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Characterization of Anthocyanins in Grape Juices by Ion Trap Liquid Chromatography−Mass Spectrometry

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A reverse phase HPLC and electrospray interface with ion trap mass spectrometer method was developed for the characterization of anthocyanins in Concord, Rubired, and Salvador grape juices. Rubired and Salvador grapes are hybrids from *Vitis vinifera* and *Vitis rupestris*. Concord grape is a grape from the native American cultivar *Vitis labrusca*. Individual anthocyanins in these three varieties were identified on the basis of UV−vis and MS spectra and further elucidated by MS/MS spectra. Anthocyanins in Salvador and Concord grapes were 3-*O*-glucosides, 3-*O*-(6″-*O*-*p*-coumaroyl)-glucosides, 3-*O*-(6″-*O*-*p*-acetyl)glucosides, 3,5-*O*-diglucosides, and 3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin. Vitisin B was detected in Salvador grape juice. Anthocyanins in Rubired grape juice were primarily anthocyanin diglucosides: peonidin 3,5-*O*-diglucoside, malvidin 3,5-*O*-diglucoside, peonidin 3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside, and malvidin 3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside are the four major anthocyanins. The presence of pelargonidin 3-*O*-glucoside, not previously reported, has been established for the first time in all three juices.

KEYWORDS: LC-MS/MS; Concord; Salvador; Rubired; grape juice; *Vitis vinifera*; *V. rupestris*; *V. labrusca*; anthocyanin; pelargonidin 3-*O*-glucoside

INTRODUCTION

Anthocyanins constitute a large family of polyphenols in plants and are responsible for many of the fruit and floral colors observed in nature. Anthocyanins were previously characterized by one- and two-dimensional paper chromatography (*1*). Subsequently, reverse phase HPLC coupled with UV−vis detection had been the standard method for the analysis of anthocyanins (*2*−*6*). Recently, the methodology evolved to coupling of HPLC with mass spectrometry for anthocyanin characterization (*7*−*9*). HPLC coupled with MS had become a very efficient tool for the characterization and elucidation of unknown and partially unknown anthocyanin pigments in fruits and vegetables (*10*−*12*).

There are several major grape species: *Vitis vinifera*, *V. labrusca*, *V. rotundifolia*, and *V. rupestris*. *V. vinifera* contains only anthocyanin monoglucosides. *V. labrusca*, *V. rotundifolia*, and *V. rupestris* contain mainly anthocyanin diglucosides (*13*). Rubired is a very dark grape variety for use in concentrate and port wine (*14*). It is derived from the cross between Tinto Cão and Alicante Ganzin. Tinto Cão is one of the most ancient varieties of *V. vinifera* in the Douro port wine region of Portugal. Alicante Ganzin, on the other hand, is an early French hybrid derived from the cross Aromon × *V. rupestris* (*14*). It is distinguished by abundant color having good stability and red hue. As a result, this gives Rubired a one-eighth *V. rupestris* parentage. The origin of Salvador also fits into the *vinifera*−*rupestris* hybrid category (personal communication with Jon Holmquist). Concord is a grape derived from a native American cultivar, *V. labrusca* (*13*).

Anthocyanin pigments from grape, especially *V. vinifera*, have been extensively studied. Anthocyanins were reported to be 3-glucosides, 3-acetylglucosides, 3-coumaroylglucosides, 3-caffeoylglucosides, 3,5-diglucosides, 3-acetyl-5-diglucosides, 3-coumaroyl-5-diglucosides, and 3-caffeoyl-5-diglucosides of cyanidin, delphinidin, peonidin, petunidin, and malvidin (*15*−*19*). Pelargonidin is the only major aglycon that has not previously been found in the grape. The structures and molecular weights of these six major aglycones are listed in **Figure 1**. Anthocyanins in Concord grape have been characterized as 3-glucosides, 3-coumaroylglucosides, and 3,5-diglucosides of delphinidin and cyanidin (*20*−*24*). In more recent work, anthocyanins in Concord grape were characterized by HPLC in combination with ES-MS and MS/MS by direct infusion (*25*). However, an individual peak from the HPLC chromatogram could not be assigned unambiguously due to lack of information of the molecular ion and product ion for that particular peak. Also, the ionization of some components of an anthocyanin mixture can be suppressed by ionization of others (*8*). Anthocyanins in Rubired grapes were analyzed by Smith and Luh (*26*). Eleven anthocyanins were elucidated by two-dimensional paper chromatography; the major anthocyanins were reported to be malvidin 3,5-diglucoside and peonidin 3,5-diglucoside. Anthocyanins in Salvador grape have not been previously reported.

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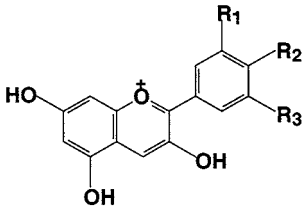

Aglycone	R_1	R_2	R_3	MW
Delphinidin	OH	OH	OH	303.2
Cyanidin	OH	OH	H	287.2
Petunidin	OH	OH	OCH_3	317.2
Pelargonidin	H	OH	H	271.2
Peonidin	OCH_3	OH	H	301.2
Malvidin	OCH_3	OH	OCH_3	331.2

Figure 1. Structure of aglycons of anthocyanins in grapes.

In the present work, the anthocyanin compositions of Rubired, Salvador, and Concord grape juice have been identified by HPLC coupled with a diode array spectrophotometer and ion trap mass spectrometer (LC/DAD/MS).

MATERIALS AND METHODS

Juice Samples. Rubired grape concentrate and Concord grape concentrate were provided by Canandaigua Concentrate (Madera, CA). Salvador concentrate was obtained from La Bordalesa, S.A. de C.V. (Aguascalientes, Ags., Mexico). Juice concentrates were diluted to single-strength juice at 16−20 °Brix. Single-strength juice was filtered through a 0.45 μm glass microfiber filter (GMF) syringe filter (Whatman Inc., Clifton, NJ) before injection onto the HPLC.

Standards. Cyanidin 3-glucoside and malvidin 3-glucoside were purchased from Indofine (Hillsborough, NJ). Malvidin 3,5-diglucoside was purchased from Aldrich (St. Louis, MO).

HPLC Analysis of Anthocyanins in Grape Juices. Five milliliters of Rubired, Concord, and Salvador grape juice was passed through a preconditioned C-18 Sep-Pak cartridge separately (Waters Associates). The adsorbed pigments were then washed with 5 mL of water and eluted by 2 mL of methanol. The eluate was stored at −20 °C prior to HPLC analysis.

HPLC/PDA Analysis (System A). The HPLC/PDA analyses were performed on a Waters 2690 Alliance separations module equipped with a 996 photodiode array detector, and data were collected on Millennium^{32} 3.2 software (Milford, MA). The column was a 250 cm × 4.6 mm i.d., 5 μm Prodigy ODS (3) (Phenomenex, Torrance, CA). Solvent A was acetic acid/phosphoric acid/acetonitrile/water (10:1:5:84, v/v/v/v), and solvent B was acetonitrile. Solvent gradient was 0−5 min, 0% B; 5−30 min, 0−10% B; 30−40 min, 10−30% B; and 40−45 min, 30−40% B at a flow rate of 1 mL/min. Detection wavelength was at 520 nm, and injection volume was 10 μL.

HPLC/DAD/ESI-MS/MS Analyses (Systems B and C). LC/ESI-MS/MS experiments were performed on an Agilent MSD SE ion trap mass spectrometer (Palo Alto, CA) equipped with an electrospray ionization (ESI) interface, 1100 HPLC, a DAD detector, and Chemstation software. The column used for system B was a 150 cm × 2.0 mm i.d., 3 μm Prodigy ODS (3) 100 Å (Phenomenex, Torrance, CA). Solvents were (A) 10% acetic acid/5% acetonitrile/85%water (v/v/v/v) and (B) acetonitrile. Solvent gradient was 0−5 min, 0% B; 5−30 min, 0−10% B; 30−40 min, 10−30% B; and 40−45 min, 30−40% B. Flow rate was 0.2 mL/min, injection volume was 3 μL, and column temperature was 25 °C. The column used for system C was a 150 cm × 2.0 mm i.d., 4 μm Synergi hydro-RP 80 Å (Phenomenex, Torrance, CA). Solvents were (A) 10% acetic acid/0.2% TFA/5% acetonitrile/84.8%water (v/v/v/v) and (B) acetonitrile. Solvent gradient was 0−30 min, 0−10% B; 30−40 min, 10−30% B; and 40−45 min, 30−40% B. Flow rate was 0.2 mL/min, injection volume was 3 μL, and column temperature was 25 °C. The ESI parameters were as follows: nebulizer, 30 psi; dry gas (N_2), 12 L/min; dry temperature, 350 °C; trap drive, 50; skim 1, 40 V; skim 2, −5.0 V; octopole RF amplitude, 150 Vpp; capillary exit, 103.4 V. The ion trap mass spectrometer was operated in positive ion mode scanning from *m*/*z* 150 to *m*/*z* 2000 at a scan resolution of 13000 amu/s. Trap ICC was 20000 units and maximal accumulation time was 200 ms. MS-MS was operated at a fragmentation amplitude of 1.2 V, and threshold ABS was 3,000,000 units.

RESULTS AND DISCUSSION

Anthocyanin pigments in Rubired, Salvador, and Concord grape juices were analyzed by HPLC using systems A−C. For all three of these juices, better separation was achieved under system A. Phosphoric acid in solvent system A was found to interfere with the ionization process due to formation of ion pairs in solution. To maximize ESI sensitivity, phosphoric acid was removed or replaced by trifluoroacetic acid (TFA) in systems B and C, respectively, for HPLC/MS analyses. A Synergi hydro-RP column was selected for mobile phase with lower pH application. Anthocyanins were eluted in the reverse phase condition in the order delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin. The polarity of the anthocyanins did not change independently from glycosylation or acylation of the aglycon, which was in agreement with previous reports (*9*, *27*). This permitted elucidation of individual anthocyanins in all three HPLC systems based on MS and MS/MS data from systems B and C.

By combination of DAD, MS, and MS/MS spectra, 23, 25, and 22 anthocyanins were elucidated from Concord, Salvador, and Rubired grape juices, respectively (**Figure 2**). Identification was achieved mainly by comparing the molecular ions and products ions of these anthocyanins with those available in the literature (*8*, *9*, *25*). Identification of malvidin 3,5-*O*-diglucoside, malvidin 3-*O*-glucoside, and cyanidin 3-*O*-glucoside was confirmed by comparison of commercially available anthocyanin standards. The molecular ions and product ions of anthocyanins in these three grape juices are summarized in **Table 1**. In Concord grape, the major anthocyanins were delphinidin 3-*O*-glucoside, cyanidin 3-*O*-glucoside, and delphinidin 3-*O*-coumaroylglucoside. The MS spectrum of peak **16** indicated that the peak had *m*/*z* 773.4, and the MS/MS spectrum showed that the ion at *m*/*z* 773.4 fragmented to three product ions at *m*/*z* 611.2 ($[M - C_6H_{10}O_5]^+$), 465.3 ($[M - C_{15}H_{16}O_7]^+$), and 303.3 ($[M - C_{15}H_{16}O_7 - C_6H_{10}O_5]^+$), corresponding to delphinidin 3-coumaroylglucose, delphinidin 5-glucose, and delphinidin, respectively. Peak 16 was thus established as delphinidin 3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside (**Figure 3A**). Peak 12, having a molecular ion at *m*/*z* 507.3 from the MS spectrum and one product ion at *m*/*z* 303.2 ($[M - C_8H_{12}O_6]^+$) from the MS/MS spectrum, indicated that the compound was delphinidin 3-*O*-(6″-*O*-*p*-acetyl)glucoside (**Figure 3B**). Peaks 22 and 23 were coeluted, and the extracted ion chromatogram (EIC) mass spectrum of the peak suggested two molecular ions at *m*/*z* 771.4 and 801.4. The MS/MS spectrum indicated the ion at *m*/*z* 771.4 (peak 22) fragmented to three product ions at *m*/*z* 609.3 ($[M - C_6H_{10}O_5]^+$), 463.3 ($[M - C_{15}H_{16}O_7]^+$), and 301.2 ($[M - C_{15}H_{16}O_7 - C_6H_{10}O_5]^+$), which corresponded to peonidin 3-coumaroylglucose, peonidin 5-glucose, and peonidin, respectively. Peak 22 was elucidated as peonidin 3-*O*-*p*-(6″-*O*-coumaroyl)-5-*O*-diglucoside. Similarly, the molecular ion at *m*/*z* 801.4 (peak 23) had three product ions at *m*/*z* 639.3 ($[M - C_6H_{10}O_5]^+$), 493.3 ($[M - C_{15}H_{16}O_7]^+$), and 331.3 ($[M - C_{15}H_{16}O_7 - C_6H_{10}O_5]^+$), which corresponded to malvidin 3-coumaroylglucose, malvidin 5-glucose, and malvidin, respectively. Peak 23 was assigned as malvidin 3-*O*-*p*-(6″-*O*-coumaroyl)-5-*O*-diglucoside. EIC also indicated that peak 8 and a peak at retention time 23.5 min had the same molecular ion at *m*/*z* 479.3 (**Figure 4A**,**B**). The MS/MS spectrum indicated peak 8 had a product ion at *m*/*z* 317.2 ($[M - C_6H_{10}O_5]^+$). The

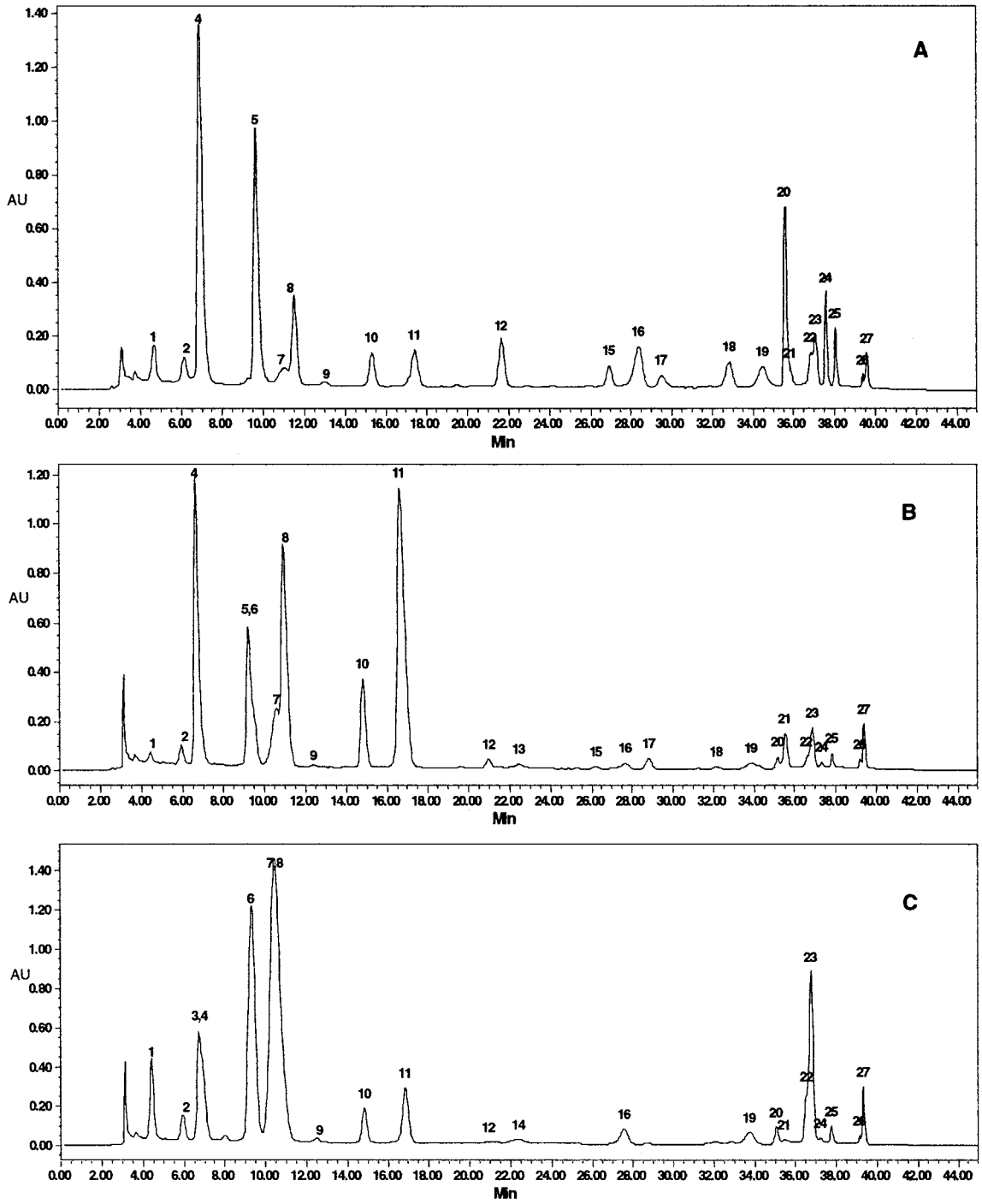

Figure 2. HPLC-DAD chromatogram of Concord (A), Salvador (B), and Rubired (C) juices at 520 nm under system A. Peaks: (1) delphinidin 3,5-diglucoside; (2) cyanidin 3,5-diglucoside; (3) petunidin 3,5-diglucoside; (4) delphinidin 3-glucoside; (5) cyanidin 3-glucoside; (6) peonidin 3,5-diglucoside; (7) malvidin 3,5-diglucoside; (8) petunidin 3-glucoside; (9) pelargonidin 3-glucoside (10) peonidin 3-glucoside; (11) malvidin 3-glucoside; (12) delphinidin 3-acetylglucoside; (13) vitisin B; (14) malvidin 3-acetyl-5-diglucoside; (15) cyanidin 3-acetylglucoside; (16) delphinidin 3-coumaroyl-5-diglucoside; (17) petunidin 3-acetylglucoside; (18) cyanidin 3-coumaroyl-5-diglucoside; (19) petunidin 3-coumaroyl-5-diglucoside; (20) delphinidin 3-coumaroylglucoside; (21) malvidin 3-acetylglucoside; (22) peonidin 3-coumaroyl-5-diglucoside; (23) malvidin 3-coumaroyl-5-diglucoside; (24) cyanidin 3-coumaroylglucoside; (25) petunidin 3-coumaroylglucoside; (26) peonidin 3-coumaroylglucoside; (27) malvidin 3-coumaroylglucoside.

UV−vis spectrum of this peak showed that the maximal absorbance was at 522 nm. From these two factors, peak 8 was elucidated as petunidin 3-*O*-glucoside. The MS/MS spectrum of a peak at retention time 23.5 min indicated that *m*/*z* 479.3 had a product ion at *m*/*z* 303.2 (**Figure 4C**). The ion at *m*/*z* 303.2 could be assigned to either delphinidin or quercetin moieties. Because no absorbance at ∼520 nm was observed in the UV−vis spectrum, the compound was presumed to be a flavonol rather than an anthocyanin. Because MS/MS cleaves only the glucosidic bonds between the flavylium ring and the sugars directly attached to it (*25*), this peak was elucidated as quercetin 3-*O*-glucuronide (**Figure 3C**), a compound reported before as a common flavonol in grape (*28*). Similarly, quercetin 3-*O*-glucuronide was also detected in Salvador grape juice.

The anthocyanin profile of Salvador grape juice was very similar to that of Concord. However, the major anthocyanin in Salvador grape juice was malvidin 3-*O*-glucoside, followed by delphinidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, cyanidin

Table 1. Molecular Ions and Product Ions of Anthocyanins in Concord, Salvador, and Rubired Grape Juices

compound	molecular ions and product ions
delphinidin 3, 5-diglucoside	627.3 (M^+), 465.3 [$M - C_6H_{10}O_5]^+$, 303.2 (delphinidin)
cyanidin 3, 5-diglucoside	611.3 (M^+), 449.3 [$M - C_6H_{10}O_5]^+$, 287.2 (cyanidin)
petunidin 3, 5-diglucoside	641.4 (M^+), 479.3 [$M - C_6H_{10}O_5]^+$, 317.2 (petunidin)
delphinidin 3-glucoside	465.3 (M^+), 303.2 (delphinidin)
cyanidin 3-glucoside	449.2 (M^+), 287.2 (cyanidin)
peonidin 3, 5-diglucoside	625.4 (M^+), 463.2 [$M - C_6H_{10}O_5]^+$, 301.2 (peonidin)
malvidin 3, 5-diglucoside	655.4 (M^+), 493.3 [$M - C_6H_{10}O_5]^+$, 331.3 (malvidin)
petunidin 3-glucoside	479.3 (M^+), 317.2 (petunidin)
pelargonidin 3-glucoside	433.3 (M^+), 271.2 (pelargonidin)
quercetin-3-glucuronide	479.3 (M^+), 303.2 (quercetin)
peonidin 3-glucoside	463.3 (M^+), 301.3 (peonidin)
malvidin 3-glucoside	493.3 (M^+), 331.3 (malvidin)
delphinidin 3-acetylglucoside	507.3 (M^+), 303.2 (delphinidin)
malvidin 3-acetyl-5-diglucoside	697.3 (M^+), 535.3 [$M - C_6H_{10}O_5]^+$, 493.3 [$M - C_8H_{12}O_4]^+$, 331.3 (malvidin)
vitisin B	517.3 (M^+), 355.3 (malvidin-acetaldehyde)
cyanidin 3-acetylglucoside	491.3 (M^+), 287.2 (cyanidin)
delphindin 3-coumaroyl-5-diglucoside	773.4 (M^+), 611.2 [$M - C_6H_{10}O_5]^+$, 465.3 [$M - C_{15}H_{16}O_7]^+$, 303.3 (delphinidin)
petunidin 3-acetylglucoside	521.3 (M^+), 317.3 (petunidin)
cyanidin 3-coumaroyl-5-diglucoside	757.4 (M^+), 595.3 [$M - C_6H_{10}O_5]^+$, 449.2 [$M - C_{15}H_{16}O_7]^+$, 287.2 (cyanidin)
petunidin 3-coumaroyl-5-diglucoside	787.4 (M^+), 625.3 [$M - C_6H_{10}O_5]^+$, 479.3 [$M - C_{15}H_{16}O_7]^+$, 317.3 (petunidin)
delphinidin 3-coumaroylglucoside	611.4 (M^+), 303.3 (delphinidin)
malvidin 3-acetylglucoside	535.3 (M^+), 331.3 (malvidin)
peonidin 3-coumaroyl-5-diglucoside	771.4 (M^+), 609.3 [$M - C_6H_{10}O_5]^+$, 463.3 [$M - C_{15}H_{16}O_7]^+$, 301.2 (peonidin)
malvidin 3-coumaroyl-5-diglucoside	801.4 (M^+), 639.3 [$M - C_6H_{10}O_5]^+$, 493.3 [$M - C_{15}H_{16}O_7]^+$, 331.3 (malvidin)
cyanidin 3-coumaroylglucoside	595.3 (M^+), 287.2 (cyanidin)
petunidin 3-coumaroylglucoside	625.4 (M^+), 317.3 (petunidin)
peonidin 3-coumaroylglucoside	609.4 (M^+), 301.3 (peonidin)
malvidin 3-coumaroylglucoside	639.4 (M^+), 331.3 (malvidin)

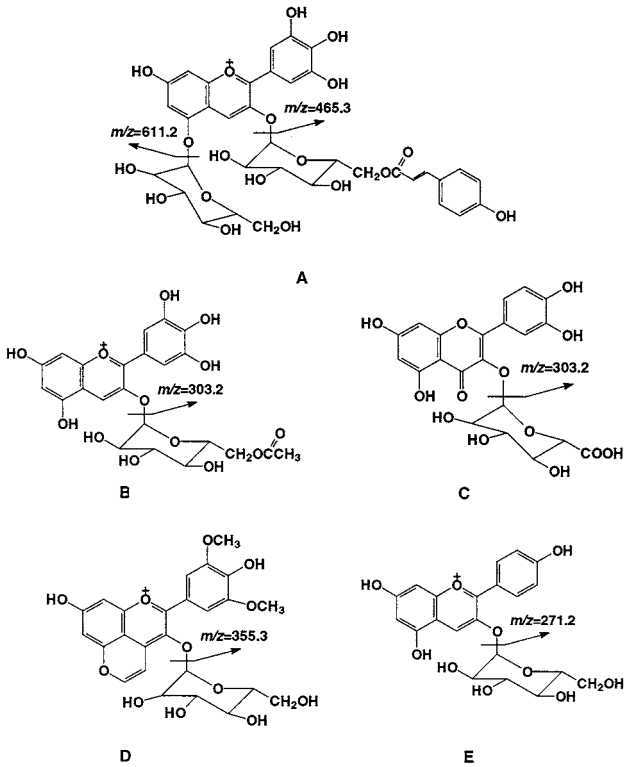

Figure 3. Structures and major cleavage of selected anthocyanins identified in grape juices.

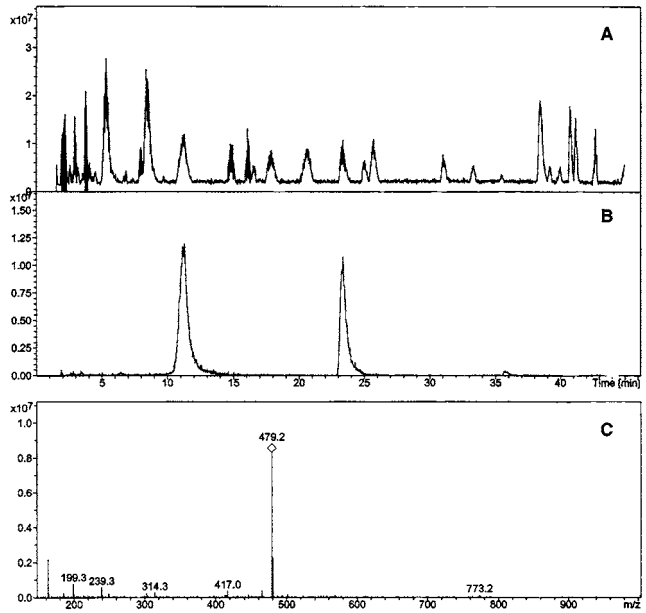

Figure 4. (A) Base peak chromatogram of Concord grape juice; (B) extracted ion chromatograms extracted at *m*/*z* 479.3; (C) MS spectrum of quercetin 3-*O*-glucuronide.

3-*O*-glucoside, and peonidin 3-*O*-glucoside (**Figure 2B**). In Salvador juice, one unique compound, normally found in fermentation products, was detected. This peak (peak 13) had a molecular ion and product ions at *m*/*z* 517.3 and 355.3, respectively. From the literature, this peak was the acetaldehyde derivative of malvidin 3-*O*-glucoside, which was assigned to vitisin B (**Figure 3D**) (*29*−*31*).

In Rubired grape juice, the major anthocyanins were malvidin 3,5-*O*-diglucoside (peak 7), peonidin 3,5-*O*-diglucoside (peak 6), peonidin 3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside (peak 22), and malvidin 3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside (peak 23). EIC revealed two peaks each at retention times of 6.75 min (peaks 3 and 4) and 10.58 min (peaks 7 and 8) that coeluted simultaneously; these were identified as petunidin 3,5-*O*-diglucoside, delphinidin 3-*O*-glucoside, malvidin 3,5-*O*-diglucoside, and petunidin 3-*O*-glucoside, respectively. Peak 9 had a weak signal in the UV−vis detector (**Figure 5B**) but showed a strong signal in the MS detector. The MS spectrum revealed that peak 9 had a molecular ion at *m*/*z* 433.3 (**Figure 5D**). The MS/MS spectrum indicated that *m*/*z* 433.3 had a product ion at

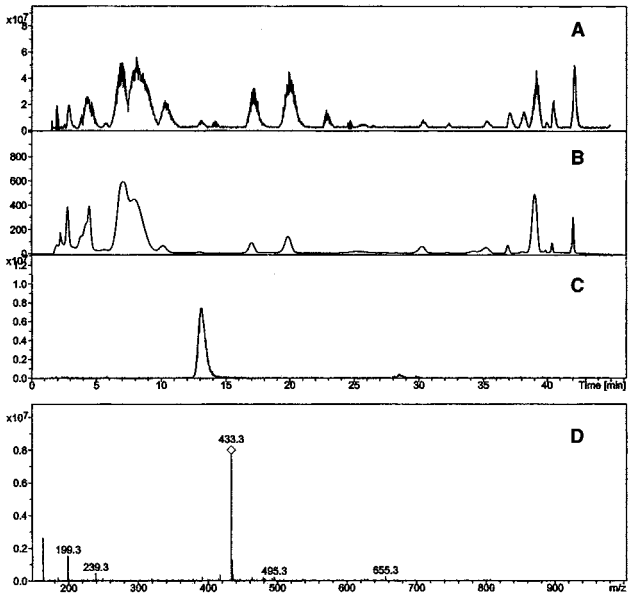

Figure 5. (A) Base peak chromatogram of Rubired grape juice; (B) HPLC-DAD chromatogram; (C) extracted ion chromatogram extracted at *m/z* 433.3; (D) MS spectrum of pelargonidin 3-*O*-glucoside.

m/z 271.2 ($[M - C_6H_{10}O_5]^+$), which corresponded to a pelargonidin or apigenin moiety. The UV−vis spectrum showed that peak 9 had a maximal absorbance at 507.3 nm. Cyanidin 3-glucoside and delphinidin 3-glucoside have maximal absorbance at 514.3 and 521.89 nm, respectively. It is known that removal of a hydroxyl group from delphinidin and cyanidin brings about a hypsochromic shift (*32*). The maximum visible absorbance of peak 9 at 507.3 nm provided further evidence that this compound had one fewer hydroxy group than cyanidin. Peak 9 was therefore elucidated as pelargonidin 3-*O*-glucoside (**Figure 3E**). Trace amounts of pelargonidin 3-*O*-glucoside were also identified in Concord and Salvador grape juices from UV−vis, MS, and MS/MS spectra, demonstrating that pelargonidin is a component common to these three grape varieties.

By coupling HPLC with ion trap mass spectrometry, anthocyanins may be monitored in high sensitivity from their molecular ion $[M^+]$. This has led to the identification of pelargonidin 3-*O*-glucoside in trace quantity in grape juices. The ion trap mass spectrometer provides MS and MS/MS spectra of the target compounds, allowing the complete and rapid identification of all the anthocyanins present in the grape juices. Because sample preparation includes membrane filtration and solid phase separation only, this method can be used for the characterization of anthocyanin pigments in other grape and wine-related products.

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