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Analytical Methods

Determination of anthocyanins in various cultivars of highbush and rabbiteye blueberries

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ABSTRACT

Anthocyanins were identified and quantified in various cultivars of blueberries grown in Australia. Those were Crunchie, Star and Sharpe (highbush, *Vaccinium corymbosum* L.) and Climax, Powderblue and Brightwell (rabbiteye, *Vaccinium ashei*). A method was developed involving liquid chromatography–mass spectrometry and UV–visible spectroscopy. The repeatability of injection was less than 2% relative standard deviation (RSD) while the repeatability of sample preparation was less than 10% RSD in most cases. The method was linear between 7% and 100% of the original concentration. The anthocyanin profile was similar in all cultivars but proportions of each compound were cultivar-dependent. Highbush had more early eluting peaks, i.e. more polar anthocyanins, than rabbiteye cultivars. Delphinidin, petunidin and malvidin were the major contributors to total anthocyanin content. Climax had the highest total anthocyanin content (13.7 ± 1.4 g cyanidin 3-glucoside equivalent kg^{-1} dry weight) and antioxidant activity (using 2,2-diphenyl-1-picrylhydrazyl as a free radical) of all tested cultivars. Rabbiteye had significantly higher total anthocyanin content than the highbush cultivars.

1. Introduction

Blueberry production in Australia has been rising since 1998. The output grew from 1274 tonnes in 1998–2060 tonnes in 2001 (Collins, Carillo, & Abraham, 2004). Two species of blueberries are grown in Australia, namely highbush (*Vaccinium corymbosum* L.) and rabbiteye (*Vaccinium ashei*) due to their low chill requirement compared to the lowbush (*Vaccinium angustifolium*). Among berries, blueberries are well known for their complex anthocyanin pattern and high total anthocyanin content (Garcia-Viguera, Zafrilla, & Tomas-Barberan, 1997; Kalt, Forney, Martin, & Prior, 1999a; Kalt, McDonald, Ricker, & Lu, 1999b; Zheng & Wang, 2003).

Anthocyanins, which are responsible for the intense red to blue colours in numerous fruits and flowers, are one of the major flavonoid classes. Anthocyanins are glycosides of 18 anthocyanidins which differ from each other by their degree of hydroxylation and methoxylation. Only six anthocyanidins are the most common in nature (Gross, 1987; Jackman & Smith, 1996) (see Fig. 1).

Anthocyanins are also known as a unique group of substances which are believed to provide a broad variety of health benefits such as prevention of heart disease, inhibition of carcinogenesis, and anti-inflammatory activity in the brain (Bagchi, Sen, Bagchi, & Atalay, 2004; Galli, Bielinski, Szprengiel, Shukitt-Hale, & Joseph, 2006; Tsang et al., 2005). Antioxidant activity of blueberries is influenced by the total anthocyanin content, the total phenolic content, genotypes, environmental variation, maturity, and the post harvest storage conditions (Conner, Luby, Hancock, Berkheimer, & Hanson, 2002a; Conner, Luby, Tong, Finn, & Hancock, 2002b; Kalt et al., 2003,1999b; Prior et al., 1998). A number of studies have reported the effect of different factors on the antioxidant activity. This paper however investigates the relative concentration of anthocyanins based on molecular polarity for different seasons of harvesting of blueberry cultivars. Also, there are extensive studies in the US and other countries such as Italy (Beccaro, Mellano, Botta, Chiabrando, & Bounous, 2006) on different cultivars of blueberries but there is no evidence of published work on Australian blueberries. It was reasonable to compare Australian harvested blueberries with those grown elsewhere.

Simple and acylated anthocyanins are found in different species of blueberries. The acylated anthocyanins are found in some highbush (Wu & Prior, 2005) and lowbush cultivars (Gao & Mazza, 1994; Wu & Prior, 2005). Different structures of anthocyanins not only give different colours but also have different antioxidant effects. For example, pelargonidin, malvidin, and peonidin which have only one hydroxyl group on the R_2 position (Fig. 1) have a lower oxygen radical absorbance capacity (ORAC) (Wang, Cao, & Prior, 1997).

The objective of this work was to develop and validate a LC/MS method to identify anthocyanins from different cultivars of blueberries locally grown in Australia harvested in different seasons and to compare the results to the total anthocyanin content

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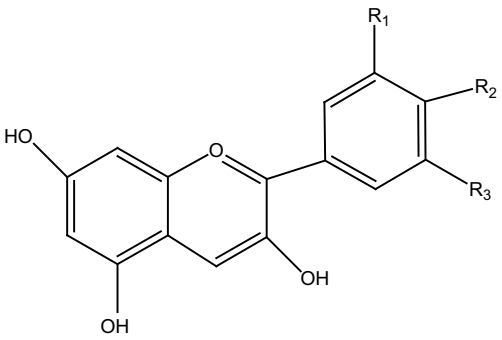

Anthocyanidin	R_1	R_2	R_3
Cyanidin	OH	OH	H
Delphinidin	OH	OH	OH
Malvidin	OCH_3	OH	OCH_3
Pelargonidin	H	OH	H
Peonidin	OCH_3	OH	H
Petunidin	OCH_3	OH	OH

Fig. 1. Structure of six most common anthocyanidins occurring in nature.

determined by spectroscopic method which was used to measure the colour quality of fresh and processed fruits. Antioxidant activity was also determined and related to total anthocyanins and total phenolics.

2. Materials and methods

2.1. Chemicals

HPLC-grade methanol (MeOH) and acetonitrile (ACN) were obtained from Merck KGaA, Darmstadt, Germany. Acetic acid, formic acid, gallic acid, and hydrochloric acid (HCl) were obtained from Ajax Chemicals Pty Ltd., Sydney, NSW, Australia. Cyanidin 3-glucoside was obtained from Polyphenols Laboratories AS, Sandnes, Norway. (*R*)-(+)-6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Aldrich Pty. Ltd., NSW, Australia. Folin-Ciocalteu's reagent was obtained from Prolabo, Paris, France.

2.2. Samples

Two *Vaccinium* species with a total of six cultivars were obtained from Blueberry Farms of Australia in Corindi, NSW. All cultivars are commercially produced at different times of the year. Crunchie, Star and Sharpe which are highbush (*V. corymbosum* L.) were harvested from August to December while Climax, Powderblue and Brightwell which are rabbiteye (*V. ashei*) were harvested later in the season, i.e. from November to April. The blueberries were kept at −20 °C and analysed within 3 months.

2.3. Analyses

2.3.1. Extraction

The extraction method was adapted from the work done by Garcia-Viguera et al. (1997). Each blueberry cultivar was blended at 5 °C for 1 min using a food processor into puree. Approximately 10 g of the well blended puree was weighed accurately then extracted using 75 mL of MeOH/water/acetic acid (25:24:1). The extract was then centrifuged at 21,900*g* (12,000 rpm) for 20 min at 15 °C. The residue, remaining after removing the supernatant, was mixed thoroughly with 75 mL of MeOH/water/acetic acid. Each sample was extracted three times. The clear liquid from three extractions was evaporated under vacuum at 35 °C. The residue from vacuum evaporation was redissolved with 5 mL of 3% (w/v) formic acid in water. This aqueous solution was purified using a C18 Sep-Pak cartridge (Waters Corp, Milford, MA, USA). The anthocyanins eluted from the cartridge were evaporated under vacuum at 35 °C until dryness. The residue was redissolved in 1.0 mL of 15% methanol and 85% of 5% (w/v) formic acid.

Total anthocyanin content determination was carried out using spectroscopic method.

The extracts were diluted to an appropriate concentration by mixing with MeOH:0.1 mol L^{-1} HCl at a ratio of 85:15 (Giusti, Rodriguez-Saona, & Wrolstad, 1999). The maximum absorbance of the samples was measured at 535 nm by UV-1601 Shimadzu UV–visible spectrophotometer (Shimadzu Corporation, Tokyo, Japan). Cyanidin 3-glucoside (molar extinction of 32,531 at 528 nm) was used as a standard to express total anthocyanin content.

2.3.2. LC/MS analysis of anthocyanins

Samples (2 μL) were analysed using a Waters Alliance 2690 HPLC pump and autosampler and Waters 996 photodiode array detector (PDA). Separation was carried out using a 2.1 mm × 150 mm Xterra™ MS C_{18} column (Waters Corp., Milford, MA, USA) with a 2.1 mm × 10 mm Xterra™ guard column. The gradient of the mobile phase A (methanol) and mobile phase B (3% formic acid) at the flow rate of 0.2 mL min^{-1} was adapted from Wu and Prior (2005). The gradient started with 15% A, increasing to 20% A at 5 min, isocratic elution at 20% A for 5 min, increasing to 25% A at 25 min, isocratic elution at 25% A for 5 min, increasing to 70% A at 46 min, isocratic elution at 70% A for 6 min, and reducing to 15% A at 55 min. Detection was carried out at 520 nm. All analyses were done in three replicates and the results were expressed as mean values.

The effluent was directed into the source of a single quadrupole mass spectrometer (Micromass ZMD). Chromatograms were recorded and processed on a Waters' Masslynx chromatographic data system. ESI mass spectra were scanned from *m*/*z* 250 to 700. The source block and desolvation temperature were 150 and 400 °C, respectively. Spectra were acquired in the positive ion mode. The capillary voltage was set at 4.5 kV. The cone voltage was maintained at 31 V. N_2 was used as drying and spraying gas at the flow rate of 500 L h^{-1}.

For this work, it was only necessary to compare different species and cultivars of blueberries; hence a comparison of absolute peak areas was possible if the data were collected under repeatable conditions. To ascertain this, several studies were carried out including linearity and precision. This method showed that uncertainties were mostly less than 10% thus allowing three significant figures for most anthocyanins.

2.3.3. Total phenolic content determination

Total phenolic content was determined using Folin–Ciocalteu reagent according to the manual method (Slinkard & Singleton, 1977) by using gallic acid as a standard.

2.3.4. Determination of antioxidant activity

The antioxidant activity was determined using DPPH as a free radical (Brand-Williams, Cuvelier, & Berset, 1995). The stock solution was prepared by dissolving 2.4 mg of DPPH in 10 ml MeOH. The stock solution was kept at −20 °C until used. The working solution was obtained by mixing 10 ml of the stock solution with 45 ml of methanol to obtain an absorbance of 1.100 ± 0.020 at 515 nm. A

volume of 0.15 ml from appropriate diluted solution of the extract was allowed to react with 2.85 ml of the working solution for 24 h in the dark at ambient temperature. The absorbance was then measured at 515 nm. The antioxidant activity was expressed as μmol Trolox equivalent g^{-1} dry weight.

2.4. Statistical analysis

Analysis of variance and Duncan's multiple range test were used to determine significant differences ($P < 0.05$) of total anthocyanin contents, total phenolic contents, and antioxidant effects.

3. Results and discussion

3.1. Method development and validation

Several parameters of the method were optimised with the aims of improving resolution, sensitivity and reducing the overall run time. These parameters included selection of the column, the gradient conditions, the percentage of formic acid and the injection volume.

Two different columns were evaluated, 4.6 × 150 mm XBridge™ C_{18} column (Waters Corp., Milford, MA, USA) with a 4.6 × 20 mm XBridge™ C_{18} guard column and 2.1 × 150 mm Xterra™ MS C_{18} column (Waters Corp., Milford, MA, USA) with 2.1 × 10 mm Xterra™ guard column. The Xterra™ column was found to provide good resolution. The column volume and the column dimensions allowed a flow rate that was compatible with the MS.

Table 1 contains a list of mobile phases tested, together with the overall number of the simple anthocyanins resolved, and the overall runtime. The gradient conditions described in the experimental section proved to provide the best resolution and allowed the runtime to be reduced from 90 min from the work done by Wu and Prior (2005) to 55 min. The optimum conditions achieved from this study were comparable to those reported in other published work (Faria et al., 2005) in terms of running time. Both studies show 15 identified anthocyanins. The method reported here demonstrated improved resolutions for peak number 3 and 4 (see Fig. 2) which are cyanidin 3-galactoside and delphinidin 3-arabinoside, respectively. However, the resolution between peonidin 3-glucoside and malvidin 3-galactoside as well as peonidin 3-arabinoside and malvidin 3-glucoside were superior in the method reported by Faria et al. (2005). For the species and cultivars of blueberries reported in this study, unidentified peaks were observed late in the chromatograms which were not present in the species studied by Faria et al. (2005). Although, these molecules could not be identified, the method chosen for the study ensured that the compounds eluted during the runtime and were not retained to elute in the following analyses.

The percentage of formic acid was tested at 1%, 2%, 3%, 4% and 5% and it was found that 3% formic acid was sufficient for this application. Below 3% the peak shape deteriorated. The injection volume was also optimised. A volume of 2 μl was found to give the best compromise between maximising sensitivity and maintaining good peak shape.

The mass spectrometry conditions were optimised using a diluted anthocyanin extract and the optimum conditions are described in the experimental section.

The method validation study included testing the repeatability of injection and sample preparation. The results for the precision tests are summarised in Table 2. The precision, which is expressed as relative standard deviation (RSD) was within 2% for the repeatability of the LC/MS injection. The repeatability of the sample preparation, with the exception of three compounds, RSD was within 10% in most cases. A 5–10% threshold is generally accepted for this type of analysis due to the complexity of the extraction method.

The linearity was also tested by diluting a single sample of blueberries over a range of 7%, 10%, 14%, 25%, 50% and 100% of the original concentration. To summarise the results, the identified peaks of delphinidin 3-galactoside, cyanidin 3-galactoside, and malvidin 3-arabinoside were selected as they were well resolved from their neighbouring peaks and represented a range of retention times spanning over the entire length of the chromatogram. The method was linear having r^2 higher than 0.9990.

3.2. LC/MS Anthocyanin profiles in different cultivars

The profile of anthocyanins from six different blueberry cultivars is shown in Table 3. The individual anthocyanins were identified using the optimised LC/MS method. By comparing the m/z of each anthocyanin molecule and its fragmentation to the values in available published works, 11–14 peaks were tentatively identified in the blueberry samples. From the above work on method development, it can be concluded that a difference in peak area which was greater than 10% could be considered as significant. High percentages of delphinidin (34–36% in highbush and 19–22% in Climax), petunidin (19–22% in highbush and 14–16% in rabbiteye), and malvidin (34–41% in highbush and 44–51% in rabbiteye) were found relative to the total anthocyanin content.

Table 1
Runtime and number of simple anthocyanins from different mobile phases and gradients used in method development

No.	Mobile phase A	Mobile phase B	Gradient	Run time (min)	No. of simple anthocyanins resolved
1	ACN	3% Formic acid	0–20 min, 92–80%B; 20–25 min, 80%B; 25–29 min, 80-20%B; 29–30, 20%B; 30–31 min, 20–92%B	31	9
			0–20 min, 95–86%B; 20–25 min, 86–80%B; 25–26 min, 80%B; 26–34 min, 80–40%B; 34–35 min, 40–20%B; 35–36 min, 20–95%B	36	12
			0–15 min, 94–86%B; 15–25 min, 86–80%B; 25–26, 80%B; 26–34 min, 80–40%B; 34–35 min, 40–20%B, 35–36 min, 20–94%B	36	11
2	Methanol	3% Formic acid	0–25 min, 85–70%B; 25–30 min, 70–50%B; 30–33 min, 50–20%B; 33–34 min, 20–85%B; 34–35 min, 85%B	35	13
			0–5 min, 82–80%B; 5–12 min, 80–77%B; 12–18 min, 77–76.5%B; 18-26 min, 76.5–75.5%B; 26–28 min, 75.5–50%B; 28–35 min, 50–20%B; 35–36 min, 20–82%B	36	13
			0–2 min, 95%B; 2–10 min, 95–80%B; 10–15 min, 80%B, 15–30 min, 80–75%B; 30–35 min, 75%B, 35–50 min, 75–67%B; 50–55 min, 67%B, 55–65 min, 67–64%B, 65–70 min, 64–55%B; 70–75 min, 55–47%B; 75–80 min, 47–45%B; 80–84 min, 45–30%B; 84–88 min, 30–95%B; 80–90 min, 95%B	90[a]	13
			0–5 min, 85–80%B; 5–10 min, 80%B; 10–25 min, 80–75%B; 25–30 min, 75%B; 30-45 min, 75–67%B; 45–50 min, 67%B; 50-60 min, 67–64%B; 60–65 min, 64–55%B; 65–68 min, 55–30%B; 68–70 min, 30–85%B	70	13
			0–5 min, 85–80%B; 5–10 min, 80%B, 10–25 min, 80–75%B; 25–30 min, 75%B; 30–46 min, 75–30%B; 46–53 min, 30%B; 53–55 min, 30–85%B	55	13

[a] Gradient condition was obtained from the work done by Wu and Prior (2005).

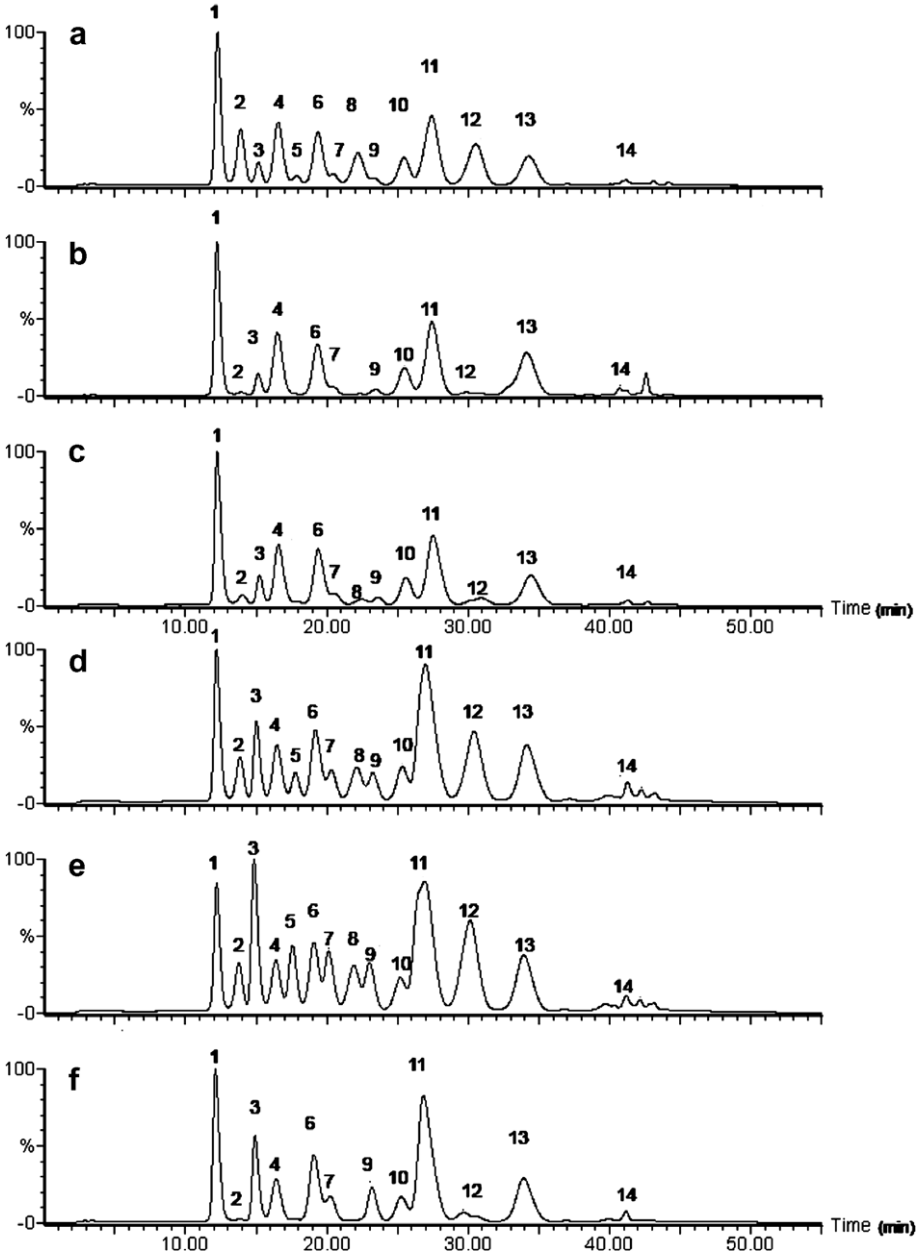

Fig. 2. Chromatograms of six cultivars of blueberries (a) Crunchie, (b) Star, (c) Sharpe, (d) Climax, (e) Powderblue and (f) Brightwell; peak numbers are according to Table 3.

Table 2
Precision of the LC/MS and extraction methods used in the analysis

Peak no.	Anthocyanin	Repeatability of sample preparation			
		RSD (%)		95% Confidence limits	
		Height	Area	Height	Area
1	Delphinidin 3-galactoside	11.1	11.3	219,363 ± 25,611	105,614 ± 12,516
2	Delphinidin 3-glucoside	15.3	15.0	31,861 ± 5126	16,948 ± 2661
3	Cyanidin 3-galactoside	7.4	8.0	272,009 ± 21,245	138,317 ± 11,673
4	Delphinidin 3-arabinoside	11.2	11.4	61,305 ± 7222	38,721 ± 4625
5	Cyanidin 3-glucoside	17.4	18.6	52,333 ± 9567	28,328 ± 5535
6	Petunidin 3-galactoside	8.5	9.6	122,523 ± 10,928	92,094 ± 9271
7	Cyanidin 3-arabinoside	7.4	8.8	83,443 ± 6513	58,007 ± 5380
8	Petunidin 3-glucoside	13.3	14.1	29,477 ± 4170	23,781 ± 3519
9	Peonidin 3-galactoside	6.5	8.0	91,086 ± 6187	67,821 ± 5691
10	Petunidin 3-arabinoside	9.0	9.2	51,762 ± 4880	43,824 ± 4222
11	Peonidin 3-glucoside coeluted with malvidin 3-galactoside	5.8	5.4	292,320 ± 17907	392,575 ± 22,423
12	Peonidin 3-arabinoside coeluted with malvidin 3-glucoside	8.8	8.7	98,620 ± 9106	136,028 ± 12,456
13	Malvidin 3-arabinoside	5.3	6.3	119,026 ± 6575	155,950 ± 10,313

Table 3
Anthocyanin profile of individual blueberry cultivars

Peak no.	RT (min)	Molecular (m/z)	Fragment (m/z)	Compound	Proportion of each compound (percentage of the total absorbance peak)					
					Crunchie	Star	Sharpe	Climax	Powderblue	Brightwell
1	12.19	465	303	Delphinidin 3-galactoside	17.5	21.6	22.5	11.0	10.8	16.3
2	13.87	465	303	Delphinidin 3-glucoside	7.7	0.3	0.9	3.5	4.6	0.2
3	15.03	449	287	Cyanidin 3-galactoside	2.1	2.7	4.4	5.4	8.7	8.9
4	16.45	435	303	Delphinidin 3-arabinoside	10.4	12.6	11.9	5.0	4.7	5.6
5	17.69	449	287	Cyanidin 3-glucoside	0.9	tr[A]	tr	1.6	3.6	tr
6	19.21	479	317	Petunidin 3-galactoside	9.5	11.8	13.4	7.5	6.2	11.1
7	20.30	419	287	Cyanidin 3-arabinoside	1.1	1.3	2.4	2.6	4.2	3.7
8	22.14	479	317	Petunidin 3-glucoside	6.7	tr	0.7	3.9	4.8	tr
9	23.28	463	301	Peonidin 3-galactoside	0.7	1.1	1.5	2.9	3.8	4.7
10	25.36	449	317	Petunidin 3-arabinoside	5.8	7.2	7.4	4.3	3.5	4.2
11[B]	27.15	463	301	Peonidin 3-glucoside	17.6	22.9	21.3	26.7	22.2	30.3
		493	331	Malvidin 3-galactoside						
12[C]	30.17	433	331	Peonidin 3-arabinoside	11.4	0.3	1.8	12.7	13.4	2.4
		493	331	Malvidin 3-glucoside						
13	34.12	463	331	Malvidin 3-arabinoside	8.5	17.8	11.3	11.1	8.4	11.4
14	41.12	521	303	Unidentified	0.1	0.4	0.5	1.8	1.1	1.2
Total anthocyanin content (g cyanidin 3-glucoside equivalent kg^{-1} dry weight)[D]					7.9 ± 0.9^{b}	5.8 ± 0.3^{a}	9.6 ± 1.0^{bc}	13.7 ± 1.4^{e}	11.9 ± 1.7^{de}	10.1 ± 0.1^{cd}
Total phenolic content (g gallic acid kg^{-1} dry weight)[D]					14.9 ± 1.2^{b}	10.8 ± 0.9^{a}	19.6 ± 2.3^{c}	21.0 ± 1.1^{c}	20.2 ± 2.0^{c}	14.4 ± 1.3^{b}
Antioxidant effect(μmol Trolox equivalent g^{-1} dry weight)[D]					84.8 ± 3.8^{b}	67.3 ± 4.8^{a}	152.9 ± 2.7^{d}	155.7 ± 9.0^{d}	140.6 ± 7.4^{c}	90.8 ± 3.7^{b}

[A] Traceable amount was detected by MS.
[B] Peonidin 3-glucoside coeluted with malvidin 3-galactoside.
[C] Peonidin 3-arabinoside coeluted with malvidin 3-glucoside.
[D] Mean of 3 replicates ± standard deviation; values with the same superscript in the same row are not significantly different ($P < 0.05$).

The proportion of five early eluted peaks in highbush was 38.6%, 37.2%, and 39.6% for Crunchie, Star, and Sharpe, respectively. In contrast, the proportion of these early eluted peaks was about 5-13% lower in all rabbiteye cultivars at 26.4, 32.3, and 31.1% for Climax, Powderblue, and Brightwell, respectively. It was noted that the early spring harvested cultivars of highbush showed a higher percentage of more polar anthocyanins which eluted at retention time (RT) below 23 minutes. It is possible that these molecules are likely to be less stable. Therefore, they are reduced during the hotter summer harvesting season. However, the percentage distribution of cyanidin glycosides in the rabbiteye (9.6%-16.5%) was higher than in the highbush (4.1%-6.8%). These more polar compounds which have two hydroxyl groups substituted at R_1 and R_2 position (Fig. 1), are more active as antioxidants than those with only one hydroxyl substituted such as pelargonidin, malvidin, and peonidin (Bors, Heller, Michel, & Saran, 1990; Fukumoto & Mazza, 2000; Wang et al., 1997).

Table 3 also shows that different amounts of total anthocyanin content are found in different cultivars. Climax had the highest total anthocyanin content (13.7 ± 1.4 g cyanidin 3-glucoside kg^{-1} dry weight) among the tested cultivars. Total anthocyanin contents in all rabbiteye cultivars were significantly higher than those in the highbush cultivars. The total anthocyanin content of the tested rabbiteye blueberries were comparable or higher than the result found in the rabbiteye blueberries from South Mississippi, the US (Stojanovic & Silva, 2007). Notably, anthocyanin content from the Climax cultivar was found higher than that of the same cultivar commercially available in the US (Prior et al., 1998). However, the total anthocyanin content found in all highbush cultivars was lower than those found in the nine different cultivars of highbush blueberries from different locations of the US (Conner, Luby, Tong, Finn, and Hancock, 2002b). There was no significant difference of total phenolic content between Sharpe, Climax and Powderblue. These three cultivars also showed higher antioxidant effects compared to those of Crunchie, Star, and Brightwell.

The cultivars which have the highest antioxidant effect are Climax and Sharpe with 155.7 ± 9.0 and 152.9 ± 2.7 μmol Trolox equivalent g^{-1} dry weight, respectively. The antioxidant activity is greatly influenced by the composition of the phenolic composition (Bors et al., 1990; Fukumoto & Mazza, 2000). Phenolic acids, catechins, flavonols, anthocyanins and proanthocyanidins are found in blueberries (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000; Naczk & Shahidi, 2006). Relating the total anthocyanin contents with the antioxidant effect gives an r^2 value of 0.6277. Meanwhile, the value of r^2 between total phenolic content and the antioxidant effect is 0.8786. These findings correlated well with the work done by Prior et al. (1998). They found that the correlation coefficient of *Vaccinium* species, which included highbush, lowbush, rabbiteye blueberries, and bilberries, was higher between ORAC and total phenolic content (0.85) compared to ORAC and total anthocyanin content (0.77).

The chromatograms of anthocyanins at 520 nm of the different cultivars are shown in Fig. 2. Anthocyanins found in the tested blueberries were galactosides, glucosides, and arabinosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin. However there were only 13 peaks of simple anthocyanins shown in the chromatograms due to two coelutions of peonidin 3-glucoside with malvidin 3-galactoside and peonidin 3-arabinoside with malvidin 3-glucoside. These coelutions was also found in bilberry and rabbiteye blueberry commercially sold in Japan. (Nakajima, Tanaka, Seo, Yamazaki, & Saito, 2004). The composition was also partly similar to other published works done (Cho, Howard, Prior, & Clark, 2004; Wu & Prior, 2005). Both studies also reported similar acylated anthocyanins such as delphinidin 3-acetylglucoside, cyanidin 3-acetylglucoside, petunidin 3-acetylglucoside, and malvidin 3-acetylglucoside. Some of these acylated anthocyanins appeared as very small peaks which were observed after 37 min. However, the m/z of molecular and fragments were different from cultivar to cultivar, hence there were not included in this study except one unidentified anthocyanin (peak 14) which was found in every cultivar.

4. Conclusion

By using the LC/MS method, it was found that all the cultivars tested contained similar types of anthocyanins but the proportion of each compound was cultivar-dependant. Rabbiteye cultivars of Climax, Powderblue, and Brightwell had significantly higher total anthocyanin content than highbush cultivars of Crunchie, Star and Sharpe. The environmental effects, such as the harvesting period, may play an important role in the existence of more antioxidant active and less stable anthocyanins such as delphinidin

glycosides. Further study could evaluate the effect of temperature during fruit development on the reduction of specific anthocyanins.

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References

Bagchi, D., Sen, C. K., Bagchi, M., & Atalay, M. (2004). Review: Antiangiogenic, antioxidant, and anticarcinogenic properties of a novel anthocyanin-rich berry extract formula. *Biokhimiya, 69*(1), 95–102.

Beccaro, G., Mellano, M. G., Botta, R., Chiabrando, V., & Bounous, G. (2006). Phenolic and anthocyanin content and antioxidant activity in fruits of bilberry (*Vaccinium myrtillus* L.) and of highbush blueberry (*V. corymbosum* L.) cultivars in North Western Italy. *Acta Horticulturae, 715*, 553–558.

Bors, W., Heller, W., Michel, C., & Saran, M. (1990). Flavonoids as antioxidants: Determination of radical-scavenging efficiencies. *Methods in Enzymology, 186*, 343–355.

Bors, W., Heller, W., Michel, C., Saran, M., Packer, Lester, & Alexander, N. G. (1990). *Flavonoids as antioxidants: Determination of radical-scavenging efficiencies*. *Methods in Enzymology* (Vol. 186). Academic Press.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Lebensmittel – Wissenschaft und Technologie, 28*(1), 25–30.

Cho, M. J., Howard, L. R., Prior, R. L., & Clark, J. R. (2004). Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. *Journal of the Science of Food and Agriculture, 84*(13), 1771–1782.

Collins, D., Carillo, L., & Abraham, L. (2004). *The Australian Horticulture Statistics Handbook*. Woolloomooloo: MODO Pty Ltd.

Conner, A. M., Luby, J. J., Hancock, J. F., Berkheimer, S., & Hanson, E. J. (2002a). Changes in fruit antioxidant activity among blueberry cultivars during cold-temperature storage. *Journal of Agricultural and Food Chemistry, 50*(4), 893–898.

Conner, A. M., Luby, J. J., Tong, C. B. S., Finn, C. E., & Hancock, J. F. (2002b). Genotypic and environmental variation in antioxidant activity, total phenolic content, and anthocyanin content among blueberry cultivars. *Journal of the American Society for Horticultural Science, 127*(1), 89–97.

de Pascual-Teresa, S., Santos-Buelga, C., & Rivas-Gonzalo, J. C. (2000). Quantitative analysis of Flavan-3-ols in Spanish foodstuffs and beverages. *Journal of Agricultural and Food Chemistry, 48*(11), 5331–5337.

Faria, A., Oliveira, J., Neves, P., Gameiro, P., Santos-Buelga, C., deFreitas, V., et al. (2005). Antioxidant properties of prepared blueberry (*Vaccinium myrtillus*) extracts. *Journal of Agricultural and Food Chemistry, 53*(17), 6896–6902.

Fukumoto, L. R., & Mazza, G. (2000). Assessing antioxidant and prooxidant activities of phenolic compounds. *Journal of Agricultural and Food Chemistry, 48*, 3597–3604.

Galli, R. L., Bielinski, D. F., Szprengiel, A., Shukitt-Hale, B., & Joseph, J. A. (2006). Blueberry supplemented diet reverses age-related decline in hippocampal HSP70 neuroprotection. *Neurobiology of Aging, 27*(2), 344–350.

Gao, L., & Mazza, G. (1994). Quantitation and distribution of simple and acylated anthocyanins and other phenolics in blueberries. *Journal of Food Science, 59*(5), 1057–1059.

Garcia-Viguera, C., Zafrilla, P., & Tomas-Barberan, F. A. (1997). Determination of authenticity of fruit jams by HPLC analysis of anthocyanins. *Journal of the Science of Food and Agriculture, 73*, 207–213.

Giusti, M. M., Rodriguez-Saona, E., & Wrolstad, R. E. (1999). Molar absorptivity and color characteristics of acylated and non-acylated pelargonidin-based anthocyanins. *Journal of Agricultural and Food Chemistry, 47*(11), 4631–4637.

Gross, J. (1987). Anthocyanins. In J. Gross (Ed.), *Pigment in fruits* (pp. 59–77). London: Academic Press.

Jackman, R. L., & Smith, J. L. (1996). Anthocyanins and betalains. In G. A. F. Hendry & J. D. Houghton (Eds.), *Natural Food Colorants* (pp. 244–280). New York: Blackie Academic & Professional.

Kalt, W., Forney, C. F., Martin, A., & Prior, R. L. (1999a). Antioxidant capacity, vitamin C, phenolics, and anthocyanins after fresh storage of small fruits. *Journal of Agricultural and Food Chemistry, 47*(11), 4638–4644.

Kalt, W., Lawand, C., Ryan, D. A. J., McDonald, J. E., Donner, H., & Forney, C. F. (2003). Oxygen radical absorbing capacity, anthocyanin and phenolic content of highbush bluberries (*Vaccinium corymbosum* L.) during ripening and storage. *Journal of the American Society for Horticultural Science, 128*(6), 917–923.

Kalt, W., McDonald, J. E., Ricker, R. D., & Lu, X. (1999b). Anthocyanin content and profile within and among blueberry species. *Canadian Journal of Plant Science, 79*(4), 617–623.

Naczk, M., & Shahidi, F. (2006). Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. *Journal of Pharmaceutical and Biomedical Analysis, 41*(5), 1523–1542.

Nakajima, J., Tanaka, I., Seo, S., Yamazaki, M., & Saito, K. (2004). LC/PDA/ESI-MS profiling and radical scavenging activity of anthocyanins in various berries. *Journal of Biomedicine and Biotechnology, 5*, 241–247.

Prior, R. L., Cao, G., Martin, A., Sofic, E., McEwen, J., O'Brien, C., et al. (1998). Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of Vaccinium species. *Journal of Agricultural and Food Chemistry, 46*(7), 2686–2693.

Slinkard, K., & Singleton, V. L. (1977). Total phenol analysis: Automation and comparison with manual methods. *American Journal of Enology and Viticulture, 28*(1), 49–55.

Stojanovic, J., & Silva, J. L. (2007). Influence of osmotic concentration, continuous high frequency ultrasound and dehydration on antioxidants, colour and chemical properties of rabbiteye blueberries. *Food Chemistry, 101*(3), 898–906.

Tsang, C., Higgins, S., Duthie, G. G., Howie, M., Mullen, W., Lean, M. E. J., et al. (2005). The influence of moderate red wine consumption on antioxidant status and indices of oxidative stress associated with CHD in healthy volunteers. *British Journal of Nutrition, 93*, 233–240.

Wang, H., Cao, G., & Prior, R. L. (1997). Oxygen radical absorbing capacity of anthocyanins. *Journal of Agricultural and Food Chemistry, 45*(2), 304–309.

Wu, X., & Prior, R. L. (2005). Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: Fruits and berries. *Journal of Agricultural and Food Chemistry, 53*(7), 2589–2599.

Zheng, W., & Wang, S. Y. (2003). Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. *Journal of Agricultural and Food Chemistry, 51*(2), 502–509.