Talanta 120 (2014) 47–54

Contents lists available at ScienceDirect

Talanta

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

Vortex-assisted liquid–liquid microextraction coupled with high performance liquid chromatography for the determination of furfurals and patulin in fruit juices

Nur-Bahiyah Abu-Bakar [a], Ahmad Makahleh [a,*], Bahruddin Saad [a,b,**]

[a] *School of Chemical Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia*
[b] *Doping Control Centre, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia*

ARTICLE INFO

Article history:
Received 21 October 2013
Received in revised form
26 November 2013
Accepted 27 November 2013
Available online 4 December 2013

Keywords:
Furfurals
Patulin
Salting out-vortex-assisted liquid-liquid microextraction
High performance liquid chromatography
Fruit juices

ABSTRACT

A fast and simple solvent microextraction technique using salting out-vortex-assisted liquid–liquid microextraction (salting out-VALLME) was developed for the extraction of furfurals (2-furfural (2-F), 3-furfural (3-F), 5-methylfurfural (5-MF) and 5-hydroxymethylfurfural (5-HMF)) and patulin (PAT) in fruit juice samples. The optimum extraction conditions for 5 mL sample were: extraction solvent, 1-hexanol; volume of extractant, 200 μL; vortex time, 45 s; salt addition, 20%. The simultaneous determination of the furfurals and PAT were investigated using high performance liquid chromatography coupled with diode array detector (HPLC–DAD). The separation was performed using ODS Hypersil C18 column (4.6 mm i.d × 250 mm, 5 μm) under gradient elution. The detection wavelengths used for all compounds were 280 nm except for 3-F (210 nm). The furfurals and PAT were successfully separated in less than 9 min. Good linearities ($r^2 > 0.99$) were obtained within the range 1–5000 μg L^{-1} for all compounds except for 3-F (10–5000 μg L^{-1}) and PAT (0.5–100 μg L^{-1}). The limits of detection (0.28–3.2 μg L^{-1}) were estimated at *S*/*N* ratio of 3. The validated salting out-VALLME-HPLC method was applied for the analysis of furfurals and PAT in fruit juice samples (apple, mango and grape).

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Furfurals are heterocyclic aldehydes which are formed by the degradation of sugars when carbohydrate-rich foods are heated [1] or via the Maillard reaction (MR) which occurred when reducing sugars react with amino acids or proteins [2]. MR is influenced by reaction time, temperature, pH and concentration of the precursors [2]. Furfurals (e.g., 5-hydroxymethylfurfural (5-HMF), 5-methylfurfural (5-MF), and 2-furfural (2-F)) are the recognized parameters of food freshness and quality. These compounds can be found in a wide variety of foodstuffs such as bread, honey, biscuit, cereal, jam, infant milk, beverages (coffee, citrus, and apple juices), etc. [3–5].

Furfurals have been used to evaluate processing methods [6], quality deterioration [7], severity of heating [8], and organoleptic characteristics of the final products [3]. Although 5-HMF is seldom found in fresh and unprocessed food, but its concentration tend to rise during heating or long term storage [9]. Furfurals, especially 5-HMF have been reported as chemical markers to evaluate the browning reactions in foods [3,9]. In vitro studies have proven that 5-HMF may exhibit cytotoxic, mutagenic, carcinogenic and genotoxic effects [10]. However, the toxicological concerns on furfurals are still under investigation. In fact, the International Federation of Fruit Juice Producers (IFFJPs) suggested a maximum concentration of 5-HMF in fruit juice as 5–10 mg L^{-1} and fruit concentrate, 25 mg L^{-1} [11]. The Codex Alimentarius of the World Health Organization and the European Union (EU Directive 110/2001) have established maximum concentration level of 5-HMF in honey (40 mg kg^{-1}) and apple juice (50 mg kg^{-1}). The high concentrations of 5-HMF in food are indicators for deterioration and heat-treatment [3]. So far, no limits have been established for the other furfurals.

Patulin (PAT) is a naturally occurring mycotoxin that is produced by fungus species of *Penicillium*, *Aspergillus*, and *Byssochlamys* [12]. PAT is mainly found in apples and apple products [13,14]. Animal studies have proven that PAT is mutagenic and can cause chronic symptoms (e.g., neurotoxic, immunotoxic, genotoxic and gastrointestinal) in rats [15]. Acute symptoms of PAT were also reported such as agitation, convulsions, edema, ulceration, intestinal inflammation and vomiting [16].

PAT is a useful quality indicator during production of apple juice. Thus, the levels of the final product are subjected to legislative

* Corresponding author. Tel.: +604 653 6018; fax: +604 657 4854.
** Corresponding author at: Doping Control Centre, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia. Tel.: +604 653 4695; fax: +604 657 4857.
E-mail addresses: makahleha@yahoo.com (A. Makahleh), bahrud@usm.my (B. Saad).

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.11.081

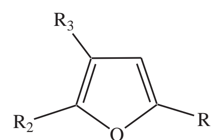
regulatory control. The European Union (EU) has established a maximum concentration of PAT: (i) 50 μg kg^{-1} in fruit (apple) juices, reconstituted fruit juices, spirit drinks, cider and other fermented drinks derived from apples or containing apple juice; (ii) 25 μg kg^{-1} in solid apple products; (iii) 10 μg kg^{-1} in apple products intended for infants and young children [17]. According to the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA), the daily consumption of PAT is estimated to 0.2 μg kg^{-1} body weight for children and 0.4 μg kg^{-1} body weight for adults [17].

High performance liquid chromatography either with ultraviolet detection (HPLC–UV) or diode array detection (HPLC–DAD) is the most commonly used method for the analysis of furfurals and PAT [11,17,18]. The simultaneous separation of furfural compounds commonly use acids in the mobile phase (e.g., sulfuric acid [19], perchloric [11], formic [20,21], and acetic acids [14]). This may sometimes affect the column performance and shortening its lifetime [19]. Furthermore, the analysis time is rather long (~31 min) [11]. The analysis of furfurals and PAT either using liquid chromatography–mass spectrometry (LC–MS) [6,22], gas chromatography (GC) [3,23,24] or capillary electrophoresis [4,5,25,26] have also been reported.

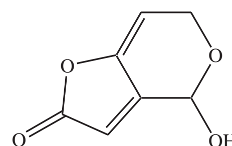
Various sample preparations have been employed for the extraction of furfurals and PAT in food stuffs. Liquid–liquid extraction (LLE) is the most commonly used [17–19,27]. Due to the differences in the solubilities of furfurals and PAT, the selection of suitable organic solvent is always problematic. Alternatively, solid phase extraction (SPE) technique has been used [3,4,6,14,21,27,28]. The consumption of large amounts of organic solvents, longer extraction time (due to the multi extraction process and involving evaporation step) are considered as the main disadvantages of the LLE and SPE techniques [17,18,27].

Recently, the use of microextraction techniques in food analysis has increased. Solid phase microextraction (SPME) (either with direct-immersion or head-space mode) has been reported for the analysis of furfurals in various types of samples such as fruits metabolites [29], honey [24], treacle [30], wines [31], vinegar [24], and palm oil [32]. An in-tube SPME method has also been reported for the extraction of PAT in dried fruit samples [33]. A headspace-liquid phase microextraction (HS-LPME) method recently been reported for the analysis of furanic compounds in coffee [34]. This technique showed satisfactory recovery for 2-F (102%) with good sensitivity (limit of detection, 10 ng g^{-1}). However, the analysis of other furfurals was not explored. Dispersive liquid–liquid microextraction (DLLME) technique has also been used for the extraction of PAT in apple juices [24,35]. Although the DLLME technique is simple, rapid and inexpensive, but toxic chemicals (e.g., chloroform) and evaporation step are still involved.

A new microextraction technique (vortex-assisted liquid–liquid microextraction (VALLME)) has recently been introduced by Yiantzi et al. [36]. An important feature of this technique is the dispersion of the extraction solvent into the aqueous sample that is obtained by vortex agitation, thus, forming a mild emulsification process [36]. Due to the shorter diffusion distance and larger specific surface area, the fine droplets formed can extract the target analytes faster [37]. The VALLME technique can also overcome the need of a disperser solvent (e.g. 2-propanol) that is mandatory in DLLME technique. Centrifugation is sometimes needed in order to speed up the separation of the aqueous and organic phases. Surfactants such as Triton X-114 [38], Tween-20 [39], Triton X-100 [40], and cetyltrimethylammonium bromide (CTAB) [41] were also used to enhance the extraction efficiency. VALLME was successfully applied for the extraction of alkyl phenols [36], pesticides [38–40,42,43], perfluooctane sulfonate [37], polychlorinated biphenyls [44], and phthalate esters [41] in water samples. The previously reported methods usually deal with



Furfural	R_1	R_2	R_3	log P
5-hydroxymethyl furfural (5-HMF)	CH_2OH	CHO	H	-0.78
2-furfural (2F)	H	CHO	H	-0.38
3-furfural (3F)	H	H	CHO	0.71
5-methylfurfural (5-MF)	CH_3	CHO	H	0.51



Patulin (log P = -0.38)

Fig. 1. Structures of the studied furfurals and patulin.

analytes that have low solubility in water and high affinity for the organic solvent ($\log P > 2$). An exception is the extraction of amines of different polarity ($\log P$, -0.47 to 2.9) in milk and beer [45]. In order to enhance the affinity of amines towards the organic solvent, an *in situ* derivatization step was used.

In the present study, the use of VALLME coupled with HPLC–DAD was applied for the first time for the simultaneous determination of furfurals (5-HMF, 5-MF, 2-F and 3-furfural (3-F)) and PAT in fruit juices. As these compounds have different solubilities ($\log P < 0.71$, Fig. 1), instead of forming derivatives as previously reported [45], the use of suitable organic solvent and the addition of salt (salting out effect) are the key strategies for achieving high extraction efficiency. As will be evident later, the proposed salting out-vortex assisted liquid–liquid microextraction (salting out-VALLME) method does not involve evaporation step, a procedure that is usually required for the extraction of furfurals and PAT using LLE [17,18,27], SPE [3,4,6,14,21,28], and DLLME [25,35] techniques. The proposed method was applied to apple, mango and grape juices.

2. Experimental

2.1. Chemicals and reagents

2-furfural (99%), 3-furfural (97%), 5-methylfurfural (99%), 5-hydroxymethylfurfural (99%), patulin (≥98%), and 1-pentanol (≥99.5%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile (≥99.9%), 1-hexanol (>98%), 1-heptanol (≥99%) and sodium chloride (≥99.5%) were obtained from Merck (Darmstadt, Germany). Ultrapure water (resistivity, 18.2 MΩ cm^{-1}) was produced from a Nanopure Diamond Analytical Water system that was purchased from Barnstead Thermo Scientific (Waltham, MA, USA), and was used throughout for the preparation of solutions. Apple, mango and grape juice samples were purchased from local supermarkets. Fresh fruits were extracted using fruit extractor in order to perform recovery studies. Sodium chloride solution was prepared by dissolving 25 g NaCl in water to a final volume of 100 mL.

2.2. Instrumentation

A HPLC system (model 2695) was obtained from Waters Alliance (Milford, MA, USA). The instrument was equipped with DAD (model 2998) set at 210 and 280 nm and scanned from 190 to 400 nm. Separations were carried out using Hypersil ODS C18

column (250 × 4.6 mm, 5 μm) purchased from Thermo Fisher Scientific (Waltham, MA, USA). The analytes (injection volume, 50 μL) were separated using acetonitrile and water as mobile phase with gradient elution. The gradient program was: linear gradient of 20% acetonitrile for 1–7 min, from 20% to 95% acetonitrile for 8–10 min and 20% acetonitrile for 12–16 min at 1 mL min^{-1}. The data were processed using licensed empower V.2 software (Milford, MA, USA).

2.3. Preparation of standards and samples

Stock solution of furfurals (50 mg L^{-1}) and PAT (1 mg L^{-1}) was prepared in water. The solution was wrapped using aluminum foil and stored in the dark at 4 °C. Working standards and solutions for calibration curve were freshly prepared by appropriate dilution of the stock solution in 5 mL volumetric flask (containing 4 mL of NaCl solution) and top-up to the mark with water. Juice sample was filtered and the filtrate (500 μL or 200 μL when the concentrations exceeded the upper limit of the calibration curves) was transferred to a 5 mL volumetric flask, NaCl solution (4 mL) was then added and the mixture was diluted with water until the mark. Standards and sample solutions were then subjected to the salting out-VALLME procedure.

2.4. Salting out-VALLME procedure

5 mL volumetric flask was used as the extraction device. 200 μL of 1-hexanol (extraction solvent) was added into the standard or sample solution (5 mL). The mixture was vigorously shaken using a vortex agitator (Mixer UZUSIO model VTX-3000 L, LMS group, Tokyo, Japan) for 45 s at 3000 rpm (maximum setting). The separation of the two phases took less than 1 min. The organic phase (upper layer) was collected and directly introduced into the chromatographic unit.

2.5. Method validation

Method validation parameters (linearity, limit of detection (LOD), repeatability, and recovery) were performed after subjected to the salting out-VALLME method. The linearity was investigated over the range of 1–5000 μg L^{-1} (5-HMF, 2-F, 3-F and 5-MF) and 0.02–100 μg L^{-1} (PAT). LOD values were estimated at S/N ratio of 3 using fresh fruit juice samples. Repeatability was studied by injecting six replicates of five different concentration levels (2, 10, 100, 1000 and 5000 μg L^{-1}) for all compounds except 3-F (10, 100, 1000 and 5000 μg L^{-1}) and PAT (1, 10, 50 and 100 μg L^{-1}) and was expressed as relative standard deviation (% RSD). The recovery test was performed by spiking fresh fruit juices (500 μL) at five different concentration levels (2, 10, 100, 1000 and 5000 μg L^{-1}) for all compounds except 3-F (10, 100, 1000 and 5000 μg L^{-1}) and PAT (1, 10, 50 and 100 μg L^{-1}). The mixture was next vortexed (1 min), NaCl solution (4 mL) was added and the mixture was then diluted to the mark with water. Each concentration was extracted in triplicates and injected twice.

3. Results and discussion

3.1. Chromatographic separation of furfurals and PAT

In order to separate furfurals and PAT, different mobile phase compositions of acetonitrile:water (20–40:60–80; v/v) and methanol:water (20–40:60–80; v/v) were studied. As the ratio of organic solvents (methanol or acetonitrile) decreased, the resolution between the peaks and the retention times were found to increase. The peaks were well separated at lower composition of organic solvents (20–25%; v/v). However, acetonitrile showed better results compared to methanol in terms of shorter run time, better peak shapes and higher resolution for all compounds (especially between 5-HMF and PAT). Therefore, acetonitrile:water (20:80; v/v) was chosen. Higher injection volume (up to 100 μL) can be achieved using this mobile phase composition without affecting the resolutions or the peak shapes.

The introduction of the extraction solvents into the HPLC unit resulted in negative effect on the reproducibility of the retention times and resolutions. It was observed that after the first injection, separation between the peaks gets progressively poorer with the subsequent injections. In order to overcome this problem, the extraction solvent was evaporated at 40 °C under gentle nitrogen stream and the residue was reconstituted with water. However, a significant loss of the analytes (especially 2-F and 5-MF) was observed. In order to avoid the loss of analytes and obtain more reproducible results, further modification of the mobile phase composition was conducted. The lack of reproducibility was probably due to the late elution of the extraction solvent which interfered the separation of the peaks in the next injection. In order to elute the extraction solvents prior to the next injection, a gradient elution was proposed after the elution of all peaks by increasing the ratio of acetonitrile to 95%. This resulted in satisfactory retention times and resolutions.

All the peaks were baseline separated and eluted in less than 9 min. This represents an improvement in the separation time compared to the previously reported method (~31 min) [11]. Unlike the earlier reported methods [11,19,21], for the first time, a simple chromatographic method that allows the simultaneous separation of 5-HMF, 5-MF, 2-F, 3-F and PAT without using acid (e.g., sulfuric and perchloric acids) in the mobile phase was feasible.

3.2. Salting out-VALLME

The optimization of the salting out-VALLME method was studied by using 5 mL of standard mixture solutions (100 μg L^{-1}). Different parameters (i.e. extraction solvent, volume of extractant, vortexing time and salt addition) that influenced the extraction efficiency were optimized.

3.2.1. Effect of the extraction solvent

The selection of an appropriate extraction solvent is one of the most important factors of the VALLME method. The extraction solvent should fulfill several criteria such as immiscibility with water, high extraction efficiency for the target analytes, lower density than water, limited solubility in water, good chromatographic behavior and easy to form emulsion after vortex agitation [43]. Organic solvents such as ethyl acetate and diethyl ether which were previously reported [27,46] for the extraction of furfurals and PAT have been tested. However, the use of small amounts of these solvents formed a miscible layer with water due to their solubility in water (6.9 and 8.3 g per 100 mL water for diethyl ether and ethyl acetate, respectively). In order to overcome the miscibility problem, different ratios of ethyl acetate and hexane mixtures were studied. It was found that the immiscibility improved as the ratio of hexane increased. However, the extraction efficiency decreased due to the low solubility of furfurals and PAT in hexane.

Since furfurals and PAT are polar compounds, the use of organic solvents with polar functional group may enhance the extraction efficiency. Therefore, alcoholic solvents were studied. The use of methanol, ethanol, propanol and butanol was avoided either due to their miscibility or high solubility in water. Therefore, longer carbon chain solvents could be viable.

1-Pentanol, 1-hexanol and 1-heptanol were next tested. It was expected that the extraction efficiency will increase as the number of carbon chain decrease due to the increase of solvent polarity. However, 1-hexanol showed the highest extraction efficiency (Fig. 2(A)). This is probably due to its high polarity index when compared to 1-heptanol. Moreover, 1-hexanol has lower solubility in water (0.59 g/100 mL) compared to 1-pentanol (2.2 g/100 mL). The high solubility of 1-pentanol in water resulted in significant loss of the solvent and reduction of the extraction efficiency. Different ratios of 1-hexanol and ethyl acetate mixtures were also studied. It was observed that the extraction efficiency decreased as the ratio of ethyl acetate increased. The best efficiency was achieved when no ethyl acetate was used. Therefore, 1-hexanol was selected for the subsequent experiments.

The use of disperser solvents (e.g., methanol, acetonitrile and acetone) was also investigated. As the volume of the disperser solvent decrease, the peak areas were found to increase (Fig. 2(B)). Best results were achieved when no disperser solvent was used.

The mass transfer of the analytes is expected to increase as the volume of 1-hexanol is increased due to the enhancement of partitioning of analytes between the two phases. However, the increase of mass transfer together with the extractant volume not necessarily resulted in increase of the analytes concentrations in the extractant phase due to the dilution factor. Different volumes of the extraction solvent (100–500 μL) were investigated. It was found that the peak areas gradually decreased as the volume of 1-hexanol increased. 200 μL produced the highest peak areas for all furfurals and PAT which is also supported by the requirement of using less amounts of organic solvents (Fig. 2(C)). The use of small volumes (<200 μL) was not considered due to the long emulsifying time and the recovered extractant volume was not enough to perform the chromatographic analysis. Therefore, 200 μL extraction solvent was chosen.

3.2.2. Effect of vortexing time and salt addition

Vortexing time affects the extraction equilibrium between the two phases and the mass transfer of the analytes, thus, influencing the extraction efficiency [43]. Different durations (15–60 s) of vortex agitation at maximum speed (3000 rpm) were studied. The peak areas of furfurals and PAT were found to slightly increase as the vortexing time increased up to 45 s (Fig. 2(D)). No significant improvements were observed thereafter (>45 s). Thus, vortexing time of 45 s (at 3000 rpm) was selected.

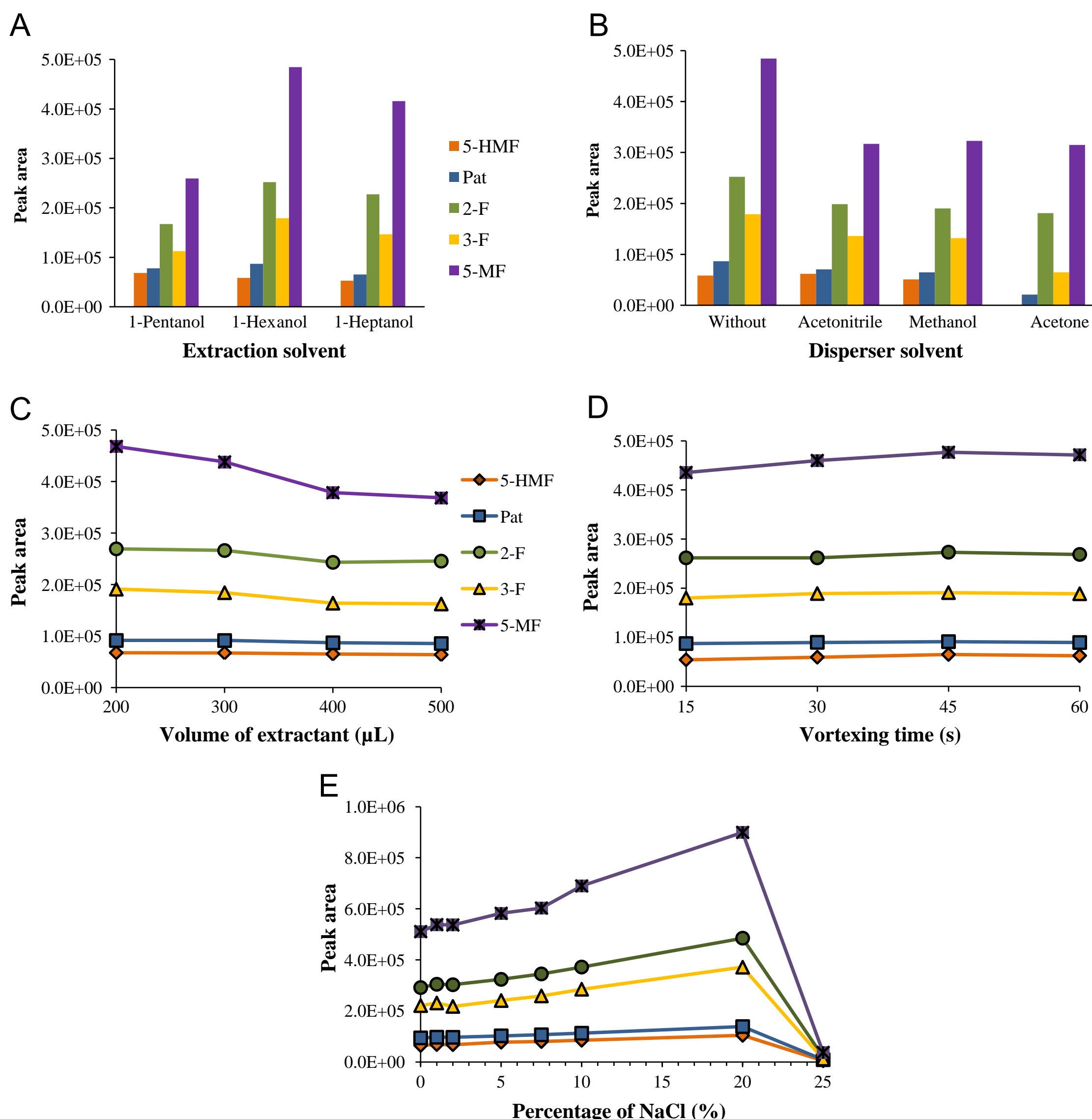


Fig. 2. Influence of: (A) types of extraction solvent, (B) disperser solvent, (C) volume of extractant, (D) vortexing time, and (E) percentage of sodium chloride.

The addition of salt to a water sample will increase the ionic strength. This results in enhancement of the solubility of hydrophobic analytes and reduces the extraction efficiency; this phenomenon is known as salting in [41]. On the other hand, addition of salt will reduce the hydrogen bonding between hydrophilic analytes and water which reduce their solubility in water and increase their mass transfer to the organic phase (salting out effect) [41]. Therefore, the influence of sodium chloride concentrations (0–25% (w/v)) on the extraction efficiency was investigated. A significant improvement in the extraction efficiency was observed as the concentration of sodium chloride was increased up to 20% but drastically dropped thereafter due to the increase of the solution viscosity (Fig. 2(E)). Thus, 20% (w/v) sodium chloride was selected as the optimum condition.

3.2.3. *Adopted extraction conditions*

The adopted extraction conditions for 5 mL sample were: extraction solvent, 1-hexanol; volume of extractant, 200 μL; vortex time, 45 s; salt addition, 20% (w/v). Typical chromatogram of the standard mixture after subjected to the optimum extraction conditions is shown in Fig. 3(A).

3.3. *Method validation*

Method validation parameters such as linearity, LOD, repeatability and recovery of the proposed salting out-VALLME method were investigated.

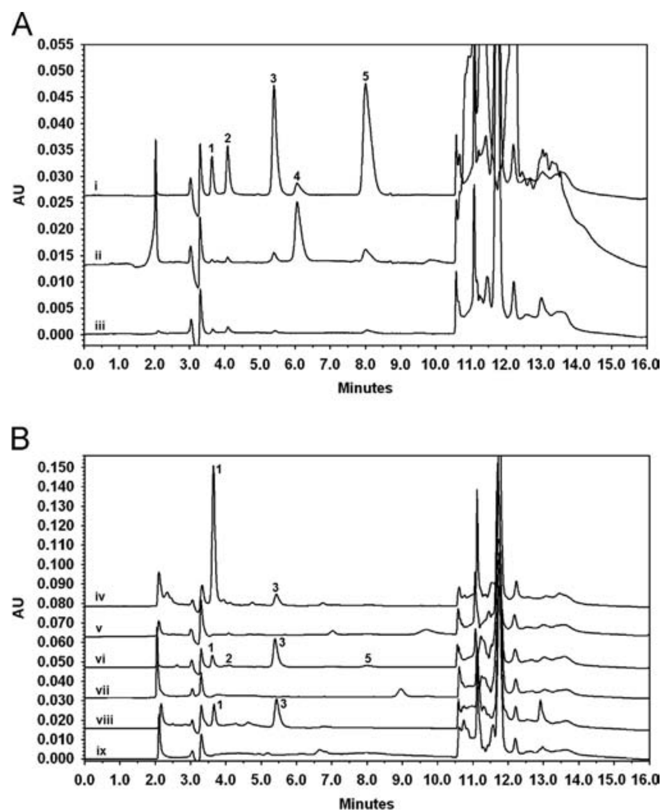


Fig. 3. Typical chromatograms of: (A) Standard mixture: 100 μg L^{-1} at 280 nm (i), 100 μg L^{-1} at 210 nm (ii) and 2 μg L^{-1} at 280 nm (iii). *HPLC conditions:* Hypersil ODS C18 column (250 × 4.6 mm^2, 5 μm); mobile phase, 20% ACN (1–7 min), 95% ACN (8–10 min) and 20% ACN (12–16 min); flow rate, 1 mL min^{-1}; injection volume, 50 μL. *Extraction conditions:* Extraction solvent, 1-hexanol; volume of extractant, 200 μL; vortex time, 45 s; salt addition, 20% (w/v). (B) Apple juice (iv), blank apple (v), mango juice (vi), blank mango (vii), grape juice (viii) and blank grape (ix) samples extracts subjected to salting out-VALLME coupled with HPLC-DAD. *Peak identity:* (1) 5-HMF, (2) PAT, (3) 2-F, (4) 3-F and (5) 5-MF.

3.3.1. *Linearity and repeatability*

Linearity was studied using nine concentrations of standard mixtures of 5-HMF, 2-F, 3-F, 5-MF and PAT. Calibration curves were established by plotting the peak area versus concentration of each analyte. 5-HMF, 2-F and 5-MF were found to be linear over the studied range (1–5000 μg L^{-1}). However, PAT and 3-F were found to be linear over the range of 0.5–100 and 10–5000 μg L^{-1}, respectively. The calibration curves were well correlated (correlation coefficients, $r^2 > 0.99$) (Table 1). Repeatability was measured by performing six extraction replicates analyses of five different concentration levels for all analytes (2, 10, 100, 1000 and 5000 μg L^{-1}) except 3-F (10, 100, 1000 and 5000 μg L^{-1}) and PAT (1, 10, 50 and 100 μg L^{-1}). Good repeatabilities were obtained for all concentrations with relative standard deviation (% RSD) less than 7.6% and 0.37% for peak area and retention time, respectively. Repeatability results for peak areas and retention times are shown in Table 2.

3.3.2. *Limit of detection (LOD)*

The LOD was estimated at *S*/*N* ratio of 3 using different fresh fruit juices (apple, mango and grape) as blank sample. The obtained LODs for furfurals were in the range 0.40–3.5 μg L^{-1} (Table 1) which is lower than the previously reported methods using SPE coupled with HPLC–UV (13–93 μg L^{-1}) [21], headspace-SPME with GC–MS (15 μg L^{-1}) [47], direct immersed-SPME with GC-flame ionization detector (1.37–8.96 μg L^{-1}) [24], headspace-LPME with GC–MS (2-F, 10 μg L^{-1}) [34] and micellar electrokinetic chromatography (MEKC)–DAD (330–700 μg L^{-1}) [5], but higher than the reported HPLC–DAD method with direct dilution (0.06–0.73 μg L^{-1}) [11].

The obtained LODs for PAT using the proposed salting out-VALLME (0.28–0.31 μg L^{-1}) was lower than the reported methods using LLE, SPE or their combination coupled with HPLC–DAD (1.3–6.6 μg L^{-1}) [14,27], DLLME with HPLC–UV (≥2 μg L^{-1}) [35] and DLLME with MEKC–DAD (0.6 μg L^{-1}) [25], while higher than the reported methods using in-tube-SPME coupled with LC–MS

Table 1
Linearity and limits of detection (LODs) of furfurals and patulin using the proposed salting out-VALLME method.

Analyte	Linear range (μg L^{-1})	Regression equation	r^2	LOD (μg L^{-1})		
				Apple juice	Mango juice	Grape juice
5-HMF	1–5000	$Y=367x+1760$	0.9997	0.43	0.45	0.40
PAT	0.5–100	$Y=591x+3871$	0.9985	0.28	0.31	0.28
2-F	1–5000	$Y=1868x-1701$	1.000	0.68	0.71	0.68
3-F	10–5000	$Y=1385x+13181$	0.9998	3.5	3.5	3.4
5-MF	1–5000	$Y=3532x-5536$	0.9995	0.51	0.55	0.50

Table 2
Repeatability for standard mixture subjected to salting out-VALLME method ($n=6$).

Spiked level[a] μg L^{-1}	RSD (%)									
	5-HMF		PAT		2-F		3-F		5-MF	
	Area	RT[b]	Area	RT	Area	RT	Area	RT	Area	RT
2	3.2	0.27	–	–	3.0	0.25	–	–	2.3	0.23
10 (1)	2.9	0.23	7.6	0.21	2.7	0.22	5.4	0.32	1.8	0.34
100 (10)	2.2	0.11	2.4	0.22	2.3	0.11	3.3	0.21	3.7	0.16
1000 (50)	1.5	0.25	4.0	0.23	2.0	0.15	3.0	0.17	1.5	0.19
5000 (100)	2.3	0.30	1.8	0.14	2.4	0.24	3.4	0.27	2.2	0.37

[a] Figures in parenthesis refer to the spiked level of PAT.
[b] Retention time.

(0.024 μg L^{-1}) [33]. Generally, the sensitivity achieved for furfurals and PAT in different matrices meets the requirement for quality control purpose for the analysis of furfurals and PAT in fruit juices (maximum permitted concentration: 5-HMF, 50 mg kg^{-1} (EU) and 5–10 mg L^{-1} (IFFJPs); PAT, 50 μg kg^{-1} (EU)).

3.3.3. *Recovery*

Good recoveries were obtained for all tested compounds in apple (90–112%), mango (82–107%) and grape (88–102%) juice samples, results are shown in Table 3. Clear juices were produced from apple and grape fruits, thus, the recoveries from these juices were better than from mango juice (cloudy). The obtained recoveries of the studied furfurals in apple (91–105%), mango (82–104%) and grape (88–102%) were better than the previous reported recoveries in apple juices and apple products using direct dilution coupled with HPLC–DAD (57–98%) [11] and SPE–HPLC–DAD (78–108%) [21], and in wine using headspace-SPME with GC–MS (56–107%) [31] and SPE–HPLC–DAD (64–106%) [21]. The results were comparable with the previously reported data in urine using direct dilution with HPLC–UV or GC–MS (89%) [2], coffee using headspace-LPME with GC–MS (102%) [34], oil using LLE–HPLC–DAD (94–100%) [20] and treacle using direct dilution with HPLC–UV (85%) [30].

The obtained recoveries for PAT in apple juice (90–112%) were better than the other reported results for the analysis of PAT in apple juices (LLE–HPLC–DAD (73–95%) [17], LLE–HPLC–UV (71–77%) [18] and DLLME–MEKC–DAD (75–80%) [25]), apple fruit (SPE–HPLC–DAD (63–80%) [28]) and apple based-baby food (LLE–HPLC–UV (70–81%) [18]). The recoveries were comparable with the reported results for the analysis of apple juice using SPE–HPLC–DAD (83–112%) [28] and in-tube-SPME–LC–MS (93–94%) [33].

3.4. *Application to apple juice samples*

Initially the method was applied using large volume of sample. However, the obtained results for some analytes exceeded the linear range. In order to avoid further dilution of the extraction solvent, the sample has been diluted before performing the extraction. 200–500 μL was found to be a suitable dilution for the tested sample. Therefore, 500 μL was used in this study. 200 μL was tested for cases when very high concentration of the analytes was expected.

The salting out-VALLME coupled with HPLC–DAD method was applied for the determination of furfurals and PAT in eighteen apple juice samples, results are summarized in Table 4. The presence of furfurals and PAT was confirmed by comparing the

Table 4
Concentrations of furfurals and PAT in tested juice samples ($n=4$) obtained using the salting out-VALLME combined with HPLC–DAD method[a].

Juice sample	Amount[b] (mg L^{-1})			
	5-HMF	PAT	2-F	5-MF
Apple				
1	17 ± 0.39	ND	2.6 ± 0.06	ND
2	14 ± 0.45	BLD	0.61 ± 0.01	ND
3	2.1 ± 0.11	0.03 ± 0.00	1.8 ± 0.01	0.12 ± 0.00
4	0.23 ± 0.01	ND	1.1 ± 0.04	ND
5	3.8 ± 0.04	ND	6.0 ± 0.13	ND
6	2.1 ± 0.02	ND	0.89 ± 0.02	ND
7	1.0 ± 0.05	BLD	ND	ND
8	2.2 ± 0.04	BLD	ND	ND
9	6.6 ± 0.11	BLD	0.12 ± 0.01	ND
10	2.1 ± 0.03	BLD	0.19 ± 0.00	0.15 ± 0.01
11	0.88 ± 0.00	BLD	0.19 ± 0.01	0.17 ± 0.01
12	11 ± 0.11	BLD	4.5 ± 0.09	0.07 ± 0.00
13	22 ± 0.76	0.02 ± 0.00	4.5 ± 0.10	ND
14	9.5 ± 0.20	BLD	4.7 ± 0.09	ND
15	5.1 ± 0.03	BLD	0.12 ± 0.00	ND
16	5.5 ± 0.22	BLD	0.18 ± 0.01	ND
17	15 ± 1.0	BLD	2.7 ± 0.24	ND
18	5.5 ± 0.16	BLD	2.2 ± 0.09	ND
Mango				
1	1.3 ± 0.02	ND	ND	ND
2	0.69 ± 0.01	ND	0.06 ± 0.00	ND
3	0.95 ± 0.01	ND	0.38 ± 0.01	ND
4	28 ± 0.25	ND	0.81 ± 0.02	ND
5	11 ± 0.11	ND	2.6 ± 0.07	ND
6	0.75 ± 0.03	ND	2.3 ± 0.07	ND
7	1.4 ± 0.03	ND	ND	ND
8	14 ± 0.06	ND	0.20 ± 0.01	ND
9	0.71 ± 0.01	ND	0.51 ± 0.03	ND
Grape				
1	4.1 ± 0.07	ND	1.9 ± 0.11	ND
2	1.8 ± 0.06	ND	0.39 ± 0.01	ND
3	0.72 ± 0.03	ND	0.10 ± 0.00	ND
4	4.5 ± 0.25	ND	0.11 ± 0.00	ND

[a] 3-F was not detected in all juice samples.
[b] Values are given as $X \pm SD$ (mg of analyte per L juice); ND, not detected; BLD, below limit of detection.

Table 3
Recoveries obtained after spiking standard mixtures to fresh apple, mango and grape juices.

Juice sample	Spiked level[a] μg L^{-1}	Recovery (%$X \pm SD$) ($n=6$)				
		5-HMF	PAT	2-F	3-F	5-MF
Apple	2	103 ± 2.5	–	96 ± 2.3	–	98 ± 4.4
	10 (1)	105 ± 1.4	90 ± 6.9	98 ± 1.5	104 ± 6.2	101 ± 2.9
	100 (10)	102 ± 2.7	101 ± 4.8	102 ± 2.8	99 ± 3.9	101 ± 3.5
	1000 (50)	96 ± 1.1	112 ± 4.0	96 ± 2.1	91 ± 4.0	93 ± 7.3
	5000 (100)	101 ± 2.2	103 ± 2.9	95 ± 2.4	95 ± 2.8	93 ± 2.7
Mango	2	101 ± 1.7	–	104 ± 3.8	–	97 ± 1.3
	10 (1)	100 ± 0.93	105 ± 2.7	103 ± 3.2	98 ± 0.89	100 ± 3.9
	100 (10)	104 ± 2.8	107 ± 6.6	102 ± 1.6	104 ± 1.6	100 ± 1.9
	1000 (50)	96 ± 4.4	99 ± 4.4	91 ± 4.2	85 ± 6.1	88 ± 8.0
	5000 (100)	96 ± 3.1	96 ± 3.1	90 ± 3.5	85 ± 3.8	82 ± 4.9
Grape	2	100 ± 3.6	–	95 ± 2.9	–	93 ± 4.8
	10 (1)	102 ± 2.9	99 ± 1.1	88 ± 3.6	93 ± 6.0	97 ± 2.9
	100 (10)	102 ± 1.6	99 ± 2.8	101 ± 0.57	99 ± 0.83	102 ± 0.84
	1000 (50)	99 ± 2.1	98 ± 2.8	100 ± 0.94	97 ± 3.0	100 ± 6.2
	5000 (100)	98 ± 2.4	99 ± 2.8	96 ± 4.9	90 ± 5.7	89 ± 5.4

[a] Figures in parenthesis refer to the spiked level for PAT.

retention time and performing photodiode array scans on the suspected peak and comparing to the standard. 5-HMF was found in all of the tested samples in the range 0.23–22 mg L^{-1}. Five of these samples were found to exceed the legal limit established by IFFJPs (10 mg L^{-1}). This revealed the bad quality of these juice samples which were either due to the bad storage conditions, involving high temperature or heating for a long time during the production process. 2-F and 5-MF were also detected in the tested samples (0.12–6.0 and 0.07–0.17 mg L^{-1}, respectively). 3-F was not detected in any of the samples. 2-F was found in most of the analyzed samples (two samples did not contain 2-F). 5-MF was found in four apple juice samples. The concentration of 2-F and 5-MF in the studied apple samples was not significant (one sample contains 2-F with concentration higher than 5 mg L^{-1}). This is the first report on the presence of 5-MF in apple juices. PAT was detected in fourteen apple juice samples, most of the samples were below the detection limit. Only two samples (0.02 and 0.03 mg L^{-1}) were quantified. The values of PAT in the tested samples were within the permitted limit established by the EU (0.05 mg kg^{-1}). Typical chromatogram of the extracted furfurals and PAT from apple juice is shown in Fig. 3(B).

Most of the previously reported methods for apple juices and apple products focused on the analysis of only PAT [17,18,25,27,28]. A few studies reported the analysis of furfurals or their simultaneous determination with PAT [11,21,48]. The presence of PAT in apple juice samples and apple products has been previously reported [11,25,33]. PAT has been reported in apple juice with concentration ranging from 0.3–14 mg L^{-1} which exceeded the permitted level by EU and higher than the results found in this study [17]. Another study reported the presence of PAT in apple juices with maximum concentration of 0.02 mg L^{-1} [33], which is lower than the concentration determined in our studies.

3.5. Application to mango and grape juices samples

To test the applicability of the salting out-VALLME-HPLC method, other juices (mango and grape) were investigated. Results are shown in Table 4. 5-HMF was detected in all mango and grape juices (0.67–28 and 0.72–4.5 mg L^{-1}, respectively). Among these samples, three mango juices exceed the maximum permitted limit set by IFFJPs (10 mg L^{-1}). Furthermore, 2-F was also found in seven out of the nine mango juice samples and all grape juices in the concentration range of 0.06–2.6 and 0.10–1.9 mg L^{-1}, respectively. Other furfurals (3-F and 5-MF) and PAT were not detected in both juices. Typical chromatograms of mango and grape juices subjected to the proposed salting out-VALLME are shown in Fig. 3(B).

The analysis of 5-HMF in grape juice was previously reported [49]. The results showed that 7 out of 9 boiled grape juices (18–200 mg L^{-1}) exceeded the IFFJPs legal limit. The reported results were also higher than the obtained results in this study. Other reports studied the presence of PAT in mango and grape juices [50]. Similar to the present study, PAT was not identified in both juices. To the best of our knowledge, the analysis of other furfurals (e.g., 2-F, 3-F and 5-MF) in the mango and grape juices sample was not previously explored.

4. Conclusion

A novel VALLME method based on the salting out effects in combination with low density solvent for the extraction of polar compounds (low log *P* values) has been demonstrated for the first time . The method was used for the simultaneous determination of 5-HMF, 2-F, 3-F, 5-MF and PAT in apple, mango and grape juice samples. The proposed salting out-VALLME method is simple, fast and requires a small amount of organic solvent (200 μL). The short extraction times (45 s) without the need of centrifugation step is another advantage of this method. Moreover, this method offers good sensitivity and does not require lengthy evaporation steps, thus preserving the integrity of the extracted compounds. The HPLC separation method itself is short and in contrary to many previous reports, do not require the use of acidic mobile phase [11,14,19,20,21]. The proposed method was successfully used in the extraction of furfurals and PAT from juice samples.

Acknowledgment

Financial support of the work by Universiti Sains Malaysia (USM) via Research University Grant (Grant no: 1001/PKIMIA/811201), postdoctoral fellowship scheme to Ahmad Makahleh and Ministry of Higher Education Malaysia via MyBrain 15 scholarship to Nur-Bahiyah Abu-Bakar are acknowledged.

References

[1] M. Rada-Mendoza, M.A.L. Sanz, A.N. Olano, M. Villamiel, Food Chem. 85 (2004) 605–609.
[2] D. Jöbstl, T. Husøy, J. Alexander, T. Bjellaas, E. Leitner, M. Murkovic, Food Chem. 123 (2010) 814–818.
[3] E. Teixidó, F.J. Santos, L. Puignou, M.T. Galceran, J. Chromatogr. A 1135 (2006) 85–90.
[4] E. Teixidó, O. Núñez, F.J. Santos, M.T. Galceran, Food Chem. 126 (2011) 1902–1908.
[5] Y.F. Wong, A. Makahleh, K.M.A. Azzam, N. Yahaya, B. Saad, S.A. Sulaiman, Talanta 97 (2012) 23–31.
[6] E. Teixidó, E. Moyano, F.J. Santos, M.T. Galceran, J. Chromatogr. A 1185 (2008) 102–108.
[7] A. Alcázar, J.M. Jurado, F. Pablos, A.G. González, M.J. Martín, Microchem. J. 82 (2006) 22–28.
[8] E. Ferrer, A. Alegría, G. Courtois, R. Farré, J. Chromatogr. A 881 (2000) 599–606.
[9] B. Fallico, E. Arena, M. Zappalà, Food Chem. 81 (2003) 569–573.
[10] L.J.K. Durling, L. Busk, B.E. Hellman, Food Chem. Toxicol. 47 (2009) 880–884.
[11] E.M.S.M. Gaspar, A.F.F. Lucena, Food Chem. 114 (2009) 1576–1582.
[12] K. Baert, F. Devlieghere, H. Flyps, M. Oosterlinck, M.M. Ahmed, A. Rajković, B. Verlinden, B. Nicolaï, J. Debevere, B.D. Meulenaer, Int. J. Food Microbiol. 119 (2007) 170–181.
[13] N.L. Leggott, G.S. Shephard, Food Control 12 (2001) 73–76.
[14] V Gökmen, J. Acar, K. Sarioğlu, Anal. Chim. Acta 543 (2005) 64–69.
[15] J. Hopkins, Food Chem. Toxicol. 31 (1993) 455–456.
[16] N. Magan, M. Olsen, Mycotoxins in Food Detection and Control, Woodhead Publishing Ltd., Cambridge, 2004.
[17] S. Marín, E.M. Mateo, V. Sanchis, F.M. Valle-Algarra, A.J. Ramos, M. Jiménez, Food Chem. 124 (2011) 563–568.
[18] C. Zaied, S. Abid, W. Hlel, H. Bacha, Food Control 31 (2013) 263–267.
[19] N. Spano, M. Ciulu, I. Floris, A. Panzanelli, M.I. Pilo, P.C. Piu, S. Salis, G. Sanna, Talanta 78 (2009) 310–314.
[20] G. Durmaz, V. Gökmen, Food Chem. 123 (2010) 912–916.
[21] G. Hu, M. Hernandez, H. Zhu, S. Shao, J. Chromatogr. A 1284 (2013) 100–106.
[22] J. Xu, J. Wu, L. Zhu, H. Shena, J. Xu, S.R. Jensen, X. Jia, Q. Zhang, S. Li, Food Chem. 135 (2012) 2277–2286.
[23] S. Manzini, C. Durante, C. Baschieri, M. Cocchi, S. Sighinolfi, S. Totaro, A. Marchetti, Talanta 85 (2011) 863–869.
[24] E.M.S.M. Gaspar, J.F. Lopes, J. Chromatogr. A 1216 (2009) 2762–2767.
[25] M.D. Víctor-Ortega, F.J. Lara, A.M. García-Campaña, M. Olmo-Iruela, Food Control 31 (2013) 353–358.
[26] V.M. Rizelio, L.V. Gonzaga, G.S.C. Borges, G.A. Micke, R. Fett, A.C.O. Costa, Food Chem. 133 (2012) 1640–1645.
[27] F.M. Valle-Algarra, E.M. Mateo, J.V. Gimeno-Adelantado, R. Mateo-Castro, M. Jiménez, Talanta 80 (2009) 636–642.
[28] G. Boonzaaijer, I. Bobeldijk, W.A. Osenbruggen, Food Control 16 (2005) 587–591.
[29] J. Pereira, J. Pereira, J.S. Câmara, Talanta 83 (2011) 899–906.
[30] A.E. Edris, M. Murkovic, B. Siegmund, Food Chem. 104 (2007) 1310–1314.
[31] S.J.P. Olivero, J.P.P. Trujillo, J. Agric. Food Chem. 58 (2010) 12976–12985.
[32] C.C. Loi, H.C. Boo, A.S. Mohamed, A.A. Ariffin, J. Am. Oil Chem. Soc. 87 (2010) 607–613.
[33] H. Kataoka, M. Itano, A. Ishizaki, K. Saito, J. Chromatogr. A 1216 (2009) 3746–3750.
[34] M. Chaichi, A. Mohammadi, M. Hashemi, Microchem. J. 108 (2013) 46–52.
[35] M. Maham, R. Karami-Osboo, V. Kiarostami, S. Waqif-Husain, Food Anal. Method. 6 (2013) 761–766.
[36] E. Yiantzi, E. Psillakis, K. Tyrovola, N. Kalogerakis, Talanta 80 (2010) 2057–2062.
[37] A. Papadopoulou, I.P. Román, A. Canals, K. Tyrovola, E. Psillakis, Anal. Chim. Acta 691 (2011) 56–61.

[38] Z. Yang, Y. Lu, Y. Liu, T. Wu, Z. Zhou, D. Liu, J. Chromatogr. A 1218 (2011) 7071–7077.
[39] R. Li, D. Liu, Z. Yang, Z. Zhou, P. Wang, Electrophoresis 33 (2012) 2176–2183.
[40] Z. Yang, P. Wang, W. Zhao, Z. Zhou, D. Liu, J. Chromatogr. A 1300 (2013) 58–63.
[41] Y. Zhang, H.K. Lee, J. Chromatogr. A 1274 (2013) 28–35.
[42] C.K. Zacharis, C. Christophoridis, K. Fytianos, J. Sep. Sci. 35 (2012) 2422–2429.
[43] C. Jia, X. Zhu, J. Wang, E. Zhao, M. He, L. Chen, P. Yu, J. Chromatogr. A 1217 (2010) 5868–5871.
[44] S. Ozcan, J. Sep. Sci. 34 (2011) 574–584.
[45] W. Chang, C. Wang, J. Jan, Y. Lo, C. Wu, J. Chromatogr. A 1248 (2012) 41–47.
[46] K. Akpınar, B. Karakas, H. Akça, M. Certel, Food Chem. 128 (2011) 790–794.
[47] L. Giordano, R. Calabrese, E. Davoli, D. Rotilio, J. Chromatogr. A 1017 (2003) 141–149.
[48] V. Gökmen, J. Acar, J. Chromatogr. A 847 (1999) 69–74.
[49] S. Kus, F. Gogus, S. Eren, Int. J. Food Prop. 8 (2005) 367–375.
[50] C.M. Sylos, D.B. Rodriguez-Amaya, Food Addit. Contam. 16 (1999) 71–74.