Comparison between HPLC and MALDI-TOF MS Analysis of Anthocyanins in Highbush Blueberries

Jian Wang,[†] Wilhelmina Kalt,[‡] and Peter Sporns*,[†]

Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada T6G 2P5, and Atlantic Food and Horticulture Research Centre, Agriculture and Agri-Food Canada, Kentville, Nova Scotia, Canada B4N 1J5

High-performance liquid chromatography (HPLC) has been widely used as a reliable technique to quantify anthocyanins in food samples. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a new technique that is having a great impact on food analysis. This study is the first to compare HPLC and MALDI-TOF MS quantifications of anthocyanins. The analyses were carried out for highbush blueberries at different stages of anthocyanin formation. In general, both techniques provided comparable quantitative anthocyanin profiles for the samples. HPLC could distinguish anthocyanin isomers, whereas MALDI-TOF MS proved to be more rapid in the accurate identification and quantification of anthocyanins with different masses. A single MALDI-TOF MS run took just 4 min. MALDI-TOF MS analysis can serve as a rapid alternative to HPLC for the analysis of anthocyanins in fruits.

Keywords: *Blueberries; anthocyanins; responses; quantitative fragmentation*

INTRODUCTION

Highbush blueberries (*Vaccinium corymbosum*) are one of several *Vaccinium* species native to North America. Highbush blueberries are grown commercially in North America, and more recently in other parts of the world, primarily for use in processed food products. Highbush blueberries, like some other commercial *Vaccinium* species (e.g., lowbush blueberry, bilberry), are noted for their high content of anthocyanin pigments. For example, highbush blueberries may contain >15 times the anthocyanin content of strawberries and >3 times the pigment level of raspberries (Kalt et al., 1999). Anthocyanins have been of great interest to the food industry because of their important contribution to food color. An extract of bilberry (*V. myrtillus*) called Myrtocyan is used for various health applications, on the basis of the reported biological activities of its anthocyanins (Marazzoni and Bombardelli, 1996).

Rapid accumulation of anthocyanin is a characteristic event during the ripening of many fruit species. In highbush blueberries, the extent of color change from white/green to blue serves as an important visual indicator of fruit maturity. Under-ripe fruit will accumulate anthocyanins when either attached to or detached from the plant (Woodruff et al., 1960; Suomalainen and Keranen, 1961). Anthocyanin analysis has been an important element in characterizing the changes that occur in fruit during ripening and after harvest.

There are 15 major anthocyanins in highbush blueberries (Sapers et al., 1984; Mazza and Miniati, 1993; Kader et al., 1996); these are the 3- monoarabinosides, 3-monogalactosides, and 3-monoglucosides of cyanidin, delphinidin, peonidin, petunidin, and malvidin (Figure 1). Gao and Mazza (1994a) have identified small amounts of acetylated anthocyanidin monoglycosides in the highbush variety Bluecrop. Detailed anthocyanin analysis has been very useful in studying the changes that occur in the maturation and storage of fruits. Ultraviolet−visible spectroscopy (UV−vis) (260−600 nm) has been widely used to study anthocyanins (Harborne, 1958, 1967; Gao and Mazza, 1994b; Baldi et al., 1995; Bakker et al., 1997). High-performance liquid chromatography (HPLC) is the main technique used to quantify anthocyanins (Wulf and Nagel, 1978; Camire and Clydesdale, 1979; Goiffon et al., 1991; Mazza and Velioglu, 1992; Gao and Mazza, 1994a; Gao et al., 1997). The coupling of HPLC and UV−vis spectroscopy (HPLC/photodiode array detection) has been the most common method to determine anthocyanin identity and quantification (Andersen, 1985, 1987; Hong and Wrolstad, 1990a,b; Goiffon et al., 1991; Dallas et al., 1996; Garcia-Viguera et al., 1997).

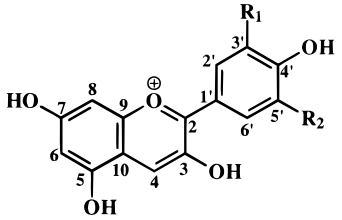

Figure 1. Structures of anthocyanidins: cyanidin, $R_1 = H$, $R_2 = OH$; delphinidin, $R_1 = R_2 = OH$; peonidin, $R_1 = OCH_3$, $R_2 = H$; petunidin, $R_1 = OCH_3$, $R_2 = OH$; malvidin, $R_1 = R_2 = OCH_3$.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was first introduced in 1987 and originally developed for large biomolecules (Karas et al., 1987). MALDI-TOF MS has several advantages over other methodologies, including speed of analysis, high sensitivity, wide applicability

* Author to whom correspondence should be addressed [telephone (780) 492-0375; fax (780) 492-4265; e-mail psporns@afns.ualberta.ca].

[†] University of Alberta.

[‡] Agriculture and Agri-Food Canada.

10.1021/jf000101g CCC: $19.00
Published on Web 07/15/2000

with a good tolerance toward contaminants, and ability to analyze complex mixtures (Karas, 1996). However, simple MALDI-TOF MS instruments cannot tell the difference between isomers, which have identical molecular mass. Recent research on applications of MALDI-TOF MS has been extensive. In food analysis, MALDI-TOF MS has been used to identify anthocyanins (Sugui et al., 1998, 1999; Sporns and Wang, 1998; Wang and Sporns, 1999). Wang and Sporns (1999) reported the potential quantitative analysis of anthocyanins in foods using MALDI-TOF MS. The objective of this study was to compare HPLC and MALDI-TOF MS analysis of anthocyanins in blueberry samples.

MATERIALS AND METHODS

Materials and Reagents. Highbush blueberries (*V. corymbosum* cv. Bergitta) were harvested when the fruit surface color was between 5 and 50% fully blue. The remaining surface area of the fruit was pink; the stem end of the fruit was largely white. Three replicate samples were collected from three different shrubs at the same production site. Fruit samples were stored in the dark in a controlled-temperature room set at 20 ± 0.5 °C. A constant vapor pressure deficit of 0.212 kPa was maintained in the chambers using solutions of glycerol−water as described by Forney and Brandl (1992). The three replicate samples were removed after 0, 2, 4, and 8 days of storage. Pelargonidin 3-glucoside, cyanidin 3-glucoside, peonidin 3-glucoside, and malvidin 3-glucoside were obtained from Extrasynthese S.A. (Genay Cedex, France). Maltotriose and maltotetraose were purchased from Sigma Chemical Co. (St. Louis, MO). 2,4,6-Trihydroxyacetophenone monohydrate (THAP) was obtained from Aldrich Chemical Co. (Milwaukee, WI). All water used was double-deionized (Milli-Q water purification system, Millipore Corp., Bedford, MA).

Extraction of Anthocyanins from Blueberries. The samples were prepared by grinding 30 g of frozen blueberries in a small food processor. Approximately 15 g of these ground samples was added with a half-teaspoon of Celite 545 (J. T. Baker Inc. Phillipsburg, NJ) to 30 mL of mixed solvent (acetone/methanol/water/formic acid, 40:40:20:0.1, v/v/v/v). This sample was blended for 2 min on a Virtis homogenizer (The Virtis Co. Inc., Gardiner, NY) on a speed setting of 4. The solid material was removed from the extract using a vacuum and a Büchner funnel lined with two sheets of Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, U.K.). The residue was rinsed with the solvent, and the filtrate was made up to 50 mL. Some samples (7.5 g), which were <15 g, were extracted with only 25 mL total volume of solvent. The solvent was removed under vacuum using a centrifugal evaporator (Savant Instruments Inc., Hicksville, NY), and then dried extracts were resolubilized in an equivalent volume (i.e., 50 or 25 mL) of water. Extract (8 mL) was passed through a Sep-Pak C_{18} cartridge (Waters Scientific, Mississauga, ON, Canada), which had been prerinsed with 2 mL of 100% methanol and 5 mL of water. Once loaded, the column was rinsed with 5 mL of water to remove sugars and organic acids. Anthocyanins were eluted with 10 mL of 0.1% formic acid in methanol and stored at −20 °C until analyzed.

HPLC Analysis of Blueberry Anthocyanins. Samples for HPLC analysis were dried, resolubilized in an equivalent volume (i.e., 10 mL) of 86% solvent A and 14% solvent B (see below), and then filtered through a 0.2 μm PVDF filter (Whatman International Ltd.). Twenty microliters of extract was injected onto the HPLC. The HPLC system consisted of a Hewlett-Packard 1100 Series HPLC [Hewlett-Packard (Canada), Mississauga, ON, Canada] coupled with an autosampler and a photodiode array detector. Anthocyanins were separated on a Zorbax SBC18 150 × 4.6 mm (5 μm) reverse phase column [Hewlett-Packard (Canada)]. The solvents used were 5% (v/v) aqueous formic acid (solvent A) and 100% HPLC grade methanol (solvent B). The flow rate was 1 mL/min, with a linear gradient profile consisting of solvent A with the following proportions (v/v) of solvent B: 0 min, 14% B; 1−10.24 min, 14−17% B; 10.24−35.28 min, 17−23% B; 35.28−64.59 min, 23−47% B; 64.59−66.59 min, 47−14% B. The total run time was 70 min. The column temperature was maintained at 26 °C. Quantification of anthocyanins was based on peak areas determined at 520 nm and compared to the absorbance of cyanidin 3-glucoside, which was used as the standard. For each storage stage, three samples were analyzed.

MALDI-TOF MS. MALDI-TOF MS was performed using a Proflex III in linear mode (Bruker Analytical Systems Inc., Billerica, MA). Anthocyanins cocrystallized with matrix on the probe were desorbed and ionized by a nitrogen laser pulse (337 nm) and accelerated under 20 kV with time-delayed extraction before entering the time-of-flight mass spectrometer. THAP was used as matrix. THAP was saturated in acetone. Matrix solution (0.3 μL) was applied on the probe first and air-dried. One microliter of final anthocyanin extracts was then spotted directly over THAP crystals and again air-dried. Laser strength was attenuated (the lower the attenuation, the higher the laser strength) to obtain the best signal-to-noise ratios while isotopic resolution was maintained. Anthocyanin extracts were diluted to obtain ~0.35 A_{520} using methanol/formic acid/water (70:2:28, v/v/v) before MALDI-TOF MS analysis so that the detector would not be saturated. When the UV−vis absorbance of dilute extracts at 520 nm was 0.35 ± 0.05, anthocyanins would not cause detector saturation problems and fit in the linear MALDI-TOF MS response range (Wang and Sporns, 1999). MALDI-TOF MS was calibrated with two point external calibration using $[\text{maltotriose} + K]^+$ (exact isotopic mass = 543.13) and $[\text{maltotetraose} + K]^+$ (exact isotopic mass = 705.19) as calibrants, resulting in a mass accuracy ≤500 ppm.

Quantification of anthocyanins using MALDI-TOF MS was achieved using a standard addition method, as reported earlier by Wang et al. (1999). MALDI-TOF MS samples contained 30 μL of dilute extract and 30 μL of solvent (methanol/formic acid/water, 70:2:28, v/v/v), whereas the standard addition samples contained 30 μL of the extract plus 30 μL of cyanidin 3-glucoside (9.5×10^{-5} M in solvent). Samples and corresponding standard addition samples were applied to the 10 positions on the probe; one probe could be used for the analysis of five samples and their standards. From each of the 10 probe positions, a single spectrum was obtained by collecting 3 × 40 or a total of 120 laser pulses from three randomly selected spots. Peak heights were determined for each anthocyanin from each spectrum. These peak heights were then scaled relative to the delphinidin 3-glycoside peak, which was arbitrarily set at a value of 1.0. Each of the five spectra for the sample were compared to a different spectrum from the five standard addition samples, and the average increase in scaled relative peak height for cyanidin 3-glucoside (standard anthocyanin added) was determined. This gave a value for the average increased response due to the addition of cyanidin 3-glucoside on a molar basis. This response factor was then used to determine the average amount of each anthocyanin in the five sample spectra. The acquisition of the MALDI-TOF MS data (5 runs, total of 10 spectra) took ~20 min. A single run represented the acquisition of two spectra (one from sample and another from standard added sample). The mean of five runs was used to calculate the average anthocyanin content of each sample. For each storage stage, three samples were analyzed. All statistics were performed using Microsoft Excel 97 (Microsoft Office 97).

RESULTS AND DISCUSSION

HPLC has been a very popular and widely used technique for the analysis of anthocyanins in foods or plants (Andersen, 1985, 1987; Hong and Wrolstad, 1990a,b; Goiffon et al., 1991; Dallas et al., 1996; Garcia-Viguera et al., 1997). Quantification of anthocyanins using HPLC was based mainly on peak areas determined at a certain wavelength such as 525 nm (Gao et al., 1997), which was usually close to the maximum

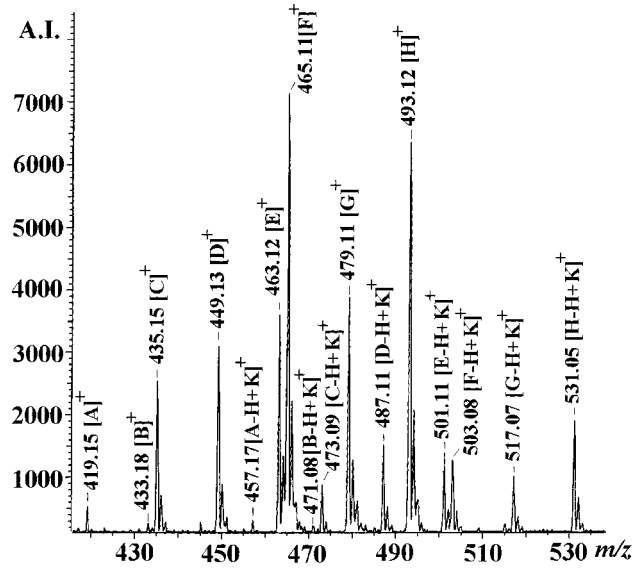

Figure 2. MALDI-TOF MS positive ion spectrum of highbush blueberry anthocyanins (stored for 4 days). Numbers in parentheses indicate corresponding peak number measured by HPLC (Figure 3): A, cyanidin 3-arabinoside (7); B, peonidin 3-arabinoside; C, delphinidin 3-arabinoside (4); D, cyanidin 3-galactoside (3) + cyanidin 3-glucoside (5) + petunidin 3-arabinoside (10); E, peonidin 3-galactoside (9) + malvidin 3-arabinoside (13); F, delphinidin 3-galactoside (1) + delphinidin 3-glucoside (2); G, petunidin 3-galactoside (6) + petunidin 3-glucoside (8); H, malvidin 3-galactoside (11) + malvidin 3-glucoside (12). Note that any anthocyanin can also be ionized in forms of potassium adducts (K^+) combined with deprotonation.

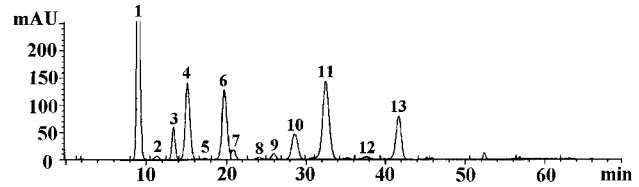

Figure 3. HPLC of anthocyanins (same sample as in Figure 2): 1, delphinidin 3-galactoside; 2, delphinidin 3-glucoside; 3, cyanidin 3-galactoside; 4, delphinidin 3-arabinoside; 5, cyanidin 3-glucoside; 6, petunidin 3-galactoside; 7, cyanidin 3-arabinoside; 8, petunidin 3-glucoside; 9, peonidin 3-galactoside; 10, petunidin 3-arabinoside; 11, malvidin 3-galactoside; 12, malvidin 3-glucoside; 13, malvidin 3-arabinoside.

absorbance wavelength (λ_{max}) of individual anthocyanins. Malvidin 3-glucoside or cyanidin 3-glucoside was usually selected as the standard for quantification (Wulf and Nagel, 1978; Boyles and Wrolstad, 1993; Gao et al., 1997). Studies have shown that anthocyanins with different chromophores have different λ_{max} values even in the same solvent system (Harborne, 1958, 1967; Francis, 1982; Hong and Wrolstad, 1990b). For example, the λ_{max} values of pelargonidin 3-glucoside, cyanidin 3-glucoside, delphinidin 3-glucoside, peonidin 3-glucoside, petunidin 3-glucoside, and malvidin 3-glucoside were 506, 523, 534, 523, 534, and 534 nm, respectively, in 0.01% HCl in methanol (Francis, 1982). Gradient elution in HPLC likely also has an effect on the anthocyanin visible absorbance characteristics due to changes in solvent composition during spectral detection. Therefore, direct comparison of spectral characteristics with those published in the literature may be inappropriate, and quantification of anthocyanins using HPLC at a fixed wavelength, for example, 525 nm, may over- or underestimate some anthocyanins. Wang and Sporns (1999) have noted considerable variation in HPLC responses at 525 nm. In this study, if cyanidin 3-glucoside was selected as the standard and its peak area was arbitrarily set at a value of 1.0, the relative molar response factors of malvidin 3,5-diglucoside, cyanidin 3-rutinoside, pelargonidin 3-glucoside, peonidin 3-glucoside, and malvidin 3-glucoside would be 0.60, 1.06, 0.69, 0.60, and 0.82, respectively. This would indicate that the amount of most individual anthocyanins and the total amount of anthocyanins might be underestimated if cyanidin 3-glucoside was used as the standard for quantification.

Table 1. Quantitative Fragmentation[a] of Anthocyanins in MALDI-TOF MS

anthocyanin[b]	MALDI-TOF MS samples 1	2	3	4	5	inter-av[d]	inter-SD[d]
pelargonidin 3-glucoside	12.4	25.5	18.4	14.3	16.9	17.5	5.0
cyanidin 3-glucoside	13.3	22.8	19.7	14.5	13.1	16.7	4.4
peonidin 3-glucoside	13.3	24.0	22.5	16.5	17.2	18.7	4.4
malvidin 3-glucoside	15.2	27.7	16.9	15.6	14.8	18.0	5.4
intra-av[c]	13.5	25.0	19.4	15.2	15.5		
intra-SD[c]	1.2	2.1	2.4	1.0	1.9		

[a] Quantitative fragmentation (percent) was expressed as ratios of the fragment ions (loss of 3-glucoside) to their unfragmented parent ions (aromatic oxonium ion form). Number of laser pulses equals 3 × 40 or total of 120 for each MALDI-TOF MS sample. [b] Concentration of individual anthocyanins in the mixture was 9.0 ± 0.5 × 10^{-5} M. [c] Average or standard deviation of individual anthocyanins within a single MALDI-TOF MS sample (in columns). [d] Average or standard deviation of each anthocyanin from five MALDI-TOF MS samples (in rows).

In MALDI-TOF MS, it has been shown that anthocyanin monoglucosides gave similar responses (Wang and Sporns, 1999). Therefore, using a single anthocyanin monoglucoside standard one can determine the correct amounts of anthocyanins present (individually and in total).

A major disadvantage for MALDI-TOF MS analysis is that it cannot distinguish anthocyanin isomers. For example, the peak at *m/z* 449 (Figure 2) could be produced by cyanidin 3-galactoside, cyanidin 3-glucoside, or petunidin 3-arabinoside. HPLC can differentiate these three anthocyanins (Figure 3, peaks 3, 5, and 10). However, because MALDI-TOF MS gives a molecular mass, it can identify anthocyanin mass directly without the use of standards.

Another concern with MALDI-TOF MS is fragmentation of anthocyanins. Using a linear MALDI-TOF MS, postsource decay, or fragmentation of ions after extraction, is not a concern. However, in-source decay can lead to the loss of a portion of the molecule such as loss of carbohydrate residues from anthocyanins, forming different mass ions. Wang and Sporns (2000) have found that fragmentation patterns and amounts of flavonol glycosides were predictable for any defined sample preparation method. Further research on anthocyanins has shown that the monoglucosides also fragment quantitatively. This fragmentation was examined by using a mixture of the monoglucosides (pelargonidin 3-glucoside, cyanidin 3-glucoside, peonidin 3-glucoside, and malvidin 3-glucoside). These glucosides fragment with loss of their 3-glucoside moieties. The amount of fragmentation of individual anthocyanins was calculated on the basis of the percentage of fragment ions (loss of 3-glucoside) to their unfragmented parent ions (aromatic oxonium ion form) in a single MALDI-TOF MS sample or MALDI-TOF MS spectrum (Table 1). The fragmentation of individual anthocyanins varied widely from MALDI-TOF MS sample to sample as indicated

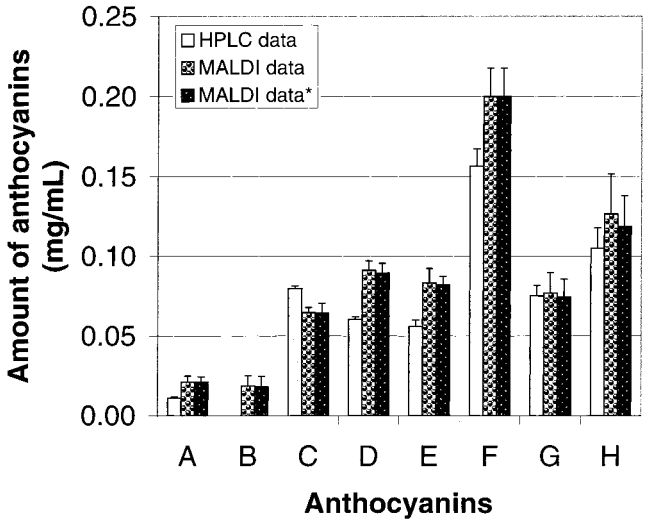

Figure 4. Comparison between HPLC and MALDI-TOF MS analysis of highbush blueberry individual anthocyanins (stored for 4 days). Numbers in parentheses indicate corresponding peak number measured by HPLC (Figure 3); percentages in parentheses represent the proportion of correspondent anthocyanins calculated from HPLC data (triplicates): A, cyanidin 3-arabinoside (7); B, peonidin 3-arabinoside; C, delphinidin 3-arabinoside (4); D, cyanidin 3-galactoside (3) (40%) + cyanidin 3-glucoside (5) (4%) + petunidin 3-arabinoside (10) (56%); E, peonidin 3-galactoside (9) (12%) + malvidin 3-arabinoside (13) (88%); F, delphinidin 3-galactoside (1) (97%) + delphinidin 3-glucoside (2) (3%); G, petunidin 3-galactoside (6) (95%) + petunidin 3-glucoside (8) (5%); H, malvidin 3-galactoside (11) (96%) + malvidin 3-glucoside (12) (4%). Each column represents the mean of three samples. Error bars indicate standard deviations ($n = 3$). * Data calculated from a single MALDI-TOF MS run.

by the large inter-standard deviation (Table 1). However, within any single MALDI-TOF MS sample, all anthocyanins exhibited a similar fragmentation percentage with small intra-standard deviation (Table 1). That is, the ratios of fragment ions to parent ions were very consistent for the monoglucosides in a single MALDI-TOF MS sample. In general, an internal standard, such as cyanidin 3-glucoside, will fragment with the same pattern or relative amount as other anthocyanins and, therefore, can be confidently used as a reference for all other anthocyanin monoglycosides. Further quantitative fragmentation studies on di- or triglycosides present in other foods would be necessary to determine the applicability of MALDI-TOF MS quantification of samples containing these anthocyanin derivatives.

Comparison between HPLC and MALDI-TOF MS Quantification on Individual Anthocyanins. Figure 3 shows an HPLC chromatogram of anthocyanins in blueberries. There were 13 anthocyanins. On the basis of their retention times, they were assigned as delphinidin 3-galactoside (1), delphinidin 3-glucoside (2), cyanidin 3-galactoside (3), delphinidin 3-arabinoside (4), cyanidin 3-glucoside (5), petunidin 3-galactoside (6), cyanidin 3-arabinoside (7), petunidin 3-glucoside (8), peonidin 3-galactoside (9), petunidin 3-arabinoside (10), malvidin 3-galactoside (11), malvidin 3-glucoside (12), and malvidin 3-arabinoside (13). Peonidin 3-arabinoside was not detected using HPLC, but it was identified in MALDI-TOF MS (Figure 2).

When analyzed by MALDI-TOF MS in positive mode, anthocyanins were detected in the aromatic oxonium ion form $[M]^+$ (Sugui et al., 1998, 1999; Sporns and

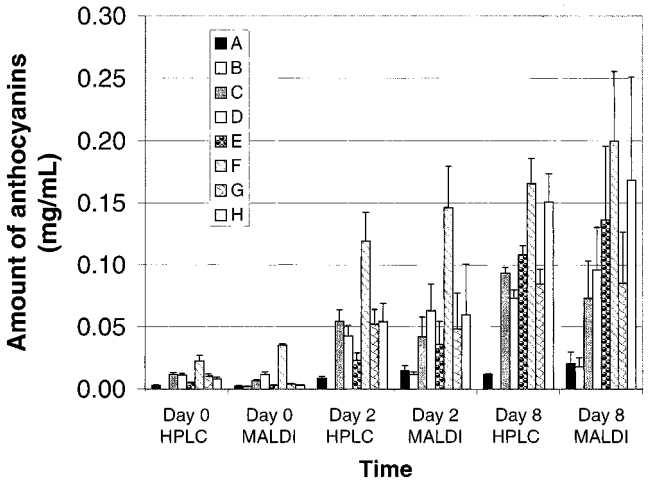

Figure 5. Comparison between HPLC and MALDI-TOF MS anthocyanin profiles at three different stages of color development. A−H denote the same anthocyanins as shown in Figure 2. Each column represents the mean of three samples. Error bars indicate standard deviations ($n = 3$).

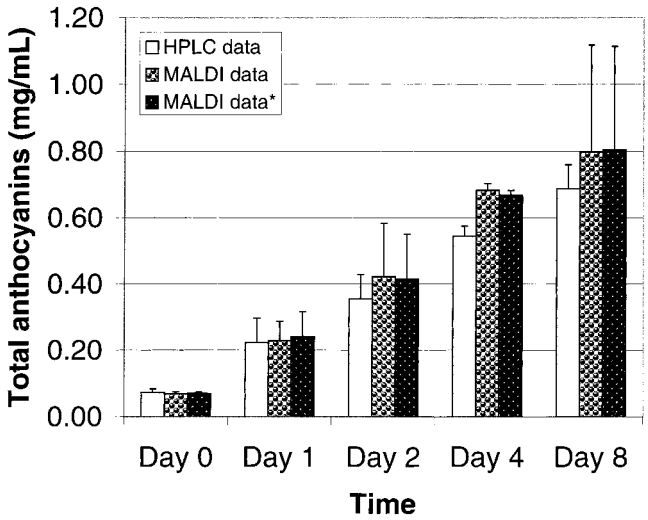

Figure 6. Comparison of total anthocyanin content determined by HPLC and MALDI-TOF MS at different stages of color development. Each column represents the mean of three samples. Error bars indicate standard deviations ($n = 3$). * Data were calculated from a single MALDI-TOF MS run.

Wang, 1998; Wang and Sporns, 1999). Our current study indicated that anthocyanins may also form single alkali metal adduct $[M - H + Na]^+$ or $[M - H + K]^+$ ions, depending on the amount of alkali metal in the MALDI-TOF MS samples. Because most plants contain large amounts of potassium, anthocyanins from blueberries can also be detected as potassium adducts (Figure 2). Also, anthocyanins showed higher affinity toward potassium than sodium (data not shown). The MALDI-TOF MS spectrum (Figure 2) shows eight anthocyanins at *m/z* 419, 433, 435, 449, 463, 465, 479, and 493, respectively. They were cyanidin 3-arabinoside (A, *m/z* 419.15), peonidin 3-arabinoside (B, *m/z* 433.18), delphinidin 3-arabinoside (C, *m/z* 435.15), cyanidin 3-galactoside or cyanidin 3-glucoside or petunidin 3-arabinoside (D, *m/z* 449.13), peonidin 3-galactoside or malvidin 3-arabinoside (E, *m/z* 463.12), delphinidin 3-galactoside or delphinidin 3-glucoside (F, *m/z* 465.11), petunidin 3-galactoside or petunidin 3-glucoside (G, *m/z* 479.11), and malvidin 3-galactoside or malvidin 3-glucoside (H, *m/z* 493.12). The potassium adduct peaks

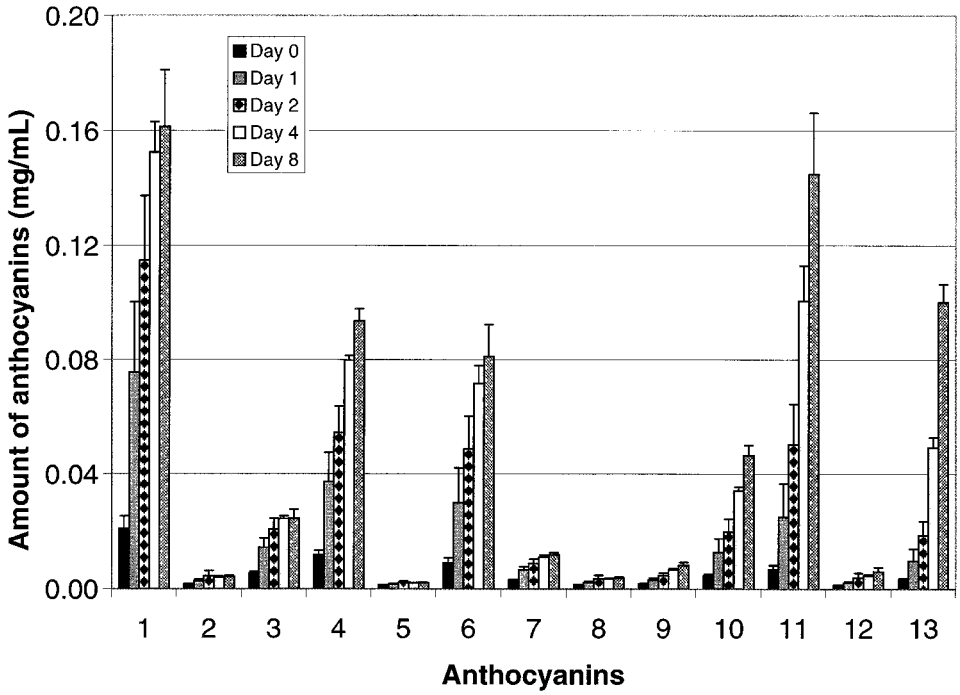

Figure 7. HPLC profile of anthocyanins in blueberries at different stages of color development. Numbers denote the same anthocyanins as shown in Figure 3. Each column represents the mean of three samples. Error bars indicate standard deviations ($n = 3$).

were also detected with 38 mass shift (addition of potassium and loss of one proton). For quantification, peak heights of both aromatic oxonium ion $[M]^+$ and potassium adduct ion $[M - H + K]^+$ were totaled. Obviously, MALDI-TOF MS cannot differentiate anthocyanins with the same mass (Figures 2−4). Generally, as expected, the amount of individual anthocyanins determined by MALDI-TOF MS was higher than that by HPLC (Figures 4 and 5), except for delphinidin 3-arabinoside. Also, total amount of anthocyanins determined by MALDI-TOF MS was higher than that of HPLC (Figure 6).

Changes of total, individual anthocyanins at different storage stages are shown in Figures 6−8. Both HPLC and MALDI-TOF MS analyses indicated that the total amount of anthocyanins increased with increased storage (Figure 6). For individual anthocyanins, HPLC results (Figure 7) illustrated changes of 13 anthocyanins at each stage, whereas MALDI-TOF MS (Figure 8) showed changes of 8 anthocyanins (on a molecular mass basis) at each stage. HPLC (Figure 7) showed that delphinidin 3-galactoside, delphinidin 3-arabinoside, petunidin 3-galactoside, malvidin 3-galactoside, and malvidin 3-arabinoside were the major anthocyanins that changed during color development. MALDI-TOF MS (Figure 8) indicated that anthocyanins F (delphinidin 3-galactoside and delphinidin 3-glucoside), C (delphinidin 3-arabinoside), G (petunidin 3-galactoside and petunidin 3-glucoside), H (malvidin 3-galactoside and malvidin 3-glucoside), and E (peonidin 3-galactoside and malvidin 3-arabinoside) followed the same trend as HPLC results. In general, HPLC and MALDI-TOF MS provided similar quantitative profiles of anthocyanins in blueberries at different stages.

Comparison of Analysis Speed. Under our experimental conditions, it took 70 min to complete one HPLC run. The total run time could be reduced, but some anthocyanins would not be resolved. The acquisition of the MALDI-TOF MS data consisted of five runs per sample and took ~20 min. It has been suggested that MALDI-TOF MS shows poor repeatability from spot to spot due to crystal inhomogeneity. Therefore, increasing the MALDI-TOF MS runs for a sample can reduce sample standard deviations. However, as shown in Figures 4 and 6, there was no significant difference between five MALDI-TOF MS runs and one MALDI-TOF MS run on both individual and total anthocyanins ($p \leq 0.05$). One MALDI-TOF MS run took just 4 min.

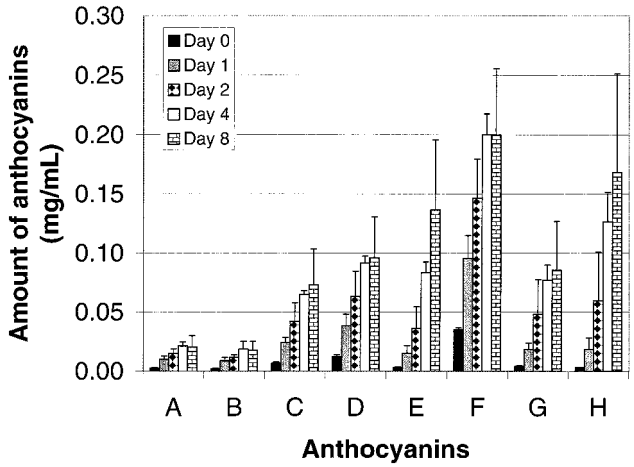

Figure 8. MALDI-TOF MS profile of anthocyanins in blueberries at different stages of color development. A−H denote the same anthocyanins as shown in Figure 2. Each column represents the mean of three samples. Error bars indicate standard deviations ($n = 3$).

In conclusion, HPLC and MALDI-TOF MS are both valuable techniques for the quantification of anthocyanins. HPLC has the power to differentiate anthocyanin isomers but may underestimate the amount of anthocyanins when cyanidin is used as the internal standard. MALDI-TOF MS is a more rapid technique to identify and quantify a group of anthocyanins with different masses. It is likely that MALDI-TOF MS has the

potential to rapidly identify and quantify anthocyanins in other foods and could make a significant contribution in developing anthocyanin "fingerprints" to explore differences in fruit varieties and maturities.

ACKNOWLEDGMENT

We gratefully acknowledge the excellent technical assistance of Jane McDonald and Carolyn Domytrak.

LITERATURE CITED

Andersen, O. M. Chromatographic separation of anthocyanins in cowberry (Lingonberry) *Vaccinium vites-idaea* L. *J. Food Sci.* **1985**, *50*, 1230−1232.

Andersen, O. M. Anthocyanins in fruits of *Vaccinium uliginosum* L. (Bog Whortleberry). *J. Food Sci.* **1987**, *52*, 665−666, 680.

Bakker, J.; Bridle, P.; Honda, T.; Kuwano, H.; Saito, N.; Terahara, N.; Timberlake, C. F. Identification of an anthocyanin occurring in some red wines. *Phytochemistry* **1997**, *44*, 1375−1382.

Baldi, A.; Romani, A.; Mulinacci, N.; Vincieri, F. F.; Casetta, B. HPLC/MS application to anthocyanins of *Vitis vinifera* L. *J. Agric. Food Chem.* **1995**, *43*, 2104−2109.

Boyles, M. J.; Wrolstad, R. E. Anthocyanin composition of red raspberry juice: influences of cultivar, processing, and environmental factors. *J. Food Sci.* **1993**, *58*, 1135−1141.

Camire, A. L.; Clydesdale, F. M. High-pressure liquid chromatography of cranberry anthocyanins. *J. Food Sci.* **1979**, *44*, 926−927.

Dallas, C.; Ricardo da Silva, J. M.; Laureano, O. Products formed in model wine solutions involving anthocyanins, procyanidin B2, and acetaldehyde. *J. Agric. Food Chem.* **1996**, *44*, 2402−2407.

Forney, C. F.; Brandl, D. G. Control of humidity in small controlled environment chambers using glycerol-water solutions. *HortTechnology* **1992**, *2*, 52−54.

Francis, F. J. Analysis of anthocyanins. In *Anthocyanins as Food Colors*; Markakis, P., Ed.; Academic Press: New York, 1982.

Gao, L.; Mazza, G. Quantification and distribution of simple and acylated anthocyanins and other phenolics in blueberries. *J. Food Sci.* **1994a**, *59*, 1057−1059.

Gao, L.; Mazza, G. Rapid method for complete chemical characterization of simple and acylated anthocyanins by high-performance liquid chromatography and capillary gas−liquid chromatography. *J. Agric. Food Chem.* **1994b**, *42*, 118−125.

Gao, L.; Girard, B.; Mazza, G.; Reynolds, A. G. Changes in anthocyanins and color characteristics of Pinot Noir wines during different vinification processes. *J. Agric. Food Chem.* **1997**, *45*, 2003−2008.

Garcia-Viguera, C.; Zafrilla, P.; Tomas-Barberan, F. A. Determination of authenticity of fruit jams by HPLC analysis of anthocyanins. *J. Sci. Food Agric.* **1997**, *73*, 207−213.

Goiffon, J. P.; Brun, M.; Bourrier, M. J. High-performance liquid chromatography of red fruit anthocyanins. *J. Chromatogr.* **1991**, *537*, 101−121.

Harborne, J. B. Spectral methods of characterizing anthocyanins. *Biochem. J.* **1958**, *70*, 22−28.

Harborne, J. B. The anthocyanin pigments. In *Comparative Biochemistry of the Flavonoids*; Harborne, J. B., Ed.; Academic Press: New York, 1967.

Hong, V.; Wrolstad, R. E. Characterization of anthocyanin-containing colorants and fruit juices by HPLC/photodiode array detection. *J. Agric. Food Chem.* **1990a**, *38*, 698−708.

Hong, V.; Wrolstad, R. E. Use of HPLC separation/photodiode array detection for characterization of anthocyanins. *J. Agric. Food Chem.* **1990b**, *38*, 708−715.

Kader, F.; Rovel, R.; Girardin, M.; Metche, M. Fractionation and identification of the phenolic compounds of highbush blueberries (*Vaccinium corymbosum*, L.) *Food Chem.* **1996**, *55*, 35−40.

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.

Karas, M. Matrix-assisted laser desorption ionization MS: a progress report. *Biochem. Mass Spectrom.* **1996**, *24*, 897−900.

Karas, M.; Bachmann, D.; Bahr, U.; Hillenkamp, F. Matrix-assisted ultraviolet laser desorption of non-volatile compounds. *Int. J. Mass Spectrom. Ion Processes* **1987**, *78*, 53−68.

Marazzoni, P.; Bombardelli, E. *Vaccinium myrtillus* L. *Fitoterapia* **1996**, *67*, 3−28.

Mazza, G.; Miniati, E. Small fruits. In *Anthocyanins in Fruits, Vegetables, and Grains*; Mazza, G., Miniati, E., Eds.; CRC Press: Boca Raton, FL, 1993.

Mazza, G.; Velioglu, Y. S. Anthocyanins and other phenolic compounds in fruits of red-flesh apples. *Food Chem.* **1992**, *43*, 113−117.

Sapers, G. M.; Burgher, A. M.; Phillips, J. G.; Jones, S. B.; Stone, E. G. Color and composition of highbush blueberry cultivars [*Vaccinium corymbosum*, anthocyanins, wax, pH, juice]. *J. Am. Soc. Hortic. Sci.* **1984**, *109*, 105−111.

Sporns, P.; Wang, J. Exploring a new frontier in food analysis using MALDI-MS. *Food Res. Int.* **1998**, *31*, 181−189.

Sugui, J. A.; Bonham, C.; Lo, S. C.; Wood, K. V.; Nicholson, R. L. MALDI-TOF analysis of mixtures of 3-deoxyanthocyanidins and anthocyanins. *J. Agric. Food Chem.* **1998**, *48*, 1063−1066.

Sugui, J. A.; Wood, K. V.; Yang, Z. Y.; Bonham, C. C.; Nicholson, R. L. Matrix-assisted laser desorption ionization mass spectrometry analysis of grape anthocyanins. *Am. J. Enol. Vitic.* **1999**, *50*, 199−203.

Suomalainen, H.; Keranen, A. J. A. The first anthocyanins appearing during the ripening of blueberries. *Nature* **1961**, *191*, 448−449.

Wang, J.; Sporns, P. Analysis of anthocyanins in red wine and fruit juice using MALDI-MS. *J. Agric. Food Chem.* **1999**, *47*, 2009−2015.

Wang, J.; Sporns, P. MALDI-TOF MS analysis of food flavonol glycosides. *J. Agric. Food Chem.* **2000**, *48*, 1657−1662.

Wang, J.; Sporns, P.; Nicholas, H. L. Analysis of food oligosaccharides using MALDI-MS: quantification of fructooligosaccharides. *J. Agric. Food Chem.* **1999**, *47*, 1549−1557.

Woodruff, R. E.; Dewey, D. H.; Sell, H. M. Chemical changes of Jersey and Rubel blueberry fruit associated with ripening and deterioration. *Proc. Am. Soc. Hortic. Sci.* **1960**, *75*, 387−401.

Wulf, L. W.; Nagel, C. W. High-pressure liquid chromatographic separation of anthocyanins of *Vitis vinifera*. *Am. J. Enol. Vitic.* **1978**, *29*, 42−49.

Received for review January 24, 2000. Revised manuscript received April 28, 2000. Accepted May 1, 2000. This research and the MALDI-TOF MS instrument used were funded by the Natural Sciences and Engineering Research Council (NSERC) of Canada. Additional financial support was provided by a Graduate Research Assistantship from the Department of Agricultural, Food and Nutritional Science and a Graduate Studies and Research Tuition Scholarship from the Faculty of Graduate Studies and Research at the University of Alberta.

JF000101G