Electrospray and Tandem Mass Spectroscopy As Tools for Anthocyanin Characterization

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The utility of electrospray and tandem mass spectroscopy (ES-MS and MS-MS) in anthocyanin characterization was tested using different anthocyanin extracts. Anthocyanins were semipurified by using a C-18 resin, washed with acidified water followed by ethyl acetate, and recovered with acidified methanol. Samples were directly injected into a mass spectrometer in either aqueous or methanolic solutions. The positive charge of anthocyanins favored fast and effective ES-MS detection of intact molecular ions. Little interference from other compounds was observed when the ethyl acetate cleaning procedure was used. Tandem mass spectroscopy provided clear and characteristic fragmentation patterns. The voltage used affected only the proportions at which these fragments were present. ES-MS may be used as a fast procedure for identification of anthocyanins, requiring minimal sample preparation. In combination with HPLC, ES-MS and MS-MS could be very powerful tools for anthocyanin characterization and monitoring the authenticity of anthocyanin-containing fruit juices and vegetable extracts.

Keywords: *Anthocyanins; ES-MS; tandem spectroscopy; MS-MS*

INTRODUCTION

Anthocyanin composition of fruits and vegetables can be used as a fingerprint to monitor the authenticity of juices. Analytical techniques for analysis of anthocyanins usually involve the use of spectrophotometric and chromatographic techniques. These techniques have proven to be very useful for routine analyses, and HPLC coupled to a diode array detector has become the method of choice for monitoring anthocyanin profiles in juices (Strack et al., 1980; Hong and Wrolstad, 1990; Wrolstad et al., 1995; Gao and Mazza, 1994). However, there are hundreds of anthocyanins in nature and the use of other detection methods is often required since these methodologies are sometimes not enough to positively discriminate between compounds that possess very similar spectral characteristics.

Most mass spectroscopic, as well as gas chromatographic, techniques require volatility of the sample, and water-soluble polar compounds would require derivatization before analysis. Ion spray mass spectrometry has been described as an ideal method for analysis of water-soluble flavonol glycosides in hops (Sagesser and Deinzer, 1996). Furthermore, electron impact ionization MS has been successfully used for determining structures of all types of flavonoids (Aramendia et al., 1995).

Electrospray ionization mass spectrometry (ES-MS) has emerged as a powerful technique for the characterization of biomolecules and is the most versatile ionization technique in existence today (Covey, 1995; Snyder, 1995). The use of ES-MS has grown in exponential proportion over the past decade (Snyder, 1995) because this soft ionization technique can produce intact ions from large and complex species in solution, even from thermally labile, nonvolatile, polar compounds (Black and Fox, 1995; Hutton and Major, 1995; Fenn et al., 1989). The only absolute prerequisite for ES ionization is that the analytes of interest be soluble in some solvent. The ability of ES to work with liquid sample introduction techniques has launched it into prominence as one of the most important detectors for high-pressure liquid chromatography and capillary zone electrophoresis (Covey, 1995).

Tandem mass spectroscopy (MS-MS) allows for the formation of low-energy collisionally induced dissociation fragments (Hutton and Major, 1995). Individual molecules are selected by the first quadrupole mass analyzer and fragmented in the collision cell using a suitable gas, usually argon, and their fragments are detected by the second quadrupole mass analyzer (Hutton and Major, 1995).

In this study we evaluate the use of ES-MS and MS-MS as tools for anthocyanin characterization and potentially for monitoring their presence and detecting the presence of adulterants in colored mixtures.

MATERIALS AND METHODS

Pigment Material. Radishes and red-fleshed potatoes were grown at the OSU Lewis-Brown horticultural farm (Corvallis, OR). Anthocyanin pigments from radish (*Raphanus sativus*) epidermal tissue and from red-fleshed potatoes (*Solanum tuberosum*) were extracted following the procedures described by Giusti and Wrolstad (1998). Frozen radish epidermal tissue was liquid nitrogen powdered by using a stainless steel Waring blender. Powdered samples were blended with 1 volume of acetone and filtered on a Buchner funnel using Whatman #1 paper. For red-fleshed potatoes, frozen slices were directly blended with 1 volume of acetone and filtered as described. The filter cake residue was re-extracted with aqueous acetone

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(30:70 v/v) until a clear solution was obtained. Filtrates were combined, shaken in a separatory funnel with 2 volumes of chloroform, and stored overnight at 1 °C. The aqueous portion was collected and placed on a Büchi rotavapor at 35 °C until all residual acetone was evaporated (5−10 min) and brought to a known volume with distilled water.

Other juice concentrates and anthocyanin extracts were obtained from their manufacturers: Red cabbage (*Brassica oleracea*) extract from Warner Jenkinson (St. Louis, MO); chokeberry (*Aronia melanocarpa*) dried concentrate from Artemis International (Fort Wayne, IN); and Concord grape (*Vitis labrusca*) juice concentrate from Welch's (Westfield, NY). Roselle (*Hibiscus sabdariffa* L.) dried powder extract was supplied by the Food Technology Center, Malaysian Agricultural Research and Development Institute (Kuala Lumpur, Malaysia).

Dried powders were dissolved in distilled water, and juice concentrates were diluted to single strength juice.

Anthocyanin Purification. Anthocyanin-containing solutions were passed through a C-18 Sep-Pak cartridge (Waters Assoc., Milford, MA), previously activated with methanol followed by 0.01% aqueous HCl (Hong and Wrolstad, 1990). Anthocyanins (and other phenolics) were adsorbed onto the mini-column; sugars, acids, and other water-soluble compounds were removed with 2 volumes of 0.01% aqueous HCl. Anthocyanins were separated from other phenolics using a modification of the procedure described by Oszmianski and Lee (1990). The non-anthocyanin phenolics were washed away from the column using 2 volumes of ethyl acetate, and anthocyanins were subsequently eluted with methanol containing 0.01% HCl (v/v). The methanolic extract was then concentrated using a Büchi rotavapor at 35 °C, and pigments were dissolved in distilled deionized water containing 0.01% HCl.

High-Performance Liquid Chromatograph (HPLC). *Apparatus.* A high-performance liquid chromatograph Perkin-Elmer Series 400, equipped with a Hewlett-Packard 1040A photodiode array detector, Gateway 2000 P5−90 computer with a Hewlett-Packard HPLC2D ChemStation software and a Beckman 501 autosampler with a 50 μL loop was used, with simultaneous detection at 280, 310, and 520 nm. The spectra (detection wavelengths from 250−600 nm) were recorded for all peaks.

Columns and Mobile Phase. A Supelcosil LC-18 column (5 μm), 250 × 5 mm i.d. (Supelco, Inc., PN), fitted with an ODS-10, 4 cm × 4.6 mm i.d., Micro-Guard column (Bio-Rad Laboratories), was used. The solvents used were (A) 100% HPLC-grade acetonitrile and (B) 1% phosphoric acid (concentrated), 10% acetic acid (glacial), 5% acetonitrile (v/v/v) in water. Flow rate: 1 mL/min. Solvents and samples were filtered through a 0.45 μm Millipore filter type HA (Millipore Corp., Bedford, MA).

HPLC Conditions for Anthocyanin Separation and Isolation. Anthocyanins were separated using a gradient from 0% to 30% A in 30 min. Peak assignments were made based on published literature and spectral data.

Electrospray Mass Spectroscopy. Low-resolution MS was done using electrospray MS. The instrument was a Perkin-Elmer SCIEX API III+ mass spectrometer equipped with an ion spray interface (ISV = 4700, orifice voltage of 80). The mass spectrometer was operated in the positive-ion mode. Samples were introduced into the ES-MS by loop injection (5 μL injection loop) dissolved in distilled water or HPLC-grade methanol containing 0.01% HCl into a flow stream of 1:1 acetonitrile:water with 0.1% TFA. Purified anthocyanins were injected directly into the system.

Tandem Mass Spectrometry (MS-MS). Collision-induced dissociation (CID) experiments were carried out using argon as the target gas. The mass of the parent ion of interest was scanned in the first quadrupole (Q1), *m/z* selected and collisionally activated in Q2, and the daughter ions were analyzed in the third quadrupole (Q3). MS-MS was performed using different voltages ranging from 15 to 30 eV to compare the fragmentation pattern of the targeted anthocyanins at different energy levels.

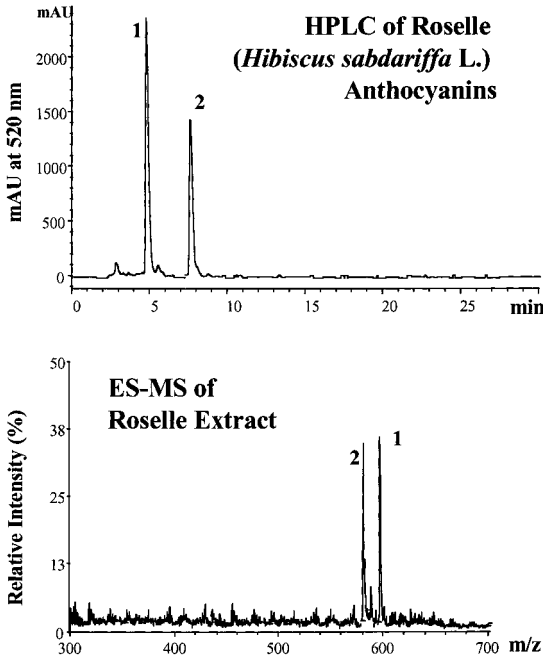

Figure 1. HPLC and ES-MS separation of Roselle (*Hibiscus sabdariffa* L.) dried powder extract anthocyanins. Tentative peak assignments: (1) dpd-3-xyl-glu, (2) cyd-3-xyl-glu.

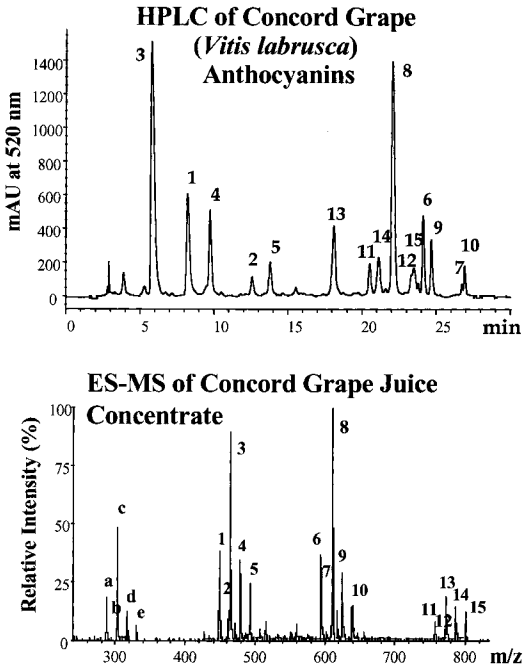

Figure 2. HPLC and ES-MS separation of Concord grape (*Vitis labrusca*) juice concentrate anthocyanins. Tentative peak assignments: (a) cyd, (b) pnd, (c) dpd, (d) ptd, (e) mvd, (1−5) their respective 3-glucosides, (6−10) their respective 3-glucosides acylated with *p*-coumaric acid, (11−15) their respective diglucosides acylated with *p*-coumaric acid.

RESULTS AND DISCUSSION

HPLC Anthocyanin Profile. HPLC analysis of the anthocyanin pigments of fruits and vegetables is very useful in characterization and authenticity testing since pigment profiles are quite distinctive for different commodities (Figures 1−5).

Different sources of anthocyanins were used for this study, including approved natural colorants and anthocyanin extracts that are being proposed as natural

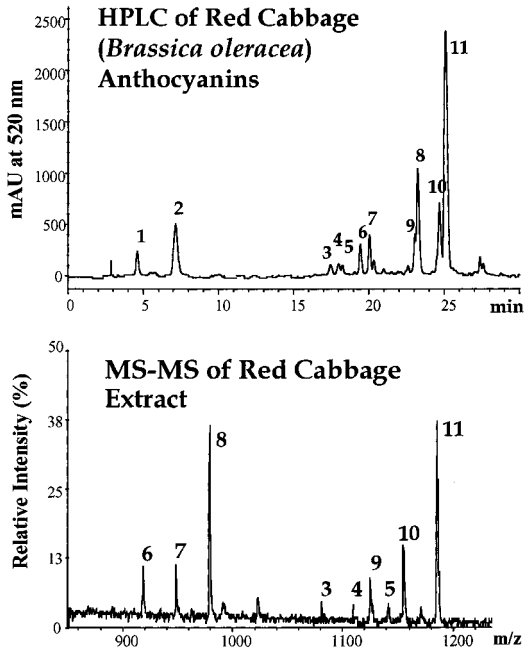

Figure 3. HPLC and ES-MS separation of red cabbage (*Brassica oleracea*) extract anthocyanins. Tentative peak assignments: (1) cyd-3-diglu-5-glu, (2) cyd-3-glu-5-glu, (3) cyd-3-triglu-5-glu acylated with *p*-coumaric acid, (4) cyd-3-triglu-5-glu acylated with ferulic acid, (5) cyd-3-triglu-5-glu acylated with sinapic acid, (6) cyd-3-diglu-5-glu acylated with *p*-coumaric acid, (7) cyd-3-diglu acylated with ferulic acid, (8) cyd-3-diglu-5-glu acylated with sinapic acid, (9) cyd-3-diglu-5-glu acylated with *p*-coumaric and sinapic acids, (10) cyd-3-diglu-5-glu acylated with ferulic and sinapic acids, (11) cyd-3-diglu-5-glu acylated with two sinapic acids.

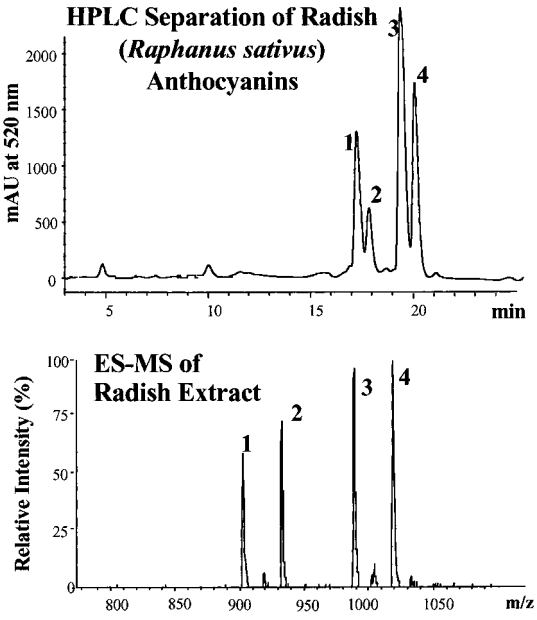

Figure 4. HPLC and ES-MS separation of radish (*Raphanus sativus*, L.) anthocyanins. (1) pgd-3-soph-5-glu acylated with *p*-coumaric acid, (2) pgd-3-soph-5-glu acylated with ferulic acids, (3) pgd-3-soph-5-glu acylated with p-coumaric and malonic acid, (4) pgd-3-soph-5-glu acylated with ferulic and malonic acids.

colorants. Peak assignments were made based on available literature on these pigments and data obtained from HPLC, ES-MS, and MS-MS. The anthocyanin profiles ranged from the simple, in the case of Roselle, with only two non-acylated major peaks (Pouget et al., 1990), to the more complex picture of Concord grape,

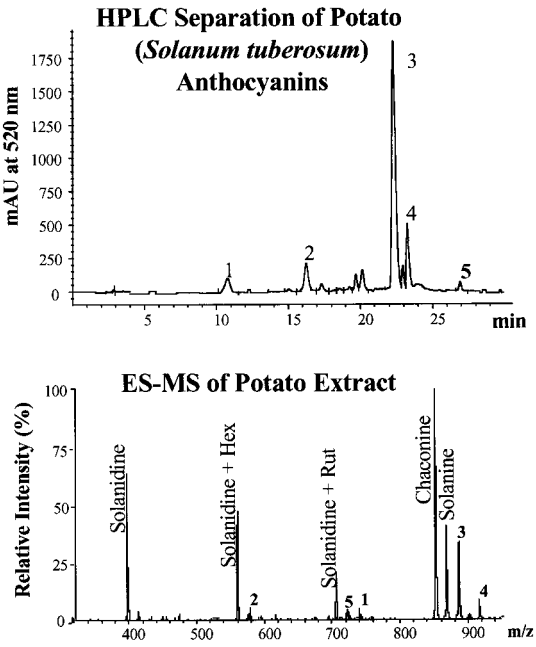

Figure 5. HPLC and ES-MS separation of red-fleshed potato (*Solanum tuberosum*) anthocyanins. (1) pgd-3-rut-5-glu, (2) pgd-3-rut, (3) pgd-3-rut-5-glu, (4) pgd-3-rut-5-glu acylated with ferulic acid, (5) pgd-3-rut-5-glu acylated with *p*-coumaric acid.

with the presence of five different anthocyanidins, and with both acylated and non-acylated pigments (Wrolstad et al., 1995; Baublis et al., 1994). We also evaluated red cabbage, with a complex anthocyanin profile that shows different acylating patterns with only one anthocyanidin group (Idaka, 1987; Baublis et al., 1994; Ichi et al., 1996). Radish and red-fleshed potatoes have been extensively studied in our laboratory (Giusti and Wrolstad, 1996, 1998; Rodríguez-Saona et al., 1998) and have been proposed as potential natural food colorants.

Depending on the HPLC conditions and the complexity of the anthocyanin profile, the length of the experimental run may range from several minutes to up to an hour. Usually, for practical reasons, the approach is to develop a systematic methodology that could be applied to a wide variety of different commodities under the same experimental conditions, and that implies the use of a longer HPLC program and in many cases also implies a decrease in peak resolution. Even then, a 30−60 min duration run is relatively short and easy compared to former methodologies applied for the separation of anthocyanin such as paper chromatography or thin-layer chromatography (TLC). For separation of commodities ranging from non-acylated to acylated anthocyanins, we developed a 30 min program that could also allow for separation of the six aglycons commonly found in nature.

A limitation of the HPLC methodology is that the retention times and resolution obtained will be very dependent on the instrument and conditions used, and changes in mobile-phase composition may result in drastic changes in separation and even in the elution order of the pigments. In the case of red cabbage, the literature agrees on the presence of cyanidin derivatives but there is no agreement on the number of pigments present, ranging from 6 to 15 anthocyanins reported. The use of a photodiode array detector allows for the analysis of spectral characteristics that give information about acylation and glycosylation patterns (Hong and Wrolstad, 1990). However, this method will not be able

Table 1. Molecular Weights of Anthocyanidins, Anthocyanins, and Acylating Groups Commonly Found in Nature[a]

anthocyanidins	pelargonidin	cyanidin	peonidin	delphinidin	petunidin	malvidin
	271	287	301	303	317	331
hexose (hex)	180.2	180.2	180.2	180.2	180.2	180.2
hex−H_2O	162.2	162.2	162.2	162.2	162.2	162.2
Acd + 1hex	433.2	449.2	463.2	465.2	479.2	493.2
Acd + 2 hex	595.4	611.4	625.4	627.4	641.4	655.4
Acd + 3 hex	757.6	773.6	787.6	789.6	803.6	817.6
pentose (pent)	150.0	150.0	150.0	150.0	150.0	150.0
pent−H_2O	132.0	132.0	132.0	132.0	132.0	132.0
Acd + pent	403.0	419.0	433.0	435.0	449.0	463.0
Acd + 1hex + 1pent	565.2	581.2	595.2	597.2	611.2	625.2
rhamnose	164.2	164.2	164.2	164.2	164.2	164.2
rutinose	326.2	326.2	326.2	326.2	326.2	326.2
rutinose−H_2O	308.2	308.2	308.2	308.2	308.2	308.2
Acd + rutinose	579.2	595.2	609.2	611.2	625.2	639.2
Acd + rutinose + 1hex	741.4	757.4	771.4	773.4	787.4	801.4
Acd + rutinose + 1pent	711.2	727.2	741.2	743.2	757.2	771.2
			Common Acylations			
p-coumaric acid	164.2	164.2	164.2	164.2	164.2	164.2
−H_2O	146.2	146.2	146.2	146.2	146.2	146.2
caffeic acid	180.2	180.2	180.2	180.2	180.2	180.2
−H_2O	162.2	162.2	162.2	162.2	162.2	162.2
ferulic acid	194.2	194.2	194.2	194.2	194.2	194.2
−H_2O	176.2	176.2	176.2	176.2	176.2	176.2
sinapic acid	224	224	224	224	224	224
−H_2O	206	206	206	206	206	206
acetic acid	82	82	82	82	82	82
−H_2O	64	64	64	64	64	64
propionic acid	96.1	96.1	96.1	96.1	96.1	96.1
−H_2O	78.1	78.1	78.1	78.1	78.1	78.1
malonic acid	104.1	104.1	104.1	104.1	104.1	104.1
−H_2O	86.1	86.1	86.1	86.1	86.1	86.1
succinic acid	118.1	118.1	118.1	118.1	118.1	118.1
−H_2O	100.1	100.1	100.1	100.1	100.1	100.1

[a] Acd, anthocyanidin; hex, hexose; pent, pentose.

to discriminate among pigments with similar retention times if they have similar spectral characteristics. That was the case with radish anthocyanins, where we separated four pigments but only two different patterns of spectral characteristics were found, suggesting the presence of isomeric forms of the same pigments. We used mass spectroscopic analysis as an additional tool for anthocyanin characterization (Giusti and Wrolstad, 1996).

ES-MS Anthocyanin Profile. ES-MS proved to be a powerful method for anthocyanin identification. Figures 1−5 show a comparative pigment profile obtained with HPLC and ES-MS. No HPLC separation was performed prior to MS analyses, and sample purification was the same used for HPLC, using C-18 Sep-Pak cartridge purification with or without the use of ethyl acetate as a sequential eluting solvent.

ES-MS uses low voltage and atmospheric pressure and is very versatile as an ionization technique. Anthocyanins are positively charged at low pH values and are very soluble in water and alcohol, with molecular weights ranging from a few hundreds to a few thousands for simple (e.g., pelargonidin-3-glucoside (Pgd-3-glu), from strawberries, with MW 433) and polyacylated ones (e.g., acylated anthocyanins in *Clitoria ternatea* flowers, MW 2107, reported by Terahara et al., 1990), respectively. These characteristics of anthocyanins would make it difficult to work with methodologies that require volatility of the compounds. ES-MS delivers the sample in a liquid phase and sprays it into a chamber where a dry gas flows opposite to the mist and a low voltage is applied, causing the disintegration of the drops into charged droplets which become smaller as the solvent vaporizes (Hesse et al., 1997). In that way ES-MS allows analysis of polar compounds from aqueous solution without derivatization (Black and Fox, 1995). Molecules that are inherently charged in solution by virtue of their chemical structure, weak association with other charged species, or by chemical reactions occurring in the solution eventually leave the liquid phase and become gas-phase ions in the atmospheric region of the ion source (Covey, 1995). The positive charge of anthocyanins at low pH values permits their easy detection using low voltages since other potentially interfering compounds are usually not ionized.

A chromatogram with only the base peak for every mass spectrum provides more readily interpretable data because of fewer interference peaks (Sagesser and Deinzer, 1996). ES-MS produces primarily intact molecular ions, although fragmentation can be accomplished by varying ionization conditions (Black and Fox, 1995). Under the conditions used in this experiment, clean and clear profiles were obtained, with the presence of intact molecular ions and very little fragmentation.

Molecular weights of common anthocyanins and typical glycosylating and acylating groups are presented in Table 1.

The ES-MS of Roselle clearly showed the molecular ions for the two major anthocyanins (Figure 1). The *m*/*z* of 580.8 and 596.8 corresponded to cyanidin-3-xylosyl-glucoside (cyd-3-xyl-glu) and delphinidin-3-xylosyl-glucoside (dpd-3-xyl-glu), as reported in the literature (Pouget et al., 1990).

In the case of Concord grape, the anthocyanin profile was very complex due to the presence of five different aglycons, malvidin (mvd), cyanidin (cyd), peonidin (pnd), petunidin (ptd), and delphinidin (dpd), and different patterns of substitution. Good HPLC resolution is

usually difficult to obtain; however, ES-MS highly simplified the results because of the similar pattern of substitutions for all anthocyanidins. Figure 2 clearly showed the pattern of anthocyanin distribution in Concord grapes. Peak assignments were done using as a reference the results reported by Baublis et al. (1994), but using the ES-MS information we were able to make further tentative assignments of some minor peaks not found or identified by those researchers. The first group of molecular ion peaks (Figure 2) corresponded to the five aglycons that were probably produced by the ionization process. A second group corresponded to the five non-acylated monoglucosylated anthocyanins, followed by five monoacylated pigments, corresponding to the 3-glucoside-*p*-coumarates of mvd, cyd, ptd, and dpd reported by Baublis et al. (1994) and the 3-glucoside-p-coumarate of peonidin. In addition, five other peaks were found and tentatively assigned as their corresponding diglucosylated-*p*-coumarate pigments.

Red cabbage ES-MS gave *m*/*z* ratios corresponding to the different cyanidin derivatives reported for this commodity (Ichi et al., 1996; Mazza and Miniati, 1993), with the presence of acylated and non-acylated pigments (Figure 3).

In the case of radish anthocyanins (Figure 4), HPLC coupled with a photodiode array detector separated the four major pigments but did not detect spectral differences between peaks 1 and 3 and peaks 2 and 4. It was only after ES-MS that we were able to detect the presence of an additional acylating group on peaks 3 and 4, malonic acid, as evidenced by the additional molecular weight of 86 units (Giusti and Wrolstad, 1996).

ES-MS may also be useful to detect other charged molecules in the sample. That was the case with potato extracts (Figure 5), where the ES-MS revealed the presence of toxic positively charged alkaloids (Rodríguez-Saona et al., 1999). The clean profile obtained with intact molecular ions facilitated the process of anthocyanin and alkaloid identification.

The use of mass spectroscopic analyses for characterization of anthocyanins has been increasing over the past decade, with most of those reports using HPLC coupled to a MS detector or isolating individual pigments prior to the mass spectroscopic analysis (Ichi et al., 1996; Baublis et al., 1994; Bakker et al., 1997; Saito et al., 1996; Shi et al., 1993). The ionization technique more often reported has been fast atom bombardment (FAB), and reports on the use of ES-MS on anthocyanins have been limited.

Some advantages of the use of ES-MS ionization and the direct injection of the anthocyanin extracts on the mass spectrometer include the simple and easy sample preparation, short run time (a few minutes), very consistent anthocyanin profiles, and generation of results which are more reproducible since no column separation or mobile-phase gradients are required, eliminating the variability due to those sources.

Mass spectroscopic techniques do not differentiate between different diastereoisomeric forms of sugars, and therefore, it will not provide information regarding the exact glycosidic substitution other than the number of carbons or presence of methylations in the sugars. That is the case for chokeberry anthocyanins (Figure 6), where the ES-MS only showed two peaks corresponding to two different anthocyanins each: cyd-3-arabinoside and cyd-3-xyloside for one and cyd-galactoside and cyd-glucoside for the other. This could represent a limitation in some instances; however, it could become an advantage when analyzing samples with a very complex anthocyanin pattern for authenticity by simplifying the spectra obtained.

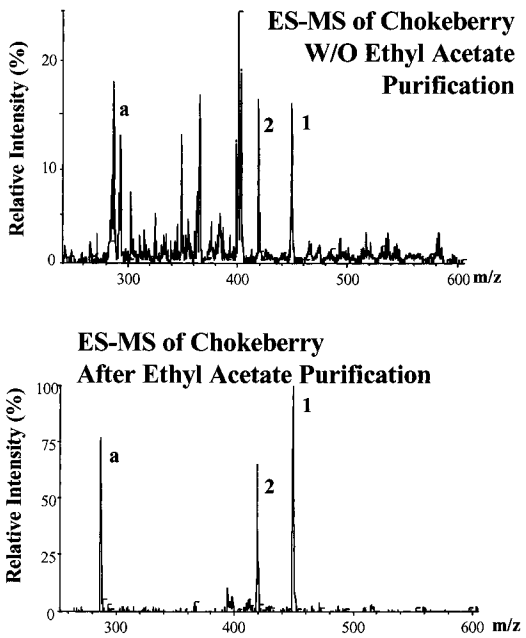

Figure 6. ES-MS spectra of chokeberry (*Aronia melanocarpa*) anthocyanins with and without the use of ethyl acetate purification. Tentative peaks assignment: (a) cyd, (1) cyd-3-arab and cyd-3-xyl, (2) cyd-3-gal and cyd-3-glu.

Utility of Ethyl Acetate Purification. Sample purification using C-18 cartridges is a very simple and fast procedure that provides efficient separation of aromatic compounds in only a few minutes. Sugars and acids have no affinity for the column and can be washed away by passing the sample through the C-18 cartridge in an aqueous solution. Anthocyanins, phenolics, and other compounds with a nonpolar character will be separated from other compounds due to their affinity to the matrix. These compounds can be recovered using methanol. However, the use of ethyl acetate as an intermediate step will remove from the column the less polar compounds such as phenolic acids and flavonoids, separating them from anthocyanins, our target compounds, which are subsequently recovered with acidified methanol.

Figure 6 shows an example of the drastic improvement in the ES-MS obtained when ethyl acetate is used as compared to the simpler C-18 procedure. Adding the ethyl acetate step does not add much time to the purification procedure but contributes substantially to generating a clean MS output. According to our experiments, samples with anthocyanins with multiple glycosylations or acylations ($m/z > 600$) did not show much of a problem in the absence of ethyl acetate purification, since most interfering peaks appeared at low *m*/*z* ranges. However, the use of ethyl acetate would still be recommended since it does not take much time and could allow for the detection of lower molecular weight anthocyanins present.

Tandem Mass Spectrometry. Tandem mass spectrometry experiments, also known as MS-MS, use two mass analyzer regions within the same instrument for the collision-induced decomposition of a chosen mass-

Table 2. Fragmentation Pattern of Different Anthocyanins Submitted to Tandem MS[a]

peak	identification	fragments
		Roselle (*Hibiscus sabdariffa* L.)
1	Dpd-3-xyl-glu	597.2 (M^+), 303.2 (dpd)
2	Cyd-3-xyl-glu	581.2 (M^+), 287.2 (cyd)
		Concord Grape
2	Pnd-3-glu	462.8 (M^+), 301.2 (pdn)
3	Dpd-3-glu	465.2 (M^+), 303.2 (dpd)
6	Cyd-3-glu + *p*-coumaric	595.2 (M^+), 287.2 (cyd)
7	Dpd-3-glu + *p*-coumaric	610.8 (M^+), 303.2 (dpd)
9	Ptd-3-glu + *p*-coumaric	624.8 (M^+), 317.2 (ptd)
13	Dpd-3-glu-5-glu + *p*-coumaric	773.2 (M^+), 610.8 (M^+ − glu), 464.8 (dpd−glu), 303.2 (dpd)
14	Ptd-3-glu-5-glu + *p*-coumaric	787.2 (M^+), 624.8 (M^+ − glu), 478.8 (ptd−glu), 317.2 (ptd)
15	Mvd-3-glu-5-glu + *p*-coumaric	801.2 (M^+), 639.2 (M^+ − glu), 492.8 (mvd−glu), 331.2 (mvd)
		Red Cabbage
6	Cyd-3-di-glu-5-glu + sinapic	979.6 (M^+), 817.6 (M^+ − glu), 449.2 (cyd−glu), 287.2 (cyd)
10	Cyd-3-di-glu-5-glu + ferulic	1155.2 (M^+), 993.6 (M^+ − glu), 449.2 (cdn−glu), 287.2 (cyd)
11	Cyd-3-di-glu-5- (2) sinapic	1185.6 (M^+), 1023.6 (M^+ − glu), 449.2 (cyd−glu), 287.2 (cyd)
		Red Radish
1	Pgd-3-soph-5-glu	757.2 (M^+), 595.2 (M^+ − glu), 433.2 (pgd−glu), 271.2 (pgd)
2	Pgd-3-soph-5-glu + *p*-coumaric	903.2 (M^+), 741.2 (M^+ − glu), 433.2 (pgd−glu), 271.2 (pgd)
3	Pgd-3-soph-5-glu + ferulic	933.2 (M^+), 771.2 (M^+ − glu), 433.2 (pgd−glu), 271.2 (pgd)
4	Pgd-3-soph-5-glu + *p*-coumaric and malonic acids	989.6 (M^+), 741.2 (M^+ − glu−mal, 518.8 (pgd−glu−mal), 271.2 (pgd)
5	Pgd-3-soph-5-glu + ferulic and malonic acids	1019.2 (M^+), 771.2 (M^+ − glu−mal), 518.8 (pgd−glu−mal), 271.2 (pgd)
		Red-Fleshed Potato
1	Pgd-3-rut-5-glu	741.4 (M^+), 578.8 (M^+ − glu), 433.2 (pgd−glu), 271.2 (pgd))
2	Pgd-3-rut	579.2 (M^+), 433.2 (M^+ − rham), 271.2 (pgd)
3	Pgd-3-rut-5-glu + *p*-coumaric	887.2 (M^+), 725.2 (M^+ − glu), 433.2 (pgd−glu), 271.2 (pgd)
4	Pgd-3-rut-5-glu + ferulic	919.6 (M^+), 755.2 (M^+ − glu), 433.2 (pgd−glu), 271.2 (pgd)
5	Pgd-3-rut + *p*-coumaric	725.4 (M^+), 433.2 (pgd−glu), 271.2 (pgd)

[a] Pgd, pelargonidin; cyd, cyanidin; pnd, peonidin; dpd, delphinidin; ptd, petunidin; mvd, malvidin; xyl, xyloside; glu, glucoside; soph, sophoroside; rut, rutinoside; +*p*-coumaric, +ferulic, +sinapic, acylated with *p*-coumaric, ferulic, or sinapic acids, respectively.

to-charge ratio ion. The first region is used as a separation device before inducing fragmentation in a collision cell followed by analysis of the fragment ion in the second (McLafferty and Turecek, 1993; Jennings, 1996; Lawson et al., 1996). Ions of a chosen mass-to-charge ratio (*m*/*z*) generated in the ionization process collide with an inert gas, leading to the formation of dissociation fragment ions (Jennings, 1996) in an energy transfer normally thought as involving primary vibrational energy transfer. This process provides structural information on the components of a mixture, which lead to the unambiguous identification of fragmentation pathways (Lawson et al., 1996).

MS-MS of individual peaks was performed (Table 2) and resulted in clear and characteristic fragmentation patterns. All glycosidic substitutions at positions C3 and C5 of the pyrilium ring were cleaved. The MS-MS resulted in cleavage of glycosidic bonds only between the flavylium ring and the sugars directly attached to it. Tandem MS of 3,5-glycosylated anthocyanins produced fragments that corresponded to the aglycon, the C3-substituted anthocyanins, and the C5-substituted anthocyanins, in addition to the molecular parent ion. In Figure 7 the cleavage of non-acylated Pg-3-soph-5-glu produced the fragments corresponding to Pg-3-soph, Pg-5-glu, and Pgd.

In the case of acylated anthocyanins (Figure 8), this fragmentation pattern may allow for a rough determination of the location of the acylating groups. In the case of radish anthocyanins, the fragments produced are consistent with the presence of one cinnamic acid acylating group attached to the C3 glycosidic substituent while the other, malonic acid, is attached to the sugars of C5. In the case of red cabbage anthocyanins, both acylating groups are attached to the sugars of C3,

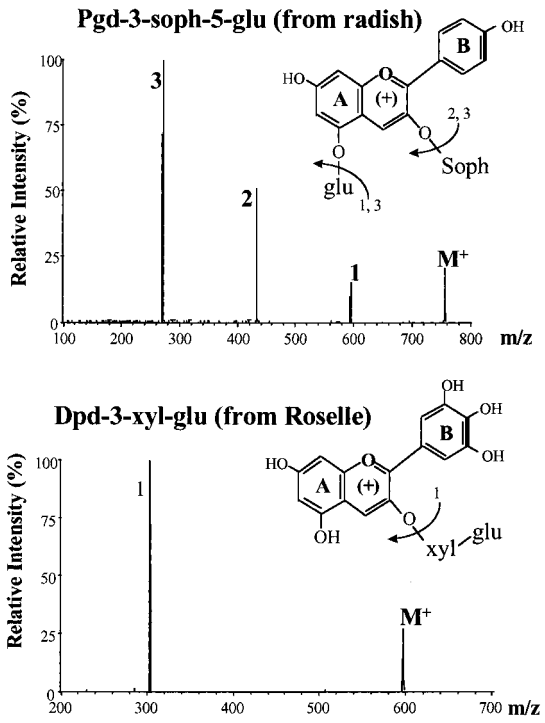

Figure 7. Fragmentation pattern of non-acylated anthocyanins, e.g., pgd-3-soph-5-glu and dpd-3-xyl-glu.

resulting in the formation of a large fragment (*m*/*z* 1023.6) corresponding to a fragment with the two acylating groups and other fragments corresponding to the aglycon and the monoglucosylated cyanidin. This same pattern of fragmentation was evidenced with most anthocyanins analyzed (Table 2).

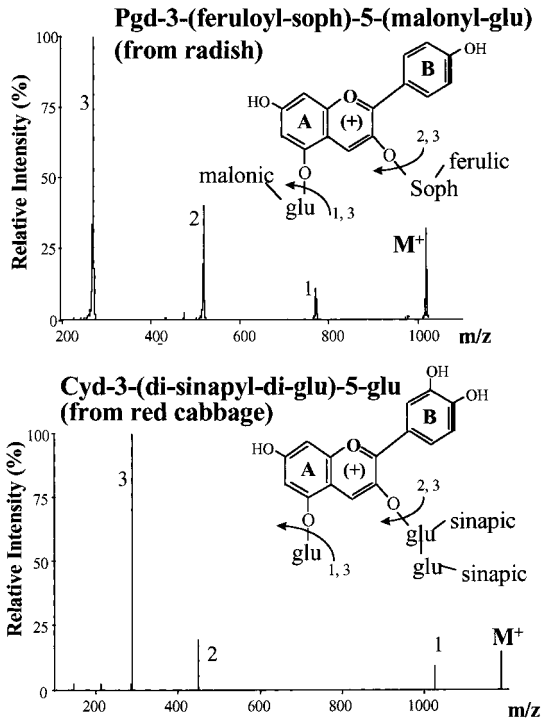

Figure 8. Fragmentation pattern of acylated anthocyanins, e.g., pgd-3-(feruloyl-soph)-5-(malonyl-glu) and cyd-3-(di-sinapyl-di-glu)-5-glu.

The fragmentation of C3-substituted anthocyanins produced only one fragment, corresponding to the *m*/*z* of the aglycon, and that was the case for the diglycosylated anthocyanins of Roselle. Pigments 1 and 2 are reported as dpd-3-sambubioside and cyd-3-sambubioside, and the fragments obtained with MS-MS were only dpd and cyd, respectively (Table 2 and Figure 7). The only exceptions to this pattern were the C3-substituted anthocyanins from potato, reported as pgd-3-rutinoside (pgd-3-rut) and pgd-3-rut acylated with *p*-coumaric acid. The linkage 1−6 between rhamnose and glucose, which form rutinose, allows for free rotation and more accessibility of the gas used to produce the fragmentation. In the case of the other anthocyanins analyzed, the disaccharides present were either sambubiose (xylose 1−2 glucose) or sophorose (glucose 1−2 glucose) with more stable sugar bonds, and tandem spectroscopy analysis did not cause its cleavage at that bond.

No cleavage of ester linkages was obtained with MS-MS on any of the acylated anthocyanins analyzed (Table 2).

The internal energy of the fragmenting ion is determined both by the initial ionization process and by the internal energy gained in the collision, and the reproducibility of the experiment depends on the control of the experimental conditions (Jennings, 1996). When experimental conditions are controlled, differences of MS-MS spectra are indicative of differences in the structures of the ions undergoing collision. Different voltages were used to evaluate the fragmentation pattern with MS-MS. Under the conditions used for this study, high reproducibility was obtained and the fragmentation patterns obtained with anthocyanins were always the same, regardless of the energy used for the experiment (in the range 15−25 eV). The only variations found were on the proportions at which these fragments were present. The use of energies higher than 25 eV (30 eV) resulted in complete fragmentation of the parent ion, with the production of only one fragment corresponding to the *m*/*z* of the aglycon.

The coupling of reversed-phase liquid chromatography to MS allows the molecules to be characterized by retention time, UV−vis response, and mass spectral information for the individual components and fragments (Hutton and Major, 1995) and is becoming a technique gaining popularity over the last few years. The development of electrospray ionization as a sensitive technique, as well as a specific and versatile detector for liquid sample introduction, has contributed enormously to the establishment of liquid chromatography, LC-MS, as an analytical technique for mixture analysis (Gaskell, 1996). ES-MS allows analysis of polar compounds from aqueous solution without derivatization (Black and Fox, 1995), which makes it well-suited for in-line analysis in conjunction with liquid chromatography. Combined information of HPLC−UV−vis spectra and mass spectra is a very powerful tool for anthocyanin identification and characterization.

Our findings indicate that the use of ES-MS and MS-MS are powerful and rapid techniques for screening and characterization of anthocyanins in samples where some background information is available or where a first compositional overview is required. Electrospray mass spectroscopy may be used as a fast procedure for identification of anthocyanins, requiring minimal sample preparation. ES-MS was fast and effective, detecting molecular ions of anthocyanins and anthocyanin extracts. Tandem mass spectroscopy has extraordinary capabilities for the structural analysis of individual components of complex mixtures (Gaskell, 1996), with the first MS working as the separation device and the second one used for chemical structure determination, and showed to be very effective for structural analysis of anthocyanins.

In combination with HPLC, ES-MS and MS-MS could be useful tools for monitoring the authenticity of anthocyanin-containing fruit juices and vegetable extracts.

ABBREVIATIONS USED

Pgd, pelargonidin; cyd, cyanidin; pnd, peonidin; dpd, delphinidin; ptd, petunidin; mvd, malvidin; acd, anthocyanidin; hex, hexose; pent, pentose; xyl, xyloside; glu, glucoside; soph, sophoroside; rut, rutinoside. HPLC, high-performance liquid chromatography; ES-MS, electrospray mass spectroscopy; MS-MS, tandem mass spectroscopy.

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