Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 109 (2008) 219–226
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical Methods

Characterisation of anthocyanins in red cabbage using high resolution liquid chromatography coupled with photodiode array detection and electrospray ionization-linear ion trap mass spectrometry

Panagiotis Arapitsas [a,b,*], Per J.R. Sjöberg [a], Charlotta Turner [a]

[a] Uppsala University, Department of Physical and Analytical Chemistry, Analytical Chemistry, P.O. Box 599, 751 24 Uppsala, Sweden
[b] University of Florence, Department of Pharmaceutical Sciences, Via Ugo Schiff 6, 50019 Sesto F.no, Florence, Italy

Received 21 October 2007; received in revised form 10 December 2007; accepted 12 December 2007

Abstract

The aim of this work was to analyse and tentatively identify anthocyanin species in red cabbage using HPLC/DAD-ESI/Qtrap MS. The extraction was realized by using a pressurized liquid technique and the separation of the pigments was achieved by a high resolution liquid chromatography system with a 1.8 μm particles C-18 column. Photodiode array detection was employed to determine the UV/Vis spectral characteristic of the pigments. Electrospray ionization-linear ion trap mass spectrometry allowed the specific determination of the fragmentation patterns of the anthocyanins, by performing different ion scan modes. Twenty four anthocyanins were separated and identified, all having cyanidin as aglycon, represented as mono- and/or di-glycoside, and acylated, or not, with aromatic and aliphatic acids. Nine anthocyanins were identified for the first time in red cabbage.

Keywords: Pigments; Vegetable; HPLC/DAD-ESI/Qtrap; Polyphenols; Pressurized liquid extraction

1. Introduction

Red cabbage (*Brassica oleracea* L. var. *capitata f. rubra*) is a native vegetable of the Mediterranean region and south-western Europe that now grows in regions all over the world. Red cabbage belongs to the family of *Brassicaceae*, which annual world production is about 68 million tons of fresh heads from 3.1 million ha, in more than 130 countries (FAOSTAT). Before being thought of as a food, cabbage was valued for medicinal purposes in treating headaches, gout, diarrhea and peptic ulcers (Cheney, 1950). In fact, epidemiological studies have stressed the capacity of *Brassica* species to prevent cardiovascular diseases as well as some types of cancer (Cooke, Steward, Gescher, & Marczylo, 2005; Singh et al., 2006; Steinmetz & Potter, 1996). Among the substances that seem to be responsible for those properties are polyphenols (Cooke et al., 2005; Singh et al., 2006), and red cabbage is a rich source of phenolic compounds, with the anthocyanins being the most abundant class (Charron, Clevidence, Britz, & Novotny, 2007; Dyrby, Westergaard, & Stapelfeldt, 2001; Mazza & Miniati, 1993; Wu & Prior, 2005; Wu et al., 2006).

Anthocyanins (in Greek *anthos* means flower, and *kyanos* means blue) are a group of plant pigments that are widely distributed in nature, among flowers, fruits and vegetable, and are responsible for their bright colors such as green, red and blue (Kong, Chia, Goh, Chia, & Brouillard, 2003; Mazza & Miniati, 1993). They have also demonstrated to play a very important role in the plant physiology and are valuable for the food industry as well as in

* Corresponding author. Address: University of Florence, Department of Pharmaceutical Sciences, Via Ugo Schiff 6, 50019 Sesto F.no, Florence, Italy. Tel.: +39 055 4573677; fax: +39 055 4573667.
E-mail address: panagiotis.arapitsas@unifi.it (P. Arapitsas).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.12.030

human health (Kong et al., 2003; Mazza & Miniati, 1993). Recently, increased attention has been given to their possible health benefits in preventing chronic and degradative diseases including heart disease and cancer, neuronal degeneration such as Alzheimer's and Parkinson's diseases, as well as being involved in preventing the process of aging (Charron et al., 2007; Chen et al., 2006; Kong et al., 2003; Middleton, Kandaswami, & Theoharides, 2000; Morse & Stoner, 1993).

Regarding the chemical structure of red cabbage anthocyanins, all of the known species have cyanidin as aglycon, glycosylated mainly with glucose and/or sophorose (diglucoside), which are acylated with various aromatic and aliphatic acids (Charron et al., 2007; Wu & Prior, 2005) (Fig. 1). Among all fruits and vegetables, the chromatographic profile of red cabbage anthocyanins is one of the most complicated, because of the high number of different anthocyanins that it contains, and the highly conjugated cyanidin glycosides with several aromatic and aliphatic acids (Charron et al., 2007; Dyrby et al., 2001; Wu & Prior, 2005).

The analysis of red cabbage anthocyanins is a difficult task, due to the lack of pure and structurally defined commercially available standards of acylated cyanidins, the large concentration range of its anthocyanins, and also the complexity of red cabbage chromatographic profile due to the big number of anthocyanins contained (Charron et al., 2007; Dyrby et al., 2001; Wu & Prior, 2005; Wu et al., 2006). For this purpose, the new developments of chromatographic columns with 1.8 μm particles show a promise in delivering higher speed, better resolution and sensitivity for high throughput analysis, when compared to conventional columns (Cooper et al., 2007; Desmet, Clicq, & Gzil, 2005). Small particle columns have already been used with good results in the field of food polyphenols (Churchwell, Twaddle, Meeker, & Doerge, 2005; Cooper et al., 2007; Zhou, Xu, Xue, Zhang, & Liang, 2006).

The objective of this work was to investigate the use of 1.8 μm C18 column coupled with DAD and ESI/Qtrap MS for the separation and identification of red cabbage anthocyanins. To identify separated polyphenolic compounds despite the lack of commercial available standard is challenging, however, to increase the confidence, we have in this work explored the possibility to combine the information acquired from the UV/Vis spectra of a diode array detector with the information obtained by the fragmentation pattern from a triple quadruple MS (Arapitsas, Menichetti, Vincieri, & Romani, 2007; Määttä-Riihinen, Kamal-Eldin, & Törrönen, 2004; Schoefs, 2004; Seeram, Schutzki, Chandra, & Nair, 2002).

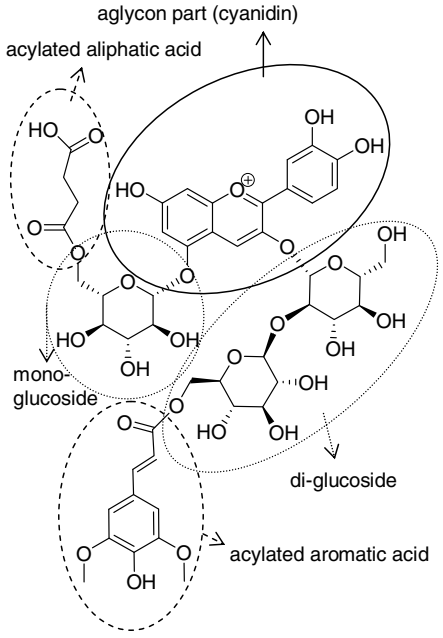

Fig. 1. Chemical structure of an acylated anthocyanin.

2. Experimental

2.1. Standards and solvents

Standards of the cyanidin chloride, cyanidin-3-*O*-glucoside chloride, cyanidin-3-*O*-galactoside chloride, cyanidin-3-*O*-rhamnoside, cyanidin-3-*O*-rutinoside chloride and cyanidin-3,5-di-*O*-glucoside chloride (HPLC grade) were obtained from Extrasynthese (Geney, France). Formic acid, *p*-coumaric acid, caffeic acid, ferulic acid, *p*-OH-benzoic acid and sinapic acid were purchased from Sigma Aldrich (St. Louis, MO). Acetonitrile (HPLC grade) and ethanol were from Merk AG (Darmstadt, Germany). Distilled water was obtained from a purification system (Milli-Q; Millipore, Bedford, MA), and nitrogen (99.996 vol%) was from AGA, Stockholm, Sweden.

2.2. Extraction procedure

For the extraction of the anthocyanins a method optimized and recently published from our group was used (Arapitsas & Turner, in press). A Dionex accelerated solvent extractor (ASE-200®, Dionex, Salt Lake City, UT, USA) was used for this study. Approximately 100 g of red cabbage leaves, obtained from the local market, were first cut into small pieces with a knife and then homogenized with a Philips cucina TYPE HR1744 blender. A portion of homogenized fresh red cabbage sample was 2.5 g of red cabbage was placed into 11 mL extraction cells containing a cellulose paper filter at the bottom. The cell containing the sample was filled with extraction solvent, pressurized, and then heated. By operating the ASE system at zero extraction time, constant pressure of 50 bar and one extraction cycle, approximately 6 min was required to heat sample/solvent from ambient temperature to 100 °C, and a short 60 s static extraction was then performed. The cell was thereafter rinsed with fresh extraction solvent (80% of the void volume) and purged with a flow of nitrogen (10 bar during 90 s). The volume of each extract was adjusted to 25 mL by using the extraction solvent, which

was a mixture of water/ethanol/formic acid, (94/5/1, v/v/v). 2 mL of the extract was filtered through a 0.45 mm Millex HV13 filter (Millipore Corp., Bedford, MA, USA) and injected to the HPLC/DAD/MS equipment.

2.3. HPLC/DAD analysis

The HPLC system used was an Agilent Technologies HP 1200 HPLC (Agilent, Palo Alto, CA), equipped with a degasser, a binary pump, an autosampler, a column oven and a diode array detector. The operating system was ChemStation for LC 3D system Rev. B.02.01-SR2. The column used was a 1.8 μm Zorbax SB-C18, 100 × 2.1 mm i.d. (Agilent, Palo Alto, CA), operating at 27 °C and with a flow rate of 0.2 mL/min. The injected volume was 10 μL.

The mobile phase was a multistep linear solvent gradient system consisting of (A) 5% formic acid aqueous solution and (B) acetonitrile. The elution profile was: 3 min 95% A, then the solvent B was increased first to 10% in 2 min, thereafter to 12% in 3 min, to 14% in 2 min, stay constant for 8 min, increased to 17% in 10 min, 20% in 7 min, to 90% in 5 min, stay constant for 5 min, and subsequently decreased to 5% B, in 5 min, and 7 min for equilibration.

2.4. Mass spectrometry analysis

A Qtrap linear ion trap mass spectrometer (Applied Biosystems, MDS Sciex. Toronto, Canada) equipped with a pneumatically assisted ESI interface was linked with the HPLC system.

Initially optimization of the parameters for the ESI/MS and ESI/MS/MS analyses of the standards and the total extract was performed by direct infusion into the ES ionisation source. For the direct infusion optimization the standards were dissolved in acetonitrile:water:formic acid (20:79:1 v:v:v) (10 μg/mL) and the total extract was diluted 1/100 with the same solution. Both samples, standards and total extract, were infused into the ES ionisation source using a syringe pump at a flow rate 10 μL/min. The optimum conditions found for the analysis of anthocyanins were: positive ion scan mode, curtain gas (CUR) 2.4 bar, collision gas (CAD) 2×10^{-5} Torr, ion source gas 1 (GC1) 3.1 bar, ion source gas 2 (GC2) 3.4 bar, ion spray voltage (IS) 4000 V, entrance potential (EP) 10 V, declustering potential (DP) 160 V, collision energy 60 V, collision cell exit potential (CXP) 3 V.

For the HPLC/MS analysis the effluent from the column after the DAD detector was split 1:10 in a Valco-tee connection (Valco International, Schenkon, Switzerland) by using two capillaries. In order to identify the different glycosidic and acylated derivatives of cyanidin, the following scan modes were performed: (i) enhanced MS scan (EMS) in a mass range of 150–1400 u; (ii) enhanced product ion scan; (iii) enhanced precursor ion scan of the 287 ion (cyanidin); and (iv) neutral loss ion scan concerning the 162 ion (hexose ion).

2.5. Identification of compounds

Compounds were identified by comparing their retention times and spectra to those of standards, when available. Identification of peaks was then confirmed by spiking samples with standard mixtures. Unknown chromatographic peaks were tentatively identified via their spectral features and by literature data (Abdel-Aal & Hucl, 2003; Brouillard, Chassaing, & Fougerousse, 2003; Charron et al., 2007; Dyrby et al., 2001; Tian, Giusti, Stoner, & Schwartz, 2005; Wu & Prior, 2005).

3. Results and discussion

Using peak spectral characteristic λ_{vis}, and λ_{acyl}, and their corresponding absorptivities (Figs. 2 and 3 and Table 1), it was possible to identify the anthocyanins as either mono- or biosides (E_{440}/E_{vis} absorptivity ratio of 29–35% indicating a monoside and a ratio of 15–24% indicating a bioside) and to determine the degree of aromatic acylation (E_{acyl}/E_{vis} absorptivity ratio of 53–69% indicating

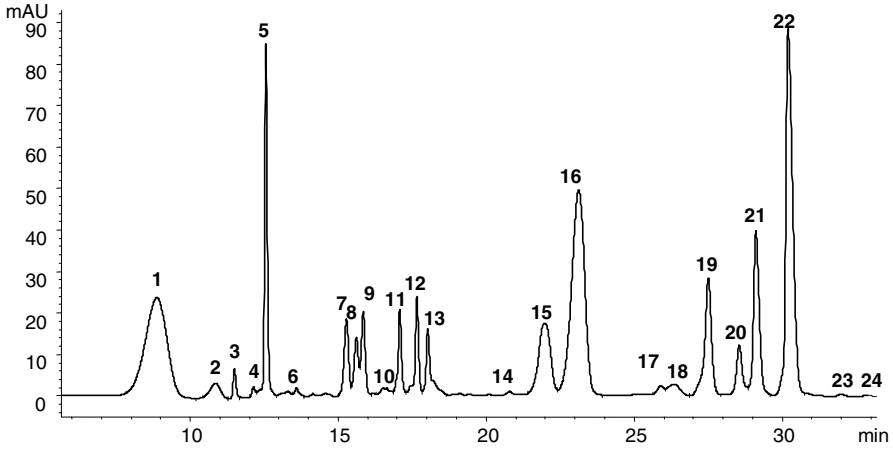

Fig. 2. HPLC/DAD chromatogram of a red cabbage extract recorded at 520 nm.

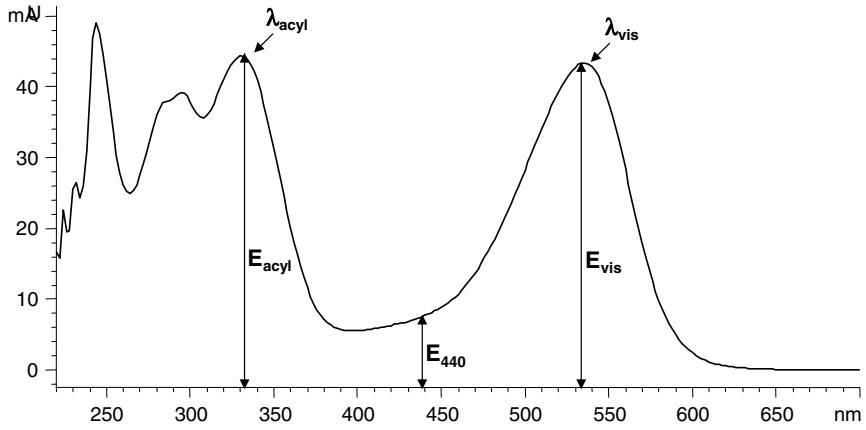

Fig. 3. UV/Vis spectrum of an acylated anthocyanin (peak 22 of Fig. 2), with the spectral information.

monoacylation and ratio of 98–128% indication diacylation) (Dyrby et al., 2001; Tian et al., 2005;). Tandem mass spectrometry (MS/MS), and in particular product-ion analysis, which acquires mass spectra from the product ions produced from the fragmentation of a selected precursor ion, and other tandem mass spectrometry techniques such as precursor ion analysis and neutral loss analysis, have been used for identification and characterization of anthocyanins. Through the correlation of the data registered from the two different detectors (DAD and MS), 24 anthocyanins, shown in the chromatographic profile reported at 520 nm in Fig. 2, were characterized (Table 1). All anthocyanins identified were having cyanidin as aglycon part (anthocyanidin), an observation supported by published data (Charron et al., 2007; Dyrby et al., 2001; Wu & Prior, 2005). Considering the sugar part, mono- and/or disaccharides of hexose (always glucose), and rarely pentoses were identified (Charron et al., 2007; Dyrby et al., 2001; Wu & Prior, 2005). For the acylated groups, both aromatic (caffeic, ferulic, *p*-coumaric, *p*-OH-benzoic and sinapic acid) and aliphatic (succinic and oxalic acid) acids were identified. Peaks 1–4, 6, 14–16, 19, 21–22 and 24 are already known anthocyanins in red cabbage (Charron et al., 2007; Dyrby et al., 2001; Mazza & Miniati, 1993; Wu & Prior, 2005).

Although a large number of anthocyanins have been identified in red cabbage, their structures are similar (Charron et al., 2007; Dyrby et al., 2001; Mazza & Miniati, 1993; Wu & Prior, 2005). One interesting particularity of these similarities, which will be thoroughly explained below, is the presence of five groups of three anthocyanins, five 'peak-triads' (peak numbers: 6–7–8, 11–12–13, 14–15–16, 17–18–20, and 19–21–22).

By combining UV/Vis, MS and MS/MS data and by calculating the possible combinations of acylated groups, peak 5 was tentatively identified as cyanidin-3-(sinapoyl)-triglucoside-5-glucoside (Table 1). UV/Vis data indicated a monoacylated bioside, and its fragment pattern a $[M]^+$ *m/z* 1141, MS/MS *m/z* 979 $[M-162]^+$, 817 $[M-2\times162]^+$, 611 $[M-2\times162-206]^+$, 449 $[M-2\times162-206-162]^+$, 287 $[cyanidin]^+$, 207 $[sinapic\ acid - H_2O]^+$.

The UV/Vis spectra of the first 'triad' (peaks 6–7–8) indicated diacylated biosides; and respect all other peaks, they show bathochromic absorption maxima in the acylated region, with $\lambda_{acyl} > 330$ nm. Their fragment patterns were for peak 6 $[M]^+$ *m/z* 1081, MS/MS *m/z* 919 $[M-162]^+$, 754 $[M-2\times162]^+$, 449 $[M-3\times162-146]^+$, 287 $[cyanidin]^+$, 163 $[caffeic\ acid - H_2O]^+$, 147 $[p\text{-coumaric acid} - H_2O]^+$; peak 7 $[M]^+$ *m/z* 1111, MS/MS *m/z* 949 $[M-162]^+$, 787 $[M-2\times162]^+$, 449 $[M-3\times162-176]^+$, 287 $[cyanidin]^+$, 177 $[ferulic\ acid - H_2O]^+$, 163 $[caffeic\ acid - H_2O]^+$; and peak 8 $[M]^+$ *m/z* 1141, MS/MS *m/z* 979 $[M-162]^+$, 817 $[M-2\times162]^+$, 449 $[M-3\times162-206]^+$, 287 $[cyanidin]^+$, 207 $[sinapic\ acid - H_2O]^+$, 163 $[caffeic\ acid - H_2O]^+$. Considering the fact that only sophorose has been identified as diglycoside in red cabbage (Charron et al., 2007; Dyrby et al., 2001; Mazza & Miniati, 1993; Wu & Prior, 2005), and that the fragment ion at *m/z* 163 is typical for the caffeic acid, the above three compounds could be attentively identified as cyanidin-3-(caffeoyl)(*p*-coumaroyl)-sophoroside-5-glucoside and cyanidin-3-(caffeoyl)(feruloyl)-sophoroside-5-glusoside, cyanidin-3-(caffeoyl)(sinapoyl)-sophoroside-5-glucoside (Table 1).

UV/Vis data of peaks 9 and 10 indicate monoacylated bioside, with λ_{acyl} at 286 nm, and UV/Vis similar to the one of peak 14. The fragmentation pattern of peak 9 was similar to peak 6, but without the fragment ion *m/z* 162, typical of caffeic acid. Peak 10 show a similar fragmentation patten to that of peak 14 but with $[M]^+$ 100 u heavier, probably because an alkyl acylation with succinic acid (Table 1). According to these observations, peaks 9 and 10 could be identified as cyanidin-3-(*p*-coumaroyl)-triglucoside-5-glucoside and cyanidin-3-(*p*-coumaroyl)-sophoroside-5-(succinoyl)-glucoside, respectively (Table 1).

Peaks 11, 12 and 13, the second 'triad', have UV–Vis spectra shaped very similar to peaks 19, 21 and 22, respectively. Their M^+ were for *m/z* 162 u heavier, compared to the anthocyanins 19, 21 and 22. The 'triad' 19-21-22 eluted about 10 min later, compared to the peaks 11-12-13, so they should be less polar. 11, 12 and 13 had M^+ *m/z* 1287, 1317 and 1347, respectively, and their fragmentation

Table 1
Chromatographic and spectroscopic properties of anthocyanins from red cabbage

Peak	t_R (min)	λ_{vis}	λ_{acyl}	E_{440}/E_{vis} (%)	E_{acyl}/E_{vis} (%)	E_{440}/E_{vis} indicating	E_{acyl}/E_{vis} indicating	M^+ m/z	Fragment ions m/z	Tentative identification
1	8.87	514		17		Bioside		773	611, 449, 287	Cyan-3-soph-5-glu
2	10.86	514		17		Bioside		611	449, 287	Cyan-3,5-diglu
3	11.49	530	272	17	55	Bioside	Monoacylated	965	803, 449, 287	Cyan-3(*p*-OH-ben)soph-5-(ox)gluc
4	12.14	514		17		Bioside		743	611, 419, 287	Cyan-3-soph-5-xyloside
5[a]	12.55	528	294	19	60	Bioside	Monoacylated	1141	979,817, 449, 287, 207	Cyan-3(sin)triglu-5-glu
6	13.59	528	330	19	98	Bioside	Diacylated	1081	919, 754, 449, 287, 163, 147	Cyan-3(caf)(*p*-coum)soph-5-glu
7[a]	15.28	528	334	16	61	Bioside	Diacylated	1111	949, 787, 449, 287, 177, 163	Cyan-3(caf)(fer)soph-5-glu
8[a]	15.61	522	336	16	103	Bioside	Diacylated	1141	979, 817, 449, 287, 207, 163	Cyan-3(caf)(sin)soph-5-glu
9[a]	15.85	522	286	19	57	Bioside	Monoacylated	1081	919, 757, 449, 287, 147	Cyan-3(*p*-coum)triglu-5-glu
10[a]	16.64	524	286	16	59	Bioside	Monoacylated	1019	919, 449, 287, 147	Cyan-3(*p*-coum)soph-5-(suc)glu
11	17.08	536	296	19	106	Bioside	Diacylated	1287	1125, 979, 449, 287, 207, 147	Cyan-3(sin)(*p*-coum)triglu-5-glu
12	17.66	538	326	20	92	Bioside	Diacylated	1317	1155, 979, 449, 287, 207, 177	Cyan-3(sin)(fer)triglu-5-glu
13	18.02	536	326	16	92	Bioside	Diacylated	1347	1185, 979, 449, 287, 207	Cyan-3(sin)(sin)soph-5-glu
14	21.06	522	288	16	56	Bioside	Monoacylated	919	757, 449, 287, 147	Cyan-3(*p*-coum)glu-5-glu
15	21.99	525	294	19	63	Bioside	Monoacylated	949	787, 449, 287, 177	Cyan-3(fer)soph-5-glu
16	23.12	522	300	17	61	Bioside	Monoacylated	979	817, 449, 287, 207	Cyan-3(sin)soph-5-glu
17[a]	25.89	526	294	21	99	Bioside	Diacylated	1287	1125, 449, 287, 207, 147	Cyan-3(gluco*p*-sin)(*p*-coum)soph-5-glu
18[a]	26.34	526	326	20	98	Bioside	Diacylated	1317	1155, 449, 287, 177, 207	Cyan-3(gluco*p*-sin)(fer)soph-5-glu
19	27.50	526	324	21	101	Bioside	Diacylated	1125	979, 449, 287, 207, 147	Cyan-3(sin)(*p*-coum)soph-5-glu
20[a]	28.55	536	324	18	102	Bioside	Diacylated	1347	1185, 1023, 979, 449, 287, 207	Cyan-3(gluco*p*-sin)(sin)soph-5-glu
21	29.11	530	324	16	100	Bioside	Diacylated	1155	993, 949, 287, 207, 177	Cyan-3(sin)(fer)soph-5-glu
22	30.20	536	324	23	103	Bioside	Diacylated	1185	1023, 979, 287, 207	Cyan-3(sin)(sin)soph-5-glu
23[a]	32.07	524	327	19	126	Bioside	Diacylated	1155	949, 449, 207, 177	Cyan-3(fer)soph-5-(sin)glu
24	33.13	538	330	18	110	Bioside	Diacylated	1185	979, 449, 287, 207	Cyan-3(sin)soph-5-(sin)glu

Abbreviations: cyan: cyanidin, glu: glucoside, triglu: triglucoside, soph: sophoroside, caf: caffeoyl, fer: feruloyl, coum: coumaroyl, sin: sinapoyl, glucop: glucopyranosyl, ben: benzenoyl, ox: oxaloyl and suc: succinoyl.

[a] Anthocyanins identified in red cabbage for first time. The absorption maxima λ_{vis} is due to the presence of the anthocyanidin chromophore while λ_{acyl} is due to the chromophore of the acylating aromatic group, and E_{vis} and E_{acyl} are the absorptivities at the corresponding maxima while E_{440} is the absorptivity at 440 nm. Peak numbering refers to the peaks of Fig. 2.

pattern show *m/z* corresponding to the M^+ ions of the peaks 19, 21 and 22, plus all their subsequent fragment ions. According to these data, peaks 11, 12 and 13 have one more hexose than peaks 19, 21 and 22 and a possible identification could be cyanidin-3-(sinapoyl)(*p*-coumaroyl)-triglucoside-5-glucoside, cyanidin-3-(sinapoyl)(feruloyl)-triglucoside-5-glucoside, and cyanidin-3-(sinapoyl)(sinapoyl)-triglucoside-5-glucoside, respectively (Table 1).

Peaks 14, 15 and 16 are all indicated as bioside and mono-acylated, with λ_{acyl} 288, 294 and 300, and M^+ *m/z* 919, 949 and 979, among their MS/MS fragment ions we found 147 [*p*-coumaric acid – $H_2O]^+$, 177 [ferulic acid – $H_2O]^+$, 207 [sinapic acid – $H_2O]^+$, respectively, and according to these data peaks 14, 15 and 16 could be identified as cyanidin-3(*p*-coumaroyl)-sophoroside-5-glucoside, cyanidin-3-(feruloyl)-sophoroside-5-glucoside and cyanidin-3-(sinapoyl)-sophoroside-5-glucoside, respectively (Table 1).

The fourth 'peaks-triad' is the 17–18–20. They have very similar UV/Vis spectra, and similar fragmentation patterns, to those of the 'triad' 11–12–13, correspondingly. These three compounds could be tentatively identified, when comparing with literature information about anthocyanins in fruits and vegetables (Charron et al., 2007; Dyrby et al., 2001; Mazza & Miniati, 1993; Seeram et al., 2002; Wu & Prior, 2005), as cyanidin-3-(glucopyranosyl-sinapoyl)(*p*-coumaroyl)-sophoroside-5-glucoside, cyanidin-3-(glucopyranosyl-sinapoyl)(feruloyl)-sophoroside-5-glucoside, and cyanidin-3-(glucopyranosyl-sinapoyl)(sinapoyl)-sophoroside-5-glucoside, respectively (Table 1). The probability that the glucose is linked in the sinapoyl moiety, of the anthocyanins 17-18-20, could also been supported by the fact that the molecular ion was less abundant, and the fragment ions at *m/z* $[M-162]^+$, were more abundant, when compared to the anthocyanins 11-12-13, where it is more likely that there is a triglucoside present (Fig. 4).

Finally, peaks 23 and 24 were having similar UV/Vis spectra and the same fragmentation patterns as peaks 21 and 22, but the retention times were longer. In the literature it has been reported that flavonoids of the family *Brassicaceae*, like kaempferol and quercetin, acylated to the glucoside at the hydroxyl group of position 5, eluted after the corresponding derivative acylated in the glucoside of the position 3 (Charron et al., 2007; Dyrby et al., 2001; Hong & Wrolstad, 1990). In respect to this consideration, and in addition to other literature data (Wu & Prior, 2005) peaks 23 and 24 could be identified as cyanidin-3-(feruloyl)-sophoroside-5(sinapoyl)-glucoside and cyanidin-3-(sinapoyl)-sophoroside-5-(sinapoyl)-glucoside, respectively (Table 1).

4. Conclusions

The use of a 1.8 μm C18 column in an HPLC system, which characteristics are suitable when working with a high-pressure and low flow rate system, gave the possibility to perform faster separations with better resolution and higher sensitivity, when compared to conventional HPLC systems and columns (Dyrby et al., 2001; Mazza & Miniati, 1993; Wu & Prior, 2005). In general, shorter chromatographic run and equilibration time could be obtained. More peaks were separated, and it was easier to couple the system

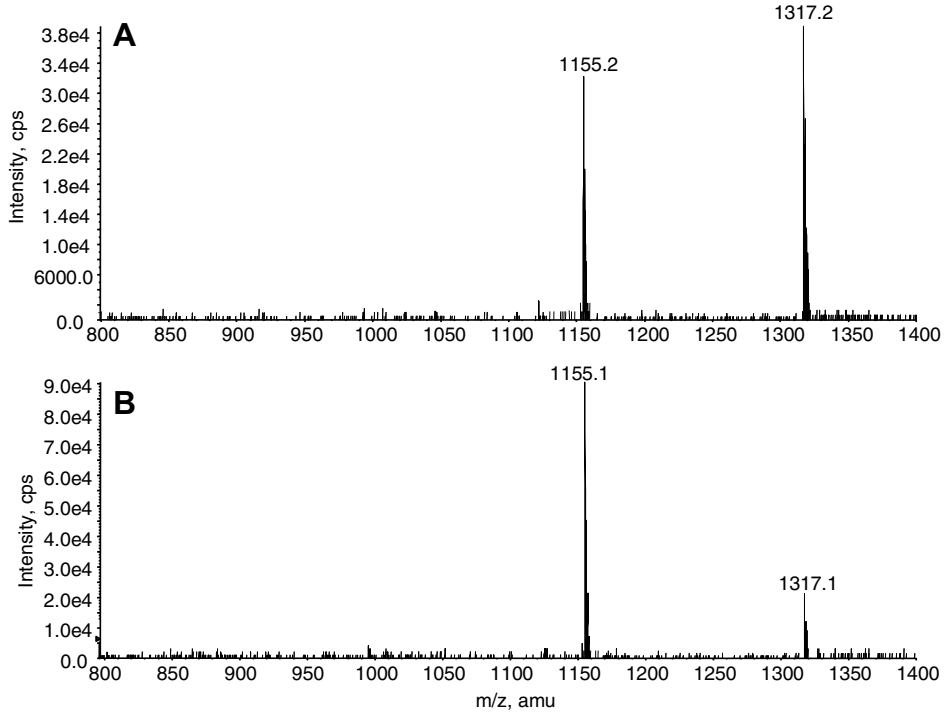

Fig. 4. The enhanced MS spectra of peak 12 (A) and peak 18 (B) of Fig. 2, registered under the same MS conditions (see Section 2).

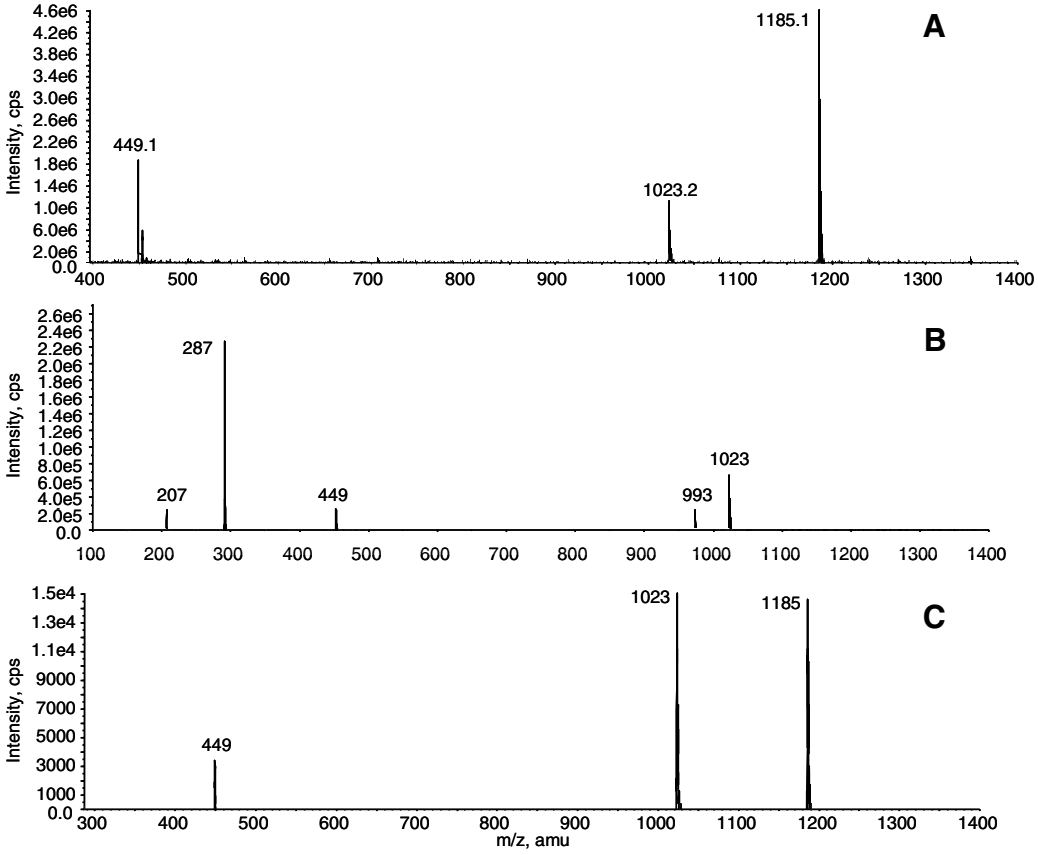

Fig. 5. Peak 22 (Fig. 2) MS spectra showing enhanced MS scan (A), enhanced product ion scan of the 1185 ion (B), and the enhanced precursor ion scan of the 287 ion (cyanidin) (C).

to a triple quadruple MS, due to the lower flow rate and more information were obtained compared to the literature data. Furthermore, in order to identify the separated peaks, with higher confidence, the association of the UV/Vis spectra information with MS and MS/MS spectra information was essential. For instance, in Fig. 5 MS spectra of peak 22 are shown, including enhanced MS scan, enhanced product ion scan of the 1185 ion (cyanidin-3(sinapoyl)(sinapoyl)-sophoroside-5-glucoside) and enhanced precursor ion scan of the 287 ion (cyanidin), which together with the UV/Vis spectra data have permitted peak identification (Figs. 3 and 5, and Table 1).

Among the 24 anthocyanins detected in the red cabbage extract, 9 of them are newly identified in this vegetable. This finding probably should be attributed more to the characteristics of the instrumentation used and less to the matrix analyzed or experimental errors of previously published studies in red cabbage anthocyanins (Dyrby et al., 2001; Mazza & Miniati, 1993; Wu & Prior, 2005). The benefits offered by the novel and alternative instruments applied, like the velocity of extraction gained from the pressurized fluid extraction, the higher resolution achieved by columns with 1.8 μm particles and the higher sensitivity and selectivity due to the DAD and QTrap MS detectors used in the this study, gave the possibility to identify a bigger number of high acylated anthocyanins.

Moreover, the use of pressurized solvent for the extraction of the pigments from the natural matrix, gave the possibility to use a fast, efficient and environmental friendly technique (Arapitsas & Turner, in press; Turner, 2006). In fact, the extraction was obtained in less than 7 min, under elevated temperature and pressures, of relatively small amounts of liquid solvents (25 mL), that was mainly composed of water (94 vol%).

It is important to point out that the regularities discussed in this work are general conclusions deduced from our limited data. They may provide some assistance in identifying anthocyanins, but unless standards or standard reference materials are available to make comparisons, it is difficult to determine the exact chemical structures. NMR study could help to approve or decline the above interpretations, but isolation of anthocyanins from a tissue is complicated and their low stability in combination with the quantities needed for that propose, makes that scope especially difficult.

In conclusion, this work establishes a simultaneous protocol for the identification of red cabbage anthocyanins, by using pressurized fluid extraction, a high resolution liquid

chromatography coupled to a DAD detector and an ESI/Qtrap mass spectrometer. The combination of spectrometric and spectroscopic characteristics registered for these two different detectors was crucial for the thorough identification of every chromatographic peak.

Acknowledgments

Panagiotis Arapitsas acknowledges the Marie Curie Fellowship for a five months grant (contract number: HPMT-CT-2001-00419).

Charlotta Turner acknowledges the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS, 209-2006-1346); the Swedish Research Council (VR, 2006-4084); the Swedish International Development Cooperation Agency (SIDA, SWE-2005-473); and the Swedish Foundation for Strategic Research (SSF, 2005:0073/13). Per Sjöberg acknowledges FORMAS (209-2006-1346).

Agilent Technologies in Sweden is greatly acknowledged for lending us the HP 1200 HPLC system and the 1.8 μm Zorbax SB-C18 HPLC column.

References

Abdel-Aal, S. M., & Hucl, P. (2003). Composition and stability of anthocyanins in blue-grained wheat. *Journal of Agricultural and Food Chemistry, 51*(8), 2174–2180.

Arapitsas, P., & Turner, C. (in press). Pressurized solvent extraction and monolithic column-HPLC/DAD analysis of anthocyanins in red cabbage. Talanta, Corrected Proof, Available online 1 September 2007.

Arapitsas, P., Menichetti, S., Vincieri, F. F., & Romani, A. (2007). Hydrolyzable tannins with the hexahydroxydiphenoyl unit and the m-depsidic link: HPLC-DAD-MS identification and model synthesis. *Journal of Agricultural and Food Chemistry, 55*(1), 48–55.

Brouillard, R., Chassaing, S., & Fougerousse, A. (2003). Why are grape/fresh wine anthocyanins so simple and why is it that red wine color lasts so long? *Phytochemistry, 64*, 1179–1186.

Charron, C. S., Clevidence, B. A., Britz, S. J., & Novotny, J. A. (2007). Effect of dose size on bioavailability of acylated and nonacylated anthocyanins from red cabbage (*Brassica oleracea* L. Var. capitata). *Journal of Agricultural and Food Chemistry, 55*(13), 5354–5362.

Chen, P. N., Chu, S. C., Chiou, H. L., Kuo, W. H., Chiang, C. L., & Hsieh, Y. S. (2006). Mulberry anthocyanins, cyanidin 3-rutinoside and cyanidin 3-glucoside, exhibited an inhibitory effect on the migration and invasion of a human lung cancer cell line. *Cancer Letters, 235*, 248–259.

Cheney, G. (1950). Anti-peptic ulcer dietary factor. *Journal of the American Dietetic Association, 26*, 668–672.

Churchwell, M. I., Twaddle, N. C., Meeker, L. R., & Doerge, D. R. (2005). Improving LC–MS sensitivity through increases in chromatographic performance: Comparisons of UPLC–ES/MS/MS to HPLC–ES/MS/MS. *Journal of Chromatography B, 825*, 134–143.

Cooke, D., Steward, W. P., Gescher, A., & Marczylo, T. (2005). Anthocyans from fruit and vegetables – Does bright colour signal cancer chemopreventive activity? *European Journal of Cancer, 41*, 1931–1941.

Cooper, K. A., Campos-Gimènez, E., Alvarez, D. J., Nagy, K., Donovan, J. L., & Williamson, G. (2007). Rapid reversed phase ultra-performance liquid chromatography analysis of the major cocoa polyphenols and inter-relationships of their concentrations in chocolate. *Journal of Agricultural and Food Chemistry, 55*(8), 2841–2847.

Desmet, G., Clicq, D., & Gzil, P. (2005). Geometry-independent plate height representation methods for the direct comparison of the kinetic performance of LC supports with a different size or morphology. *Analytical Chemistry, 77*(13), 4058–4070.

Dyrby, M., Westergaard, N., & Stapelfeldt, H. (2001). Light and heat sensitivity of red cabbage extract in soft drink model systems. *Food Chemistry, 72*(4), 431–437.

FAOSTAT. On-line and multilingual database currently covering international statistics. <http://faostat.fao.org/foastat>.

Hong, V., & Wrolstad, R. E. (1990). Use of HPLC separation/photodiode array detection for characterization of anthocyanins. *Journal of Agricultural and Food Chemistry, 38*(3), 708–715.

Kong, J., Chia, L., Goh, N., Chia, T., & Brouillard, R. (2003). Analysis and biological activities of anthocyanins. *Phytochemistry, 62*, 923–933.

Määttä-Riihinen, K. R., Kamal-Eldin, A., & Törrönen, R. A. (2004). Identification and quantification of phenolic compounds in berries of *fragaria* and *rubus* species (family *Rosaceae*). *Journal of Agricultural and Food Chemistry, 52*(20), 6178–6187.

Mazza, G., & Miniati, E. (1993). *Anthocyanins in fruit, vegetables, and grains*. Boca Raton, FL: CRC Press.

Middleton, E. J., Kandaswami, C., & Theoharides, T. C. (2000). The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. *Pharmacological Reviews, 52*, 673–751.

Morse, M. A., & Stoner, G. D. (1993). Cancer chemoprevention: Principles and prospects. *Carcinogenesis, 14*, 1743–1746.

Schoefs, B. (2004). Determination of pigments in vegetables. *Journal of Chromatography A, 1054*(1–2), 217–226.

Seeram, N. P., Schutzki, R., Chandra, A., & Nair, M. G. (2002). Characterization, quantification, and bioactivities of anthocyanins in Cornus species. *Journal of Agricultural and Food Chemistry, 50*(9), 2519–2523.

Singh, J., Upadhyay, A. K., Bahadur, A., Singh, B., Singh, K. P., & Rai, M. (2006). Antioxidant phytochemicals in cabbage (*Brassica oleracea* L. var. capitata). *Scientia Horticulturae, 108*, 233–237.

Steinmetz, K. A., & Potter, J. D. (1996). Vegetables, fruits, and cancer prevention: A review. *Journal of the American Dietetic Association, 96*(10), 1027–1039.

Tian, Q., Giusti, M. M., Stoner, G. D., & Schwartz, S. J. (2005). Screening for anthocyanins using high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry with precursor-ion analysis, product-ion analysis, common-neutral-loss analysis, and selected reaction monitoring. *Journal of Chromatography A, 1091*(1–2), 72–82.

Turner, C. (2006). *Modern Extraction Techniques – Food and Agricultural Samples ISBN-13: 9780841239401*. American Chemical Society (pp. 3–19).

Wu, X., Beecher, G. R., Holden, J. M., Haytowitz, D. B., Gebhardt, S. E., & Prior, R. L. (2006). Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *Journal of Agricultural and Food Chemistry, 54*(11), 4069–4075.

Wu, X., & Prior, R. L. (2005). Identification and characterization of anthocyanins by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry in common foods in the United States: Vegetables, nuts, and grains. *Journal of Agricultural and Food Chemistry, 53*(8), 3101–3113.

Zhou, D., Xu, Q., Xue, X., Zhang, F., & Liang, X. (2006). Identification of *O*-diglycosyl flavanones in Fructus aurantii by liquid chromatography with electrospray ionization and collision-induced dissociation mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis, 42*(4), 441–448.