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Screening for anthocyanins using high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry with precursor-ion analysis, product-ion analysis, common-neutral-loss analysis, and selected reaction monitoring

Qingguo Tian [a], M. Monica Giusti [a], Gary D. Stoner [b], Steven J. Schwartz [a,*]

[a] *Department of Food Science and Technology, 110 Parker Food Science and Technology Building, The Ohio State University, 2015 Fyffe Road, Columbus, OH 43210, USA*
[b] *Division of Environmental Health Sciences, School of Public Health and Ohio State University Comprehensive Cancer Center, Suite 1148, 300 West 10th Avenue, Columbus, OH 43210, USA*

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Abstract

A systematic method for anthocyanin identification using tandems mass spectrometry (MS/MS) coupled to high-performance liquid chromatography (HPLC) with photo-diode array detection (PDA) was developed. Scan for the precursor ions of commonly found anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, petunidin, and peonidin) using LC/MS/MS on a triple quadrupole instrument allows for the specific determination of each category of anthocyanins. Further characterization of each anthocyanin was performed using MS/MS product-ion analysis, common-neutral-loss analysis, and selected reaction monitoring (SRM). The method was demonstrated for analysis of anthocyanins in black raspberries, red raspberries, highbush blueberries, and grapes (*Vitis vinifera*). Previous reported anthocyanins in black raspberries and red raspberries are confirmed and characterized. Common-neutral-loss analysis allows for the distinction of anthocyanin glucosides or galactoside and arabinosides in highbush blueberries. Separation and identification of anthocyanin glucosides and galactosides were achieved by LC/MS/MS using SRM. Anthocyanin isomers such as cyanidin sophoroside and 3,5-diglucoside were differentiated by their fragmentation pattern during product-ion analysis. Fifteen anthocyanins (all possible combinations of five anthocyanidins and three sugars) were characterized in highbush blueberries. Pelargonidin 3-glucoside and pelargonidin 3,5-diglucoside were detected and characterized for the first time in grapes. The present approach allows mass spectrometry to be used as a highly selective detector for rapid identification and characterization of anthocyanins and can be used as a sensitive procedure for screening anthocyanins in fruits and vegetables.
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Keywords: Anthocyanin; Tandem mass spectrometry (MS/MS); High-performance liquid chromatography (HPLC); Characterization; Fruits

1. Introduction

Anthocyanins are a group of *O*-glycosides of 3,5,7,3′-tetrahydroxyflavylium cation responsible for the red, blue, and violet colors of most berries and other fruits and vegetables [1]. There is an increasing interest in anthocyanins because of their use as natural food colorants [2,3] and potential health-promoting properties. Numerous studies have shown the positive therapeutic effects of anthocyanins such as antioxidative [4], anti-inflammatory [5], DNA cleavage and cardiovascular protective properties [6]. In addition, anthocyanin composition of many fruits is distinctive and anthocyanin profiles have been used as fingerprint to clarify plants and to detect the adulteration of fruit juices [7]. Cyanidin (3,5,7,3′,4′-pentahydroxyflavylium), delphinidin (3,5,7,3′,4′,5′-hexahydroxyflavylium), malvidin (3,5,7,4′-tetrahydroxy-3′,5′-dimethoxyflavylium), pelargonidin (3,5,

* Corresponding author. Tel.: +1 614 292 2934; fax: +1 614 292 4233.
E-mail address: schwartz.177@osu.edu (S.J. Schwartz).

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7,4′-tetrahydroxyflavium), peonidin (3,5,7,4′-tetrahydroxy-3′-methoxyflavylium), and petunidin (3,5,7,3′,4′-pentahydroxy-5′-methoxyflavylium) are the six most commonly found anthocyanin aglycones (Fig. 1). However, different types and numbers of sugars that are conjugated to the aglycones form numerous structures of anthocyanins and as such more than 600 different anthocyanins have been isolated from plants to date [8]. The most prevalent glycosylation in anthocyanins is glucose, however, rhamnose, galactose, xylose, and arabinose are also present in anthocyanins [2]. In addition, many anthocyanins have sugar residues acylated with aromatic or aliphatic acids such as *p*-coumaric, caffeic, ferulic substituents, etc. (Fig. 1).

Numerous methods have been developed for anthocyanin characterization. The most commonly used techniques are high-performance liquid chromatography (HPLC) coupled to photodiode-array detection [7], liquid chromatography–mass spectrometry (LC/MS) using continuous-flow fast atom bombardment (CF-FAB) [9,10], electrospray ionization (ESI) [11–14], atmospheric pressure chemical ionization (APCI) [15], and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) techniques [16]. Tandem mass spectrometry (MS/MS), particularly product-ion analysis that acquires a mass spectrum of the product ions produced from the fragmentation of the selected precursor ion, has been extensively used for identification and characterization of anthocyanins [11,17]. However, other tandem mass spectrometric techniques such as precursor-ion analysis and common-neutral-loss analysis are particularly important for analysis of mixtures and screening for the presence of specific compounds in complex matrices [18]. A precursor-ion analysis detects all the precursor ions in a sample that fragment to a common product ion, whereas a common-neutral-loss analysis detects those precursor ions that fragment to product ions with a common difference in *m*/*z* produced by loss of a neutral fragment. Quadrupole mass spectrometers have been estimated to be the most widely used devices among mass analyzers in chemical research and industrial laboratories [19]. The implementation of tandem mass spectrometric techniques using a quadrupole mass analyzer facilitates the conduction of various MS/MS experiments. Furthermore, the precursor-ion analysis on a triple quadrupole mass spectrometer can specifically detect the precursors of a given indicative fragment and can be used as filters for chemical noise, significantly increasing the sensitivity of detection [20].

In this study, we report the use of precursor-ion analysis, product-ion analysis, common-neutral-loss analysis, and selected reaction monitoring (SRM) tandem mass spectrometry to screen known anthocyanins in biological samples. These techniques are demonstrated in the identification and characterization of anthocyanins in fruit samples of black raspberries (*Rubus occidentalis*), red raspberries (*Rubus idaeus*), highbush blueberries (*Vaccinium corymbosum*), and grapes (*Vitis vinifera*) since these fruits contain a complete mixture of six types of anthocyanins.

2. Experimental

2.1. Materials and chemicals

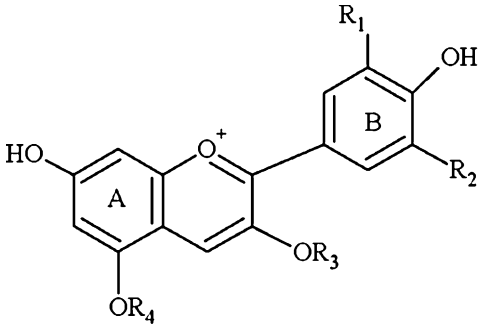
Black raspberries (*R. occidentalis*), red raspberries (*R. idaeus*), and blueberries (*V. corymbosum*) used for a clinical study were purchased from the Dale Stokes Berry Farm (Wilmington, OH). Fruit samples were ground using a Brown pulper-finisher (Brown International Corp., Covina, CA) and frozen at −20 °C before being lyophilized using a Virtis freeze-drying unit (Virtis Company, Gardiner, NY). A commercial Rubired grape extract (*V. vinifera*) was provided by Polyphenolics (Madera, CA). Freeze-dried fruit samples (∼100 mg) were extracted with 10 mL 90% methanol containing 0.5% formic acid. The solution was sonicated for 10 min, and the supernatant was recovered by centrifugation at 2000 rpm for 5 min. After extracting three times, the combined supernatants were evaporated using a rotary evaporator (<40 °C) to remove the methanol. An aliquot of the remaining aqueous solution was further purified using a C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA) that has been activated with 5 mL acidified methanol (0.1% formic acid) followed by 5 mL acidified water (0.1% formic acid). The cartridge with the adsorbed extract was washed with 10 mL acidified water (0.1% formic acid). Anthocyanins were then eluted with 5 mL acidified methanol (0.1% formic acid). After drying under nitrogen gas, the samples were dissolved in 1 mL acidified water (0.1% formic acid) and filtered through a 0.2 μm nylon filter for LC/MS/MS analysis. All reagents and solvents were of HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ).

2.2. Anthocyanin standards

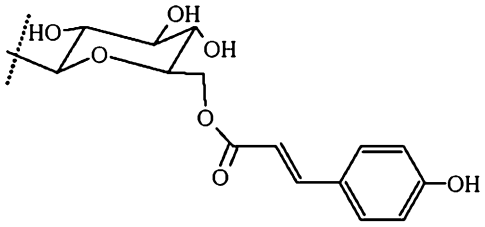
Cyanidin 3-glucoside, cyanidin 3-sambubioside, cyanidin 3-rutinoside, and cyanidin 3,5-diglucoside, purchased from Polyphenols Laboratories (Hanaveien, Norway), were used to tune the mass analyzer for each MS/MS experiment. These standards were also used to further confirm the identities of anthocyanins whenever these compounds were found in the fruit extracts.

2.3. HPLC–ESI/MS/MS

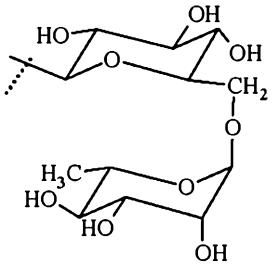
Separation of anthocyanins was conducted on a Waters Symmetry C_{18} column (4.6 mm × 75 mm, 3.5 μm) using a HPLC system consisted of a Waters 2695 separation module and a 996 photodiode-array (PDA) detector (Waters Associates, Milford, MA). Three HPLC procedures were used for anthocyanin analyses in different fruit extracts. For black and red raspberries, a linear gradient from 100% A to 10% B in 20 min, to 100% A in 5 min was used. For blueberries, a linear gradient from 100% A to 30% B in 20 min, to 100% A in 5 min was used. For grapes, a linear gradient from 100% A to 20% B in 13 min, to 30% B in 7 min, then returned to 100% A in 5 min. Solvent A was water containing 10% formic acid



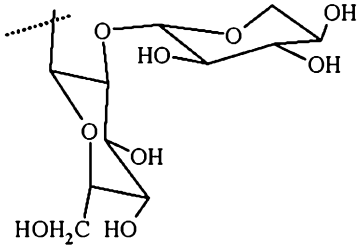
Anthocyanidin



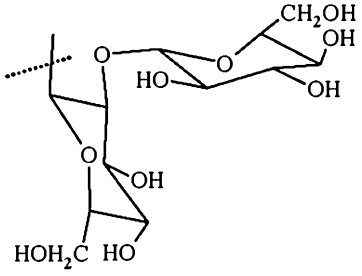
p-coumaroylglucoside



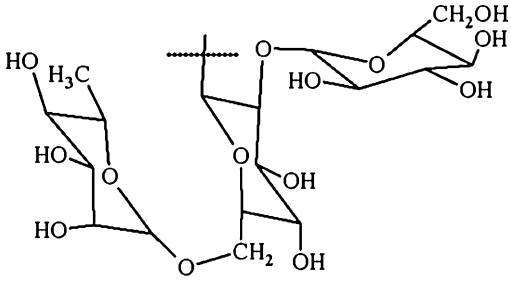
Rutinoside



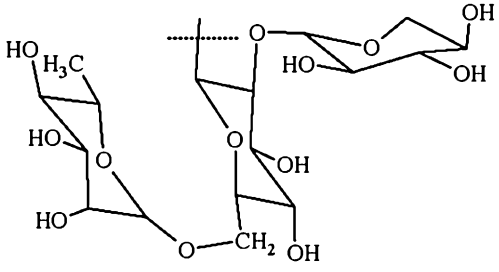
Sambubioside



Sophoroside



2^{G}-glucosylrutinoside



2^{G}-xylosylrutinoside

Fig. 1. Structures of anthocyanins: cyanidin, R_1 = OH, R_2, R_3, R_4 = H; delphinidin: R_1, R_2 = OH, R_3, R_4 = H; peonidin: R_1 = OCH_3, R_2, R_3, R_4 = H; petunidin: R_1 = OCH_3, R_2 = OH, R_3, R_4 = H; malvidin: R_1, R_2 = OCH_3, R_3, R_4 = H; pelargonidin: R_1, R_2, R_3, R_4 = H; anthocyanin 3-coumaroylglucoside: R_3 = *p*-coumaroylglucoside; anthocyanin 3,5-diglucoside: R_3, R_4 = glucoside; anthocyanin 3-rutinoside: R_3 = rutinoside; anthocyanin 3-sambubioside: R_3 = sambubioside; anthocyanin 3-sophoroside: R_3 = sophoroside; anthocyanin 3-(2^{G}-glucosylrutinoside): R_3 = 2^{G}-glucosylrutinoside; anthocyanin 3-(2^{G}-xylosylrutinoside): R_3 = 2^{G}-xylosylrutinoside.

and solvent B was acetonitrile. The flow rate was 1 mL/min. Absorption spectra of anthocyanins were recorded from 200 to 600 nm with the in-line PDA detector.

Mass spectra were obtained on a triple quadrupole ion-tunnel mass spectrometer equipped with Masslynx V3.5 software and a Z-spray ESI source (Quattro Ultima, Micromass UK Limited, Manchester, UK). Approximately 100 μL/min of the HPLC eluate separated by a micro-splitter valve (Upchurch Scientific, Oak Harbor, WA) were delivered to the ESI source. The quadrupole instrument was operated at the following settings: capillary voltage, 3.0 kV; cone voltage, 35 V; RF lense 1, 50 V; desolvation gas temperature, 420 °C at a flow of 17 L/min; source temperature, 105 °C; collision gas (argon) pressure, 7 psi; collision energy was set at 25 eV. For anthocyanin diglycosides, triglycosides, and coumaroylglycosides, a collision energy of 35 eV was used to produce relatively higher abundant fragment ions during product-ion analysis experiments. A dwell time of 0.1 s was used for all SRM experiments.

3. Results and discussion

The methodology was established based on direct LC/MS/MS analysis of a mixture of four standard cyanidin anthocyanins (~3 μmol/mL each). Screening the precursors of *m/z* 287 (cyanidin) detected four ions at *m/z* 449, 581, 595, and 611 which corresponded to the molecular cations of cyanidin 3-glucoside, cyanidin 3-sambubioside, cyanidin 3-rutinoside, and cyanidin 3,5-diglucoside, respectively. Common-neutral-loss analysis by monitoring loss of glucose (162 U), rhamnose (146 U), and xylose (132 U) as substituents detected the molecular cations of cyanidin 3-glucoside and cyanidin 3,5-diglucoside (loss of 162 U) and a much less abundant signal for cyanidin 3-rutinoside (loss of 146 U). MS/MS product-ion analysis of cyanidin 3,5-diglucoside produced the aglycone cation and a fragment ion at *m/z* 449 corresponding to 3-substituted or 5-substituted anthocyanin, indicating that either the C-3 or C-5 glucose substituent was fragmented. Cyanidin 3-rutinoside was fragmented to the aglycone cation (*m/z* 287, cyanidin) and a fragment at *m/z* 449 that corresponded to loss of rhamnose substituent, indicative of the fragmentation of the glycosidic bond between glucose and rhamnose. However, cyanidin 3-sambubioside only produced the aglycone cation during product-ion analysis, illustrating that the glycosidic bond between xylose and glucose did not fragment during MS/MS.

For precursor-ion analysis experiment, the precursors of all six anthocyanidins, including cyanidin (MW 287), delphinidin (MW 303), malvidin (MW 331), peonidin (MW 301), pelargonidin (MW 271), and petunidin (MW 317), were scanned simultaneously during analysis of all fruit samples. Fig. 2 shows a LC–ESI/MS/MS analysis of anthocyanins in black raspberries. UV–vis detection (520 nm) showed the presence of four major and one minor anthocyanins (Fig. 2A). Increasing formic acid concentration from 10% to 15% in the mobile phase helped the separation of peaks 1 and 2, 3 and 4 (Fig. 2A). However, base-line separation of peaks 1 and 2 could not be achieved using the current column. Scanning the precursors of *m/z* 287 (Fig. 2B) detected four molecular cations at *m/z* 449, 581, 727, and 595 that corresponded to the four major anthocyanins of peaks 1–4 (Fig. 2A), respectively, indicative of cyanidin anthocyanins. The minor anthocyanin (*m/z* 579, peak 5 in Fig. 2A) was identified as a pelargonidin anthocyanin which was a precursor of *m/z* 271 (Fig. 2C). During MS/MS product-ion analysis, one common fragment ion at *m/z* 287 (cyanidin) was observed for *m/z* 449 and 581 which corresponded to loss of hexose from *m/z* 449 and loss of 294 (xylose + hexose) from *m/z* 581, respectively (data not shown). In comparison, MS/MS of *m/z* 727 and 595 produced fragment ions at *m/z* 581 and 449 (loss of a rhamnose substituent from each corresponding molecular cation, $[M - \text{rhamnose}]^+$), respectively, in addition to the common aglycone cation at *m/z* 287 (Fig. 2D and E). The molecular cations, aglycone cation, fragmentation pattern, and UV–vis spectra of these anthocyanins matched previously reported cyanidin 3-glucoside, cyanidin 3-sambubioside, cyanidin 3-(2^G-xylosylrutinoside), and cyanidin 3-rutinoside, respectively [21–23]. In addition, the identities of cyanidin 3-glucoside, cyanidin 3-sambubioside, and cyanidin 3-rutinoside were further confirmed using their respective standards. The minor pelargonidin anthocyanin producing fragment ions at *m/z* 433 ($[M - \text{rhamnose}]^+$) and *m/z* 271 (pelargonidin) during product-ion analysis (Fig. 2F) was recently isolated and identified as pelargonidin 3-rutinoside using nuclear magnetic resonance (NMR) and mass spectrometry in our laboratory [24].

Eight previous reported [21,25–27] and recently confirmed [28] anthocyanins in red raspberry extract were detected during LC/MS/MS analyses (Fig. 3). Precursor-ion analysis detected two categories of anthocyanins (cyanidin and pelargonidin) in the red raspberry extract (Fig. 3B and C). Scan for the precursors of *m/z* 287 (Fig. 3B) detected four cyanidin anthocyanins with molecular cations of *m/z* 611, 449, 757, and 595 which corresponded to the four major peaks 1, 2, 3, and 5 (Fig. 3A), respectively. Scan for precursors of *m/z* 271 (Fig. 3C) detected four pelargonidin anthocyanins with molecular cations of *m/z* 595, 433, 741, and 579 that corresponded to the four minor peaks 4, 6, 7, and 8 (Fig. 3A), respectively. The two anthocyanin monoglycosides (*m/z* 449 and 433) produced their corresponding aglycones (cyanidin and pelargonidin) during product-ion analysis which matched previously reported cyanidin 3-glucoside and pelargonidin 3-glucoside, respectively (data not shown) [21,28]. The pair of anthocyanin isomers (equivalent molecular weight of 595) produced differentiated fragment ions. The cyanidin anthocyanin (*m/z* 595) was identified as cyanidin 3-rutinoside based on fragment ions observed at *m/z* 449 ($[M - \text{rhamnose}]^+$) and *m/z* 287 (cyanidin) during MS/MS (Fig. 3D), which is consistent with the fragmentation pattern of standard cyanidin 3-rutinoside. In addition, identical UV–vis spectrum and HPLC retention time of this compound

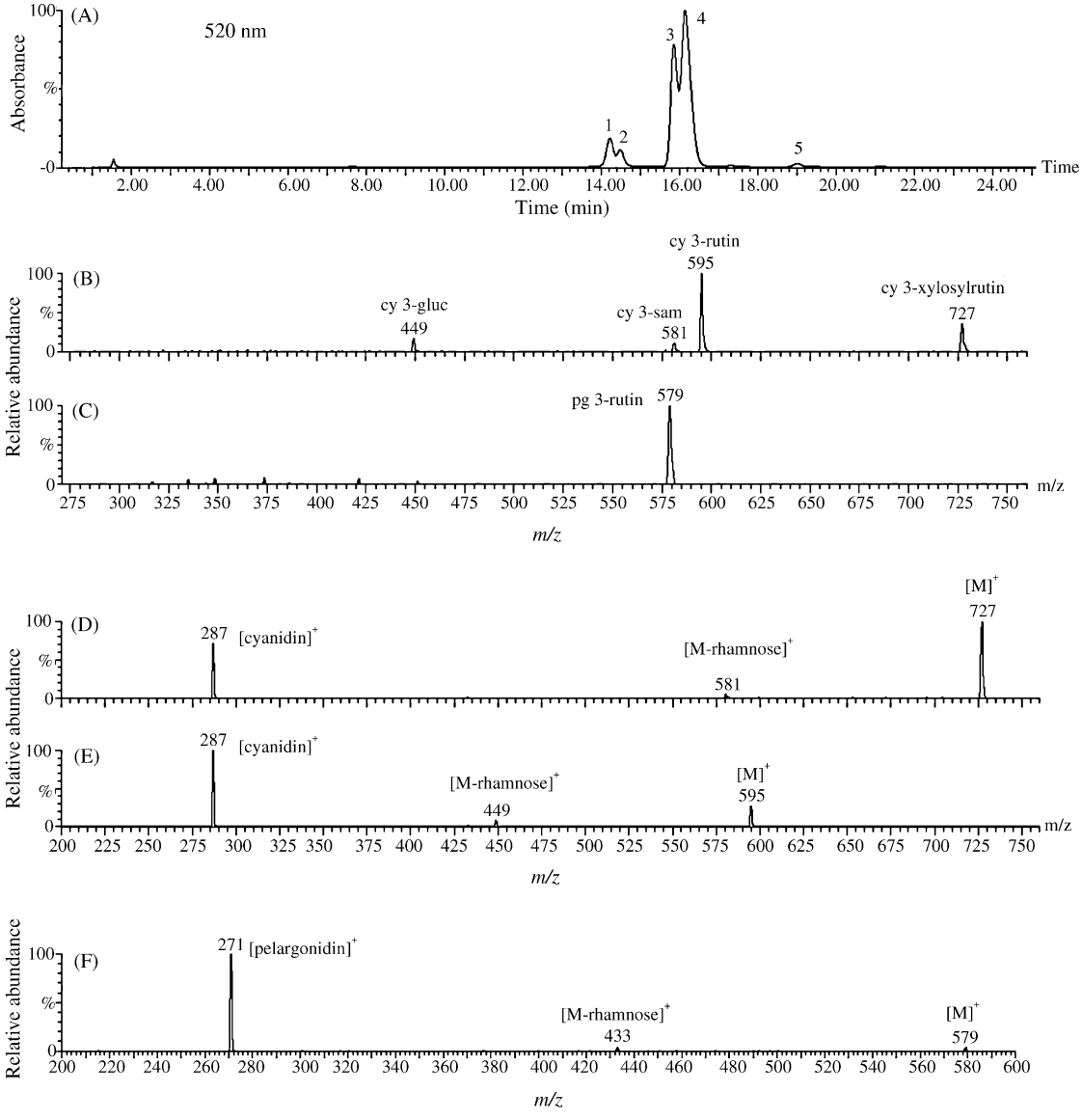


Fig. 2. LC–ESI/MS/MS of anthocyanins in black raspberries (*Rubus occidentalis*). (A) HPLC chromatogram of anthocyanins (520 nm). Peak labels: (1) cyanidin 3-glucoside (cy 3-gluc); (2) cyanidin 3-sambubioside (cy 3-sam); (3) cyanidin 3-(2^G-xylosylrutinoside) (cy 3-xylosylrutin); (4) cyanidin 3-rutinoside (cy 3-rutin) and (5) pelargonidin 3-rutinoside (pg 3-rutin). Precursor-ion analysis of (B) *m/z* 287 (cyanidin) and (C) *m/z* 271 (pelargonidin). Product-ion analysis of (D) cyanidin 3-xylosylrutinoside (*m/z* 727); (E) cyanidin 3-rutinoside (*m/z* 595) and (F) pelargonidin 3-rutinoside (*m/z* 579).

as standard cyanidin 3-rutinoside further confirmed its identity (data not shown). In comparison, the pelargonidin anthocyanin (*m/z* 595) only produced the aglycone cation at *m/z* 271 (pelargonidin) (Fig. 3E) which matched previously reported pelargonidin 3-sophoroside in red raspberries [28]. Another pelargonidin anthocyanin (*m/z* 579) was characterized as pelargonidin 3-rutinoside based on fragment ions at *m/z* 433 ($[M - \text{rhamnose}]^+$) and *m/z* 271 (pelargonidin) (Fig. 3F) as well as by comparing the elution time and UV–vis spectra to this compound identified in black raspberries. Both cyanidin 3-sophoroside and cyanidin 3,5-diglucoside (equivalent molecular weight of 611) have been reported in red raspberries [21,25,29,30]. However, the cyanidin anthocyanin exhibiting a molecular cation of *m/z* 611 detected during precursor-ion analysis (Fig. 3B) was identified as cyanidin 3-sophoroside. This compound only produced one fragment ion at *m/z* 287 (cyanidin) during product-ion analysis (Fig. 3G), however, standard cyanidin 3,5-diglycoside produced either 3-glycosylated or 5-glycosylated anthocyanin by loss of one glucose substituent ($[M - 162]^+$) from the molecular cation (data not shown). Similar fragmentation pattern was also reported in previous studies [11]. The other two higher molecular weight anthocyanins (*m/z* 741 and 757) matched previously reported [21,31] and recently confirmed pelargonidin 3-(2^G-glucosylrutinoside) and cyanidin 3-(2^G-glucosylrutinoside), respectively [28]. During MS/MS product-ion analysis, pelargonidin 3-(2^G-glucosylrutinoside) (*m/z* 741) produced

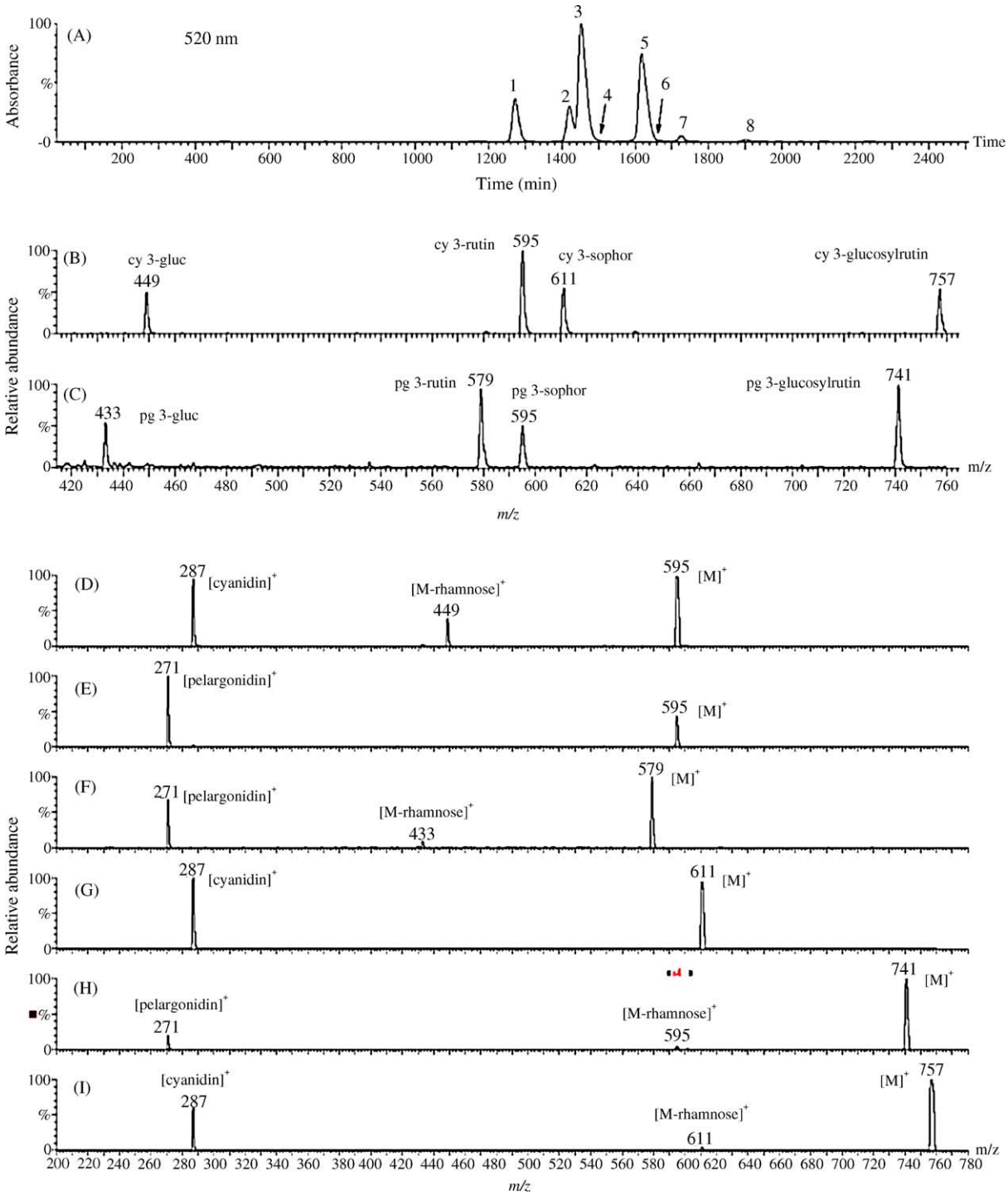


Fig. 3. LC–ESI/MS/MS of anthocyanins in red raspberries (*Rubus idaeus*). (A) HPLC chromatogram of anthocyanins (520 nm). Peak labels: (1) cyanidin 3-sophoroside (cy 3-sophor); (2) cyanidin 3-glucoside (cy 3-gluc); (3) cyanidin 3-(2^G-glucosylrutinoside) (cy 3-glucosylrutin); (4) pelargonidin 3-sophoroside (pg 3-sophor); (5) cyanidin 3-rutinoside (cy 3-rutin); (6) pelargonidin 3-glucoside (pg 3-gluc); (7) pelargonidin 3-(2^G-glucosylrutinoside) (pg 3-glucosylrutin) and (8) pelargonidin 3-rutinoside (pg 3-rutin). Precursor-ion analysis of (B) *m/z* 287 (cyanidin) and (C) *m/z* 271 (pelargonidin). Product-ion analysis of (D) cyanidin 3-rutinoside (*m/z* 595); (E) pelargonidin 3-sophoroside (*m/z* 595); (F) pelargonidin 3-rutinoside (*m/z* 579); (G) cyanidin 3-sophoroside (*m/z* 611); (H) pelargonidin 3-glucosylrutinoside (*m/z* 741) and (I) cyanidin 3-glucosylrutinoside (*m/z* 757).

the aglycone cation (*m/z* 271) and a fragment at *m/z* 595 corresponding to loss of rhamnose from the molecular cation (Fig. 3H). Similarly, cyanidin 3-(2^G-glucosylrutinoside) (*m/z* 757) was fragmented to *m/z* 611 ($[M - \text{rhamnose}]^+$) and *m/z* 287 (cyanidin, loss of one rhamnosyl and two glucosyl units from *m/z* 757) (Fig. 3I). The fragmentation behavior of these two anthocyanin glucosylrutinosides is similar to that previously reported using a single quadrupole mass spec-

trometer [28]. However, tandem mass spectrometry using a Q_1qQ_2 analyzer produced much cleaner and distinctive spectra than that obtained from a single quadrupole mass analyzer in which some fragments were hardly recognizable because of the background noise [28].

Numerous anthocyanins have been reported in blueberries, however, it is well documented that only anthocyanins monoglycosylated with three sugar species (galactose, glucose, and arabinose) have been found in highbush blueberries [32–34] which facilitates the conduction of a common-neutral-loss analysis experiment. Therefore, a neutral loss MS/MS experiment by monitoring loss of glycosylated substituents of galactose or glucose (loss of 162) and arabinose (loss of 132) will detect all known anthocyanin monoglycosides in highbush blueberries. Five categories of anthocyanins corresponding to the arabinosides, galactosides or glucosides of cyanidin, delphinidin, malvidin, peonidin, and petunidin were detected during precursor-ion analysis of six antho-

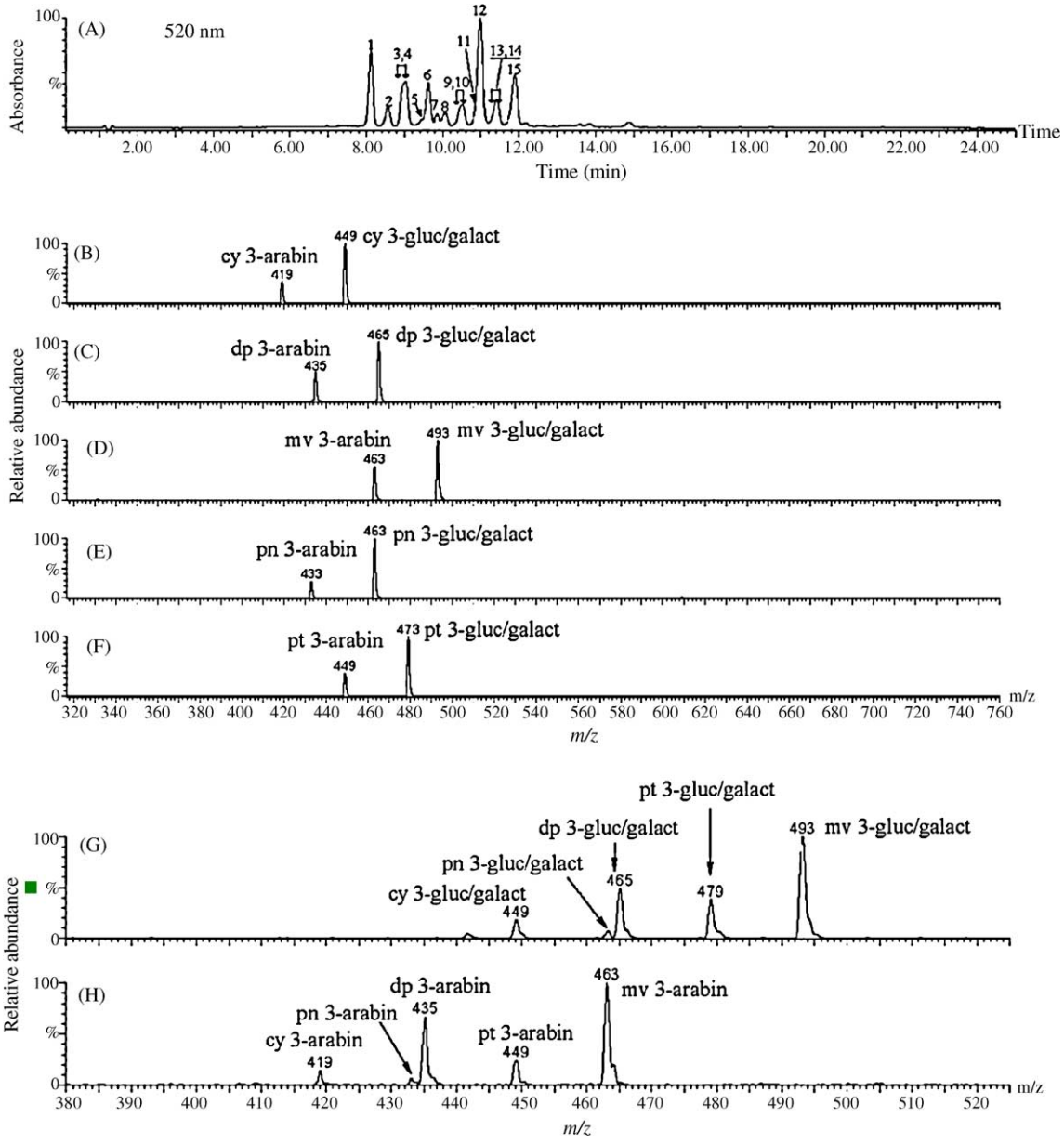


Fig. 4. LC–ESI/MS/MS of anthocyanins in highbush blueberries (*Vaccinium corymbosum*). (A) HPLC chromatogram of anthocyanins (520 nm). Peak labels: (1) delphinidin 3-galactose (dp 3-galact); (2) delphinidin 3-glucoside (dp 3-gluc); (3) cyanidin 3-galactoside (cy 3-galact); (4) delphinidin 3-arabinoside (dp 3-arabin); (5) cyanidin 3-glucoside (cy 3-gluc); (6) petunidin 3-galactoside (pt 3-galact); (7) cyanidin 3-arabinoside (cy 3-arabin); (8) petunidin 3-glucoside (pt 3-gluc); (9) peonidin 3-galactoside (pn 3-galact); (10) petunidin 3-arabinoside (pt 3-arabin); (11) peonidin 3-glucoside (pn 3-gluc); (12) malvidin 3-galactoside (mv 3-galact); (13) peonidin 3-arabinoside (pn 3-arabin); (14) malvidin 3-glucoside (mv 3-gluc); (15) malvidin 3-arabinoside (mv 3-arabin). Precursor-ion analysis of (B) *m/z* 317 (petunidin); (C) *m/z* 331 (malvidin); (D) *m/z* 303 (delphinidin); (E) *m/z* 301 (peonidin) and (F) *m/z* 287 (cyanidin). Common-neutral-loss analysis of (G) loss 162 U (glucose or galactose substituent) and (H) loss 132 U (arabinose substituent).

cyanidins (Fig. 4B–F). Because anthocyanin galactosides and glucosides of the same anthocyanin species such as cyanidin galactoside and cyanidin glucoside exhibited identical molecular weight and backbone structure, precursor-ion analysis cannot differentiate these anthocyanin isomers. As a result, all anthocyanin galactosides and glucosides of the same anthocyanin type were detected as one peak (Fig. 4B–F). Common-neutral-loss analysis separated all anthocyanins into two groups according to their sugar substituents, anthocyanin glucosides or galactosides (loss of 162) and anthocyanin arabinosides (loss of 132) (Fig. 4G and H). Compared to MALDI-TOF MS which was unable to differentiate anthocyanin isomers, such as cyanidin 3-glucoside/galactoside and petunidin 3-arabinoside (equivalent molecular weight of 449); peonidin 3-galactoside/glucoside and malvidin 3-arabinoside (equivalent molecular weight of 463) [32], precursor-ion analysis and common-neutral-loss analysis experiments conducted on a Q_1qQ_2 analyzer were particularly useful to distinguish these anthocyanin isomers with identical molecular weight but different backbone structures or different sugar substituents. However, precursor-ion analysis and common-neutral-loss analysis alone cannot discriminate glucosides and galactosides of same anthocyanin species which can be separated by HPLC. Fig. 5 shows the complete separation of anthocyanins in highbush blueberries using LC/MS/MS with SRM detection. Identical elution sequence (anthocyanin 3-galactoside > anthocyanin 3-glucoside > anthocyanin 3-arabinoside within one anthocyanin species) as reported previously using reversed-phase HPLC [32,33] was obtained during LC/MS/MS analyses using SRM detection. However, previous studies only identified part of these anthocyanins in highbush blueberries due to the coelution of anthocyanins during HPLC or anthocyanin isomers present which made it difficult to identify all anthocyanins by HPLC or mass spectrometry alone [32,33]. In comparison, we have been able to identify and characterize fifteen anthocyanins in highbush blueberries of all 15 possible combinations of five anthocyanin aglycones and three sugars using LC/MS/MS with precursor-ion analysis, common-neutral-loss analysis, and SRM detection.

Anthocyanins in grapes have been extensively studied and 3-glucosides, 3-acetylglucosides, 3-coumaroylglucosides, 3,5-diglucosides of cyanidin, delphinidin, malvidin, peonidin, and petunidin have been reported in *V. vinifera* species, pelargonidin derivatives are the only anthocyanin species that have not been found in grapes [35–39]. However, MS/MS precursor-ion analysis confirmatively identify the presence of two minor pelargonidin anthocyanins (Fig. 6C) in addition to the previously reported 3-glucosides, 3,5-diglucosides of delphinidin (Fig. 6B), petunidin (Fig. 6D), malvidin (Fig. 6E), peonidin (Fig. 6F), and cyanidin (Fig. 6G). A minute pelargondin 3-glucoside was also recently identified in Concord, Rubired, and Salvador grape species [40]. Moreover, three previously reported coumaroylglucosides of delphinidin (Fig. 6B), petunidin (Fig. 6D), and malvidin (Fig. 6E) were also found in the grape extract. Present in

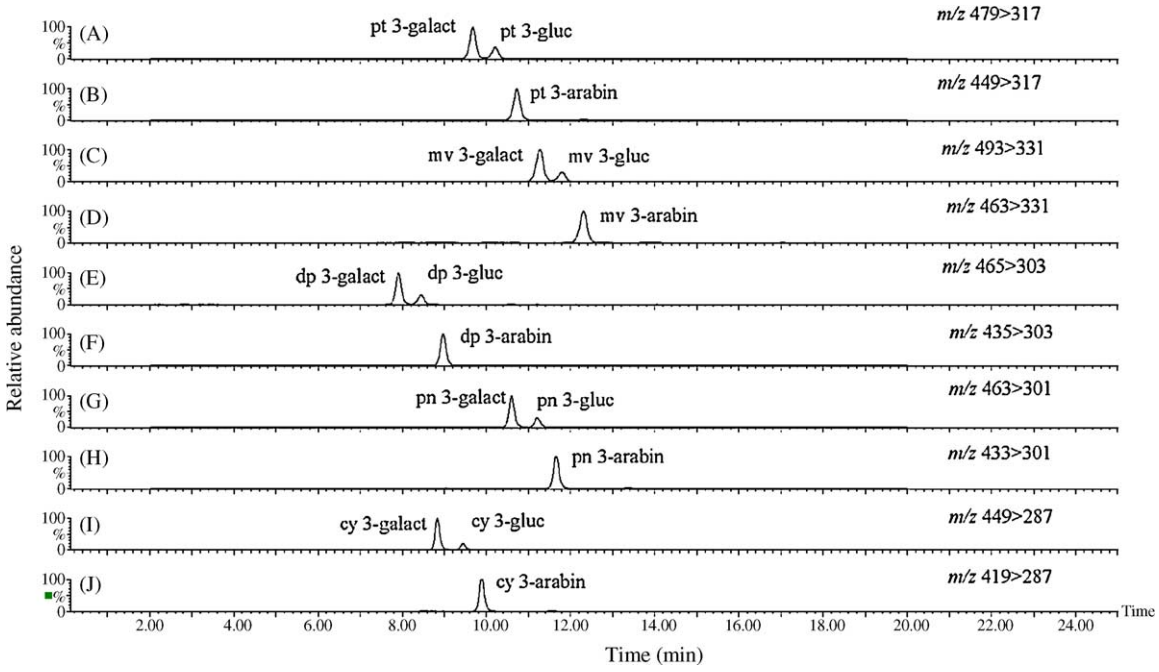


Fig. 5. LC–ESI/MS/MS of anthocyanins in highbush blueberries (*Vaccinium corymbosum*) using selected reaction monitoring (SRM). SRM of (A) *m/z* 479 > 317 for petunidin 3-galactoside and 3-glucoside; (B) *m/z* 449 > 317 for petunidin 3-arabinoside; (C) *m/z* 493 > 331 for malvidin 3-galactoside and malvidin 3-glucoside; (D) *m/z* 463 > 331 for malvidin 3-arabinoside; (E) *m/z* 465 > 303 for delphinidin 3-galactoside and delphinidin 3-glucoside; (F) *m/z* 435 > 303 for delphinidin 3-arabinoside; (G) *m/z* 463 > 301 for peonidin 3-galactoside and peonidin 3-glucoside; (H) *m/z* 433 > 301 for peonidin 3-arabinoside; (I) *m/z* 449 > 287 for cyanidin 3-galactoside and cyanidin 3-glucoside and (J) *m/z* 419 > 287 for cyanidin 3-arabinoside.

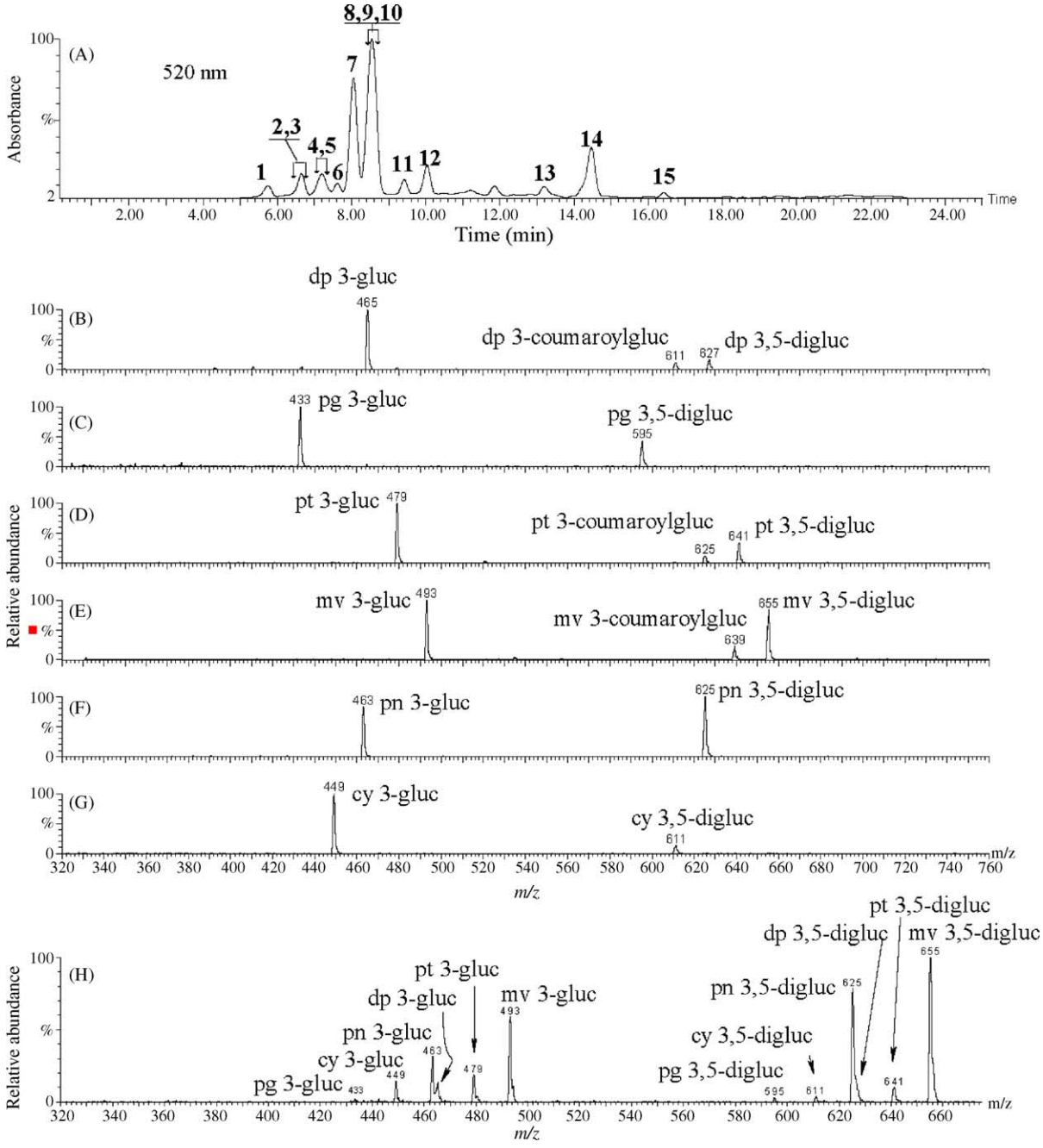


Fig. 6. LC–ESI/MS/MS of anthocyanins in grapes (*Vitis vinnifera*). (A) HPLC chromatogram of anthocyanins (520 nm). Peak labels: (1) delphinidin 3,5-diglucoside (dp 3,5-digluc); (2) cyanidin 3,5-diglucoside (cy 3,5-digluc); (3) delphinidin 3-glucoside (dp 3-gluc); (4) petunidin 3,5-diglucoside (pt 3,5-digluc); (5) pelargonidin 3,5-diglucoside (pg 3,5-digluc); (6) cyanidin 3-glucoside (cy 3-gluc); (7) peonidin 3,5-diglucoside (pn 3,5-digluc); (8) petunidin 3-glucoside (pt 3-gluc); (9) malvidin 3,5-diglucoside (mv 3,5-digluc); (10) pelargonidin 3-glucoside (pg 3-gluc); (11) peonidin 3-glucoside (pn 3-gluc); (12) malvidin 3-glucoside (mv 3-gluc); (13) delphinidin 3-coumaroylglucoside (dp 3-coumarylgluc); (14) petunidin 3-coumaroylglucoside (pt 3-coumarylgluc) and (15) malvidin 3-coumaroylglucoside (mv 3-coumarylgluc). Precursor-ion analysis of (B) *m/z* 303 (delphinidin); (C) *m/z* 271 (pelargonidin); (D) *m/z* 317 (petunidin); (E) *m/z* 331 (malvidin); (F) *m/z* 301 (peonidin) and (G) *m/z* 287 (cyanidin). (H) Common-neutral-loss analysis of loss 162 U (glucose substituent).

minute quantities in comparison to the other anthocyanins and co-eluted with other anthocyanins, it is difficult to detect the two pelargonidin anthocyanins by most chromatographic and mass spectrometric analyses since their signals are normally under the background noise. However, precursor-ion analysis enhances the signal-to-noise ratio and allows a more sensitive screening for anthocyanins in complex matrices which permits us to detect and tentatively identify these two minor pelargonidin anthocyanins not found or reported by previous researchers. Common-neutral-loss analysis experiments by monitoring loss of glucose (loss of 162) is also very useful to characterize anthocyanin 3-glucosides and 3,5-diglucosides

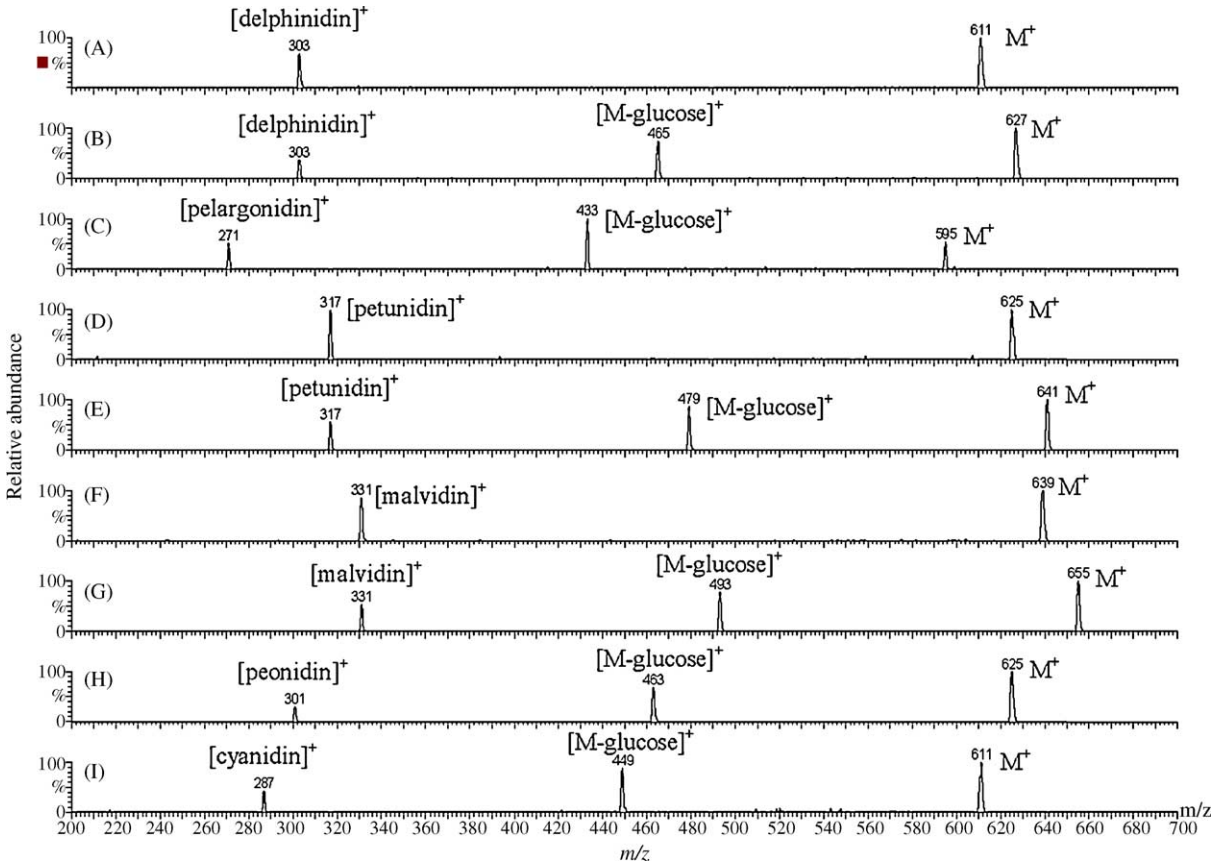


Fig. 7. LC–ESI/MS/MS of anthocyanin 3,5-diglucosides and anthocyanin 3-coumaroylglucosides in grapes (*Vitis vinnifera*). Product-ion analysis of (A) delphinidin 3-coumaroylglucoside (*m/z* 611); (B) delphinidin 3,5-diglucoside (*m/z* 627); (C) pelargonidin 3,5-diglucoside (*m/z* 595); (D) petunidin 3-coumaroylglucoside (*m/z* 625); (E) petunidin 3,5-diglucoside (*m/z* 641); (F) malvidin 3-coumaroylglucoside (*m/z* 639); (G) malvidin 3,5-diglucoside (*m/z* 655); (H) peonidin 3,5-diglucoside (*m/z* 625) and (I) cyanidin 3,5-diglucoside (*m/z* 611).

in the grape extract, and all anthocyanin monoglucosides and 3,5-diglucosides were detected and separated based on the difference of these molecular weights (Fig. 6H). Since glucose is the only glycosidic substituents in the six types of anthocyanins, these anthocyanins were thus separated based on their corresponding aglycones. However, due to the glycosidic linkage between glucoside and coumaric acid was not fragmented during MS/MS, the anthocyanin coumaroylglucosides were not detected during neutral loss analysis.

Product-ion analysis experiments were also conducted to further identify the anthocyanin 3,5-diglucosides and coumaroylglucosides detected during precursor-ion and common-neutral-loss analyses. Our initial studies using standard anthocyanins showed that cyanidin 3,5-diglucoside fragmented into the aglycone cation (*m/z* 287) and a fragment at *m/z* 449 which corresponded to 3-glycosylated or 5-glycosylated anthocyanin by loss of one glucose substituent during MS/MS product-ion analysis. Similar fragmentation behavior was observed for delphinidin 3,5-diglucoside (Fig. 7B), pelargonidin 3,5-diglucoside (Fig. 7C), petunidin 3,5-diglucoside (Fig. 7E), malvidin 3,5-diglucoside (Fig. 7G), peonidin 3,5-diglucoside (Fig. 7H), and cyanidin 3,5-diglucoside (Fig. 7I). In comparison, delphinidin 3-coumaroylglucoside (Fig. 7A), petunidin 3-coumaroylglucoside (Fig. 7D), and malvidin 3-coumaroylglucoside (Fig. 7F) only produced the corresponding aglycone cations which matched previously reported fragmentation behavior of anthocyanin coumaroylglucosides [11].

4. Conclusion

The data presented here demonstrate that the combinational use of precursor-ion analysis, common-neutral-loss analysis, product-ion analysis, and SRM on a Q_1qQ_2 instrument is a viable technique for screening anthocyanins in complex matrices. The use of HPLC coupled to ESI/MS/MS allowed us to identify and characterize a number of anthocyanins in fruit samples. By employing these techniques, we have confirmed and characterized previously reported anthocyanins in black raspberries and red raspberries; detected fifteen anthocyanins in five categories of all possible combinations of five anthocyanidins and three sugars in highbush blueberries. Moreover, these techniques allowed us to tentatively identify and characterize two pelargonidin antho-

cyanins in grapes not previously reported. In conclusion, these techniques represent an important tool for systematic identification and characterization of anthocyanins in complex biological samples by mass spectrometry.

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