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# Anthocyanins in Wild Blueberries of Quebec: Extraction and Identification

EUGÉNE ÉMILE NICOUÉ,† SYLVAIN SAVARD,‡ AND KHALED BELKACEMI*,†

Department of Food Sciences and Nutrition and Department of Soil Science and Agri-Food Engineering, Université Laval, FSAA, Sainte-Foy, Québec G1K 7P4, Canada, and Centre de Recherche Industrielle du Québec (CRIQ), 333, rue Franquet, Québec, Québec G1P 4C7, Canada

Anthocyanins were extracted from a mixture of berries of *Vaccinium angustifolium* and *Vaccinium myrtilloïdes* at 7.7 °C, 26 °C, and 79 °C using ethanol alone or ethanol acidified with hydrochloric, citric, tartaric, lactic, or phosphoric acids at a solvent to solid ratio of 10. The effect of these parameters on extracted anthocyanins stability was investigated. The pH-differential and HPLC-DAD methods were used to determine anthocyanin contents. Extracted anthocyanins were purified on a C-18 solid-phase extraction cartridge and characterized by HPLC/electrospray ionization/mass spectrometry (HPLC-ESI-MS/MS). Anthocyanins were identified according to their HPLC retention times, elution order, and MS fragmentation pattern and by comparison with standards and published data. Anthocyanin extractions gave different yields depending on the type of added acid and the extraction temperature. High yields of monomeric and total anthocyanins (26.3 and 28.9 mg/g of dry matter) were obtained at 79 °C using phosphoric acid. Extraction using tartaric acid at 79 °C provided the lowest degradation index (1.05). Anthocyanins were stable and browning by polyphenol oxidase was inhibited under these conditions. Of the six common anthocyanindins, five were identified in the extracts, namely, delpinidin, cyanidin, peonidin, petunidin, and malvidin; pelargonidin was not found. In addition to well-known major anthocyanins, new anthocyanins were identified for the first time in extracts of wild blueberries from Quebec.

**KEYWORDS:** ***Vaccinium angustifolium*; *Vaccinium myrtilloïdes*; anthocyanins; extraction; solid-phase extraction; pH differential; HPLC-DAD; degradation index; HPLC-ESI-MS/MS**

## INTRODUCTION

Fruits and vegetables contain a wide variety of phytonutrients, many of which have antioxidant properties and are thus believed to have certain beneficial effects on the health of animals and humans, based on scavenging of free radicals generated by metabolism and environmental factors. Studies have shown that in addition to the well-known vitamins C, E, and A ($\beta$-carotene), fruits and vegetables contain other antioxidants that contribute significantly to their total antioxidant capacity (*1*). Several studies have shown that fruits and vegetables contain phenolic compounds with significant dietary antioxidant activity and that many of these, particularly flavonoids, have antioxidant capacity (*2*) and stability (*3*) exceeding that of ascorbic acid. Fruits of the genus *Vaccinium* are especially rich in flavonoids (anthocyanins, flavonols, and proanthocyanidins) and other phenolic compounds (*2*). Members of this genus have high anthocyanin pigment content, which is responsible for the deep violet coloration of blueberries, for example (*4*). Anthocyanins are found in nature in various plants as well as fruits and vegetables, providing a broad range of colors varying from red to violet. Besides being pigments, anthocyanins appear to fulfill other biological functions, of which antioxidant capacity may be one of the most significant (*4*). Kalt and co-workers (*5*) showed that the antioxidant capacity of highbush blueberries (*Vaccinium corymbosum* L.) and lowbush blueberries (*Vaccinium angustifolium* Aiton) is strongly correlated with the total anthocyanin content and that the antioxidant capacity of berries of these two species is about three times higher than for strawberries and raspberries. Compared to anthocyanins, ascorbic acid makes only a small contribution (0.4−9.4%) to the total antioxidant capacity of blueberries (*5*).

Anthocyanins are heterosides whose aglycone or anthocyanidin is derived from the flavylium or 2-phenylbenzopyrilium cation. Among the 21 anthocyanidins described in the literature, six are widespread in fruits and vegetables: pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin. These are reported to be the major bilberry anthocyanidins linked to glucose, arabinose, or galactose, forming a total of 15 anthocyanins (*6*). Lowbush blueberries are reported to contain these anthocyanins (*7*), with the exception of pelargonidin (*8*).

* To whom correspondence should be addressed. Telephone: (418) 656-2131 ext. 6511. Fax: (418) 656-3723. E-mail: khaled.belkacemi@sga.ulaval.ca.
† Université Laval.
‡ Centre de Recherche Industrielle du Québec.

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The polar character of anthocyanins makes them soluble in several types of polar solvents such as methanol, ethanol, acetone, and water. Solvent extraction of anthocyanins is the initial step in the determination of total and individual anthocyanins prior to quantification, purification, separation, and characterization (*9*) and generally involves the use of acidified methanol or ethanol. Even though ethanol is less efficient and more difficult to eliminate later, it would be preferred for food use, because methanol is toxic. The use of acid stabilizes anthocyanins in the flavylium cation form, which is red at low pH (*8*). However, solvent acidified with hydrochloric acid may hydrolyze acylated anthocyanins, which explains why it has been overlooked in the past that many anthocyanins are acylated with aliphatic acids (*10*). To avoid or at least minimize the breakdown of acylated anthocyanins, organic acids such as acetic, citric, or tartaric acids, which are easier to eliminate during anthocyanin concentration, have been preferred (*10*).

The biological properties of lowbush blueberries are believed to have a strong correlation with their anthocyanin content (*4*). The anthocyanin compositions of highbush blueberry (*V. corymbosum* L), lowbush blueberry (*V. angustifolium* Ait.), and bilberry (*Vaccinium myrtillus*) are qualitatively very similar (*7*).

To our knowledge, there are few reports on wild blueberries in Québec, and their anthocyanin composition has never been described. The aim of this study was to determine optimal conditions, particularly with respect to temperature and type of acid, for ethanol extraction of anthocyanins from a mixture of wild blueberries (*V. angustifolium* and *Vaccinium myrtilloides*) commonly gathered in Quebec.

Yield and monomer stability were measured, and anthocyanin composition was analyzed in detail using HPLC-electrospray ionization (ESI) coupled with ion trap mass spectrometry.

## MATERIALS AND METHODS

**Plant Material.** A mixture of wild blueberries harvested in the Lac-Saint-Jean area and immediatetly frozen for 24 h was provided frozen by Les Bleuets Sauvages du Québec Inc. (Saint-Bruno, Québec, Canada) and stored at −20 °C until use. The fruits were 80% from *V. angustifolium* and 20% from *V. myrtilloides*.

**Chemicals.** All chemicals were reagent grade unless otherwise stated. Hydrochloric, citric, tartaric, formic, and lactic acids as well as sodium acetate, potassium chloride, methanol, and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Ottawa, Ontario, Canada). Phosphoric acid was purchased from Laboratoire Mat (Beauport, Québec, Canada). Ethanol (95% v/v) was purchased from Les Alcools de Commerce, Inc. (Boucherville, Québec, Canada). Cyanidin 3,5-diglucoside (cyanin) and malvidin 3,5-diglucoside (malvin) standards were purchased from Fluka, Sigma-Aldrich (Oakville, Ontario, Canada). Cyanidin 3-glucoside (kuromanin) was purchased from Extrasynthese (Genay, France). Deionized water was used to prepare all solutions.

**Anthocyanin Extraction.** Anthocyanins were extracted from frozen berries using ethanol at 7.7 °C, 26 °C, or 79 °C without acid (pH = 5.4) or acidified with hydrochloric (pH = 4.1), citric (pH = 4.9), tartaric (pH = 5.0), lactic (pH = 4.8), or phosphoric acid (pH = 4.6; 0.02% v/v) in 3 × 6 factorial experiments. The 18 extractions were done in random order and repeated twice. Samples of 150 g were first ground in a Moulinex HV6 food processor and then homogenized in 1.5 L of solvent at 7.7 °C, 26 °C, or 79 °C for 2 h using a Premier Mill 1.5 HP laboratory homogenizer (Nortec S.G.S., Inc., Montreal, Québec, Canada) at 3000 rpm. Homogenates were filtered on Whatman no. 415 paper, and the residue was washed with additional solvent. The filtrates were stored at 4 °C until analysis.

To determine the total anthocyanin content potentially present in the fruits, frozen blueberries were also extracted at room temperature for 2 h with methanol acidified with hydrochloric acid (0.02% v/v) and filtered as described above. All filter residues were oven-dried at 40 °C for 24 h and then extracted for 2 h at room temperature with HCl-acidified methanol at a solvent/solids ratio of 20. The extracts were filtered through Whatman no. 4 paper and stored at 4 °C until analysis.

**Determination of Monomeric and Total Anthocyanins and Degradation Index.** The spectrophotometric pH differential method (*11*) was used to determine monomeric and total (monomeric plus polymerized) anthocyanins in the ethanol extracts. Two dilutions of the same sample were prepared in 0.025 M potassium chloride solution and in 0.4 M sodium acetate solution adjusted respectively to pH 1.0 and 4.5 with HCl. The absorbance of each dilution was measured at 520 and 700 nm against a distilled water blank using a Lambda-EZ210 UV−visible spectrophotometer (Perkin-Elmer, Markham, Ontario, Canada). Absorbance (*A*) was calculated as follows:

$$A = (A_{\lambda_{max}} - A_{700})_{pH=1.0} - (A_{\lambda_{max}} - A_{700})_{pH=4.5} \quad (1)$$

where $\lambda_{max} = 520$ nm.

The monomeric anthocyanin concentration in the original sample was obtained from eq 2:

$$[\text{monomeric anthocyanins}]\ (\text{mg/L}) = \frac{A \times MW \times DF \times 1000}{\epsilon \times 1} \quad (2)$$

The total anthocyanin concentration was obtained from eq 3:

$$[\text{total anthocyanins}]\ (\text{mg/L}) = \frac{A' \times MW \times DF \times 1000}{\epsilon \times 1} \quad (3)$$

where

$$A' = (A_{\lambda_{max}} - A_{700})_{pH=1.0} \quad (4)$$

The degradation index or DI (*10*) was obtained from eq 4:

$$DI = \frac{[\text{total anthocyanins}]_{\text{single pH method}}}{[\text{monomeric anthocyanins}]_{\text{pH differential method}}} \quad (5)$$

**Separation of the Anthocyanin Fraction.** Anthocyanins were separated from non-pigments by solid-phase extraction as described by Durst and Wrolstad (*12*). The extracts were passed through a C-18 DSC-18Lt 60 mL, 10 g cartridge (Supelco, Bellefonte, PA) activated with acidified methanol followed by 0.01% HCl (v/v) in deionized water. Anthocyanins were adsorbed onto the column while sugars, acids, and other water-soluble compounds were removed by flushing with 0.01% HCl. Anthocyanins were recovered with methanol containing 0.01% HCl (v/v). The acidified methanol fractions were evaporated using a rotary evaporator at 40 °C and the solids were stored at 4 °C until analysis. Cartridges were washed with ethyl acetate to remove phenolic compounds other than anthocyanins.

**Anthocyanin Quantification.** Total anthocyanins were quantified by HPLC as described by Durst and Wrolstd (*13*) using an HP1090 liquid chromatography system (Agilent Technologies, Inc., Mississauga, Ontario, Canada) equipped with a 250 × 4.6 mm i.d., 4 $\mu$m LC-18 reverse-phase column (Supelco, Bellefonte, PA) and a photodiode array detector (DAD). Anthocyanins extracts were dissolved in 4% phosphoric acid and filtered through 0.45 $\mu$m Teflon filters. Samples of 25 $\mu$L were injected by an autosampler at ambient temperature. Absorbance was recorded at 520 nm for all peaks. An elution gradient with acetonitrile as solvent A and 4% phosphoric acid (v/v) in water as solvent B was used with the following profile: 0 min, 94% B; 55 min, 75% B; 65 min, 75% B; and 70 min, 94% B. The total solvent flow rate was 1 mL/min. A calibration curve obtained for a cyanidin 3-glucoside standard was used to calculate the anthocyanin concentration represented by each peak. The total concentration was obtained by summing these concentrations.

**Anthocyanin Identification.** Individual anthocyanin compounds were identified by HPLC ESI tandem mass spectrometry using an HP 1100 series HPLC (Hewlett-Packard, Saint-Laurent, Québec, Canada) with a UV detector coupled to an ion trap mass spectrometer (Agilent, Saint-Laurent, Québec, Canada) equipped with an Agilent 1100 Series LS/MSD ESI interface. Chromatographic separation of anthocyanins was performed using the same column as for quantification and eluting

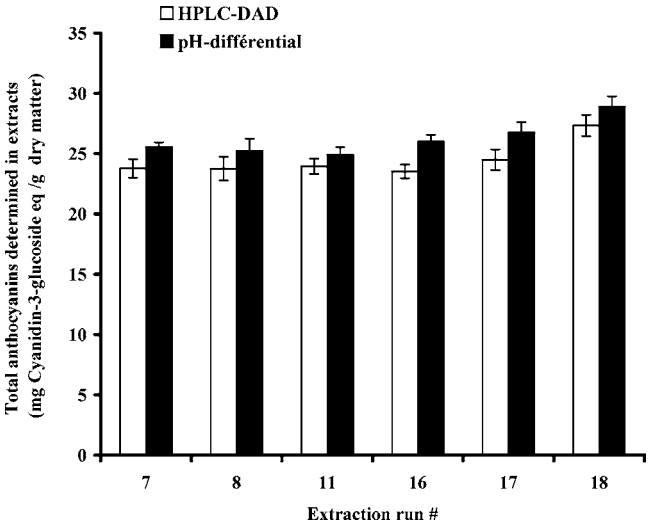


**Figure 1.** Comparison of total anthocyanins determined by the pH differential method and by the HPLC-DAD method for extraction extractions run nos. 7, 8, 11, 16, 17, and 18.

with 5% formic acid and methanol in a gradient described by Wu and Prior (*13*). The solvent flow rate was 1 mL/min, sample injection volume was 100 $\mu$L, and detection was based on absorbance at 520 nm. Extracts were filtered through a 0.45 $\mu$m Teflon filter before analysis. ESI interface conditions: nebulizer, 50 psi; dry gas ($N_2$), 12 L/min; dry temperature, 350 °C; average, −5; trap drive level, 55; skim 1, 59.0 V; skim 2, 6.0V; capillary exit, 146.0V; cap exit offset, 87.0 V. The ion trap mass spectrometer was operated in positive ion mode scanning from *m*/*z* 100 to *m*/*z* 800. Mass spectra data were collected by LC-MSTRAP control Software Version 5.2 from Brüker (Daltonik GmbH).

**Statistical analysis.** The analysis of the variance (ANOVA) procedure of Statistical Analysis System (version 9.1.3, Service pack 2, SAS Institute, Inc., 2004) was used to analyze the treatment effects for significant differences and to distinguish the type of effect (linear or quadratic) at the $\alpha = 0.05$ level.

## RESULTS AND DISCUSSION

**Extraction Experiments.** The effects of acid type and of temperature on the efficiency of anthocyanin extraction from blueberries with ethanol were investigated using carboxylic (citric, tartaric, and lactic) acids, two mineral (hydrochloric and phosphoric) acids, and three temperatures (7.7 °C, 26 °C, and 79 °C). In this study, "degradation" refers to the loss of monomeric compounds to the pool of polymerized forms, which, although the are of organoleptic value, are considered of lesser biological value because of their lower absorbability by the digestive tract. The impact of deacylation is not considered in the DI. Total anthocyanins and monomeric anthocyanins recovered for the various extraction conditions are shown in **Figure 1**.

It has been reported that high extraction temperature, while not destroying the desired phytochemicals and nutraceutical ingredients, increases compound solubility and extraction into the solvent (*14*). It has also been previously shown that anthocyanins are more stable in acidic conditions (*15*), and Kalt et al. (*16*) demonstrated that blueberry juice contained more anthocyanins at lower pH. We therefore decided to use acidic conditions to maximize pigment recovery.

The recovery of total anthocyanins from the berries ranged from 21.1 to 28.9 mg/g dry matter (**Table 1** and **Figure 1**) while recovery of total monomeric anthocyanins ranged from 17.8 to 26.3 mg/g dry matter (**Figure 1**). Extraction run no. 18 (phosphoric acid, 79 °C) gave the highest yield of total and monomeric anthocyanins (26.7 and 28.2 mg/g dry matter). Extractions runs no. 7 (citric acid, 26 °C), no. 8 (citric acid, 7.7 °C), no. 11 (lactic acid, 7.7 °C), no. 16 (phosphoric acid, 26 °C), and no. 17 (phosphoric acid, 7.7 °C) performed nearly as well as extraction run no. 18, while extraction run no. 9 (citric acid, 79 °C) extracted the least anthocyanin. The DI varied little with extraction conditions, ranging from 1.05 to 1.17 (values not shown). The lowest DI was obtained when the extraction was carried out with tartaric acid at 79 °C.

The greatest amounts of total monomeric and total anthocyanins recovered were respectively 26.3 and 28.9 mg/g dry matter, determined by the pH differential method as cyanidin-3-glucoside equivalent (**Figure 1**). These amounts are higher than those reported for highbush bluecorp (*17*), lowbush Blomindon, lowbush Cumberland, lowbush clonal mix, lowbush Fundy (*4*), and Rubbel berries (*2*) as well as highbush blueberry (*18*). The amount extracted under extraction run no. 18 conditions falls within the range of 21.4−49.8 mg/g dry matter reported by Gao and Mazza (*19*) for *V. myrtillus* L. (European blueberry).

The analysis of variance (**Table 3**) indicates that blueberry anthocyanin yield was markedly affected by both the extraction temperature and type of acid. Monomeric and total anthocyanins extracted cannot be explained in terms of separate effects of temperature and type of acid. There is thus a highly significant interaction between these two parameters. This interaction was more linear than quadratic for both monomeric and total anthocyanins. The effect of temperature changed according to the type of acid for anthocyanins extraction. Acid increased anthocyanin yield under the conditions of extractions run nos. 4, 7, 8, 10, 11, 13, 14, 15, 16, 17, and 18 but not in the conditions of extractions run nos. 5, 6, 9, and 12. High yields of anthocyanin were obtained under the conditions of extraction run nos. 16, 17, and 18 (phosphoric acid at 26 °C, 7.7 °C, and 79 °C), extraction run nos. 7 and 8 (citric acid at 26 °C and 7.7 °C) and extraction run no. 11 (7.7 °C, tartaric acid).

The most anthocyanin was extracted at 79 °C using phosphoric acid. This temperature likely affected both equilibrium and mass transfer. A higher temperature should generally favor higher solubility of phytochemicals in the solvent and thus a larger equilibrium constant. According to Fick's Second Law, temperature plays an important role in the rate of extraction, because diffusion of the solute through the particle to the particle surface is the rate-limiting step (*14*). The diffusion coefficient and hence the rate of extraction increase with temperature, which is limited by the solvent boiling point, 79 °C for ethanol.

The detrimental effect of high temperature on anthocyanins is well-known. A half-life of 1 h for strawberry pigment at 100 °C has been reported (*20*). However, because anthocyanins are found in vacuoles and often in berry skin tissue, a high temperature could help extraction by breaking down these structures (*21*). Increased anthocyanin recovery has been reported to result from increased permeability of membranes in macerated blueberry tissue, which would facilitate pigment release during extraction (*22*).

Frozen blueberries were ground to small particles before extraction. Extraction runs no. 15 and no. 18, both at 79 °C, yielded more anthocyanins compared to the control (no acid addition). These results are in agreement with those of Kalt et al. (*16*). The contribution of phosphoric acid to increased anthocyanin extraction may be due partly to its polyacidity. Adding acid keeps the compounds protonated, and in this state they are more soluble in the solvent.

**Table 1.** Anthocyanins[a] Recovered by Ethanol Extraction of Blueberries under Various Experimental Conditions

| extraction run number | temperature (°C) | acid used | ethanol filtrate (g) | filter residue[b] (g) | total[c] (g) | yield[d] (%) | loss[d] (%) |
|---|---|---|---|---|---|---|---|
| 1 | 26 | none | 22.52 | 0.37 | 22.89 | 81.05 | 18.94 |
| 2 | 7.7 | none | 22.67 | 0.79 | 23.46 | 83.07 | 16.93 |
| 3 | 79 | none | 21.05 | 1.61 | 22.66 | 80.24 | 19.76 |
| 4 | 26 | hydrochloric | 23.98 | 0.30 | 24.28 | 85.97 | 14.03 |
| 5 | 7.7 | hydrochloric | 22.29 | 0.79 | 23.08 | 81.72 | 18.28 |
| 6 | 79 | hydrochloric | 21.22 | 1.04 | 22.26 | 78.82 | 21.18 |
| 7 | 26 | citric | 25.58 | 0.28 | 25.86 | 91.57 | 8.43 |
| 8 | 7.7 | citric | 25.74 | 0.74 | 25.99 | 92.03 | 7.97 |
| 9 | 79 | citric | 19.34 | 1.73 | 21.07 | 74.61 | 25.38 |
| 10 | 26 | tartaric | 24.57 | 0.44 | 25.01 | 88.56 | 11.44 |
| 11 | 7.7 | tartaric | 27.09 | 0.86 | 27.95 | 98.97 | 1.03 |
| 12 | 79 | tartaric | 22.41 | 0.93 | 23.34 | 82.64 | 17.36 |
| 13 | 26 | lactic | 24.46 | 0.24 | 24.70 | 87.46 | 12.54 |
| 14 | 7.7 | lactic | 23.33 | 0.97 | 24.30 | 86.04 | 13.96 |
| 15 | 79 | lactic | 24.19 | 1.24 | 25.43 | 90.04 | 9.96 |
| 16 | 26 | phosphoric | 26.00 | 0.26 | 26.26 | 92.98 | 7.02 |
| 17 | 7.7 | phosphoric | 26.76 | 1.03 | 27.79 | 98.40 | 1.60 |
| 18 | 79 | phosphoric | 28.88 | 0.02 | 28.90 | 100.00 | 0.00 |

[a] Includes degraded (polymerized) and nondegraded anthocyanins expressed as cyanidin 3-glucoside equivalents in mg/g whole berry dry matter. [b] Methanol extraction. [c] Sum of filtrate and residue anthocyanin contents. [d] On the basis of the amount extractable using methanol–HCl, 28.2 mg/g of whole berry dry matter.

**Table 2.** Dry Matter Recovery from Ethanol Extractions of Blueberries under Various Experimental Conditions

| extraction | temperature (°C) | acid | ethanol filtrate (g) | filter residue (g) | total[a] (g) | yield[b] (%) | loss[b] (%) |
|---|---|---|---|---|---|---|---|
| 1 | 26 | none | 14.18 | 5.76 | 19.94 | 94.95 | 5.05 |
| 2 | 7.7 | none | 13.01 | 7.88 | 20.89 | 99.47 | 0.53 |
| 3 | 79 | none | 12.82 | 6.79 | 19.61 | 93.38 | 6.62 |
| 4 | 26 | hydrochloric | 13.04 | 7.68 | 20.72 | 98.66 | 1.34 |
| 5 | 7.7 | hydrochloric | 13.33 | 7.23 | 20.56 | 97.90 | 2.10 |
| 6 | 79 | hydrochloric | 13.32 | 7.33 | 20.65 | 98.33 | 1.67 |
| 7 | 26 | citric | 12.47 | 7.07 | 19.54 | 93.04 | 6.96 |
| 8 | 7.7 | citric | 12.87 | 7.96 | 20.83 | 99.19 | 0.81 |
| 9 | 79 | citric | 14.26 | 6.33 | 20.59 | 98.04 | 1.96 |
| 10 | 26 | tartaric | 13.23 | 6.99 | 20.22 | 96.28 | 3.72 |
| 11 | 7.7 | tartaric | 13.59 | 7.33 | 20.92 | 99.61 | 0.39 |
| 12 | 79 | tartaric | 13.33 | 7.05 | 20.37 | 97.04 | 2.96 |
| 13 | 26 | lactic | 12.76 | 8.04 | 20.80 | 99.04 | 0.96 |
| 14 | 7.7 | lactic | 14.82 | 5.86 | 20.69 | 98.47 | 1.53 |
| 15 | 79 | lactic | 13.59 | 6.73 | 20.32 | 96.76 | 3.24 |
| 16 | 26 | phosphoric | 13.24 | 7.55 | 20.79 | 99.00 | 1.00 |
| 17 | 7.7 | phosphoric | 13.04 | 7.21 | 20.25 | 96.42 | 3.58 |
| 18 | 79 | phosphoric | 12.91 | 6.86 | 19.77 | 94.14 | 5.86 |

[a] Sum of filtrate dry matter and residue dry matter. [b] On the basis of the dry matter content of 150 g of whole berries, 21 g.

The low DI may be explained by inhibition of native enzymes involved in anthocyanin degradation. Blueberry pigments have been found to degrade quickly in the presence of chlorogenic acid and polyphenol oxidase (PPO) (*23*). Chlorogenic acid, a phenolic acid, contributes about 10% to the total acid concentration of lowbush blueberry (*24*). According to Pifferi and Cultrera (*25*) the oxidation of chlorogenic acid by PPO produces quinones that subsequently polymerize into brown pigments and which are responsible for anthocyanin degradation. Quinones can in fact oxidize anthocyanins to form brown polymers (*26*). During extraction, some enzymatic oxidation may occur because the enzyme comes into contact with its substrate. Of course, the enzyme may be inhibited by the low pH or by the high temperature. The enzymes responsible for anthocyanin degradation have temperature optima ranging between 20 and 40 °C, which could explain the degradation indices of 1.15 to 1.17 for extractions run nos. 1, 4, 7, and 16, all at 26 °C, and the indices of 1.11−1.14 for the extractions carried out at 7.7 °C. The lowest DI (1.05) was obtained with extraction run no. 12 (tartaric acid, 79 °C), which might be explained by the decreased solubility of oxygen at the higher temperature (*22*) because oxygen has been reported to play an important role in destabilizing pigments in processed products (*27*) and is required for browning by PPO. At elevated temperature, these enzymes are susceptible to denaturing and thus loss of activity.

**Extraction of Anthocyanin from Whole Blueberry Filter Residue.** The amount of total and monomeric anthocyanins extracted from the filter residue ranged from 0.0 to 1.7 mg/g dry matter, which is substantially lower than from whole blueberry. The pigment in the filter residue represents 0.0 to 8.2% of the total anthocyanins in the starting blueberry fruits. Filter residue thus does not appear to be a significant source of anthocyanins. The DI for extract of filter residue ranged from 1.2 to 6.7 (data not shown), which was substantially higher than for whole blueberry. Its higher DI may be explained by the fact that extractions were carried out at room temperature (27 to 30 °C), which lies within the optimal temperature range for PPO activity. Furthermore, the residue was dried in an oven at 40 °C, which put it in contact with oxygen. Polyphenols would oxidize quickly under these conditions and lead to significant anthocyanin degradation.

**Table 3.** Anthocyanins Identified[a] in a Mixture of *V. Angustifolium* and *V. Myrtilloides* Berries

| peak | peak area (%) | retention time (min) | compound | $[M]^+$ (*m/z*) | MS/MS (*m/z*) | anthocyanin[d] |
|---|---|---|---|---|---|---|
| 1 | 6.5 | 16.8 | 1 | 465 | 303 | Dp 3-galactoside |
| 2 | 8.2 | 20.6 | 2 | 465 | 303 | Dp 3-glucoside |
| **3** | **7.6** | **22.0** | **3**[b] | **465** | **303** | ***Dp 3-hexose*** |
| **4** | **8.8** | **24.3** | **4**[b] | **465** | **303** | ***Dp 3- hexose*** |
| 5 | 4.8 | 26.6 | 5[c] | 449 | 287 | Cy 3-galactoside |
| 26.6 | 6[c] | 479 | 317 | Pt 3-galactoside | | |
| 26.6 | 7[c] | 465 | 303 | ***Dp 3-hexose*** | | |
| 26.6 | 8[c] | 493 | 331 | Mv 3-galactoside | | |
| 6 | 5.6 | 28.1 | 9[c] | 435 | 303 | Dp 3-arabinoside |
| 28.1 | 10[c] | 479 | 317 | Pt 3- glucoside | | |
| 7 | 4.8 | 29.6 | 11[c] | 449 | 287 | Cy 3-glucoside |
| 29.6 | 12[c] | 479 | 317 | Pt 3-glucoside | | |
| 8 | 5.5 | 31.8 | 13[b] | 479 | 317 | ***Pt hexose*** |
| 9 | 2.9 | 32.9 | 14[c] | 419 | 287 | Cy 3-arabinoside |
| 32.9 | 15[c] | 493 | 331 | Mv 3-glucoside | | |
| 10 | 6.7 | 34.9 | 16[b] | 479 | 317 | ***Pt hexose*** |
| 11 | 2.6 | 36.7 | 17[c] | 463 | 301 | Pn 3-galactoside |
| 36.7 | 18[b] | 493 | 331 | ***Mv 3-hexose*** | | |
| 12 | 5.1 | 39.8 | *c* | 493 | 331 | ***Mv 3-hexose*** |
| 13 | 3.1 | 41.4 | 19[b] | 463 | 301 | Pn 3-glucoside |
| 41.4 | 20[c] | 493 | 331 | ***Mv hexose*** | | |
| 14 | 5.4 | 42.6 | 21[b] | 493 | 331 | ***Mv hexose*** |
| 15 | 8.4 | 45.8 | *c* | 493 | 331 | ***Mv hexose*** |
| 16 | 2.4 | 50.0 | **22**[b] | 463 | 331 | Mv 3-arabinose |
| **17** | **1.0** | **52.2** | **23**[b] | **535** | **465/303** | **Dp -oxalyl-hexose** |
| 24 | **435/303** | **Dp -succinyl-pentose** | | | | |
| **52.2** | **25** | **535** | **449/287** | **Cy -malonyl-hexose** | | |
| **419/287** | **Cy -malyl-pentose** | | | | | |
| **52.2** | **26** | **535** | **479/317** | **Pt -propionyl-hexose** | | |
| **449/317** | **Pt -malonyl-pentose** | | | | | |
| **52.2** | **27** | **535** | 493/331 | Mv acetyl-hexose | | |
| **463/331** | **Mv oxalyl-pentose** | | | | | |
| **18** | **0.8** | **53.5** | **28** | **535** | **465/303** | **Dp oxalyl-hexose** |
| **435/303** | **Dp succinyl-pentose** | | | | | |
| **53.5** | **29** | **535** | **449/287** | **Cy malonyl-hexose** | | |
| **419/287** | **Cy malyl-pentose** | | | | | |
| **53.5** | **30** | **535** | **479/317** | **Pt propionyl-hexose** | | |
| **449/317** | **Pt -malonyl-pentose** | | | | | |
| **53.5** | **31** | **535** | 493/331 | Mv acetyl-hexose | | |
| **463/331** | **Mv oxalyl-pentose** | | | | | |
| 19 | 0.7 | 55.9 | 33[a] | 515 | 303 | unknown |
| **20** | **1.2** | **57.0** | **34**[a] | **507** | **449/287** | **Cy 3-propionyl-galactoside** |
| 21 | 1.4 | 64.3 | 35[c] | 535 | 449/287 | Cy 3-malonyl-galactoside |
| **64.3** | **36**[a,c] | **535** | **479/317** | **Pt 3-propionyl-galactoside** | | |
| 64.3 | 37[c] | 535 | 493/331 | Mv 3-acetyl-galactoside | | |
| 22 | 0.5 | **65.3** | **38**[a,c] | **535** | **479/317** | **Pt 3-propionyl-glucoside** |
| 65.3 | 39[c] | 535 | 493/331 | Mv 3-acetyl-glucoside | | |
| **65.3** | **40**[a,c] | **535** | **463/331** | **Mv 3-oxalyl-arabinoside** | | |
| **23** | **0.8** | **67.2** | **41**[b,c] | **535** | **479/317** | ***Pt 3-propionyl-hexose*** |
| **67.2** | **42**[b,c] | **535** | **493/331** | ***Mv 3-acetyl-hexose*** | | |
| 24 | 1.0 | 70.2 | 43 | 521 | 479/317 | Pt 3-acetyl-glucoside |
| 25 | 1.5 | **73.2** | **44**[a,c] | **535** | **433/301** | **Pn 3-succinyl-arabinoside** |
| **73.2** | **45**[a,c] | **535** | **463/331** | **Mv 3-oxalyl-xyloside** | | |
| **26** | **0.8** | **73.8** | **46**[a,c] | **535** | **463/301** | **Pn 3-oxalyl-galactoside** |
| **73.8** | **47**[b,c] | **535** | **493/331** | ***Mv 3-acetyl-hexose*** | | |
| **27** | **1.6** | **74.6** | **48** | **535** | **493/331** | ***Mv 3-acetyl-hexose*** |
| **463/331** | ***Mv 3-oxalyl-xylose*** | | | | | |
| **28** | **0.7** | **75.2** | **49** | **535** | **493/331** | ***Mv 3-acetyl-hexose*** |
| **463/331** | ***Mv 3-oxalyl-pentose*** | | | | | |

[a] By HPLC-EI-MS/MS. [b] Compounds identified for the first time are in bold type. Those in italic type are linked to unusual hexoses or pentoses identified for the first time. [c] Compounds coeluting at the same retention time. [d] Note: Cy, cyanidine; Dp, delphinidin; Mv, malvidin; Pn, peonidin; Pt, petunidin.

**Anthocyanin Content and Dry Matter Mass Balances.** Anthocyanin yield and loss under the various extraction conditions are summarized in **Table 1**. Frozen whole blueberries contained 28.2 mg of anthocyanin per gram of fruit dry weight, as determined by methanol/HCl extraction, in comparison to the content obtained when methanol/phosphoric acid was used as the extraction solvent (28.1 mg of anthocyanin/g dry matter). Ethanol extraction yields ranged from 74.2 to 100%, and losses ranged from 0 to 25.7%.

Dry matter recovery ranged from 93.0 to 99.8% (**Table 2**). There are few reports in the literature about blueberry anthocyanin content and dry matter balance. Extraction run no. 9 produced the highest loss of anthocyanins (25.7%).

The higher anthocyanin extraction yield from blueberries strongly supports the hypothesis that anthocyanins can be extracted efficiently using polar solvents and mechanical disruption of intact berries. The dry matter yields of the

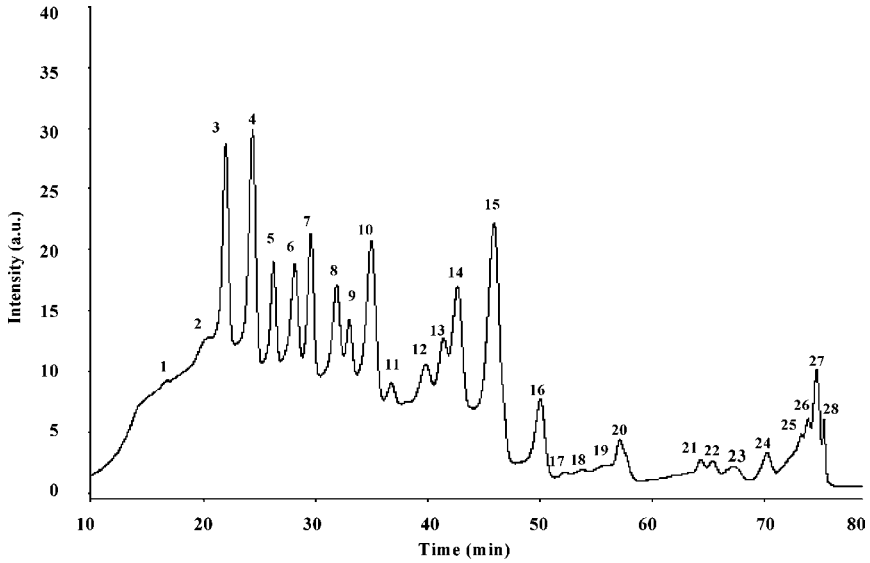


**Figure 2.** Reversed-phase HPLC chromatogram of the anthocyanin fraction purified by solid-phase extraction of blueberries showing relative absorbance of 28 peaks at 520 nm.

extractions were generally higher than the anthocyanin yields, which is not surprising because dry matter cannot be lost by oxidation.

**Comparison of Total Anthocyanins Determined by pH Differential and by HPLC-DAD.** Total anthocyanins were determined by HPLC-DAD for extractions run nos. 7, 8, 11, 16, 17, and 18, which yielded the highest amounts of anthocyanins as measured by the pH differential method. The two methods of anthocyanin quantification are compared in **Figure 2**. Total anthocyanins determined by HPLC-DAD ranged from 23.7 to 27.3 mg/g dry matter compared to 25.5 to 28.8 mg/g dry matter by the pH differential method. However, both analytical methods produced the same general trend. According to Lee et al. (*28*), the differences could be due to different effects of the two solvent systems on absorbance at 520 nm by anthocyanins. Moreover, the determination by HPLC-DAD is based on the summation of individual peaks of absorbance, while the pH differential method measures the difference between absorbance by the whole sample at pH 1.0 and that at pH 4.5. It is also possible that polymerized anthocyanins in the filtrate were retained in the HPLC-DAD column and never reached the diode array detector (*28*). Both methods showed that extraction run no. 18 produced the greatest extraction of anthocyanins. The amounts of anthocyanin extracted in this study are higher than in other published works. This could be due to the nature of compound used as a standard and its molar absorption coefficient. The contents determined by the two methods of analysis are in agreement with previous reports, which also mention large variations of anthocyanins in blueberries (*19*).

**Anthocyanin Peak Identification.** The anthocyanin chromatographic profile at 520 nm obtained from reversed-phase HPLC is shown in **Figure 3**. Although 28 peaks are visible, mass spectrometry analysis revealed that peaks 6, 7, 9, 11, 13, 21, 23, and 24 corresponded to two different coeluted anthocyanins, while peaks 21 and 22 and peaks 5, 17, and 18 were assigned to three and four anthocyanins, respectively. This represents a total of 49 different anthocyanins in the wild blueberry mixture (**Table 3**). The coeluted anthocyanins are structurally different but have the same retention time. The anthocyanins in peaks 1−16 are major, and each represent 2.7−8.9% of the total antocyanin content, while peaks 17−28 contain minor components representing less than 2% of the total (**Figure 3**; **Table 3**).

The individual anthocyanins were identified mainly by their HPLC retention times, elution order, comparison of MS spectra to standards, previously reported data (*13*, 29), and guidelines previously introduced by Wu and Prior (*13*) and Guisti et al. (*30*). The determination of the molecular weights by ESI-MS/MS showed that only five of the six most widespread anthocyanidins, namely, delphinidin (*m*/*z* 303), cyanidin (*m*/*z* 287), petunidin (*m*/*z* 317), peonidin (*m*/*z* 301), and malvidin (*m*/*z* 331) were found in the mixture of the two wild blueberries (**Table 3**). Pelargonidin (*m*/*z* 271) was not found. These results are in agreement with those reported in the literature.

Among the 49 anthocyanins detected in this study, 19 are identical to those previously reported (*14*). These are compounds 5, 6, and 8 (peaks 1, 2, and 5), compounds 9 and 10 (peak 6), compound 12 (peak 7), compounds 14 and 15 (peak 9), compound 17 (peak 11), compound 20 (peak 13), compound 24 (peak 16), compound 28 (peak 17), compound 32 (peak 18), compound 37 (peak 21), compound 39 (peak 22), and compound 43 (peak 24; **Table 3**).

Peaks 1 and 2 were identified as delphinidin 3-galactoside and delphinidin 3-glucoside. Peaks 3 and 4 have the same mass spectrometry profile but differ in retention time (22.0, and 24.3 min; **Table 3**). Mass spectrometry data indicated that these two compounds contain delphinidin linked to one hexose ($M^+$ *m*/*z* = 465; MS/MS *m*/*z* = 303; **Figure 4A**,**B**). The retention times were slightly longer than for common anthocyanin-hexosides like delphinidin 3-galactoside (peak 1, $t_R$ = 16.8 min) and delphinidin 3-glucoside (peak 2, $t_R$ = 20.6 min). The elution order based upon anthocyanin polarity on HPLC reversed-phase was not altered (*8*). The anthocyanins of peaks 3 and 4 eluted later than delphinidin 3-galactoside and delphinidin 3-glucoside. To our knowledge, this is the first report of unusual hexosides in lowbush blueberries, the most common sugars being galactose and glucose. The two unusual hexoses are epimers of galactose and glucose, each presenting different polarities, based on retention time and structure. As a result of the limited information available, the exact chemical structures of these hexoses could not be determined.

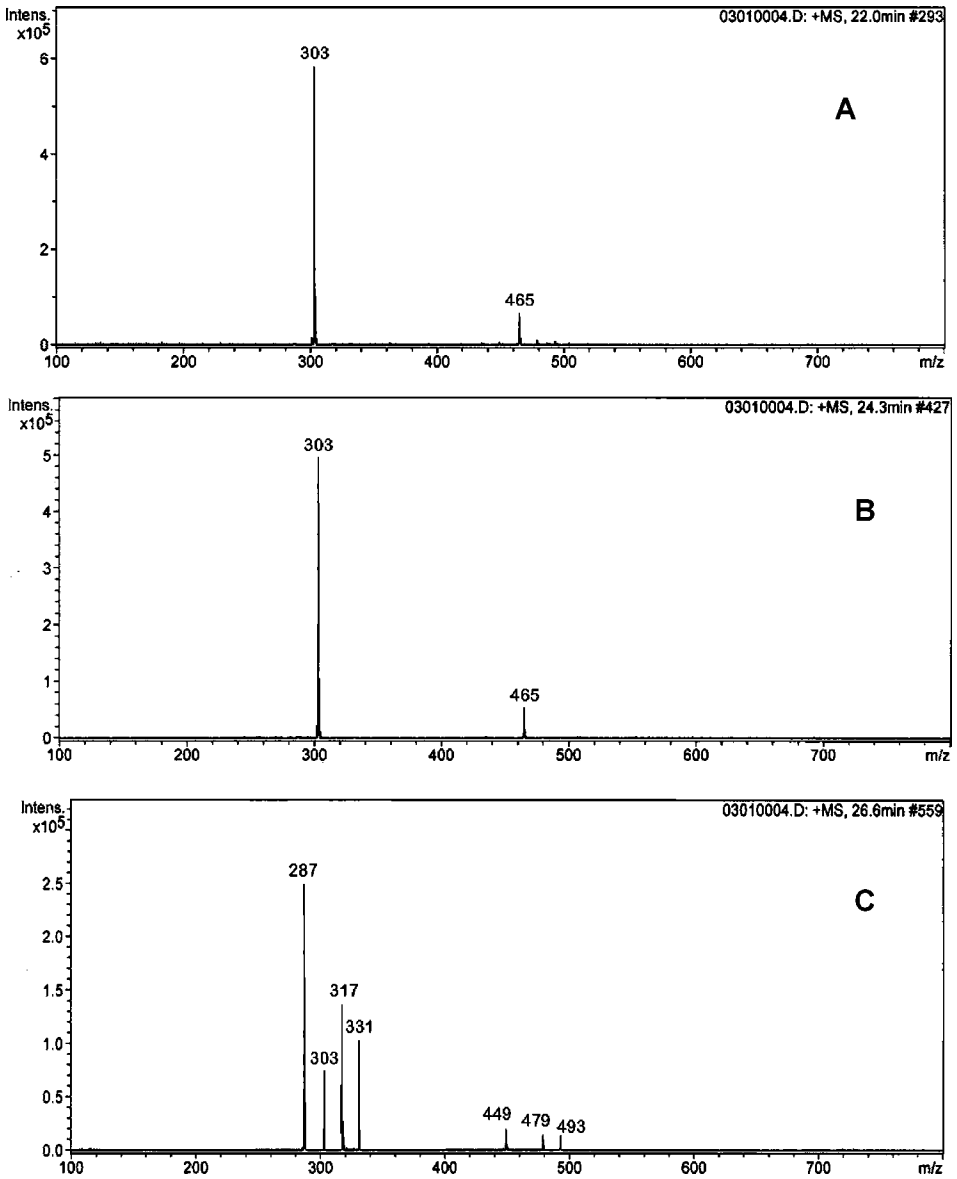


**Figure 3.** MS spectra of (A) peak 3, (B) peak 4, and (C) peak 5 (coeluted compounds 5, 6, 7, and 8).

Similar MS fragmentation patterns were observed from peak 5 (compound 7, $M^+$ $m/z$ = 465; MS/MS $m/z$ = 303 for delphinidin), peak 8 (compound 13), peak 10 (compound 16, $M^+$ $m/z$ = 479; MS/MS $m/z$ = 317 for petunidin), peak 11 (compound 18, $M^+$ $m/z$ = 493; MS/MS $m/z$ = 331 for malvidin), peak 12 (compound 19), peak 14 (compound 22), and peak 15 (compound 23, $M^+$ $m/z$ = 493; MS/MS $m/z$ = 331 for malvidin; **Table 3**), also revealing unusual hexosides (epimers of galactose and glucose) linked to delphinidin, petunidin, and malvidin. These unusual hexoses were found linked to a not previously reported acylated anthocyanin in peak 23, namely, compound 41 ($M^+$ $m/z$ = 535; MS/MS $m/z$ = 479/317) and two known acylated anthocyanins, compound 42 ($M^+$ $m/z$ = 535; MS/MS $m/z$ = 493/331) in peak 23 and compound 47 ($M^+$ $m/z$ = 535; MS/MS $m/z$ = 493/331) in peak 26. They were identified as petunidin 3-propionyl-hexose, malvidin 3-acetyl-hexose ($t_R$ = 67.7 min), and malvidin 3-acetyl-hexose ($t_R$ = 73.8 min). However, the structural identification of these hexoses is not possible with the information available.

Acylation of the sugar moieties of the anthocyanins causes a loss of polarity, increasing the retention time (*8*). On the basis of the retention times (respectively 52.2 and 53.5 min), peaks 17 and 18 should be acylated anthocyanins. These two peaks have the same unusual fragmentation pattern: a molecular ion at $m/z$ 535 fragmented to four product ions matching the molecular masses of aglycons at $m/z$ 287 (cyanidin), 303 (delphinidin), 317 (petunidin), and 331 (malvidin) resulting in the loss of hexose or pentose (**Figure 4A**,**B**). However, the MS data did not clearly indicate that these anthocyanins contained hexose or pentose and suggested that each peak corresponded to four different coeluted anthocyanins.

Because anthocyanins are composed of anthocyanidin (aglycone), one or more sugars and optional acylated groups, their identification was in each case based on two hypotheses. In the case of compound 25 (peak 17), MS/MS $m/z$ = 465/303 (delphinidin-hexose) or 435/303 (delphinidin-pentose) suggest respectively delphinidin 3-oxalyl-hexose or delphinidin 3-succinyl-pentose. In the case of compound 26, MS/MS $m/z$ = 449/287 (cyanidin-hexose) or 419/287 (cyanidin-pentose), the compound may be identified as cyanidin 3-malonyl-hexose or cyanidin 3-malonyl-pentose (possibly the first observation of a malonyl group in an anthocyanin). For compound 27, with MS/MS $m/z$ = 479/317 (petunidin-hexose) or 449/317 (petunidin-pentose), the structure could be petunidin 3-propionyl-hexose

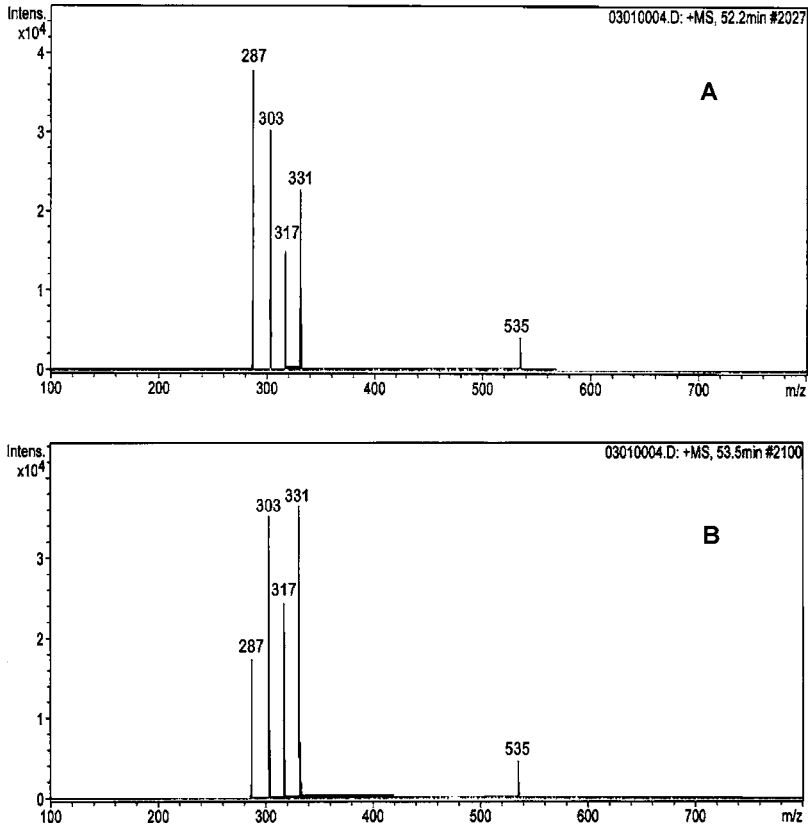


**Figure 4.** MS fragmentation patterns of (A) peak 17 and (B) peak 18.

or petunidin 3-malonyl-pentose, while MS/MS $m/z = 493/331$ (malvidin-hexose) or 463/331 (malvidin-pentose) suggesting that compound 28 could be either malvidin 3-acetyl-hexose or malvidin 3-oxalyl-pentose, possibly a new acylated anthocyanin which has not been previously reported. Mass spectra of compound 33 (peak 19) showed an unusual molecular ion of $m/z$ 515 and one fragment of $m/z$ 303 suggesting that this anthocyanin contains delphinidin.

A pigment recently identified by Wu and Prior (*13*), cyanidin 3-(malonyl) galactoside, was also found by our analyses (peak 21, compound 35, $M^+ = m/z$ 535, MS/MS $m/z = 449/287$). New acylated anthocyanins identified for the first time were present in peaks 20, 21, 22, 23, 25, and 26: compound 34 ($M^+$ $m/z = 507$, MS/MS $m/z = 449/287$; **Figure 5**A), compound 36 ($M^+ = m/z$ 535, MS/MS $m/z = 479/317$; **Figure 5B**), compounds 38 and 40 ($M^+$ $m/z = 535$; MS/MS $m/z = 479/317$ and 463/331; **Figure 5C**,**D**), compounds 44 and 45 ($M^+$ $m/z = 535$, MS/MS $m/z = 433/301$ and 463/331; **Figure 5G**,**E**), and compound 46 ($M^+$ $m/z = 535$, MS/MS $m/z = 463/301$; **Figure 5F**). These compounds could be identified respectively as cyanidin 3-(propionyl) galactoside, petunidin 3-(propionyl) galactoside, petunidin 3-(propionyl) glucoside, malvidin 3-(oxalyl) arabinoside, peonidin 3-(succinyl) galactoside, malvidin 3-(oxalyl) xyloside, and peonidin 3-(oxalyl) galactoside. To our knowledge, this is the first reported observation of propionyl, succinyl, and oxalyl groups on anthocyanins in lowbush blueberry.

MS spectra of peaks 27 and 28 (respectively compounds 48 and 49) showed unusual fragmentation patterns: $M^+$ $m/z = 535$, MS/MS $m/z = 331$ for delphinidin$^+$ (**Table 3**). There was no fragment that indicated the presence of hexose or pentose. The same reasoning used to identify coeluted compounds in peaks 17 and 18 was adopted to identify those of peaks 27 and 28 (**Table 3**). For peak 27, the first hypothesis was that if MS/MS $m/z = 493/331$ (malvidin-hexose), the anthocyanin would be identified as malvidin 3-acetyl-hexose, with an unusual hexose, while another hypothesis was that if MS/MS $m/z = 463/331$ (malvidin-xyloside), the pigment would be malvidin 3-oxalyl-xyloside, a new acylated anthocyanin not previously identified. For peak 28, if MS/MS $m/z = 493/331$ (malvidin-hexose), the anthocyanin would be identified as malvidin 3-acetyl-hexose, with an unusual hexose, while if MS/MS $m/z = 463/331$ (malvidin-pentose), the pigment would be malvidin 3-oxalyl-pentose with an unusual pentose. Unusual pentoses are epimers of arabinose and xylose.

It was observed that anthocyanins did not always follow the general rule of eluting from the reversed phase HPLC as a function of compound polarity. For example, four anthocyanins eluted together in peak 5 (**Figure 4C**) rather than individually, even though their polarities differ, following the order cyanidin 3-galactoside > petunidin 3-galactoside > delphinidin 3-hexose > malvidin 3-galactoside. Coelution in reversed-phase HPLC peaks is common among the complex anthocyanins in fruits such as blueberries, and these can be detected and distinguished only in mass spectra (*16*), as illustrated by **Figure 4C**. Coupling reversed-phase HPLC with ion trap mass spectrometry provides additional structural information based on molecular mass and MS/MS fragmentation profiles, which may be sufficient to identify pigments coeluted in a single peak. It is seldom possible to identify a compound with only the ionic masses. Furthermore, colored flavynium cations can be selectively detected in the visible region at 520 nm, avoiding the interference of other flavonoids that may be present in the same extract. Consequently, the photometric detection is easy (*8*).

To conclude, it is considered that the temperature and the acid choice can be optimized to improve anthocyanin extraction from a mixture of berries of *V. angustifolium* and *V. myrtilloides*. In this study, phosphoric acid at 79 °C gave the best extraction

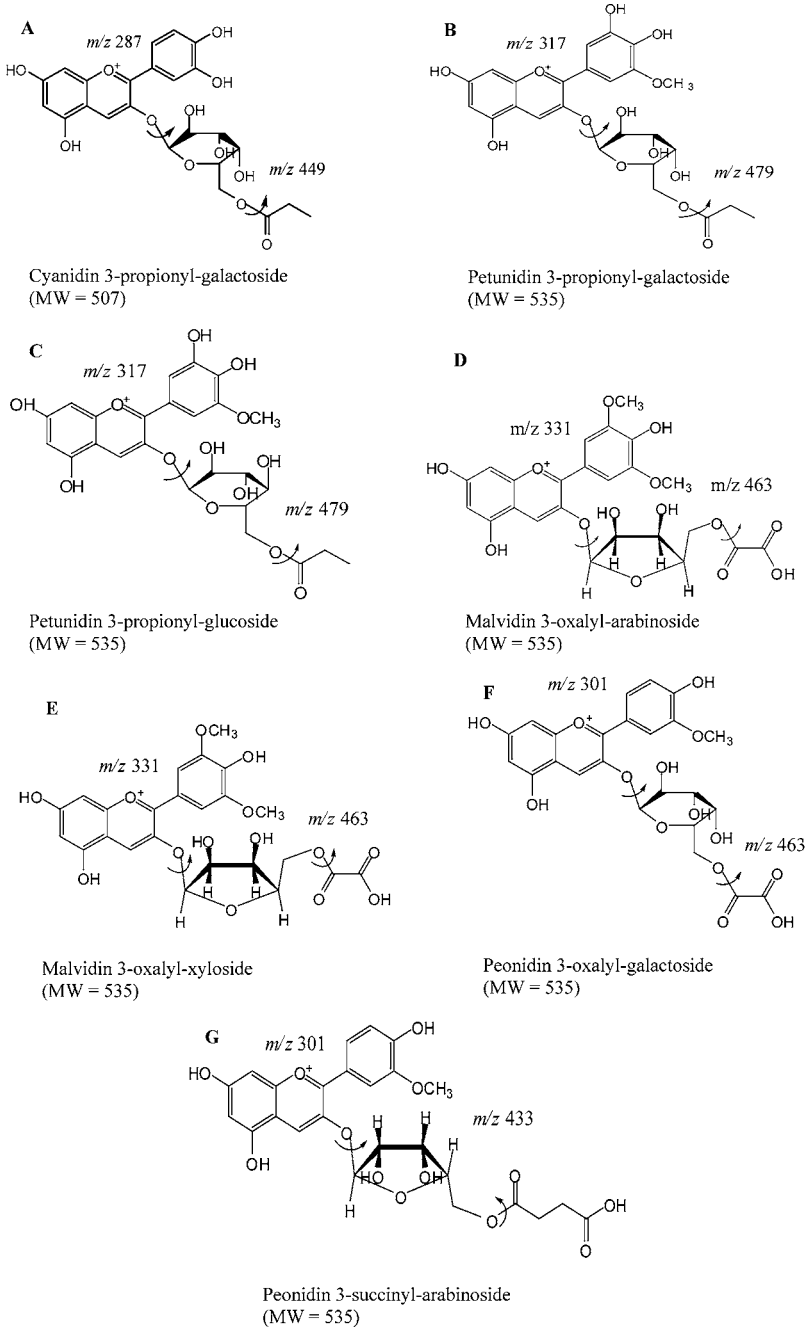


**Figure 5.** Structures and major cleavage of new selected anthocyanins.

approaching nearly the yield obtained using HCl/methanol for the quantification of the total anthocyanin content potentially extractable. Anthocyanins with a low DI were efficiently extracted from whole blueberries. The filter residue obtained from the extraction was very poor in pigments, and these had a relatively high DI, making the residue not valuable as starting material for additional extraction of nutraceutical substances. Anthocyanin and dry matter mass balance clearly demonstrated that there were no significant losses from the starting blueberries as a result of extraction. Among the 49 anthocyanins detected in the extract by HPLC-ESI-MS/MS, at least seven with malonyl, oxalyl, and succinyl groups have been identified for the first time in these wild blueberries, as well as simple and acylated anthocyanins linked to unusual hexoses and pentoses that differ from the usual galactose and glucose moieties in ways that remain to be determined.

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## LITERATURE CITED

(1) Wang, H.; Cao, G.; Prior, L. R. Total antioxidant capacity of fruits. *J. Agric. Food Chem.* **1996**, *44*, 701−705.

(2) Prior, R. L.; Martin, A.; Cao, G.; Sofic, E.; McEwan, J.; O'Brien, C.; Lischner, N.; Ehlenfeldt, M.; Krewer, G.; Mainland, C. M. Antioxidant capacity as influenced by total phenolics and anthocyanin content, maturity and variety of *Vaccinium* species. *J. Agric. Food Chem.* **1998**, *46*, 2686−2693.

(3) Miller, N. J.; Rice-Evans, C. The relative contributions of ascorbic acid and phenolic antioxidant activity of orange and apple juices and blackcurrant drink. *Food Chem.* **1997**, *60*, 331−337.
(4) Kalt, W.; Dufour, D. Health functionality of blueberries. *Hortic. Technol.* **1997**, *7*, 216−221.
(5) Kalt, W.; Forney, C. F.; Martin, A.; Prior, R. L. Antioxidant capacity, vitamin C, phenolics, and anthocyanins after fresh storage of small fruits. *J. Agric. Food Chem.* **1999**, *47*, 4638−4644.
(6) Martinelli, E. M.; Scilingo, A.; Pifferi, G. Computer-aided evaluation of the relative stability of *Vaccinium myrtillus* anthocyanins. *Anal. Chim. Acta* **1992**, *259*, 109−113.
(7) Francis, F. J.; Harbone, J. B.; Barker, W. G. Anthocyanins in the lowbush blueberry, *Vaccinium angustifolium*. *J. Food Sci.* **1966**, *31*, 583−587.
(8) Rivas-Gonzalo, J. Analysis of polyphenols. In *Methods in Polyphenols Analysis*; Santos-Buelga, C., Williamson, G., Eds.; Royal Society of Chemistry (Athenaeum Press, Ltd.): Cambridge, U.K., 2003; pp 95−98, 338−358.
(9) Fuleki, T.; Francis, J. F. Quantitative methods for anthocyanins. 2. Determination of total anthocyanins and degradation index for cranberry juice. *J. Food Sci.* **1968**, *33*, 78−83.
(10) Strack, D.; Wray, V. Anthocyanins. In *Methods in Plants Biochemistry*; Dey, P. M., Harbone, J. B., Eds.; Academic Press: San Diego, CA, 1989; Vol. 1: Plant Phenolics, pp 325−359.
(11) Giusti, M. M.; Wrolstad, R. E. Unit F1.2: Anthocyanins. Characterization and Measurement with UV-Visible Spectroscopy. In *Current Protocols in Food Analytical Chemistry*; Wrolstad, R. E., Eds.; John Wiley and Sons: New York, 2001; pp 1−13.
(12) Dust, R. W.; Wrolstad, R. E. Unit F1.3: Anthocyanins*:* Separation and Characterization of Anthocyanins by HPLC. In *Current Protocols in Food Analytical Chemistry*; Wrolstad, R. E., Eds.; John Wiley and Sons: New York, 2001; pp 1−13.
(13) Wu, X.; Prior, R. L. Systematic Identification and Characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: fruits and berries. *J. Agric. Food Chem.* **2005**, *53*, 2589−2599.
(14) Shi, J.; Mazza, G.; Le Maguer, M. Functional Foods: Biochemical and Processing aspects. *Functional Foods and Nutraceuticals Series*; CRC Press: Boca Raton, 2002; pp 331−365.
(15) Wolstad, R. E. Anthocyanins. In *Naturals Food Colorants*; Lauro, G. J., Francis, F. J., Eds.; Marcel Decker: New York, 2000; pp 237−252.
(16) Kalt, W.; McDonald, J. E.; Donner, H. Anthocyanins, phenolics and antioxidant capacity of processed lowbush blueberry product. *J. Food Sci*. **2000**, *5*, 390−393.
(17) Gao, L.; Mazza, G. Quantification and distribution of simple and acylated anthocyanins and other phenolics in blueberries. *J. Food Sci*. **1994**, *59*, 1057−1059.
(18) Ehlenfeldt, M. K.; Prior, R. L. Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf tissues of highbush blueberry. *J. Agric. Food Chem.* **2001**, *49*, 2222−2227.
(19) Gao, L.; Mazza, G. Quantification and distribution of simple and acylated anthocyanins and other phenolics in blueberries. *J. Food Sci*. **1994**, *59*, 1057−1059.
(20) Meschter, E. E. Fruit color loss, effects of carbohydrates and other factors on strawberry products. *J. Agric. Food Chem.* **1953**, *1*, 574−579.
(21) Lee, J.; Wrolstad, R. E. Extraction of anthocyanins and polyphenolics from blueberry processing waste. *J. Food Sci.* **2004**, *69*, C564−C573.
(22) Spanos, G. A.; Wrolstad, R. E.; Heatherbell, D. A. Influence of processing and storage on the phenolic composition of apple juice. *J. Agric. Food Chem.* **1990**, *38*, 1572−1589.
(23) Kader, F.; Rovel, B.; Girardin, M.; Metche, M. Mechanism of browning in flesh highbush blueberry fruit (*Vaccinium corymbosum*). Role of blueberry polyphenoloxidase, chlorogenic acid and anthocyanins. *J. Sci. Food Agric.* **1997**, *74*, 31−34.
(24) Kalt, W.; McDonald, J. E. Chemical composition of lowbush blueberry cultivars. *J. Am. Soc. Hortic. Sci.* **1996**, *12*, 142−146.
(25) Pifferi, P. G.; Cultrera, R. Enzymatic degradation of anthocyanins: the role of sweet cherry polyphenol oxidase. *J. Food Sci.* **1997**, *39*, 786−791.
(26) Mathew, A. G.; Parpia, H. A. G. Food browning as a polyphenol reaction. *Adv. Food Res.* **1971**, *19*, 76−145.
(27) Francis, F. J. Food colorants: Anthocyanins. *Crit. Rev. Food Sci. Nutr*. **1989**, *28*, 273−314.
(28) Lee, J.; Durst, R. W.; Wrolstad, R. E. Impact of juice processing on blueberry anthocyanins and polyphenolics: Comparison of two pretreatments. *J. Food Sci.* **2002**, *67*, 1660−1667.
(29) Wu, X.; Prior, R. L. Identification and Characterization of anthocyanins by high-performance liquid chromatography-electro-spray ionization-tandem mass spectrometry in common foods in the United States: Vegetables, Nuts, and Grains. *J. Agric. Food Chem.* **2005**, *53*, 3101−3113.
(30) Giusti, M. M.; Rodriguez-Saona, L. E.; Griffin, D.; Wrolstad, R. E. Electrospray and tandem mass spectroscopy as tools for anthocyanin characterization. *J. Agric. Food Chem.* **1999**, *47*, 4657− 4664.

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