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BLUEBERRY ANTHOCYANINS ANALYZED BY ABSORPTION SPECTROSCOPY AND HPLC-UV-MS

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ABSTRACT

Anthocyanins are found in various kinds of blueberry fruits. Since there are reported benefits of anthocyanins, there are numerous blueberry products available. We present a study of total anthocyanin quantification and individual anthocyanins examination of various blueberry samples. Total anthocyanins were found to be higher in two blueberry powder capsules (1.38±0.02 mg/g for WN, and 0.64±0.03 mg/g for WQ) than in blueberry juice (300±2 μg/mL) using the pH differential method. Individual anthocyanins were characterised analytically using LC-UV-MS. Twenty-three anthocyanins were identified by HPLC-MS in 19 min. Interestingly, 4 new species were found and they were derived from two anthocyanidins: delphinidin and petunidin.

Keywords: anthocyanins, blueberry, visible absorption spectroscopy, liquid chromatography-mass spectrometry.

INTRODUCTION

Blueberries, which are the fruits of the flowering plants in the genus of *Vaccinium*, have been reported to contain numerous polyphenol compounds such as flavonoids (e.g. anthocyanins, flavonols and proanthocyanidins) (Prior, 1998). Anthocyanins are naturally occurring glycosides and acylglycosides of anthocyanidins, as shown in figure 1 (Mazza, 1993). Anthocyanidins vary with different hydroxyl or methoxyl substitutions in their basic structure, flavylium (2-phenylbenzopyrillium). The sugar substituents in anthocyanins are usually hexoses (galactose and glucose) and pentoses (arabinose), as shown in figure 2. Owing to the differences in chemical structure at various levels of pH, anthocyanins (*in Greek*, anthos = flower; kyanos = blue) change colors from red in acids to blue in bases (Giusti, 2001). Laboratory studies have shown that anthocyanins inhibit promotion and progression of tumor cells (Wada, 2002). In fact, these naturally occurring compounds have several functions, such as acting as a sunscreen to protect plant cells from photo damages and free radicals damages (Lohachoompol, 2004).

In this study, we have conducted a comparative chemical analysis of various blueberry samples, namely juice and powder capsules. The total anthocyanin content was determined using absorption spectroscopy, and the individual anthocyanin species were examined using HPLC-UV-MS.

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MATERIALS AND METHODS

Chemicals and materials

Pure blueberry juice (Bremner, 100%) was purchased from London Drugs (Surrey, BC, Canada). The blueberry juice was labeled not to contain additional water, sugar, additives and preservatives. The juice was stored at 4°C after opening. Blueberry powder capsules (Webber Naturals or WN and WellQuest or WQ) were obtained from Pharmasave (Burnaby, BC, Canada). The blueberry powder in the capsules contain the following fillers: rice starch, silica, magnesium stearate (lubricant), gelatin.

Ethyl acetate, methanol, hydrochloric acid and acetic acid were obtained from BDH Co. (Toronto, ON). HPLC-grade acetonitrile and methanol were from Fisher Scientific, and formic acid from EM Science™ (Analar grade). Pure anthocyanin standards (Antho5), which were obtained from Polyphenols/Cerilliant, consists of cyanidin glucoside (Cy3Gl), cyanidin galactoside (Cy3Ga), cyanidin arabinoside (Cy3Ar), peonidin galactoside (Pe3Ga) and peonidin arabinoside (Pe3Ar). Moreover, malvidin galactoside (M3Ga) and cyanidin/peonidin (Cy/Pe) were obtained from Chromadex (Irvine, CA). The concentrations of Antho5, M3Ga and Cy/Pe were 400, 320 and 200 ppb, respectively. In addition, cranberry cocktail (Ocean Spray) was used as a low-cost standard for cyanidin-containing anthocyanins (Durst, 2001).

Anthocyanin extraction and purification

Blueberry juice (30 mL) was filtered using a 0.45-μm syringe filter. Anthocyanins were extracted from the juice using a C18 solid-phase extraction column (Sorbent

Technologies, Atlanta, GA) based on a published procedure (Rodriguez-Saona, 2001). Briefly, the blueberry sample was first introduced to the C18 column for anthocyanin retention. Then, acidified water (0.01% HCl) was used to remove matrix interferences, such as sugars and acids. Afterwards, ethyl acetate was used to pass polyphenolics (e.g. proanthocyanidins) to the waste. Finally, the retained anthocyanins were eluted using methanol acidified with 0.01% HCl.

Blueberry powders were released from the capsules and weighed. Then the powders were dissolved in 10 ml of methanol. After filtration, the extracts were collected for subsequent analysis.

Determination of total anthocyanins by absorption spectroscopy

A UV-visible spectrometer equipped with a photodiode array detector (HP 8453) was used to determine the total monomeric anthocyanin content. The anthocyanin pigments undergo reversible structural transformations at different pH values (Giusti, 2001). For instance, at pH 1, the predominant form of anthocyanin is the flavylium cation, which is red (see Fig. 1). But at pH 4.5, the major structural form is the neutral cabinol, which is colorless. Therefore, the monomeric anthocyanins are determined at pH 1, to be corrected by other absorbing species at pH 4.5, and this method is called pH differential method (Giusti, 2001). The advantage of the pH-differential method is that it permits an accurate and rapid measurement of the total monomeric anthocyanins even in the presence of polymerized degraded pigments (e.g. proanthocyanidins) and other possible interfering compounds (Wada, 2002).

The sample was diluted by 15 times using (a) 0.025-M potassium chloride solution at pH 1.0, and (b) 0.4-M sodium acetate solution at pH 4.5. The absorbances of these 2 sample solutions were measured at 520 and 700 nm against the de-ionized water blank to give the A_{520} and A_{700} values, respectively. The absorbance (A) due to monomeric anthocyanins was calculated as follows:

$$A = (A_{520} - A_{700})_{pH=1.0} - (A_{520} - A_{700})_{pH=4.5} \qquad \text{(Eq. 1)}$$

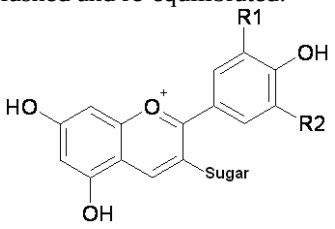
The anthocyanin concentration in the original sample was determined using the following equation:

$$[anthocyanin]\left(\frac{\mu g}{mL}\right) = \frac{A \times MW \times DF \times 1000}{\varepsilon \times b} \qquad \text{(Eq. 2)}$$

where DF is dilution factor equal to 15; b is pathlength (1 cm); ε is extinction coefficient (26,900 $dm^3\ mol^{-1}$); MW is molecular weight of the cyanidin-3-glucoside equivalent (449.2 g mol^{-1}), and it is used since cyanidin-3-glucoside is the most abundant anthocyanin in nature (Francis, 1982).

Separation and structural analysis of blueberry anthocyanins

HPLC analyses were performed using a Waters Alliance Separations Module (Fleshner, 2005). Separation was carried out with a C18 column (Phenomonex Gemini, 2x150mm, 3µm) at 40°C. A methanol gradient of 10-50-80% from 1-15-17.5 min., with 1% formic acid present throughout the run, was used, which was previously employed for cranberry juice analysis. Thereafter, the column was flushed and re-equilibrated.



Aglycon	R1	R2	MW (g/mol)
Pelargonidin	H	H	271
Cyanidin	OH	H	287
Peonidin	OMe	H	301
Delphinidin	OH	OH	303
Petunidin	OMe	OH	317
Malvidin	OMe	OMe	331

Fig. 1. General structure for anthocyanins.

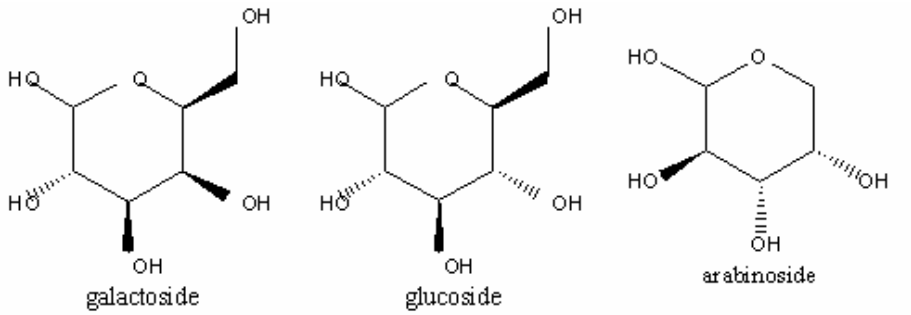


Fig. 2. Common monosaccharides (sugar) that bind to anthocyanidins to form anthocyanins (see the position of the sugar in figure 1).

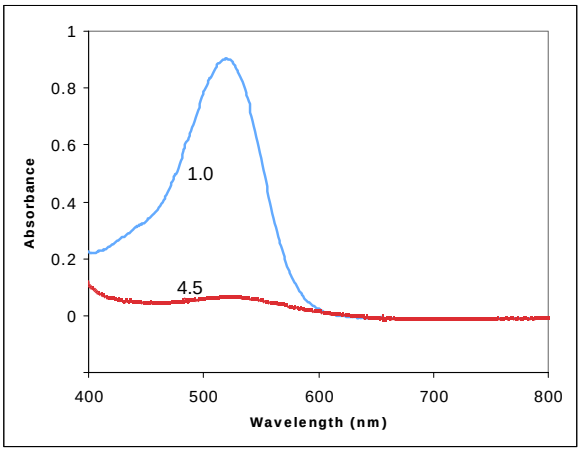


Fig. 3. Spectroscopic data for blueberry extract (from WN capsule) obtained at pH 1.0 and 4.5

With a photodiode array (PDA) detector, absorbance data was collected from 210-600 nm. With a coupled Quattro Micro detector, mass spectrometric (MS) electrospray positive scan (ES+) was employed. All MS data were collected using this single quadrupole detector, with mass range of m/z 150-750, and using two cone voltages, 25V (1: Scan ES+) and 55V (2: Scan ES+). The positive charge of the anthocyanin (see Fig. 1) favors the fast and effective ES+ detection. Desolvation and source temperatures were 350°C and 120°C, respectively; desolvation and cone gas flows were 600 l h^{-1} and 50 l h^{-1}, respectively; capillary and multiplier voltages were 3000V and 650 V respectively.

RESULTS AND DISCUSSION

Determination of total anthocyanin content by absorption spectroscopy

The absorption spectra for the blueberry extracts at two different pH values are shown in figure 3. Based on the pH differential method, by measuring the absorbances of two solutions at pH values of 1.0 and 4.5, and at 2 wavelengths (520 nm and 700 nm), the anthocyanin concentrations were determined using Equations 1 and 2. The anthocyanin content in blueberry juice is 300±2 μg/mL. The values for blueberry powders are 1.38±0.02 mg/g for WN, and 0.64±0.03 mg/g for WQ.

Analysis of anthocyanins using HPLC-UV

Anthocyanins extracted from blueberry samples was analyzed by HPLC, as shown in figure 4a and 4b. The two chromatograms obtained from blueberry juice and blueberry extract are very similar, with the major peaks occurring at retention times of 14.5-15.2 min. A comparison with the anthocyanin standard shown in figure 4c reveals that one of these peaks could be malvidin-3-galactoside (Table 1). A comparison of figure 4a and 4b was also made with the chromatogram of cranberry juice, for which the HPLC conditions were

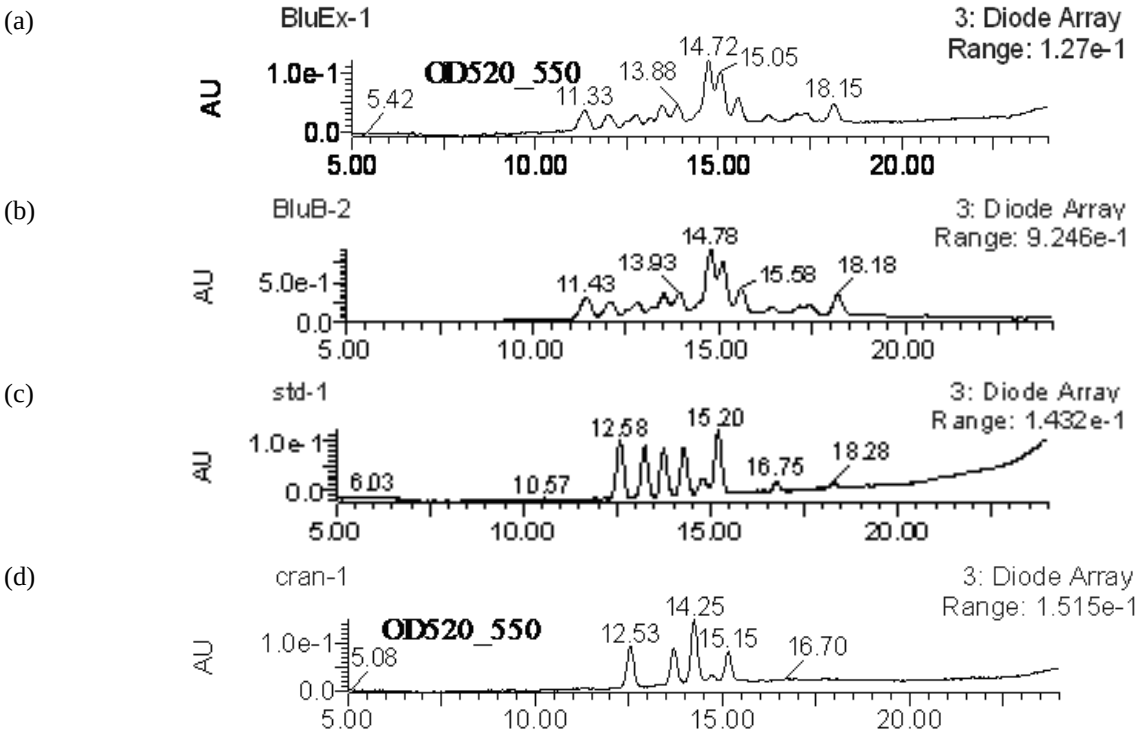


Fig. 4. HPLC chromatograms for (a) Blueberry extract (b) Blueberry juice with 10-fold dilution (c) Anthocyanin standards (see Experimental for concentrations), and (d) Cranberry juice; all monitored at a wavelength of 520 nm.

optimized (Fig. 4d). The cranberry juice is widely used as a low-cost standard for cyanidin- and peonidin-based anthocyanins (Rodriguez-Saona, 2001). It was observed that the major peaks in the blueberry samples could contain peonidin-3-glucoside (Pe3Gl), which was eluted at 14.77 min in figure 4d. The comparison of the UV retention times, among the MS data were listed in Table 1. However, such a comparison is inconclusive and confirmation is required using MS data.

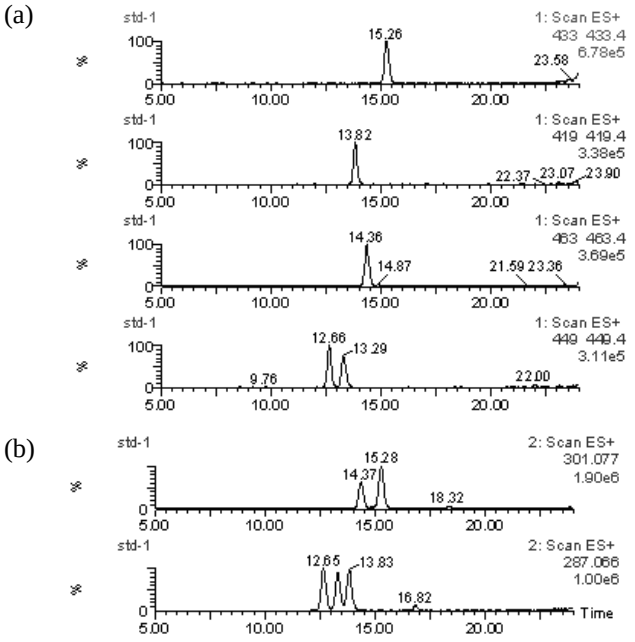


Fig. 5. Extracted ion chromatograms of anthocyanin standards (a) low cone voltage MS data (b) high cone voltage MS data.

Analysis and identification of anthocyanins in blueberry using HPLC-MS

Six different anthocyanin standards were used to identify anthocyanins in blueberry samples (Fig. 5). In figure 5a, the single peak at 13.82 min. in the MS chromatogram (m/z=419) corresponds to Cy3Ar, and the 2 peaks at 12.66 min. and 13.29 min. (m/z=449) are assigned to Cy3Ga and Cy3Gl, respectively. It is because of the presence of cyanidin (Cy) after anthocyanin fragmentation, as confirmed by the occurrence of 3 peaks in the MS chromatogram (m/z=287), in Fig. 5b, at similar retention times as mentioned above. The loss of mass of 132 (=419-287) corresponds to the fragmentation of arabinoside (Ar), and the mass change of 162 (=449-287) corresponds to the loss of the hexose, either galactose or glucose (Ga or Gl). Since it is well known that glucosides are eluted at a later retention time than galactosides (Rivas-Gonzalo, 2003) the peaks at 12.66 min. and 13.29 min. (m/z=449) are assigned to Cy3Ga and Cy3Gl, respectively.

Similarly, in figure 5a, the peak at 15.26 min. in the MS chromatogram (m/z at 433) corresponds to Pe3Ar, and the peak at 14.36 min. in the MS chromatogram (m/z at 463) corresponds to Pe3Ga. It is because the appearance of the m/z 301 peaks at similar retention times in the MS chromatogram (Fig. 5b) confirms the presence of peonidin (Pe).

The HPLC-MS analysis was then applied to blueberry juice and extracts. All peaks were identified by comparing with the anthocyanin standards, MS data and previously reported data (Nicoue, 2007; Wu 2005). The HPLC retention times, mass data and peak assignments are tabulated in table 2. Peaks 3, 5, 7, 9, 12 and 14 were identified by matching them to the LC-MS data for the six anthocyanins standards; whereas peaks 3, 7, 9, 11, and 14 were matched by comparison with the cranberry juice standard (see Table 1).

Table 1. Anthocyanins from Craneberry juice and Standards

Cranberry juice

Peak	t_R (UV) (min)	t_R (MS) (min)	$[M]^+$ (m/z)	fragments (m/z)	anthocyanins
C1	12.53	12.6	449	287	cyanidin 3-galactoside
C2	13.71	13.78	419	287	cyanidin 3-arabinoside
C3	14.25	14.32	463	301	Peonidin 3-galactoside
C4	14.77	14.84	463	301	Peonidin 3-glucoside
C5	15.15	15.22	433	301	Peonidin 3-arabinoside

Anthocyanin Standards

Peak	t_R (UV) (min)	t_R (min)	[M]+ (m/z)	fragments (m/z)	anthocyanins
A1	12.58	12.65	449	287	cyanidin 3-galactoside
A2	13.22	13.29	449	287	cyanidin 3-glucoside
A3	13.75	13.82	419	287	cyanidin 3-arabinoside
A4	14.3	14.37	463	301	peonidin 3-galactoside
A5	14.73	14.80	493	331	malvidin 3-galactoside
A6	15.2	15.27	433	331	peonidin 3-arabinoside

(The MS retention times are 0.07 min later than the UV retention times.)

Twenty three anthocyanins (peaks 1-15, 17, and 19-25) were identified within 19 min. They were tabulated according to the mass number information associated with the retention times (see Table 3). These anthocyanins were derived from delphinidin, cyanidin, petunidin, peonidin, and malvidin. None were derived from pelargonidin, as previously noted (Wu, 2005). Among these, we have identified peonidin 3-arabinoside (peak 14), which has not been identified in previous reports (Wu, 2005).

Examination of the MS chromatogram collected at m/z =287 reveals all the anthocyanins that contain cyanidin (see Fig. 6a). Other than peaks 3, 5 and 7 that are described above, there is an additional peak 21 at 17.24 min., which could potentially be cyanidin 3-acetylated glucoside. Since a peak at m/z=491 also occurs at the same retention time, the mass difference (i.e. 204) suggests the presence of the acetylated hexose. The longer retention time of peak 21 is also consistent with a decrease in polarity after acylation of the glucose moiety (Rivas-Gonzalo, 2003).

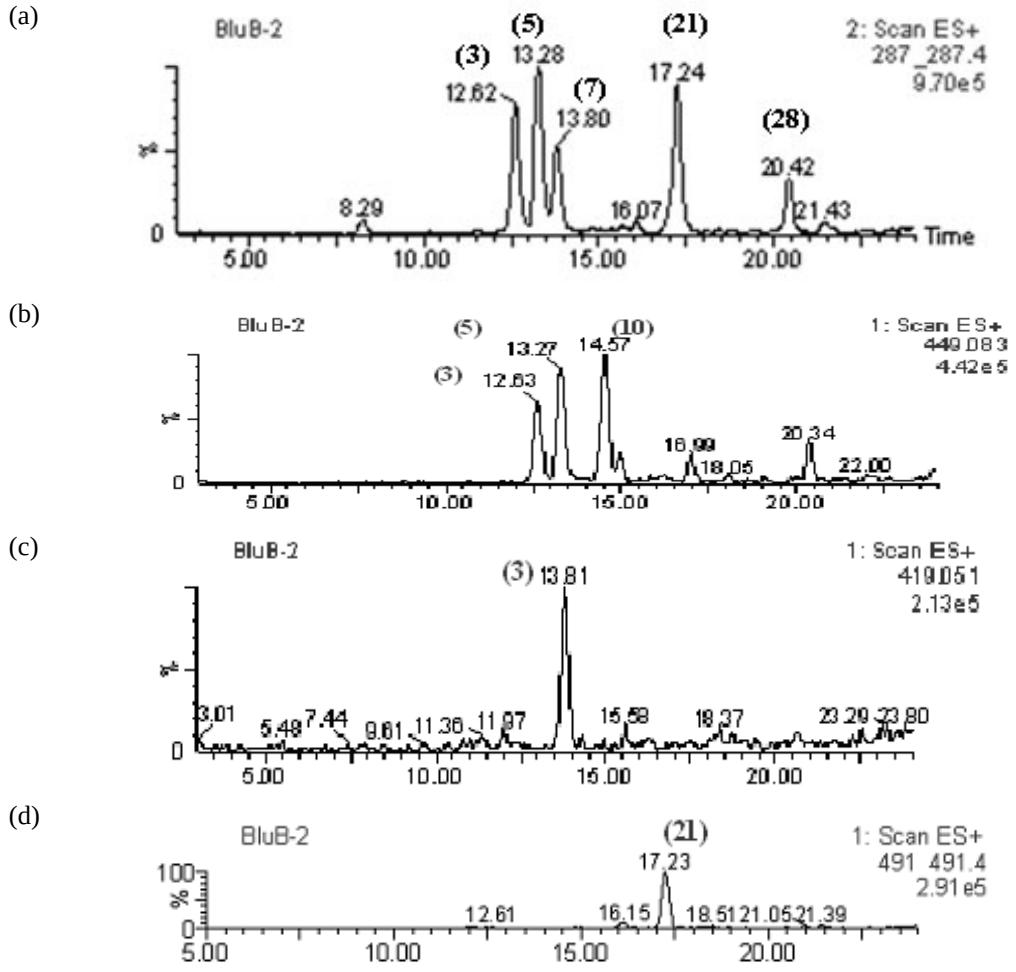


Fig. 6. LC-MS data of blueberry juice. (a) MS data of fragments with m/z=287 showing cyanidin-containing compounds. (b) MS data of compounds 3, 5, 10 that have MW = 449 cyanidin 3-galactoside, cyanidin 3-glucoside, and petunidin 3-arabinoside. (c) MS data of compound 7 that has MW of 419, cyanidin 3-arabinoside (d) MS data of compound 21 that has MW of 491, cyanidin 3acetylated glucoside.

Identification of malvidin 3-acetylated galactoside (compound 22, RT 17.4 min) and malvidin 3-acetylated glucoside (compound 25, RT 18.3 min) is shown in figure 7. We see spectral similarities to those observed above in that if we consider a corresponding mass difference of 204 for peaks representing m/z 535 and 331, we might postulate that we have acetylation of a hexose moiety as we also see a longer retention as expected for the glucoside (Fig. 7a and 7b: m/z 331 and 535 monitor malvidin and malvidin-acetylated hexose, respectively). In addition, the mass spectra and chemical structures shown in figure 7c and d illustrate the fragmentation of the acetylated galactose and glucose respectively, where the lower spectrum of each pair is collected with a higher cone voltage, thus greater fragmentation.

Separation of the common blueberry anthocyanins (peaks 1-25) in this work has been achieved within 19 min., as shown in figure 8. All MS data were collected using a single quadrupole detector. Methods described in two other reports were achieved in much longer times, namely, 74 min. (Wu, 2005) and 59 min. (Kalt, 1999). In addition, 4 new chemical species were found (peaks 26-29) (see Fig. 9). Although they are associated with delphinidin and petunidin, their full structures remain to be elucidated.

CONCLUSIONS

Total anthocyanins were found to be higher in two blueberry powder capsules than in blueberry juice using the pH differential method. Individual anthocyanins were characterised analytically using LC-MS. Twenty-three anthocyanins were confirmed from the mass data alone.

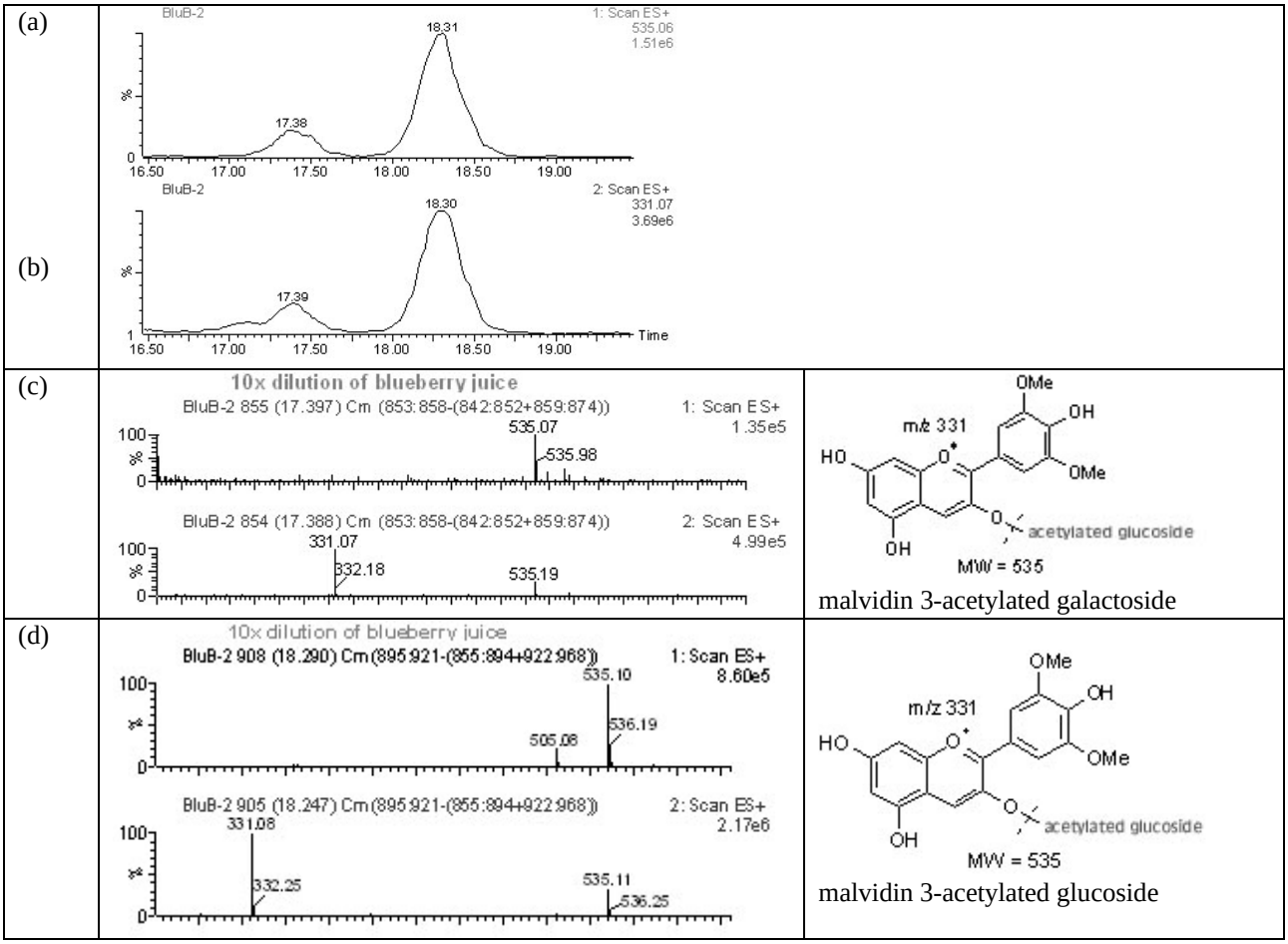


Fig. 7. MS chromatograms and MS spectra of malvidin 3-acetylated galactoside (compound 22) and malvidin 3-acetylated glucoside (compound 25). (a) MS chromatogram monitored at m/z = 535, (b) MS chromatogram of fragment with m/z = 331, (c) mass spectrum at t_R = 17.4 min. showing the major parent ion (m/z 535) and fragment ion (m/z 331); the inset shows the chemical structure of malvidin 3-acetylated galactoside and the fragmentation site, (d) mass spectrum at t_R = 18.3 min. showing the major parent ion of MS (m/z 535) and fragment ion (m/z 331), the inset shows the chemical structure of malvidin 3- acetylated glucoside and the fragmentation site.

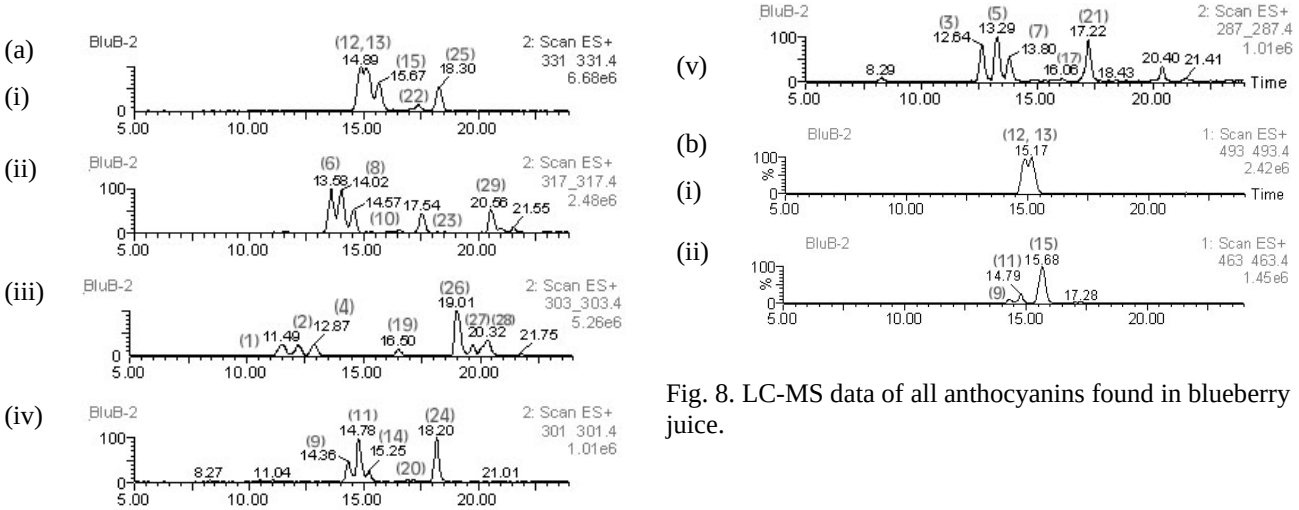


Fig. 8. LC-MS data of all anthocyanins found in blueberry juice.

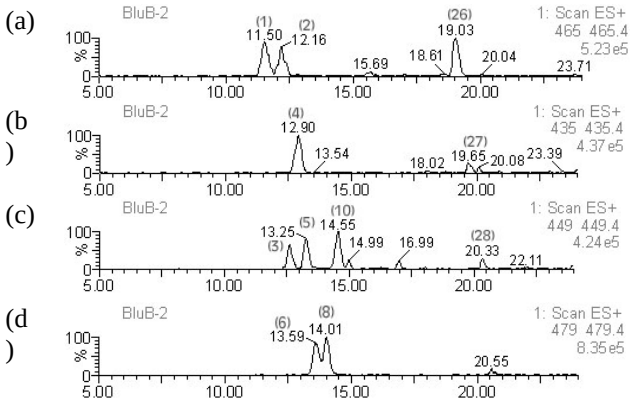


Fig. 9. LC-MS data of 4 new anthocyanins found in blueberry.

Malvidin 3-acetylated galactoside and malvidin 3-acetylated glucoside were assigned speculatively to two of the anthocyanins detected. Interestingly, 4 new species were found, which were derived from delphinidin and petunidin.

Table 2. Identification of Anthocyanins from Blueberry samples

Peak	t_R (min)	$[M]^+$ (m/z)	t_R (min)	fragments (m/z)	anthocyanins
1	11.50	465	11.49	303	delphinidin 3-galactoside
2	12.16	465	12.16	303	delphinidin 3-glucoside
3	12.62	449	12.64	287	cyanidin 3-galactoside
4	12.90	435	12.87	303	delphinidin 3-arabinoside
5	13.25	449	13.29	287	cyanidin 3-glucoside
6	13.59	479	13.58	317	petunidin 3-galactoside
7	13.80	419	13.80	287	cyanidin 3-arabinoside
8	14.01	479	14.02	317	petunidin 3-glucoside
9	14.36	463	14.36	301	peonidin 3-galactoside
10	14.55	449	14.57	317	petunidin 3-arabinoside
11	14.79	463	14.78	301	peonidin 3-glucoside
12	14.80	493	14.89	331	malvidin 3-galactoside
13	15.17	493	15.17	331	malvidin 3-glucoside
14	15.25	433	15.25	301	peonidin 3-arabinoside
15	15.68	463	15.67	331	malvidin 3-arabinoside
16	15.85	579	-	331	a
17	16.15	491	16.06	287	cyanidin 3-acetylated galactoside
18	16.41	551	-	303	b
19	16.40	507	16.50	303	delphinidin 3-acetylated glucoside
20	17.19	505	17.19	301	peonidin 3-acetylated galactoside
21	17.23	491	17.22	287	cyanidin 3- acetylated glucoside
22	17.40	535	17.40	331	malvidin 3-acetylated galactoside
23	17.53	521	17.54	317	petunidin 3-acetylated glucoside
24	18.17	505	18.20	301	peonidin 3-acetylated glucoside
25	18.30	535	18.30	331	malvidin 3-acetylated glucoside
26	19.03	465	19.01	303	delphinidin + hexose
27	19.65	435	19.65	303	delphinidin + pentose
28	20.33	449	20.32	303	delphinidin + pentose + methyl
29	20.55	479	20.56	317	petunidin + hexose

a and b could not confirmed to be malvidin 3-(malonoyl)galactoside and malvidin 3-(malonoyl)glucoside, respectively, as previously reported

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Table 3. Identification of Anthocyanins from Blueberry

	Glycosides or acylglycosides							
	Galactoside	Glucoside	hexose	Arabinoside	pentose	pentose +methyl	acetylated galactoside	acetylated glucoside
Anthocyanidin	+162	+162	+162	+132	+132	+146	+204	+204
Delphinidin +303	11.5 (1)	12.16 (2)	19.03 (26)	12.9 (4)	19.65 (27)	20.33 (28)	16.5 (19)	
Cyanidin +287	12.62 (3)	13.28 (5)		13.8 (7)			16.07 (17)	17.2 (21)
Petunidin +317	13.61 (6)	14.01 (8)	20.55 (28)	14.57 (10)				17.53 (23)
Peonidin +301	14.36 (9)	14.79 (11)		15.25 (14)			17.19 (20)	18.17 (24)
Malvidin +331	14.8 (12)	15.17 (13)		15.68 (15)			17.4 (22)	18.3 (25)

retention time t_R are given in minutes; the plus numbers refer to added mass number; the numbers in parentheses refer to peak numbers in Table 2.

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