botting, & Adlercreutz, 2005; Sicilia et al., 2003; Smeds et al., 2007). The mass spectra of the silylated lignans identified in pomegranate samples are reported in Fig. 1. Pinoresinol was identified by comparing the retention time and the mass spectra with those of authentic standards. The fragment ion at *m/z* 209 is attributable to the stable tropylium ion structure substituted with $-OCH_3$ and –OTMS groups (Meagher et al., 1999; Sicilia et al., 2003). This ion is strong and is often the base peak in lignans. Medioresinol and syringaresinol can be considered substituted pinoresinols and molecular peaks at *m/z* 532 for medioresinol and 562 for syringaresinol, are in agreement with the addition, respectively, of one and two methoxy group to the pinoresinol structure (Penalvo et al., 2005). Moreover, the addition of a second methoxy group to the aromatic rings causes a shift of the ion *m/z* 209–239. This ion is present in medioresinol spectrum and becomes the base peak in syringaresinol. A further confirmation of the presence of medioresinol and syringaresinol in

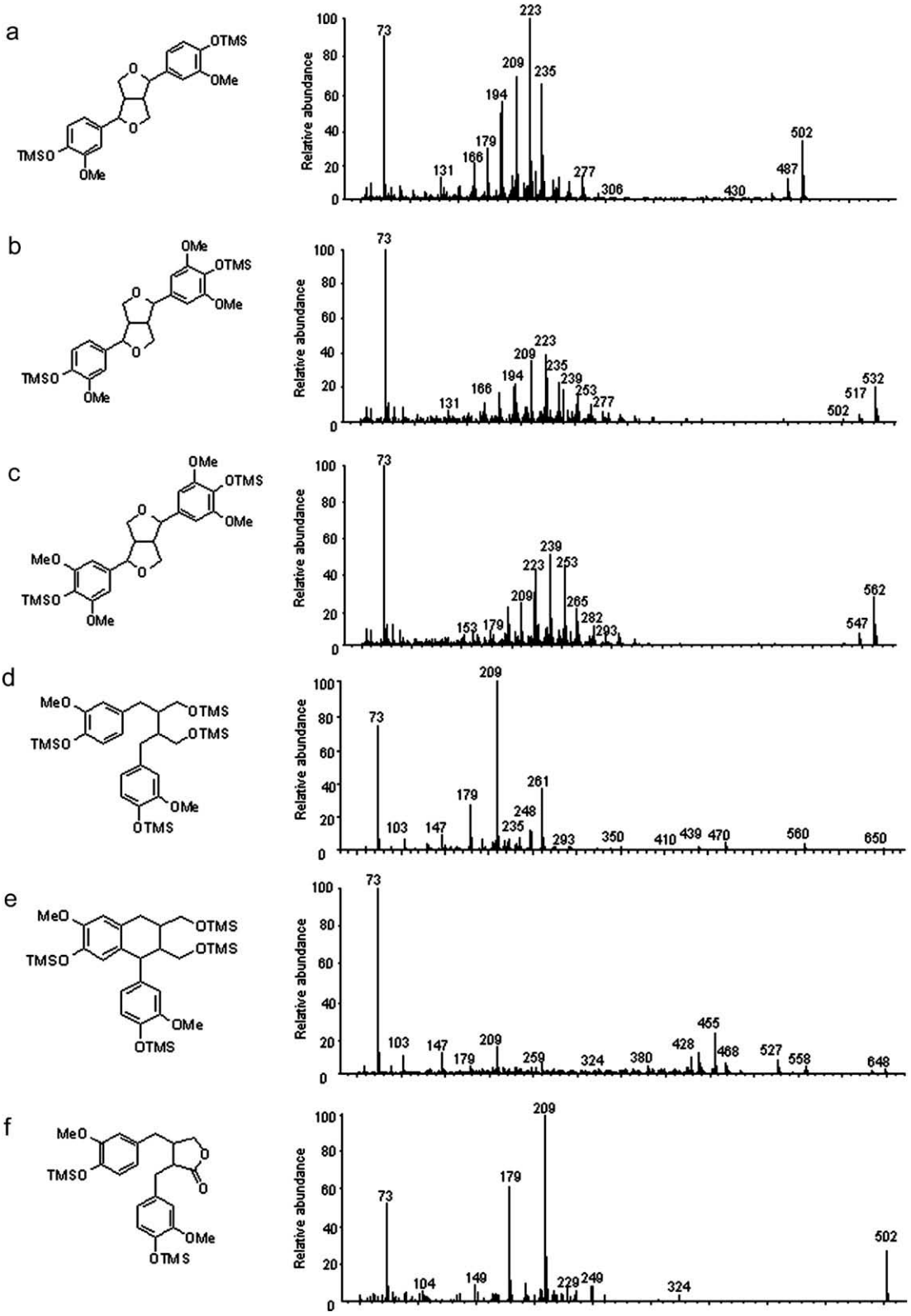

Fig. 1. Mass spectra of TMS derivatives of lignans characterised in pomegranate extracts: (a) pinoresinol, (b) medioresinol, (c) syringaresinol, (d) secoisolariciresinol, (e) isolariciresinol and (f) matairesinol.

pomegranate extract was obtained analysing in the same conditions the sesame seeds (*Sesamum indica* L.), a known source of these lignans (Smeds et al., 2007). Secoisolariciresinol spectrum is in agreement with those reported in literature for *Linum usitatissumum* (Sicilia et al., 2003). In most GC–MS chromatograms it was observed also a signal identified as anhydrosecoisolariciresinol, an artefact of the acid hydrolysis, resulting from the dehydration of secoisolariciresinol (Meagher et al., 1999). Matairesinol and

isolariciresinol spectra are in agreement with literature data (Sicilia et al., 2003).

3. Results and discussion

Lignans are produced by dimerization and subsequent rearrangement of two identical phenylpropane units, most frequently coniferyl alcohol. They are mainly stored in plant cell vacuoles in form of glycosides, to increment their solubility in aqueous media and reduce the risk of autotoxicity. Their distribution in the Plant Kingdom is scattered and do not seem to follow an unequivocal pattern allowing to predict their presence or absence in a peculiar specie or food (Umezawa, 2003). The distribution of lignans with different structures (Fig. 1), namely furofuran (a, b and c), dibenzylbutane (d and e) and dibenzylbutyrolactol (f) in *P. granatum* is described in Table 1. With respect to their selective presence in different plant parts and organs, lignans were almost absent in the inner teguments of the balausta, in which only a small amount of pinoresinol was detected, while their presence was relevant in both seeds and arils. The remarkable presence of these substances in wood knots is in accordance with previous reports evidencing an elective allocation of lignans in young woody tissues rather than in stemwood and branches; a distribution likely consequent to their strong relationship with lignin biosynthesis (Piispanen et al., 2008). The relevant presence in seeds may be also in line with previous observations, evidencing that seeds are an elective site of lignan accumulation also in soft-red berries and in most seeds of drupaceous fruits, likely as a consequence of the lignification of the seed testa. Such distribution seems to be in accordance with the fact that the drugs most abundant in lignans are seeds, i.e. sesame, buckwheat and linseed or seed-like fruits as caryopsis and brans of rye and wheat. Such an elective distribution may be a consequence of specific roles played by lignans in the plant's defensive system, as antifungals, antioxidants and in particular as feed deterrent for insects, roles that may be of particular value in oil-rich seeds that have to spend the whole winter almost defenceless before sprouting and thus may be palatable for insects (Schroeder, del Campo, Grant, Weiber, & Smedley et al.,2006). All the lignans detected in *P. granatum* share the same biosynthetic pathway, stemming from coniferyl alcohol and then modified following the sequent order: pinoresinol→lariciresinol→secoisolariciresinol and isolariciresinol→matairesinol (Umezawa, 2003). Their simultaneous presence in a single species is thus not surprising.

Concentrated juices and commercial beverages made of pure pomegranate juice have been also evaluated, evidencing an inferior presence of secoisolaricresinol, pinoresinol and syringaresinol (Table 2). Both amounts of total lignan aglycones and the qualitative profile were however rather different between the tested samples. Natural variability of plant sources (in terms of both phylogenetic and ontogenetic variability) and different industrial protocols due to dissimilar squeezing techniques and dilution rate may account for these fluctuations. A remarkable difference was noticed between concentrated and commercial fruit juices. It must be noticed, however, that most pomegranate juices available on the market are produced by dilution of concentrates, a step that is obviously detrimental in terms of lignan content. Nevertheless, a serving of filtered, freshly squeezed pomegranates (250 ml) may provide up to 0.5 mg of phytoestrogen convertible lignans.

Table 2
Amount of lignans (μg/g) in different pomegranate fruit juices, obtained by extraction under reflux in acetone–water.

	CdJ[a] – Tunisia	CdJ – Wonderful	CJ[b] brand #1	CJ brand #2	CJ brand #3
Isolariciresinol	n.d	0.3 ± 0.1[d]	n.d	0.7 ± 0.1	n.d.
Secoisolariciresinol[c]	n.d	0.7 ± 0.1	0.4 ± 0.1	0.9 ± 0.1	n.d.
Matairesinol	n.d	n.d.	n.d.	n.d.	n.d.
Pinoresinol	2.1 ± 0.2	1.5 ± 0.2	n.d.	0.9 ± 0.1	n.d.
Medioresinol	0.5 ± 0.2	n.d.	n.d.	n.d.	n.d.
Syringaresinol	1.8 ± 0.1	1.4 ± 0.1	n.d.	n.d.	0.6 ± 0.1
Total	**4.4 ± 0.**1	**3.9** ± 0.2	**0.4** ± 0.1	**2.5** ± 0.3	**0.6** ± 0.1

[a] Concentrated juice.
[b] Commercial juice.
[c] Obtained as a sum of secoisolariciresinol and anhydrous secoisolariciresinol.
[d] The results reported are the mean of three independent extractions and three chromatographic analyses for each extraction.

Regarding the possible contribution of pomegranate-derived foods to dietary intake of lignans it must be noticed that in absence of a clear RDI/RDA for lignans, general nutritional suggestions rely on the epidemiologic correlations between intake and the onset of diseases. Recent surveys in representative European populations evidenced a mean total lignan daily intake of approximately 1 mg (Milder et al., 2005; Touillaud, Thiébaut, Fournier, Niravong, & Boutron-Ruault et al., 2007), which would be almost doubled by the assumption of less than 50 g of fresh pomegranate arils with seeds or home made pomegranate squeeze or approximately 200 ml of pomegranate concentrated juice. In terms of nutraceutical value, it must be noticed that higher dietary intakes of lignans were associated with a reduction in the risk of postmenopausal breast cancers, in particular when the daily intake of lignans is above 1.5 mg (McCann et al., 2006; Touillaud et al., 2007).

A particularly high value was recorded for seeds of Wonderful variety, in which the most abundant lignan was syringaresinol. This compound however cannot be considered a true phytoestrogen. Its conversion rate into enterolactone and enterodiol by human gut flora is in fact extremely low and thus syringaresinol may not be a relevant contributor of the purported phytoestrogenic effect of *P. granatum* fruits (Heinonen et al., 2001). Nevertheless, syringaresinol is however known for its involvement in the inhibition of nitric oxide in the inflammatory cascade and for its activity against *Helycobacter pilori* (Miyazawa et al., 2006).

Table 1
Distribution of ligans (μg/g) in different plant part of *Punica granatum*, obtained by extraction under reflux in acetone–water.

	Wood knots	Endocarp wonderful	Endocarp tunisia	Seeds wonderful	Seeds tunisia	Pulp wonderful
Isolariciresinol	0.9 ± 0.1[a]	n.d.	n.d.	0.5 ± 0.1	0.6 ± 0.1	1.2 ± 0.1
Secoisolariciresinol[b]	2.3 ± 0.2	n.d.	n.d.	2.6 ± 0.2	1.9 ± 0.1	1.1 ± 0.1
Matairesinol	2.7 ± 0.3	n.d.	n.d.	n.d.	n.d.	n.d.
Pinoresionol	8.9 ± 0.3	3.3 ± 0.1	0.9 ± 0.1	4.4 ± 0.2	7.7 ± 0.1	7.4 ± 0.2
Medioresionol	0.8 ± 0.1	n.d.	n.d.	5.1 ± 0.2	3.1 ± 0.1	n.d.
Syringaresinol	2.2 ± 0.1	n.d.	0.5 ± 0.1	23.5 ± 0.4	2.5 ± 0.1	1.5 ± 0.2
Total	**17.8** ± 0.2	**3.3** ± 0.1	**1.4** ± 0.1	**36.1** ± 0.3	**15.8** ± 0.1	**11.2** ± 0.2

[a] The results reported are the mean of three independent extractions and three chromatographic analyses for each extraction.
[b] Obtained as a sum of secoisolariciresinol and anhydrous secoisolariciresinol.

4. Conclusions

Besides the renowned abundance of flavonoids, anthocyanins, catechins and specific hydrolysable ellagic derivatives named punicalagins, pomegranates proved to be rich also in lignans. As a consequence of their significant presence, lignans may act in synergy with other healthy polyphenolics of the plant's phytocomplex and represent a potential contributor to the purported oestrogenic functional properties of pomegranate juice. Their abundance may constitute a relevant marker in quality definition of *P. granatum* fruit and its nutraceutical byproducts. At the same time, the considerable presence of lignans in waste material from *P. granatum* fruit industrial processing may suggest the development of new lignan-rich food additives or herbal supplements and represent an intriguing and economic renewable source of these healthy compounds.

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The main objectives of this study were, therefore, to screen the antioxidants in *H. cordata* and to further identify and quantify those compounds using LC–MS coupled with DPPH assay.

2. Materials and methods

2.1. Materials and reagents

The following herbal teas were examined: (1) *Orthosiphon grandiflorus* Bolding (Lamiaceae), (2) *Houttuynia cordata* Thunb. (Saurraceae), (3) *Morus alba* L. (Moraceae), and (4) *Vernonia cinerea* L. (Asteraceae). The samples were obtained from Bangkratum hospital, Phitsanulok, Thailand.

The standard compounds, catechin, kaempferol were purchased from Wako (Pure Chemical Industries Ltd., Japan). Chlorogenic acid, quercetin dihydrate, ascorbic acid (vitamin C), quinic acid, butylated hydroxyanisole (BHA) and 1,1 diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma–Aldrich Chemie (Steinheim, Germany). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was purchased from Fluka Chemie AG (Buchs, Switzerland). Procyanidin B2 was purchased from Indofine (Somerville, NJ, USA). Methanol (LC/MS reagent) was purchased from JT Baker (Mallinckrodt Baker Inc., Phillipsburg, NJ, USA). Formic acid (analytical grade) was purchased from Merck (Darmstadt, Germany). Water was purified using Elga USF system (Bucks, England).

2.2. Sample preparation

For the off-line antioxidant activity determination, each sample (dry leaves) 250 mg was extracted with 2 × 10 ml 70% (v/v) methanol by sonication for 15 min then filtered through Whatman No. 1 filter paper (Kent, England). The filtrate was adjusted to a volume of 25 ml. The extracts were directly tested for antioxidant activity using DPPH assay.

For the analysis using on-line LC–ESI–MS coupled with DPPH assay. *H. cordata* dry leaves (2 g, 1 sachet) were infused in boiling water (100 ml) for 10 min, filtered through Whatman No. 1. The filtrate volume was readjusted to 100 ml and further filtered through a 0.2 μm Nylon syringe filter (Chrom Tech, Inc., MN, USA) prior to injection to the LC–ESI–MS coupled with DPPH assay system.

2.3. Off-line antioxidant activity determination

The potential antioxidant activity of a plant extract was assessed on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. Different concentrations (10–10,000 μg/ml in 70% (v/v) methanol) of test samples were prepared. The reaction mixtures consisting of 75 μl of test samples and 150 μl of 0.2 mM DPPH in methanol were mixed in 96 well plates and incubated for 30 min. The absorbance (OD) was measured at 515 nm. The antioxidat activity was obtained from the following equation: antioxidant activity (%) = [(OD control-OD sample)/(OD control)] × 100. Percent antioxidant activity was plotted against log concentration (μg/ml).The antioxidant activity of plant extracts was expressed as EC_{50}, which was defined as the concentration in μg/ml. The measurement was performed in triplicate. Trolox, ascorbic acid and BHA were used as positive controls.

2.4. LC–ESI–MS coupled with DPPH assay

The HPLC was coupled on-line to MS (line A) and a continuous flow DPPH assay (line B) as described in the previous study (Nuengchamnong & Ingkaninan, 2009). The set up system was shown in Fig. 1. Line A; Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) is coupled to a PE SCIEX API 4000 triple quadrupole tandem mass spectrometer (Applied Biosystem, Foster city, CA) equipped with electrospray ionization interface. The chromatographic separation was achieved by a phenomenex Gemini column (5 μm, 250 × 4.6 mm i.d.) (Phenomenex, Torrance, CA) protected with an ODS C18 guard column, operated at 25 °C. The mobile phase consisted of solvent A (1 ml formic acid in 1 l of deionized water) and solvent B (methanol). The elution program started from 90:10 solvent A: solvent B for 4 min, then changed to 80:20 solvent A: solvent B in 6 min, and linearly increased to 10:90 solvent A: solvent B in 30 min and the ratio of solvent A: solvent B was constant at 10:90 for 5 min then changed to 90:10 solvent A: solvent B in 5 min and was kept constant at 90:10 solvent A: solvent B for 5 min for reconditioning of the column. Mass spectra were recorded within 55 min. The injection volume was 5 μl. The flow rate was set at 600 μl/min. The Analyst 1.3.2 software was used for data acquisition and processing. The full scan mass spectra from *m/z* 100–1000 amu were acquired both in positive and negative ion modes. The optimum conditions of the interface were as follows: ESI-positive; ion spray voltage of 4500 V, curtain gas of 69 Kpa (10 psi), ion source gas 1 of 450 Kpa (65 psi), ion source gas 2 of 380 Kpa (55 psi). The interface temperature was set at 400 °C. The entrance and declustering potential were 10 and 80 V, respectively. ESI-negative; the condition was similar to ESI positive except the voltage was set in negative mode. Line B represents the continuous flow system for antioxidant activity detection. It consisted of an HPLC pump, LC20AD prominence (Shimadzu, Kyoto, Japan), home-made knitted reaction coil PEEK tubing with an inner diameter of 180 μm and a total reaction coil volume of 100 μL. The flow of 0.1 mM DPPH was set to 200 μl/min and the reduction was detected as a negative peak at 515 nm using the UV–VIS detector (SPD 20AV, Shimadzu, Kyoto, Japan). The residence time in the coil is 0.45 min. The LC solution software was used for data acquisition and processing. The polarity of the signal output was reversed in order to obtain positive signals. The system was operated at 25 °C. Line A coupled to line B with a 24 cm length of 0.17 mm i.d. PEEK tubing by a *Y*-connector. The eluent flow was split in a ratio of 8:2 between the MS ion source and DPPH line. For the characterization of antioxidant peaks, the fragment ions from their corresponding parent ions in negative mode were induced with collision gas (CAD) of 41 Kpa (6 psi), collision energy (CE) between −5 and −50 V and collision cell exit potential (CXP) of −6 V, DP in the range of −20 to −110 V. In positive mode, the ions were induced with CAD of 48 Kpa (7 psi). CE, CXP and DP were set in the range of 5–25, 3–13 and 20–100 V, respectively. Quinic acid and kaempferol at a final concentration of 100 μg/ml were added to the sample to synchronise an antioxidant peak in line B with the MS peak in line A.

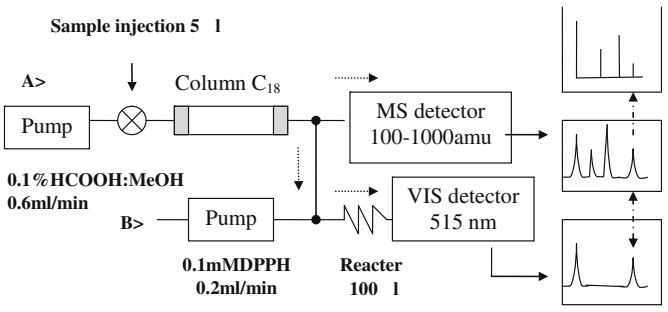

Fig. 1. Scheme of the HPLC on-line coupled to ESI-MS and DPPH assay. The arrows indicate flow directions.

2.5. Peak identification

Peak identification was performed by comparison of the retention time, mass spectra and fragmentation patterns with reference compounds and published data.

2.6. Quantitative analysis

For quantitative analysis of the antioxidants in *H. cordata* herbal tea, the same column was used and the gradient system was changed to shorten analysis time. The system was employed from solvent A:B (90:10) to (10:90) in 10 min, followed by isocratic elution with solvent A:B (10:90) for 5 min and linear gradient elution from solvent A:B (10:90) to (90:10) for 2 min and equilibration with solvent A:B (90:10) in 3 min before injecting a new sample. The flow rate was set at 600 μl/min. The injection volume was 5 μl. The MS parameters were operated in negative multiple reactions monitoring (MRM) scan mode. Optimal operating parameter of ESI–MS with maximum signal intensity of molecular ions and fragment ions were obtained by direct infusion of the standard solution at concentration 10 μg/ml in methanol, using a Harvard syringe pump (Syringe Pump 11 plus, Harvard apparatus Inc., Holliston, USA) at a flow rate of 5 μl/ml. The optimum conditions of the interface were as follows: ion spray voltage of −4500 V, curtain gas of 138 Kpa (20 psi), ion source gas 1 of 380 Kpa (55 psi), ion source gas 2 of 310 Kpa (45 psi). The interface temperature was set at 400 °C. The entrance potential was −10 V. The dwell time per transition was set at 100 ms. The individual optimized parameters for detection of each compound are as follows. For chlorogenic acid, the transition at *m/z* 353.2 → 190.8 amu with DP, CE and CXP of −120, −22 and −30 V were set. Catechin was detected at two transitions. At *m/z* 288.9 → 245.0, DP, CE and CXP were set at −150, −22 and −12 V, respectively. At *m/z* 288.9 → 125.1 amu, DP, CE and CXP were set at −150, −30 and −20 V. For quercetin, the two transitions were set at *m/z* 300.9 → 151.0 amu and *m/z* 300.9 → 178.9 amu. The DP and CE for both transitions were set at −150 and −30 V. The CXP was set at −22 for *m/z* 300.9 → 151.0 and at −26 V for *m/z* 300.9 → 178.9 detection. Chlorogenic acid, catechin and quercetin in various concentrations were used for preparation of calibration curves. The curves were generated by linear regression based on the peak area. Analyses were performed in triplicate. Other standards were not available; hence neo-chlorogenic acid and crypto-chlorogenic acid were quantified as chlorogenic acid equivalents and also procyanidin B and quercetin hexoside were quantified as catechin and quercetin equivalents, respectively. The limit of detection (LOD) was determined as the analyte signal having a peak area equal to three times of that of noise (S/N = 3) using Analyst software.

3. Results and discussions

3.1. Off-line antioxidant activity determination

The antioxidant activities of four herbal teas were measured using DPPH assay on 96 welled microplate. This assay is based on a measurement of the scavenging ability of antioxidants towards a stable radical, DPPH. DPPH, which shows absorption at 515 nm, is reduced to the corresponding hydrazine when it reacts with hydrogen donors and this can be detected as a decrease of absorption (Brand-Williams, Cuvelier, & Bersec, 1995). The antioxidant activities of the methanolic extracts of *O. grandiflorus*, *H. cordata*, *M. alba* and *V. cinerea* were expressed as EC_{50} values of 217.2 ± 12.6, 341.5 ± 17.2, 874.9 ± 83.4 and 886.9 ± 53.1 μg/ml, respectively. The positive controls BHA, ascorbic acid and Trolox gave EC_{50} values of 4.5 ± 2.1, 6.7 ± 0.2 and 7.9 ± 1.3. The results showed that the methanolic extracts of *O. grandiflorus* and *H. cordata* had the highest activity among all the teas tested.

3.2. LC–ESI–MS coupled with DPPH assay for the rapid identification of antioxidants

An aqueous extract of *H. cordata* was subjected to the on-line LC–ESI–MS coupled with DPPH assay. The DPPH based antioxidant activity profile (Fig. 2a) exhibited that at least ten compounds showed antioxidant activity peak. The deprotonated molecular ion peaks of standard quinic acid and kaempferol were found at 5.7 and 39.3 min, respectively. The delay time between the read out from the MS detector and the corresponding peak from the antioxidant activity was then calculated as 0.6 min. Negative ionization gave a better result than positive ionization (Fig. 2b). The negative ions of the major active compounds are listed in Table 1 and the identification of these compounds is proposed. The aqueous extract was used in this study as it will provide the same form of antioxidants as that present in the tea.

Peak 1 (t_R = 5.8 min) was a standard quinic acid that mixed in the sample to a concentration of 100 μg/ml as a delay time marker for the two detectors. Peak 2 has a mass spectra and fragmentation pattern nearly identical to standard quinic acid but difference in retention time (t_R 9.3 min). So, peak 2 was tentatively identified as quinic acid derivative.

Peaks 3–5 (t_R = 12.3, 14.7 and 17.9 min) with *m/z* 371.2 and fragmentation at *m/z* 197.3 [caffeic acid-H+18]$^-$, *m/z* 191.0 [quinic acid-H]$^-$, *m/z* 179.0 [caffeic acid −H]$^-$ and *m/z* 173.3 [quinic acid-H–H_2O]$^-$.These *m/z* are characteristic of caffeic acid. Therefore, these three compounds were identified as caffeic acid derivatives.

Peak 6 (t_R = 20.3 min) exhibited [M−H]$^-$ base ion at *m/z* 577.3. It gave secondary fragment at *m/z* 289.0 [M−288.3]$^-$ due to the cleavage of the inter-flavanoid C–C linkages with losses of 288 amu and *m/z* of 425 [M−152]$^-$ evolved from retro Diels–Alder fragmentation of the heterocyclic ring. The *m/z* of 407 [M−152–18]$^-$ resulted from water elimination of *m/z* 425. When comparing with standard procyanidin B2 (t_R 23.13 min), the fragmentation pattern of this compound is nearly identical but the retention time was different. This compound might be one of procyanidin B group, but stereochemistry of this compound cannot be indicated by this MS technique.

Peak 8 (t_R = 23.04 min) with *m/z* 289.0 [M−H]$^-$ and fragmentation at *m/z* 245.0 and 125.1(cleavage of the C ring) was identified as catechin by the comparison of the authentic standard.

Peak 9 (t_R = 24.8 min) with *m/z* 353.6 and fragmentation at *m/z* 191.0 [quinic acid-H]$^-$, *m/z* 179.0 [caffeic acid –H]$^-$ and *m/z* 173.3 [quinic acid-H–H_2O]$^-$. By comparing its mass spectra and t_R with standard compound, this compound was assigned to be chlorogenic acid (5-*O*-caffeoyl quinic acid). Compounds 7 and 10 were most likely naturally occurring isomers of chlorogenic acid. According to their elution order, neo-chlorogenic acid (3-*O*-caffeoyl quinic acid) was eluted prior to crypto-chlorogenic acid (4-*O*-caffeoyl quinic acid) (Carini, Facino, Aldini, Calloni, & Colombo, 1998). Therefore, peaks 7 and 10 were assigned as neo-chlorogenic acid and crypto-chlorogenic acid, respectively (Fig. 3a).

Peak 11 (t_R = 32.5 min) with a molecular ion at *m/z* 463.3 and fragment ions at *m/z* 300.0 and *m/z* 301.0 [M−162]$^-$ (loss of a hexose unit) was tentatively identified as quercetin hexoside. Standard quercetin was eluted at 37.2 min (Fig. 3c).

Peak 12 (t_R = 39.3 min) with *m/z* 285 was kaempferol standard at concentration 100 μg/ml was used as delay time marker for the two detectors.

The general structures of the antioxidant compounds identified in *H. cordata* are shown in Fig. 4.

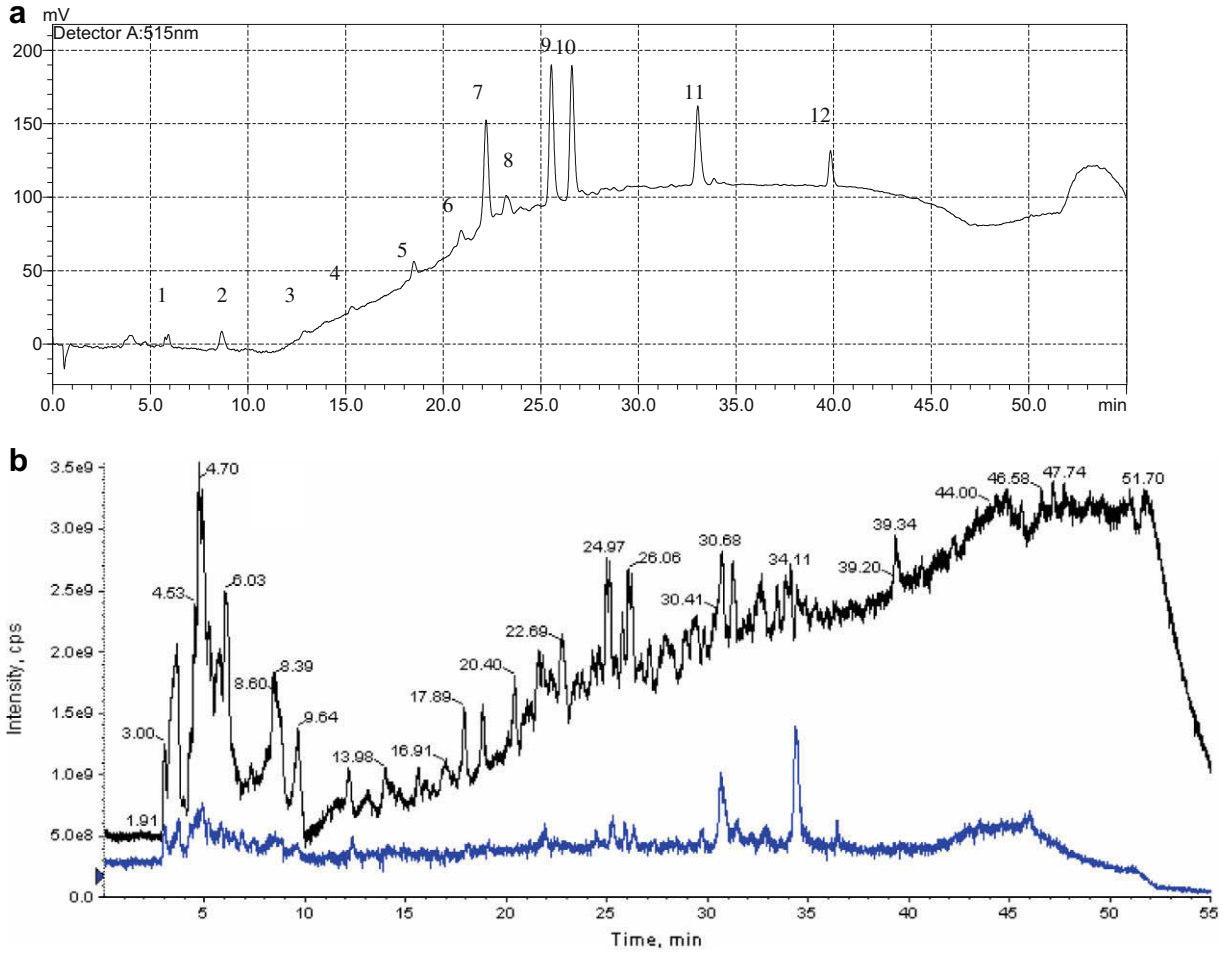

Fig. 2. Analysts of *H. cordata* aqueous extract using LC–ESI–MS coupled with DPPH assay. (a) The chromatogram from the antioxidant activity assay detection at 515 nm. (b) The total ion current (TIC) output from the ESI–MS in negative mode (upper line) and positive mode (lower line). Conditions are described in the text. For peak assignments, see Table 1. Peak 1 and 12 are quinic acid and kaempferol added to the extract for delay time measurement.

Table 1
Identification of antioxidant compounds in water extracts of *H. cordata* by using their LC–ESI–MS–DPPH assay; data in negative ionization.

Peak no.	t_R (min)	ESI–MS (m/z)		Identification
		MS	MS/MS	
1	5.8	190.9	127.3	Quinic acid*
2	9.3	190.9	127.3, 111.1	Quinic acid derivative
3	12.3	371.2	197.3, 191.0, 179.0, 173.3	Caffeic acid derivative
4	14.7	371.2	197.3, 191.0, 179.0, 173.3	Caffeic acid derivative
5	17.9	371.2	197.3, 191.0, 179.0, 173.3	Caffeic acid derivative
6	20.3	577.3	425.0, 407.2, 289.0	Procyanidin B**
7	22.2	353.6	191.0, 179.0, 173.3	Neo-chlorogenic acid
8	22.7	289.2	245.0, 125.1	Catechin**
9	24.8	353.6	191.0, 179.0, 173.3	Chlorogenic acid**
10	26.5	353.6	191.0, 179.0, 173.3	Crypto-chlorogenic acid
11	32.5	463.3	301.3, 300.0	Quercetin hexoside
12	39.8	285.0	–	Kaempferol*

* Standard compound added.
** Compared with standard compound.

3.3. Quantitative analysis of antioxidants using LC–MS/MS

Antioxidant compounds in *H. cordata* can be quantitatively analyzed using LC–MS/MS in MRM mode. In order to shorten the run time, the elution profile was changed and the analysis was finished in 20 min. In our studies, chlorogenic acid, catechin and quercetin at various concentrations were used for the preparation of calibration curves. The regression equation of chlorogenic acid at concentrations of 5–100 μg/ml was $y = 4.37e + 005x - 2.3e + 006$; with r value of 0.9985. The regression equation of catechin at concentrations of 1–100 μg/ml was $y = 2.36e + 004x + 7.86e + 003$; with r value of 0.9991. The regression equation of quercetin at concentrations of 1–100 μg/ml was $y = 9.26e + 004x + 6.6e + 004$; with r value of 0.9977. The limits of detection (LOD) with signal

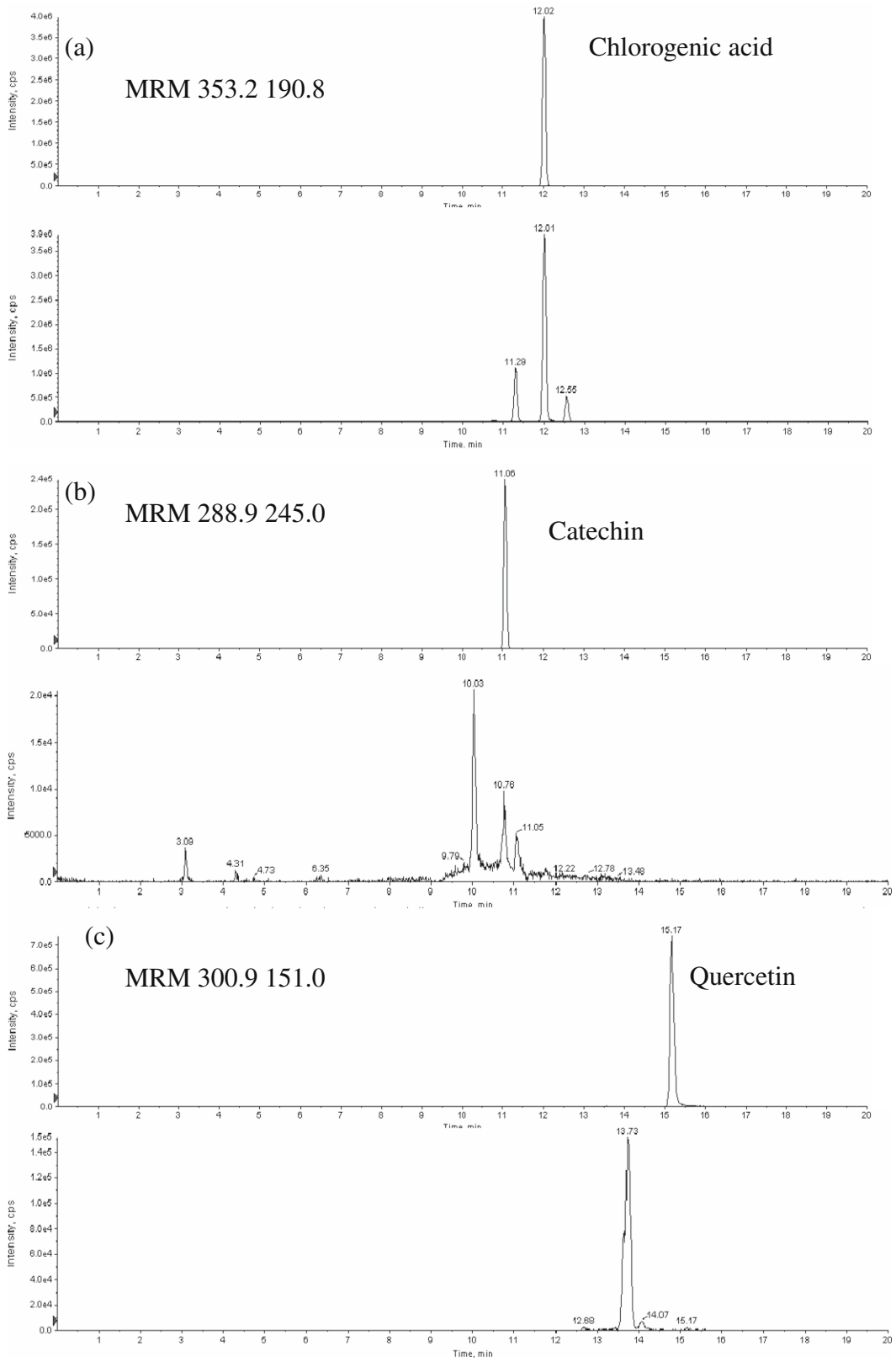

Fig. 3. Chromatograms of (a) chlorogenic acid (upper pane) and *H. cordata* extract (lower pane) obtained from the extract mass in MRM mode at transition of *m/z* 353.2 → 190.8, (b) catechin (upper pane) and *H. cordata* extract (lower pane) obtained from the extract mass in MRM mode at transition of *m/z* 288.9 → 245.0, (c) quercetin (upper pane) and *H. cordata* extract (lower pane) obtained from the extract mass in MRM mode at transition of *m/z* 300.9 → 151.0.

to noise ratio of three were determined. LOD of chlorogenic acid, catechin and quercetin was 2.5, 2.5 and 5.0 ng per injection, respectively. Neo-chlorogenic acid and crypto-chlorogenic acid were quantified by using a linear regression line of chlorogenic acid. Procyanidin B and quercetin hexoside were also quantified as catechin and quercetin. The extracts mass in MRM quantification

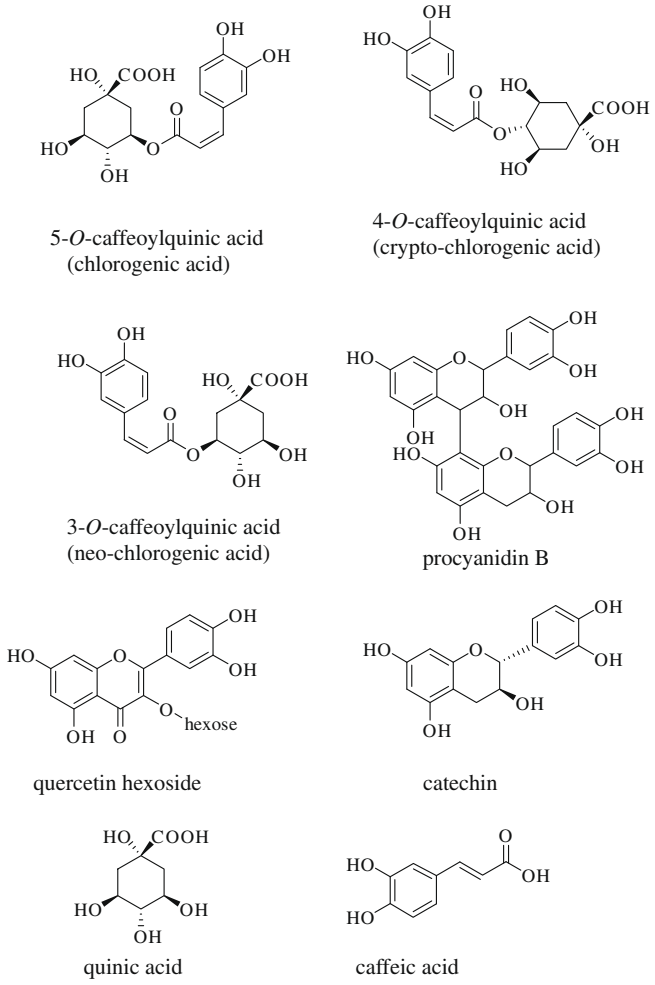

Fig. 4. Structure of phenolic compounds identified in the *H. cordata* tea.

of the standard compounds and compound equivalent in the *H. cordata* tea aqueous extracts were shown in Fig. 3. The amounts of antioxidant compounds found in *H. cordata* tea were shown in Table 2. Quinic acid derivative and caffeic acid derivatives were not quantified due to contain in a small amount.

The major antioxidative components in this plant were found to be chlorogenic acid and its derivatives. Several reports showed that chlorogenic acid exhibited antivirus, anticancer, anti-inflammation activities, and anxiolytic-like effects (Bouayed, Rammal, Dicko, Younos, & Soulimani, 2007; Jiang, Satoh, Watanabe, Kusama, & Sakagami, 2001; Jin et al. 2005; Nakamura et al. 1997). Some of these compounds such as chlorogenic acid and quercetin glycoside have already been reported in *H. cordata* (Meng et al., 2005). However, this is the first time that the presence of catechin, and procyanidin B in the aqueous *H. cordata* tea extract are reported. The results suggest that these compounds could be partly responsible for antioxidant activity of *H. cordata* tea.

Table 2
Content of phenolic antioxidant found in *H. cordata* aqueous extract analysis by LC–MS/MS in MRM mode.

Compound	Content (μg/g)
Chlorogenic acid	2808.7 ± 7.5
Neo-chlorogenic acid	921.7 ± 2.9
Crypto-chlorogenic acid	603.5 ± 2.9
Catechin	28.0 ± 0.8
Procyanidin B*	52.4 ± 5.2
Procyanidin B*	189.5 ± 0.6
Quercetin hexoside	730.0 ± 5.0

* The stereochemistry of procyanidin B could not be identified.

4. Conclusions

The preparative isolation of the active compounds of herbal extracts in adequate quantities for off-line spectral and biological analysis is a laborious and time consuming. In this study, the use of on-line rapid screening of the antioxidants in herbal tea and their subsequent identification are reported. Antioxidants in the aqueous extract of *H. cordata* mainly consisting of chlorogenic acids and its derivatives, catechin and procyanidin B were characterized using on-line LC–MS coupled with DPPH assay for the first time.

Acknowledgement

This work was financially supported by the Thailand Research Fund (TRF) under the grant DBG-4980011. The Center of Excellence for Innovation in Chemistry (PERCH-CIC), Thailand is acknowledged for the support for the last author.

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Mn content can increase in foods packed in glazed ceramic containers (Cabrera et al., 1996).

In general, estimates of the total element content in food are insufficient and its bioavailability also needs to be considered. Mineral bioavailability has gained increasing interest in the field of nutrition. *In vivo* studies are both expensive and laborious, and the possibility of measuring certain parameters during the experiments is often limited (Cabañero, Madrid, & Cámara, 2007; Perales, Barberá, Lagarda, & Farré, 2007). *In vitro* methods of simulated digestion are an alternative for calculating the percentage that is transformed into absorbable forms in the digestive tract (bioaccessibility) (Perales et al., 2007). These procedures are rapid, usually inexpensive, and they allow individual experimental variables to be easily controlled. The results are usually expressed as dialyzable fraction under given experimental conditions such as pH, enzyme addition and temperature (Cabañero et al., 2007; Cabrera et al., 1996; Velasco-Ryenold, Navarro-Alarcón, López-García de la Serrana, Perez-Valero, & López-Martinez, 2008).

On the other hand, Cr and Mn analysis in food are difficult because of low presence and problems in sample collection, storage, processing and final determination without outside contamination. Electrothermal atomic absorption spectrometry (ETAAS) is the most widely used technique and gives reliable results (Cabrera et al., 2003a; López et al., 2002; Taylor et al., 1997).

In this study, Cr and Mn content in convenience and fast food and its dialyzable fraction after simulated *in vitro* the digestion process was determined by ETAAS. The present findings are of potential use in food composition tables and to estimate the total dietary intake of these elements in accordance with the actual dietary habits. Modern societies appear to consume large amounts of convenience and fast food. For example, its consumption in Spain has been increased in recent years and has been established approximately as 32 kg person^{-1} year (Ministry of Agriculture, Fisheries, & Food, 2006).

2. Materials and methods

2.1. Sample collection

A total of 170 samples of 43 different convenience and fast food including beef-, chicken-, fish-, and pork-based products, as well as samples containing egg as major constituent and several types of sauces, were analyzed. Several brand names of each product, representing the most widely accepted and consumed in Spain, was selected for testing. The selection was specially made to reflect the popular types consumed by the different income groups. All samples are commercially available and made in Spain. Appropriate quality assurance procedures and precautions were carried out to ensure reliability of the results. Samples were carefully handled to avoid contamination.

2.2. ETASS instrumentation and conditions

A Perkin–Elmer 1100B double beam atomic absorption spectrometer equipped with deuterium-arc-background correction (Perkin–Elmer, Norwalk, CT, USA) and a Perkin–Elmer HGA-700 graphite furnace atomizer were used. Pyrolytically coated graphite tubes (Ref. B013–5653) and Pyrolytic graphite platforms (Ref. B012–1092) were obtained from Perkin–Elmer. Wall tube atomization system was used for Cr determination and platform atomization system for Mn. Argon of 99.999% purity (Sociedad Española de Oxígeno, Barcelona, Spain) at 300 mL/min was used as internal gas during all stages except atomization when the flow was stopped. A 10-μL volume of analytical solution and 10 μL of $Mg(NO_3)_2$ was used as chemical modifier were injected into the furnace. Measurements were performed at 357.9 nm for Cr and 279.5 nm for Mn using a hollow cathode lamp (Perkin–Elmer) with a slit width of 0.7 and 0.2 nm, respectively. Background-corrected integrated absorbance was used as analytical signal. A Selecta digestion block (Selecta SA, Barcelona, Spain) and Pyrex tubes were used for sample mineralization. A Moulinex microwave oven (Moulinex, Bagnolet, France) was used to dry samples. A Moulinex blender (Moulinex, Bagnolet, France) was used to sample homogenization. A shaking and thermostatic water bath Selecta (Selecta SA, Barcelona, Spain) was used to *in vitro* assays. Bidistilled deionized water from a Milli-Q system (Millipore, Milford, MA) was used.

2.3. Material

In order to decrease the risk of contamination, the use of glassware was reduced to minimum and plastic (polypropylene) vessels and pipette tips were used. All the materials were nitric acid-washed and rinsed several times with bidistilled deionized water.

2.4. Chemicals

Standard solutions of Cr and Mn (1.00 ± 0.002 g) (Tritisol, Merck, Darmstadt, Germany) were used and diluted as necessary to obtain working standards. High quality concentrated nitric acid (65%), perchloric acid (70%) and vanadium pentaoxide (Merck) were used for sample mineralization. Magnesium nitrate (Merck) was used as chemical modifier for Cr and Mn determination. Ammonium molybdate (Merck) was used to precondition the furnace tubes. All reagents used were of analytical grade. Pepsin (Sigma P7000 *porcine pancreas*, Sigma–Aldrich Chemie Gmbh, Steinheim, Germany), pancreatin (Sigma P1500 *porcine pancreas*, Sigma–Aldrich Chemie Gmbh, Steinheim, Germany) and bile salts (Sigma B8756) were used to simulate gastric and intestinal digestion. A pepsin solution was prepared dissolving 16 g of pepsin in 100 mL of 0.1 mol/L HCl; pancreatin-bile extract mixture was prepared by dissolving 4 g of pancreatin and 25 g of bile extract in 1L of 0.1 mol/L $NaHCO_3$. Sodium hydroxide (0.5 M) and hydrochloric acid (6 M) were used to adjust pH. Dialysis tubing Spectra with a molecular mass cut-off of 10–12 kDa ('Visking size 3–20/32́ Medicell International, London, GB) was used. The dialysis tubing was freed of trace metal impurities by boiling in a 2% (w/v) $NaHCO_3$, 0.1% (w/v) sodium dodecyl sulphate and 0.01 M ethylenediaminetetraacetic acid disodium salt (EDTA) for 30 min, followed by thoroughly washing with bidistilled deionized water and preserved in 20% ethanol solution.

Table 1
Analytical characteristics for Cr and Mn determination in convenience and fast foods.

Analytical parameter	Chromium	Manganese
Detection limit, pg	4.0	2.0
Sensitivity, pg	10.0	2.5
Accuracy,%	98.80 ± 1.20	98.6 ± 0.50
Precision, RSD%[a]	4.5–4.7	4.5–4.8
Slope ratio blank/addition	0.999–1.010	0.998–0.999
Against certified reference material (*Mixed human diet* IAEA-H9)		
Measured value[b], mg/kg	0.148 ± 0.015	11.75 ± 0.50
Certified value[b], mg/kg	0.150 ± 0.054	11.80 ± 0.20
Δ_m[c]	0.02	0.05
U_Δ[d]	0.112	1.077

[a] RSD, relative standard deviation for 10 replicate determinations in each of five randomly chosen samples.
[b] Mean ± S.D. at 95% CI interval about the mean (n = 10), refereed to dry weight.
[c] Absolute difference between mean measured value and certified value.
[d] Expanded uncertainty of difference between result and certified value. $\Delta_m \leqslant U_\Delta$, no significant difference between measurement result and certified value at 95% confidence level.

2.5. Sample preparation and analysis

Preliminary assays established the appropriate amount of sample for analysis to ensure homogeneity between samples and that they were representative. Food samples were subjected to a simulated eating procedure. Different food items were sliced and inedible parts thrown away. The remaining parts were homogenized in a blender and the total amount of food weighed. Three aliquots of approximately 100 g were dried in a microwave oven under controlled temperature conditions (García et al., 2001a). Each dried aliquot sample was again homogenized in the blender and three portions of 0.250 g of sample was treated with 5 mL of 65% HNO_3 and a few micrograms of V_2O_5 (as a catalyst) in Pyrex tubes placed in an acid digestion block, first it was heated at 60 °C for 30 min and then, at 120 °C for 60 min. The solutions were cooled to room temperature, treated with 1 mL 65% $HClO_4$, and heated at 120 °C for 90 min. The solutions were cooled to room temperature, transferred to a calibrated flask and diluted to a final volume of 25 mL with bidistilled deionized water. Cr and Mn were determined in this solution by ETAAS. Temperature selected for the ashing stage was 1650 °C and 1400 °C for Cr and Mn, respectively. For the atomization stage the selected temperature was 2500 °C and 2200 °C for Cr and Mn, respectively. All analyses were done in triplicate.

CAUTION: Perchloric acid should not be allowed to evaporate to dryness and should only be used in an appropriate fume hood.

2.6. In vitro method for estimating Cr and Mn dialyzable fraction

On order to simulate the gastrointestinal digestion, a portion of 10 g of homogenized sample was added to 80 mL of bidistilled deionized water and pH was adjusted to 2.0 with 6 M HCl. 3 mL of freshly prepared pepsin solution were added to the sample and the mixture was made up to 100 g with bidistilled deionized water. Then, the mixture was incubated in a shaking and thermo-

Table 2
Chromium levels in convenience and fast foods.

Sample	*n*	Cr content, (μg/g)[a]		Portion size (g)	Mean Cr supplied per portion (μg)
		Mean	Range		
Beef-based foods					
Hamburger	6	0.91	0.89–1.10	150	136.50
Cheese burger	4	0.80	0.79–0.81	150	120.00
Lasagne meat	5	0.48	0.47–0.51	200	96.00
Lasagne meat and pâté	3	0.61	0.60–0.62	200	122.00
Pasta Bolognese	4	0.28	0.21–0.29	200	56.00
Beef in ale	3	0.84	0.80–0.85	300	252.00
Minced beef and onion pies	3	0.71	0.70–0.72	150	106.50
Frankfurter	5	0.83	0.74–0.86	150	124.25
Chicken-based foods					
Noodle soup with chicken	6	0.20	0.19–0.22	125	25.00
Chicken croquettes	5	0.31	0.29–0.32	150	46.50
Chicken and vegetable soup	5	0.20	0.19–0.21	125	25.00
Chicken with rice	4	0.36	0.35–0.38	250	90.00
Cooked chicken	5	0.41	0.39–0.42	200	82.00
Chicken sandwiches	5	0.21	0.19–0.22	150	31.50
Chicken with aromatic herbs	3	0.57	0.53–0.58	250	142.50
Chicken with mushroom	5	0.41	0.39–0.43	250	102.50
Fish-based food					
Sole with white sauce	4	0.06	0.05–0.09	300	18.00
Hake with green sauce	3	0.07	0.07–0.08	250	17.50
Salmon cottage pie	3	0.05	0.04–0.06	300	15.00
Mariner's pie	3	0.04	0.03–0.05	250	10.00
Tuna pasties	3	0.03	0.02–0.06	200	6.00
Cod croquettes	4	0.04	0.03–0.08	150	6.00
Hake croquettes	4	0.04	0.03–0.06	150	10.00
Hake fish fingers	3	0.05	0.04–0.06	200	21.00
Rice with shellfish	3	0.07	0.06–0.08	300	21.90
Pork-based foods					
Pork with mushroom sauce	4	0.81	0.79–0.84	250	202.50
Ham-cheese-tomato pizza	5	0.42	0.40–0.45	200	84.00
Pork pie	3	0.73	0.69–0.75	200	146.00
Frankfurter	4	0.53	0.50–0.56	150	79.50
Hamburger	3	0.65	0.60–0.71	150	97.50
Ham croquettes	4	0.57	0.52–0.60	150	85.50
Egg-based foods					
Cheese-ham quiche	3	0.74	0.70–0.74	200	144.00
Broccoli quiche	2	0.47	0.49–0.50	200	94.00
Pudding (chocolate)	3	0.62	0.58–0.67	125	77.50
Custard	6	0.58	0.52–0.67	125	72.50
Crème caramel	6	0.12	0.11–0.18	125	15.00
Sauces					
Ketchup	4	0.04	0.01–0.07	30	1.20
Tomato	3	0.06	0.05–0.07	50	3.00
Mayonnaise	5	0.09	0.08–0.14	25	2.25
Béchamel	4	0.07	0.06–0.09	50	3.50
Roquefort	4	0.05	0.04–0.08	25	1.25
Sweet and sour	3	0.03	0.02–0.05	25	0.75
Mustard	3	0.07	0.06–0.09	25	1.75

[a] Referred to fresh weight of edible portion.

static water bath at 37 °C for 2 h. After the simulated gastric digestion, duplicate 20 g samples of pepsin digest were transferred to 250 mL Erlenmeyer flask. The dialysis tubing containing 25 mL of bidistilled deionized water and an amount of $NaHCO_3$ equivalent to the titrable acidity measured previously were placed in the Erlenmeyer flask and incubated in the shaking bath at 37 °C for 30 min. Then, 5 mL of the pancreatin–bile extract mixture were added to each Erlenmeyer flask and incubated for 2 h. The dialysis tubing was removed and the dialyzate was weighed. In this dialyzate, Cr and Mn content were determined by ETAAS in the same conditions above described. Titrable acidity was defined as the number of equivalents 0.5 M NaOH required to titrate the amount of combined pepsin digests pancreatin-bile extract mixture to pH 7.5. This was determined on a 20 g aliquot of the pepsin digest to which 5.0 mL of pancreatin-bile extract mixture has been added with 0.5 M NaOH at 20 °C. All digestions were done in triplicate and blanks were prepared and analyzed in the same procedure.

2.7. Statistical analysis

Each value of all the statistical variables studied corresponds to the average of three independent measurements of a sample food. The amount of Cr (μg/g), Mn (μg/g), Cr per portion (μg), Mn per portion (μg), Cr dialyzable fraction (%), Mn dialyzable fraction (%) and food group were the statistical variables taken into account. The numerical variables were studied as marginals, as a vector of dimension six and conditionally to the qualitative one. The analysis implemented has been mainly descriptive analysis, correlation, regression, analysis by subsets and non parametric analysis. Statistical analysis was performed by Statgraphics Plus package (1999).

3. Results and discussion

3.1. Method validation

A rapid and accurate analytical method is reported for the determination of Cr and Mn by ETAAS in convenience and fast food, useful for food routine analysis. In addition, the sample mineralization procedure provides a set of advantages including rapid and complete mineralization without analyte losses or additional contaminations. For method validation, detection limit (corresponding to three times the standard deviation of 10 replicates of the blank), sensitivity (expressed as characteristic mass for 0.0044 absorbance units), precision and accuracy (recovery assays of five randomly chosen samples) were tested for each element (Horwitz, Albert, Deutsch, & Thompson, 1990). Standard addition method was applied to five randomly chosen samples. A comparison of the slopes of aqueous and standard addition calibration graphs was performed. To check the similarity of slopes, the Student's *t*-test was applied and the results showed that the values were similar with an absence of matrix effects (slope ratios approaching 1). Thus, calibration was performed using aqueous standard. The reliability of the method was further corroborated by using a certified reference material of the International Atomic Energy Analytical Quality Services of Vienna (IAEA-H-9 *Mixed human diet*). Comparison of the measurement result with the certified value indicates that there is no significant difference between the measurement result and the certified value at a confidence level of about 95% (Linsinger, 2005). Results are summarized in Table 1.

3.2. Cr levels in convenience and fast foods

Cr levels in the analyzed samples ranged from 0.01 to 1.10 μg/g referred to fresh weight of the edible portion. The foodstuffs were classified according to their major constituent (beef, chicken, fish, pork and egg); also some sauces were included. Data are summarized in Table 2. With regard to the size of the portions (see Table 2), in most cases the weight of the packed product was taken even although in some cases it cannot be assumed that the whole portion would be eaten by one person all at once. In certain cases, however, the portion was estimated on the basis of several observations and literature (López et al., 2002).

Cr presence has been detected in all the samples and the content in a specific class of food did not appear to vary markedly among the brands. The most elevated Cr concentrations were found in beef and pork-based-food, but there is a high variability inside of each one of these food groups (Fig. 1). A higher content appears in products that contain spices and aromatic herbs, whole cereals, dry fruits, cheese and mushrooms. Some mushroom species seemed to be a major source of Cr, Mn and other elements (López et al., 2002). Wilplinger, Schonsleben, and Pfannhauser (1995) and Ashton, Barclay, Louie, Wu, and Di (2003) indicated that the best sources of Cr in diet are yeast products, chocolate derived products, prawns, processed meats, soy-based meat substitutes and breakfast cereals. In the same way, Mateos, Aguilar, and Martínez-Para (2003) found high Cr values that ranged from 0.09 to 0.55 μg/g in breakfast cereals. According to the bibliography, meat products showed a wide range of Cr content, generally lower than fresh meat, which was dependent on product composition and added materials, e.g. the high fat content in sausages (Ashton et al., 2003; Bratakos et al. 2002).

In previous studies, we analyzed several foods and beverages widely consumed in Spain, in order to estimate its possible contribution to the total Cr dietary intake (García, Cabrera, Lorenzo, & López, 2000; García, Cabrera, Sánchez, Lorenzo, & López, 1999). A high Cr presence in dairy products, meat, stimulant drinks and infusion (especially tea and coffee), whole cereals, brown sugar, spices and aromatic herbs was observed (Cabrera et al. 2003a, 2003b; Lendínez, Lorenzo, Cabrera, & López 2001). The presence of Cr in convenience and fast foods is similar to Cr content of potatoes (0.028–0.071 μg/g fresh weight of edible portion), and legumes and nuts (0.05–0.06 and 0.25–1.05 μg/g dry weight, respectively). On other hand, a moderate increase of Cr presence

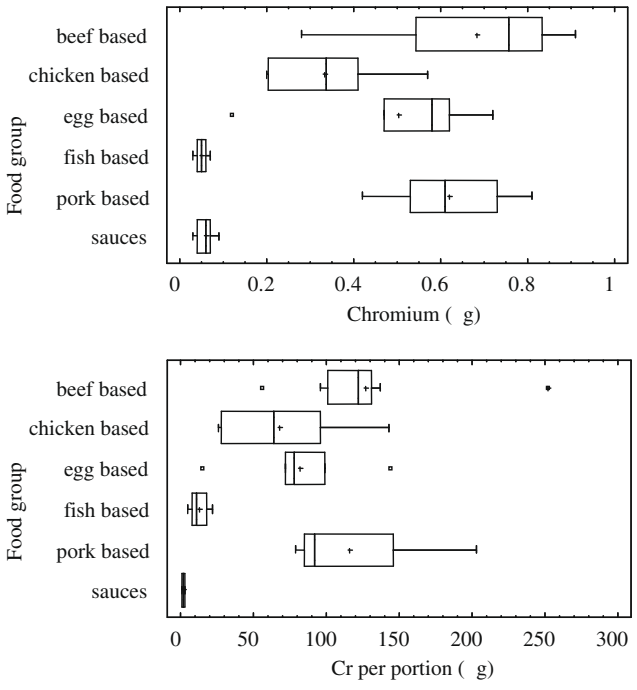

Fig. 1. Box and Whisker plot for Cr and Cr per portion by food group analyzed.

in the samples of beef in ale, chicken with aromatic herbs, chicken with mushrooms and sole with white sauce that are packed in aluminium-containers was observed. These type of containers are sometimes used as packaging material in convenience and fast foods, and often used for direct heat food cooking (e.g. during grilling or baking). In the same way, Lendínez, López, Cabrera, and Lorenzo (1998) reported significant higher Cr levels in canned beer than in bottled beers.

With regard to the Cr levels found in the present study, the mean Cr supplied per normal portion (125–300 g) of the convenience and fast foods could oscillated between 6 and 252 μg (Table 2). For sauces, a portion (25–50 g) could contribute a Cr dietary intake of 0.75–3.50 μg. The most elevated levels of Cr per portion were found in beef and pork-based food (see Fig. 1). It is important to distinguish between Cr amount and Cr amount per portion, these are different variables and they behave differently in each food group. Even so, data show that there is a strong positive relationship between Cr and Cr per portion. In fact, a regression analysis was made and it was found out that a square root-Y model describes very well the relationship between both variables. The equation model is Cr/portion = $(2.2615 + 13.2481\ Cr)^2$ given a correlation coefficient of 0.9386. A Kruskal–Wallis test has been applied to both variables, Cr and Cr per portion. Even without the sauce group, the most different one, there is no statistical evidence of similarity between the medians of the food groups (p-value = 0.000028 and 0.00019, respectively) what could be intuitively expected observing Fig. 1. García et al. (2001a) measured Cr dietary intake in southern Spain by sampling duplicate diets for seven consecutive days in different population groups that ranged between 9.39 and 205.16 μg/day with a mean of 100 μg/day. These results are similar to levels found in other countries (Ashton et al., 2003; Bocio, Nadal, & Domingo, 2005; Santos, Lauria, & Da Silveira, 2004) but higher to the range recommended by the USA Institute of Medicine (2002). These data indicate that convenience and fast food could contribute a large amount of this essential element.

Table 3
Manganese levels in convenience and fast foods.

Sample	*n*	Mn content (μg/g)[a]		Portion size (g)	Mean Mn supplied per portion (mg)
		Mean	Range		
Beef-based foods					
Hamburger	6	1.09	1.00–1.12	150	163.50
Cheese burger	4	0.76	0.68–0.82	150	114.00
Lasagne meat	5	0.80	0.70–1.00	200	160.00
Lasagne meat and pâté	3	1.10	0.90–1.60	200	220.00
Pasta Bolognese	4	0.75	0.68–0.83	200	150.00
Beef in ale	3	0.90	0.85–1.30	300	270.00
Minced beef and onion pies	3	1.05	0.98–1.15	150	157.50
Frankfurter	5	0.88	0.80–0.96	150	132.00
Chicken-based foods					
Noodle soup with chicken	6	1.95	1.80–2.30	125	243.75
Chicken croquettes	5	2.40	2.00–2.70	150	360.00
Chicken and vegetable soup	5	1.95	1.80–2.82	125	243.75
Chicken with rice	4	1.50	1.20–1.80	250	375.00
Cooked chicken	5	2.10	1.75–2.40	200	420.00
Chicken sandwiches	5	2.00	1.85–2.30	150	300.00
Chicken with aromatic herbs	3	2.10	2.00–2.30	250	525.00
Chicken with mushroom	5	2.60	2.30–2.90	250	650.00
Fish-based food					
Sole with white sauce	4	0.75	0.60–0.80	300	225.00
Hake with green sauce	3	0.90	0.67–1.00	250	225.00
Salmon cottage pie	3	0.76	0.65–0.87	300	228.00
Mariner's pie	3	0.79	0.65–0.88	250	197.50
Tuna pasties	3	1.80	1.50–1.95	200	360.00
Cod croquettes	4	0.90	0.70–1.30	150	135.00
Hake croquettes	4	1.45	1.10–1.67	150	217.50
Hake fish fingers	3	2.42	2.00–2.58	200	484.00
Rice with shellfish	3	1.33	1.00–1.46	300	399.00
Pork-based foods					
Pork with mushroom sauce	4	1.60	1.50–1.90	250	400.00
Ham-cheese-tomato pizza	5	1.70	1.66–1.95	200	340.00
Pork pie	3	1.45	1.30–1.89	200	290.00
Frankfurter	4	1.95	1.80–2.40	150	292.50
Hamburger	3	1.88	1.70–2.50	150	282.00
Ham croquettes	4	1.93	1.88–2.50	150	289.50
Egg-based foods					
Cheese-ham quiche	3	0.96	0.90–1.12	200	192.00
Broccoli quiche	2	1.05	0.98–1.13	200	210.00
Pudding (chocolate)	3	0.98	0.87–1.05	125	122.50
Custard	6	0.65	0.55–0.99	125	81.25
Crème caramel	6	0.18	0.15–0.22	125	22.50
Sauces					
Ketchup	4	2.25	2.10–2.50	30	67.50
Tomato	3	2.00	1.80–2.90	50	100.00
Mayonnaise	5	2.10	1.75–2.00	25	52.50
Béchamel	4	1.80	1.70–1.95	50	90.00
Roquefort	4	1.40	1.10–1.50	25	35.00
Sweet and sour	3	1.75	1.40–2.10	25	43.75
Mustard	3	2.00	1.50–2.38	25	50.00

[a] Referred to fresh weight of edible portion.

3.3. Mn levels in convenience and fast foods

Mn levels in the analyzed samples ranged from 0.15 to 2.90 μg/g referred to fresh weight of the edible portion. Data are summarized in Table 3. Mn presence was detected in all the samples but notable differences from sample to sample even in each food group were observed. The most elevated Mn concentrations were found in chicken-based foods (mean of 2.07 μg/g), pork-based foods (mean of 1.75 μg/g) and sauces (mean of 1.90 μg/g) (Fig. 2). Among each group, elevated levels were detected in dairy products that contain cocoa or chocolate, nuts, egg-yolk, whole cereals, and green leaf vegetables. Stobbaerts, Robberecht, and Deelstra (1995) determined the Mn content of several duplicate meals consumed by different population groups in Belgium; these authors reported that the intake level of vegetarians is similar to that of omnivorous adults, 2.9 ± 2.0 and 2.5 ± 0.3 mg/day, respectively. These authors indicated that a lower energy intake may in part be associated with a lower Mn intake. Bocio et al. (2005) estimated the mean dietary intake of Mn in the general population of Tarragona (Spain) as 2.42 mg/day, Santos et al. (2004) in Rio de Janeiro (Brazil) as 2.5 mg/day, and Velasco-Ryenold et al. (2008) in south eastern Spain as 3.05 ± 0.61 mg/day.

According to our results, a normal portion of convenience and fast foods (125–300 g) supplied between 22.5 and 650 μg of Mn (crème caramel and chicken with mushroom, respectively) whereas the mean supplied by a sauce portion (25–50 g) could ranged between 35 and 100 μg. There is not a good linear correlation between Mn and Mn per portion ($r = 0.4298$) and a deeper regression study did not find any good model to fit the data. It is important to note that the highest levels of Mn per portion were found in chicken based food and the lowest in sauces (Fig. 2) in contradiction to Mn levels (Fig. 2). The Kruskal–Wallis tests for Mn and Mn per portion indicate that there is no statistical evidence of similarity between the medians of the food groups (p-value = 0.00019 and 0.00011, respectively) what could be intuitively expected observing Fig. 2. These data indicates that certain convenience and fast food could contribute a notable amount of Mn, whereas the dietary intake repercussion of others is scarce. In accordance with Cabrera et al. (1996), it is found a low moderate increase of Mn presence in pudding, custard and crème caramel packed in glazed ceramic containers but the number of samples with this type of container was scarce. The most usual containers are the aluminium ones. It is interesting to compare the Mn content between convenience and fast food and a dish of the raw components; generally, foods produced from Mn-rich ingredients were themselves high in Mn. However, food processing and additional ingredients of the instant meal contribute to a higher final Mn concentration or conversely they may further dilute the initial low Mn content. Taking into account the differences between the metal content and its content per portion, let us remark that the Cr per portion mean was 64.93 μg, its 95% confidence interval (46.17 μg, 83.70 μg), the Mn per portion mean was 240.56 μg and its 95% confidence interval (191.02 μg, 290.09 μg). It is also interesting to note that there was no correlation between Cr and Mn or Cr per portion and Mn per portion.

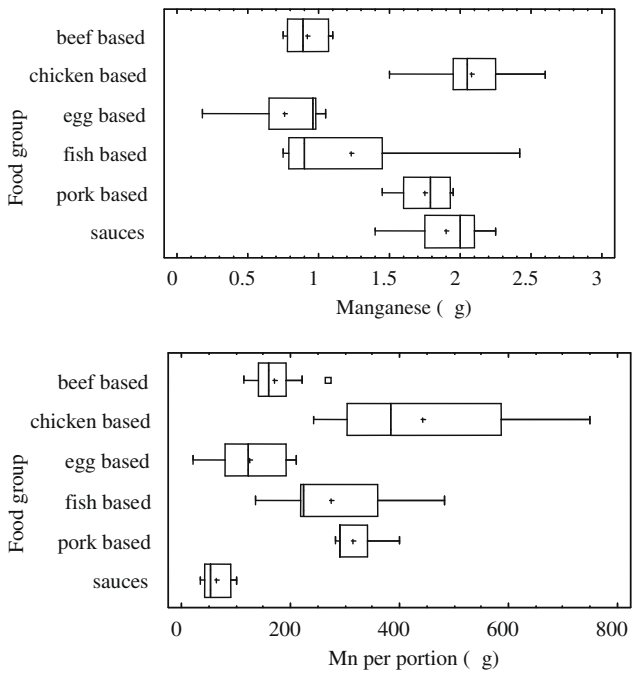

Fig. 2. Box and Whisker plot for Mn and Mn per portion by food group analyzed.

3.4. In vitro Cr and Mn dialyzable fraction

Table 4 shows the results of Cr and Mn dialyzable fraction after simulated *in vitro* the digestion process. *In vitro* analyses were done with 10 randomly chosen samples. Cr and Mn dialyzable fraction ranged from 0.38% to 1.05% and from 7.75% to 15.60%, respectively. These percentages express the element fraction that would be available for absorption by intestinal cells (bioaccessible Cr and Mn). The statistical analysis found that the data variability decreases considerably from a variation coefficient of 90% for Cr to 35.46% for Cr dialyzable fraction and from 35.92% for Mn to 23.83% for Mn dialyzable fraction. It may point out that the absorption level is limited. Cr dialyzable fraction of is quite positively correlated with Cr levels ($r = 0.7706$). However, there is no correlation between Mn dialyzable fraction and Mn levels ($r = 0.2444$). Then, data suggests that the relation between dialyzable-total Cr content in food is stronger than in Mn case. In addition, the latter is more irregular. As what happened with Cr and Mn, there is no good correlation between absorbable Cr and absorbable Mn. Cr bioaccessibility may depend on interactions with other dietary components, including other minerals, and both competitive and cooperative interdependence have been reported. Van Cauwenberg, Hendrix, Robberecht, and Deelstra (1996) suggested that Cr intake may increase parallel to energy intake. García, Cabrera, Lorenzo, López, and Sánchez (2001b) estimated Cr bioaccessibility from the diet by an *in vitro* method as 0.4–1.6% for a total dietary intake from 16 to 117 μg/day. These authors observed the influence of the total Cr content in the diet on the Cr dialyzable fraction. Cr absorption was higher for low levels of daily dietary intake (<40 μg) than for levels of 40–80 μg; for high levels (>80 μg) there was an increase in the dialyzable fraction. Cabrera et al. (1996) determined the mean absorbable fraction of Cr and Mn in dairy products using an *in vitro* method as 2.0 ± 1.0% and 5.0 ± 2.0%, respectively. Velasco-Ryenold et al. (2008) reported that Mn absorption (1–16%) is affected by dietary factors, such as element concentration, chemical form, source and oxidation state, lumen intestinal content, endogenous physiological factors, interaction with other nutrients and meal composition. Phytate, ascorbic acid, proteins and other dietary constituents interacts with Mn in absorption process. These authors indicated that foods with higher protein, carbohydrate and therefore energy contents, e.g. cereals, legumes, vegetables and fruits, would be primary sources of bioaccessible Mn in the diet and established the mean bioaccessible fraction of Mn from duplicate meals as 22.0 ± 8.93%. These findings suggest the need for further studies aimed at identifying the factors that can modify the bioavailability of Cr and Mn in the diet. The resulting data will also be useful to estimate the nutritional requirements of these elements.

Table 4
Cr and Mn dialyzable fraction in convenience and fast foods, estimated by an *in vitro* method.

Sample	Cr total content(μg/g)[a]	Cr dialyzablefraction (%)[b]	Mn total content(μg/g)[a]	Mn dialyzablefraction (%)[b]
Hamburger (beef)	0.89 ± 0.05	1.05 ± 0.06	1.09 ± 0.06	8.50 ± 0.10
Lasagne meat	0.47 ± 0.04	0.60 ± 0.02	0.75 ± 0.02	10.75 ± 0.15
Chicken and vegetable soup	0.20 ± 0.03	0.54 ± 0.03	2.05 ± 0.07	12.10 ± 0.10
Salmon cottage pie	0.05 ± 0.005	0.67 ± 0.02	0.78 ± 0.02	9.50 ± 0.15
Hake with green sauce	0.07 ± 0.01	0.54 ± 0.02	1.00 ± 0.03	8.90 ± 0.12
Pork with mushroom sauce	0.81 ± 0.02	1.02 ± 0.07	1.90 ± 0.06	7.80 ± 0.10
Ham-cheese-tomato-pizza	0.42 ± 0.02	0.38 ± 0.02	1.66 ± 0.07	15.60 ± 0.20
Pudding (chocolate)	0.65 ± 0.03	0.90 ± 0.04	0.99 ± 0.04	10.35 ± 0.10
Tomato sauce	0.06 ± 0.007	0.65 ± 0.02	1.80 ± 0.08	8.90 ± 0.08
Roquefort sauce	0.47 ± 0.03	0.40 ± 0.02	1.50 ± 0.07	7.75 ± 0.05

[a] Mean ± standard deviation of three replicate of each sample.
[b] Mean ± standard deviation of three assays in each sample.

4. Conclusion

This study contributes new data on Cr and Mn content in foods that are potentially useful as supplementary and/or previously unavailable information to current food composition tables and also to estimate reliably the total dietary intake of these elements in accordance with the actual dietary habits. Periodical determinations and additional studies on Cr and Mn bioaccessibility are advisable because the growing popularity and the large amount of these products that are consumed as part of the habitual diet, especially elder people who live alone, young people and children. It also derives information about the bioaccessibility of these essential elements.

Acknowledgements

We thank A.L. Tate and Elise Little for revising our English text. This work was partially supported by project MTM2007-63793 of Dirección General de Investigación of the Ministerio de Ciencia e Innovación, Spain.

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Analytical papers related to the microbiological, sensory, nutritional, physiological, authenticity and origin aspects of food. Papers should be primarily concerned with new or novel methods (especially instrumental or rapid) provided adequate validation is described including sufficient data from real samples to demonstrate robustness. Papers dealing with significant improvements to existing methods, or data from application of existing methods to new foods, or commodities produced in unreported geographical areas, will also be considered.

- Methods for the determination of both major and minor components of food especially nutrients and non-nutrient bioactive compounds (with putative health benefits) will be considered.
- Results of method inter-comparison studies and development of food reference materials for use in the assay of food components;
- Methods concerned with the chemical forms in food, nutrient bioavailability and nutritional status;
- General authentication and origin [e.g. Country of Origin Labelling (COOL), Protected Designation of Origin (PDO), Protected Geographical Indication (PGI), Certificate of Specific Character (CSC)] determination of foods (both geographical and production including commodity substitution, and verification of *organic, biological* and *ecological* labelling) providing sufficient data from authentic samples should be included to ensure that interpretations are meaningful.

FOOD CHEMISTRY

Aims and Scope

Food Chemistry publishes original research papers dealing with the chemistry and biochemistry of foods and raw materials covering the entire food chain from *'farm to fork.'* Topics include:

- Chemistry relating to major and minor components of food, their nutritional, physiological, sensory, flavour and microbiological aspects;
- Bioactive constituents of foods, including antioxidants, phytochemicals, and botanicals. Data must accompany sufficient discussion to demonstrate their relevance to food and/or food chemistry;
- Chemical and biochemical composition and structure changes in molecules induced by processing, distribution and domestic conditions;
- Effects of processing on the composition, quality and safety of foods, other bio-based materials, by-products, and processing wastes;
- Chemistry of food additives, contaminants, and other agro-chemicals, together with their metabolism, toxicology and food fate.

Analytical Section

Analytical papers related to the microbiological, sensory, nutritional, physiological, authenticity and origin aspects of food. Papers should be primarily concerned with new or novel methods (especially instrumental or rapid) provided adequate validation is described including sufficient data from real samples to demonstrate robustness. Papers dealing with significant improvements to existing methods, or data from application of existing methods to new foods, or commodities produced in unreported geographical areas, will also be considered.

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