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Systematic Identification and Characterization of Anthocyanins by HPLC-ESI-MS/MS in Common Foods in the United States: Fruits and Berries

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Anthocyanins were systematically identified and characterized by HPLC-ESI-MS/MS coupled with diode array detection in common fruits from U.S. food markets and other commercial sources. Of the 25 different fruits that were screened, 14 fruits were found to contain anthocyanins; the number of anthocyanins varied from 2 in peaches and nectarines to 31 in Concord grape. The individual anthocyanins were identified by comparing their mass spectral data and retention times with those of standards and published data. In all of the samples analyzed, only 6 common anthocyanidins, delphinidin, cyanidin, pelargonidin, petunidin, peonidin and malvidin, were found. In addition to the well-known major anthocyanins, a number of minor anthocyanins were identified for the first time. Some possible guidelines that help to identify anthocyanins in foods with complex anthocyanin composition were deduced and discussed. For the first time, this paper presents complete anthocyanin HPLC profiles and MS spectral data of common fruits using the same uniform experimental conditions.

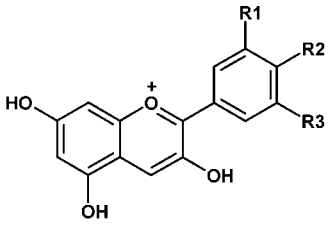
KEYWORDS: Anthocyanin; HPLC-ESI-MS/MS; apple; black plum; black raspberry; blackberry; blueberry; Concord grape; cranberry; marionberry; nectarine; peach; plum; raspberry; red grape; strawberry; sweet cherry

INTRODUCTION

Anthocyanins are a group of widespread natural phenolic compounds in plants. They are mainly distributed among flowers, fruits (particularly in berries), and vegetables and are responsible for their bright colors such as orange, red, and blue. Anthocyanins are glycosides and acylglycosides of anthocyanidins. Anthocyanidins vary with different hydroxyl or methoxyl substitutions in their basic structure, flavylium (2-phenylbenzopyrilium) **(Figure 1**) (*1*). There are >600 naturally occurring anthocyanins (*2*), and all are O-glycosylated with different sugar substitutes and acylated groups (*3*).

Anthocyanins are believed to play an important role in plant function (*4*). As a major group of secondary metabolites in plants commonly consumed as food, they are of importance in both the food industry and human nutrition. Anthocyanins have been regarded as potential food colorants used to replace synthetic colorants. Recently, increased attention has been given to their possible heath benefits in preventing chronic and degradative diseases including heart disease and cancer (*5*, *6*). These effects were partly attributed to their antioxidant capacity (*7*, *8*).

For research to progress in this area, it is critical to know the distribution and actual chemical structures of anthocyanins in foods. Many common foods containing anthocyanins have been studied (*1*, *9*, *10*). However, information on structures and concentrations is still incomplete, due in part to limitations in analytical instrumentation. Advancement of new technology has provided us additional and more powerful ways to identify minor or uncommon anthocyanins in fruits. Different anthocyanins may have significantly different chemical or physiological properties. The major anthocyanins may not necessarily be the most active compounds biologically. In food colorant studies, acylated anthocyanins have been shown to be better candidates compared to nonacylated anthocyanins due to the possible intra- and



Anthocyanidin	R1	R2	R3	MW
Pelargonidin (Pg)	H	OH	H	271
Cyanidin (Cy)	OH	OH	H	287
Delphinidin (Dp)	OH	OH	OH	303
Peonidin (Pn)	OMe	OH	H	301
Petunidin (Pt)	OMe	OH	OH	317
Malvidin (Mv)	OMe	OH	OMe	331

Figure 1. Chemical structures and molecular weights (MW) of six common anthocyanidins.

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intermolecular copigmentation (*4*). However, acylated anthocyanins are generally minor anthocyanins except in some vegetables and are easily neglected or overlooked. In absorption/metabolism studies of anthocyanins in human and experimental animals, different anthocyanidin and glycoside patterns were demonstrated to act differently (Wu and Prior, unpublished data). For instance, the apparent absorption rate of pelargonidin 3-glucoside is almost 8 times higher than that of cyanidin 3-glucoside (*11*). In the food industry, anthocyanin composition has been used as a good indicator of possible adulteration of food products (*12*).

One of the objectives of this study was to identify and characterize the anthocyanins in common foods using HPLC-ESI/MS/MS. Food samples for this study were part of the U.S. Department of Agriculture's National Food and Nutrient Analysis Program (NFNAP). The food samples were sampled directly from the U.S. market using statistically validated methods (*13*, *14*). The intent was not to study factors that might affect anthocyanin composition of foods (i.e., genetics, processing, and environmental factors such as drought, pests, diseases, etc.), but to provide data on foods with anthocyanins that are being consumed by the U.S. population.

Identification of anthocyanins was complicated by the fact that there are a large number of anthocyanins found in nature, and standards are not readily available for most of them. Even though a large amount of published data is available, different investigators have used different experimental conditions, which can make comparison of anthocyanins in different foods more difficult. Thus, a second objective of this study was to analyze anthocyanins from fruits using standardized experimental conditions, which facilitates the comparison of anthocyanin content in different foods. In this process we have introduced some possible guidelines that may help other investigators in the identification of anthocyanins in unknown or less common foods.

MATERIALS AND METHODS

Standards and Solvents. Standards of the 3-*O*-β-glucosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (six mixed anthocyanin standard, HPLC grade), cyanidin 3-*O*-β-glucoside, and peonidin 3,5-di-*O*-β-glucoside (HPLC grade) were obtained from Polyphenols Laboratories (Sandnes, Norway). Formic acid was purchased from Aldrich (St. Louis, MO). All other solvents were purchased from Fisher (Fair Lawn, NJ).

Sample Preparation. The sources of most food samples were used as described previously (*15*). Freeze-dried powders of black raspberry and marionberry were provided by the Oregon Raspberry and Blackberry Commission (ORBC, Corvallis, OR). A strawberry freeze-dried powder was provided by the Oregon Strawberry Commission (OSC, Corvallis, OR). All powders were kept at −70 °C until analyzed.

The freeze-dried powders were extracted by methanol/water/acetic acid (85:15:0.5, v/v, MeOH/H_2O/AcOH) as reported previously (*16*). The solutions from the extracted samples were then diluted with acidic methanol as necessary to obtain concentrations in a detectable range (mAU from 1 to 250) and filtered using a 0.22 μm Teflon syringe filter (Cameo, MN) for anthocyanin analysis.

HPLC-DAD-ESI/MS/MS Analysis of Anthocyanins. Chromatographic analyses were performed on an HP 1100 series HPLC (Hewlett-Packard, Palo Alto, CA) equipped with an autosampler/injector and diode array detector. A Zorbax Stablebond Analytical SB-C_{18} column (4.6 × 250 mm, 5 μm, Agilent Technologies, Rising Sun, MD) was used for separation. Elution was performed using mobile phase A (aqueous 5% formic acid solution) and mobile phase B (methanol). The flow rate was 1 mL/min, and detection was at 520 nm. Three gradient systems were used for different samples. Gradient I [described as "gradient 1" in a previous paper (*16*)] was used to separate most of the samples except for blueberry, Concord grape, red grape, and black raspberry. Gradient II ["gradient 2" from an earlier paper (*16*)] was used for black raspberry analysis. Gradient III was used for blueberry, Concord grape, and red grape analysis, which is described as follows: 0−2 min, 5% B; 2−10 min, 5−20% B; 10−15 min, 20% B; 15−30 min, 20−25% B; 30−35 min, 25% B; 35−50 min, 25−33% B; 50−55 min, 33% B; 55−65 min, 33−36% B; 65−70 min, 36−45% B; 70−75 min, 45−53% B; 75−80 min, 53−55% B; 80−84 min, 55−70% B; 84−88 min, 70−5% B; 88−90 min, 5% B. Low-resolution electrospray mass spectrometry was performed with an Esquire 3000 ion trap mass spectrometer (MS) (Bruker Daltoniks, Billerica, MA). The experimental conditions were as follows: ESI interface, nebulizer, 45 psi; dry gas, 11.0 psi, dry temperature, 340 °C; MS/MS, scan from *m*/*z* 350 to 1500; ion trap, scan from *m*/*z* 100 to 1500; maximum accrual time, 100.00 ms; average, 10; smart parameter setting (SPS), compound stability, 50%; trap drive level, 60%.

RESULTS AND DISCUSSION

Peak Identification and Assignment. Identification and peak assignment of anthocyanins in all foods was based on comparison of their retention times and mass spectral data with those of standards and published data. Two fruits, lowbush blueberry and Concord grape, both of which have a great diversity of anthocyanins and have been studied extensively, served as references for identification purposes. Only one representative chromatogram from every different type of fruit is presented unless a different chromatogram was observed in terms of anthocyanin composition. Anthocyanins found in the given fruits and berries for the first time are indicated in **Tables 1**−**3**.

Blueberry. Different blueberry cultivars were found to contain 20−27 anthocyanins. Lowbush blueberry was found to contain 27 anthocyanins (**Figure 2A**; **Table 1**), 22 of them were identified by comparing their MS data and retention times with published data (*17*−*19*). Five other anthocyanins were found in lowbush blueberry for the first time. Peaks 15, 19, and 20 shared the same mass spectral pattern in that they all had the molecular weight of anthocyanidins (cyanidin, *m*/*z* 287; delphinidin, *m*/*z* 303; and malvidin, *m*/*z* 331) plus 248. According to one study (*20*), 248 most likely represents the residue composed of hexose and malonic acid. Thus, these three anthocyanins were tentatively identified as cyanidin 3-(malonoyl)glucoside (peak 15), delphinidin 3-(malonoyl)glucoside (peak 19), and malvidin 3-(malonoyl)glucoside (peak 20). This is the first reported observation of the malonoyl group on anthocyanins in lowbush blueberry. Peak 17 had the same mass data as peak 24 and 27 ($[M]^+$, *m*/*z* 535; MS/MS, *m*/*z* 331), which indicates that malvidin, acetoyl, and hexose groups are in the structure. Peak 18 was shown to be a pentoside of petunidin ($[M]^+$, *m*/*z* 449; MS/MS, *m*/*z* 317). The exact structures of these anthocyanins could not be identified on the basis of the limited information.

Concord Grape. Concord grape juice has been previously studied, and 27 anthocyanins were identified in a recently published paper (*21*). We were able to detect 31 anthocyanins in Concord grape (**Figure 2B**; **Table 1**). Among them, 26 were identical to what was found in the former paper (*21*). Of the 5 unreported anthocyanins, 4 anthocyanins, peak 1 ($[M]^+$, *m*/*z* 435; MS/MS, *m*/*z* 303), peak 3 ($[M]^+$, *m*/*z* 449; MS/MS, *m*/*z* 317), peak 10 ($[M]^+$, *m*/*z* 463; MS/MS, *m*/*z* 331), and peak 15 (MS, *m*/*z* 419; MS/MS, *m*/*z* 287) shared similar mass spectral patterns in terms of having an anthocyanidin plus a pentose. The retention times were much shorter than that of common pentosides, such as arabinoside or xyloside, and the order of elution based upon the anthocyanidin was altered, with petunidin and malvidin eluting much earlier than cyanidin. Thus, their exact structures could not be determined on the basis of the

Table 1. Identification of Anthocyanins from Blueberry, Concord Grape, and Grape (Gradient III)

peak	t_R (min)	$[M]^+$ (*m*/*z*)	MS/MS (*m*/*z*)	anthocyanin	peak	t_R (min)	$[M]^+$ (*m*/*z*)	MS/MS (*m*/*z*)	anthocyanin
				Blueberry					
1	20.4	465	303	delphinidin 3-galactoside	15[a]	51.1	535	287	cyanidin 3-(malonoyl)glucoside
2	22.7	465	303	delphinidin 3-glucoside	16	51.4	491	287	cyanidin 3-(6″-acetoyl)galactoside
3	24.9	449	287	cyanidin 3-galactoside	17[a]	53.5	535	331	malvidin + acetoyl + hexose
4	26.7	435	303	delphinidin 3-arabinoside	18[a]	53.6	449	317	petunidin + pentose
5	28.2	449	287	cyanidin 3-glucoside	19[a]	54.7	551	303	delphinidin 3-(malonoyl)glucoside
6	30.3	479	317	petunidin 3-galactoside	20[a]	55.3	579	331	malvidin 3-(malonoyl)glucoside
7	31.8	419	287	cyanidin 3-arabinoside	21	56.1	507	303	delphinidin 3-(6″-acetoyl)glucoside
8	33.5	479	317	petunidin 3-glucoside	22	62.4	505	301	peonidin 3-(6″-acetoyl)galactoside
9	35.7	463	301	peonidin 3-galactoside	23	64.3	491	287	cyanidin 3-(6″-acetoyl)glucoside
10	38.3	449	317	petunidin 3-arabinoside	24	66.7	535	331	malvidin 3-(6″-acetoyl)galactoside
11	40.5	463	301	peonidin 3-glucoside	25	69.2	521	317	petunidin 3-(6″-acetoyl)glucoside
12	41.4	493	331	malvidin 3-galactoside	26	73.1	505	301	peonidin 3-(6″-acetoyl)glucoside
13	45.0	493	331	malvidin 3-glucoside	27	74.2	535	331	malvidin 3-(6″-acetoyl)glucoside
14	49.1	463	331	malvidin 3-arabinoside					
				Concord Grape					
1[a]	12.4	435	303	delphinidin + pentose	17	60.5	773	611/465/303	delphinidin 3-(6″-coumaroyl)-5-diglucoside
2	14.6	627	465/303	delphinidin 3,5-diglucoside	18	64.6	491	287	cyanidin 3-(6″-acetoyl)glucoside
3[a]	15.9	449	317	petunidin + pentose	19	67.2	757	595/449/287	cyanidin 3-(6″-coumaroyl)-5-diglucoside
4	17.4	611	449/287	cyanidin 3,5-diglucoside	20[a]	68.0	611	303	delphinidin + coumaroyl + hexose
5	19.9	641	479/407/317	petunidin 3,5-diglucoside	21	68.6	787	625/317	petunidin 3-(6″-coumaroyl)-5-diglucoside
6	22.9	465	303	delphinidin 3-glucoside	22	69.4	521	317	petunidin 3-(6″-acetoyl)glucoside
7	24.5	625	463/301	peonidin 3,5-diglucoside	23	72.6	801	639/493/331	malvidin 3-(coumaroyl)-5-diglucoside
8	26.7	655	493/331	malvidin 3,5-glucoside	24	72.7	771	609/463/301	peonidin 3-(coumaroyl)-5-diglucoside
9	28.4	449	287	cyanidin 3-glucoside	25	73.0	505	301	peonidin 3-(6″-acetoyl)glucoside
10[a]	30.5	463	331	malvidin + pentose	26	74.0	535	331	malvidin 3-(6″-acetoyl)glucoside
11	33.7	479	317	petunidin 3-glucoside	27	74.4	611	303	delphinidin 3-(6″-coumaroyl)glucoside
12	34.8	433	271	pelargonidin 3-glucoside	28	76.5	595	287	cyanidin 3-(6″-coumaroyl)glucoside
13	40.7	463	301	peonidin 3-glucoside	29	77.2	625	317	petunidin 3-(6″-coumaroyl)glucoside
14	45.2	493	331	malvidin 3-glucoside	30	78.9	609	301	peonidin 3-(6″-coumaroyl)glucoside
15[a]	48.2	419	287	cyanidin + pentose	31	78.9	639	331	malvidin 3-(6″-coumaroyl)glucoside
16	56.4	507	303	delphinidin 3-(6″-acetoyl)glucoside					
				Red Grape					
1	22.8	465	303	delphinidin 3-glucoside	7	74.4	35	331	malvidin 3-(6″-acetoyl)glucoside
2	28.3	449	287	cyanidin 3-glucoside	8	76.5	595	287	cyanidin 3-(6″-coumaroyl)glucoside
3	33.6	479	317	petunidin 3-glucoside	9	77.2	625	317	petunidin 3-(6″-coumaroyl)glucoside
4	40.4	463	301	peonidin 3-glucoside	10	78.9	609	301	peonidin 3-(6″-coumaroyl)glucoside
5	44.9	493	331	malvidin 3-glucoside	11	78.9	639	331	malvidin 3-(6″-coumaroyl)glucoside
6	74.1	611	303	delphinidin 3-(6″-coumaroyl)glucoside					

[a] Anthocyanins identified in these foods for the first time.

information available. This is first time that these unusual pentosides were found in Concord grape. Peak 20 had a similar MS as peak 27 [delphinidin 3-(6″-coumaroyl)glucoside]; we assume that the coumaroyl group is linked to a different position on glucose, but the actual substitute position cannot be determined. To our knowledge, Concord grape is among the very few fruits that contain all six common anthocyanidins.

Red Grape. Eleven anthocyanins were found in red grape (**Figure 2C**). By comparing their mass spectral data and retention times with those of Concord grape and published data (*21*, *22*), they were identified as 3-glucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin and 3-(6″-coumaroyl)glucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin as well as malvidin 3-(6″-acetoyl)-glucoside (**Table 1**).

Cranberry. Cranberry is widely used to make juice and is used as a food colorant. In this study, 13 anthocyanins were found in cranberry (**Figure 3A**; **Table 2**). Six of them, peaks 2, 4, 5, 7, 8, and 12, were reported previously. They were identified by comparison to published data (*19*, *23*). Seven other anthocyanins were found in cranberry for the first time. Peaks 3, 6, 10, 11, and 13 were identified by comparing their mass spectral data and retention times with those of anthocyanins in either blueberry or Concord grape as delphinidin 3-arabinoside, petunidin 3-galactoside, peonidin 3-glucoside, malvidin 3-galactoside, and malvidin 3-arabinoside. Peak 1 had a molecular weight of 625 ($[M]^+$, *m*/*z*) and two MS/MS fagment ions of *m*/*z* 463 and *m*/*z* 301. MS data indicated that this anthocyanin was peonidin plus two hexoses. According to a former study (*20*), if these two hexoses appeared in one position (3-position), MS/MS would show only a fragment ion of the aglycon. Thus, these two hexoses were likely linked to different positions of peonidin, most likely at the 3- and 5-positions. Because its retention time was shorter than that of peonidin 3,5-diglucoside in Concord grape (comparison under same gradient, data not shown), this anthocyanin was tentatively identified as peonidin 3,5-digalactoside. Peak 9 had a molecular ion *m*/*z* 403 and a fragment ion *m*/*z* 271, which indicated that this anthocyanin was a pentoside of pelargonidin. Because arabinose is the only pentose in cranberry, this anthocyanin was identified as pelargonidin 3-arabinoside. Delphinidin was found in cranberry for the first time. Thus, cranberry is another fruit that contains all six common anthocyanidins, although only cyanidin and peonidin anthocyanidins predominant.

Strawberry. Strawberry cultivars were found to contain pelargonidin as the major anthocyanidin. We and others (*11*, *24*) have shown that pelargonidin has an extremely high apparent absorption rate compared to those of other anthocyanidins. Thus, pelargonidin may exert more significant health effects in vivo. However, this conclusion is confounded by the fact that

Table 2. Identification of Anthocyanins in Other Fruits except Black Raspberry (Gradient I)

peak	t_R (min)	$[M]^+$ (*m*/*z*)	MS/MS (*m*/*z*)	anthocyanin	peak	t_R (min)	$[M]^+$ (*m*/*z*)	MS/MS (*m*/*z*)	anthocyanin
				Cranberry					
1[a]	16.0	625	463/301	peonidin 3,5-digalactoside	8	31.5	463	301	peonidin 3-galactoside
2	24.1	449	287	cyanidin 3-galactoside	9[a]	33.4	403	271	pelargonidin 3-arabinoside
3[a]	25.9	435	303	delphinidin 3-arabinoside	10[a]	34.3	463	301	peonidin 3-glucoside
4	26.9	449	287	cyanidin 3-glucoside	11[a]	34.7	493	331	malvidin 3-galactoside
5	28.5	433	271	pelargonidin 3-galactoside	12	37.0	433	301	peonidin 3-arabinoside
6[a]	28.6	479	317	petunidin 3-galactoside	13[a]	40.4	463	331	malvidin 3-arabinoside
7	29.2	419	287	cyanidin 3-arabinoside					
				Strawberry–OSC[b]					
1[a]	22.9	611	287	cyanidin 3-sophoroside	5[a]	32.9	479	317	petunidin 3-glucoside
2	26.6	449	287	cyanidin 3-glucoside	6	33.9	579	433/271	pelargonidin 3-rutinoside
3	29.4	595	449/287	cyanidin 3-rutinoside	7	45.9	519	433/271	pelargonidin 3-(malonoyl)glucoside
4	30.9	433	271	pelargonidin 3-glucoside	8	52.1	475	271	pelargonidin 3-(6″-acetoyl)glucoside
				Strawberry					
1	26.7	449	287	cyanidin 3-glucoside	4	34.1	579	433/271	pelargonidin 3-rutinoside
2	29.6	595	449/287	cyanidin 3-rutinoside	5	46.3	519	433/271	pelargonidin 3-(malonoyl)glucoside
3	31.2	433	271	pelargonidin 3-glucoside	6	52.5	475	271	pelargonidin 3-(6″-acetoyl)glucoside
				Marionberry–ORBC[c]					
1	26.5	449	287	cyanidin 3-glucoside	5[a]	39.4	419	287	cyanidin 3-xyloside
2	29.7	595	449/287	cyanidin 3-rutinoside	6	41.8	535	449/287	cyanidin 3-(6″-malonoyl)glucoside
3[a]	31.0	433	271	pelargonidin 3-glucoside	7[a]	45.0	593	287	cyanidin 3-dioxaloylglucoside
4[a]	36.7	609	463/301	peonidin 3-rutinoside					
				Blackberry					
1	26.8	449	287	cyanidin 3-glucoside	6[a]	34.3	463	301	peonidin 3-glucoside
2	29.2	419	287	cyanidin 3-arabinoside	7	40.0	419	287	cyanidin 3-xyloside
3	29.6	595	449/287	cyanidin 3-rutinoside	8	42.1	535	449/287	cyanidin 3-(6″-malonoyl)glucoside
4	31.2	433	271	pelargonidin 3-glucoside	9	45.3	593	287	cyanidin 3-dioxaloylglucoside
5[a]	32.3	535	287	cyanidin 3-(3″-malonoyl)glucoside					
				Raspberry					
1	23.3	611	287	cyanidin 3-sophoroside	5	29.8	595	449/287	cyanidin 3-rutinoside
2[a]	25.4	757	611/433/287	cyanidin 3-sophoroside-5-rhamnoside	6	31.4	433	271	pelargonidin 3-glucoside
3	27.0	449	287	cyanidin 3-glucoside	7	34.4	579	433/271	pelargonidin 3-rutinoside
4[a]	28.3	727	581/433/287	cyanidin 3-sambubioside-5-rhamnoside					
				Sweet Cherry					
1	26.9	449	287	cyanidin 3-glucoside	4	34.1	579	433/271	pelargonidin 3-rutinoside
2	29.6	595	449/287	cyanidin 3-rutinoside	5	34.1	463	301	peonidin 3-glucoside
3	31.0	433	271	pelargonidin 3-glucoside	6	37.1	609	463/301	peonidin 3-rutinoside
				Apple (Cv. Red Delicious Fuji,[d] Gala[e])					
1 (1,[d] 1[e])	24.0	449	287	cyanidin 3-galactoside	4[a]	31.7	463	301	peonidin 3-galactoside
2	26.2	449	287	cyanidin 3-glucoside	5	37.9	419	287	cyanidin 7-arabinoside
3 (2,[d] 2[e])	29.1	419	287	cyanidin 3-arabinoside	6	40.9	419	287	cyanidin 3-xyloside
				Peach and Nectarine					
1	26.7	449	287	cyanidin 3-glucoside	2	29.9	595	449/287	cyanidin 3-rutinoside
				Black Plum					
1[a]	24.3	449	287	cyanidin 3-galactoside	5[a]	33.2	565	287	cyanidin 3-(maloyl)glucoside
2	26.6	449	287	cyanidin 3-glucoside	6	33.9	463	301	peonidin 3-glucoside
3	29.3	595	449/287	cyanidin 3-rutinoside	7[a]	40.0	419	287	cyanidin 3-xyloside
4[a]	31.2	433	271	pelargonidin 3-glucoside	8	49.2	491	287	cyanidin 3-(6″-acetoyl)glucoside
				Plum					
1[a]	24.3	449	287	cyanidin 3-galactoside	4[a]	40.1	419	287	cyanidin 3-xyloside
2	26.8	449	287	cyanidin 3-glucoside	5	49.0	491	287	cyanidin 3-(6″-acetoyl)glucoside
3	29.4	595	449/287	cyanidin 3-rutinoside					

[a] Anthocyanins identified in these foods for the first time. [b] Sample provided by the Oregon Strawberry Commission. [c] Sample provided by the Oregon Raspberry and Blackberry Commission. [d] Found in Fuji apple. [e] Found in Gala apple.

pelargonidin has only one hydroxyl group on the B-ring, which may make it less reactive biologically, particularly as an antioxidant. In this study, two different anthocyanin profiles were found in strawberries. One was from the NFNAP food database samples (*15*) (**Figure 3B**; **Table 2**), which had a light pink color and represented the strawberries commonly consumed. Six anthocyanins were found in this group of strawberries by comparing their mass spectral data and retention times (*25*). These anthocyanins were cyanidin 3-glucoside, cyanidin 3-rutinoside, pelargonidin 3-glucoside, pelargonidin 3-rutinoside, pelargonidin 3-(malonoyl)glucoside, and pelargonidin 3-(6″-acetoyl)glucoside. The other strawberry sample with dark red color was from the OSC (**Figure 3C**; **Table 2**). Besides the same six anthocyanins found in other strawberries, two other anthocyanins were also found. Among them, peak 1 had a molecular ion *m*/*z* 611 and a fragment ion *m*/*z* 287, which indicated peak 1 was cyanidin diglucoside. According to previous work (*20*), diglycosides, except for rutinosides, attached at one position exhibit only one fragment ion from MS/MS data. Thus, this anthocyanin was identified as cyanidin 3-sophoroside. Peak 5 was identified as petunidin 3-glucoside by comparing its MS data and retention time to those of standards. This

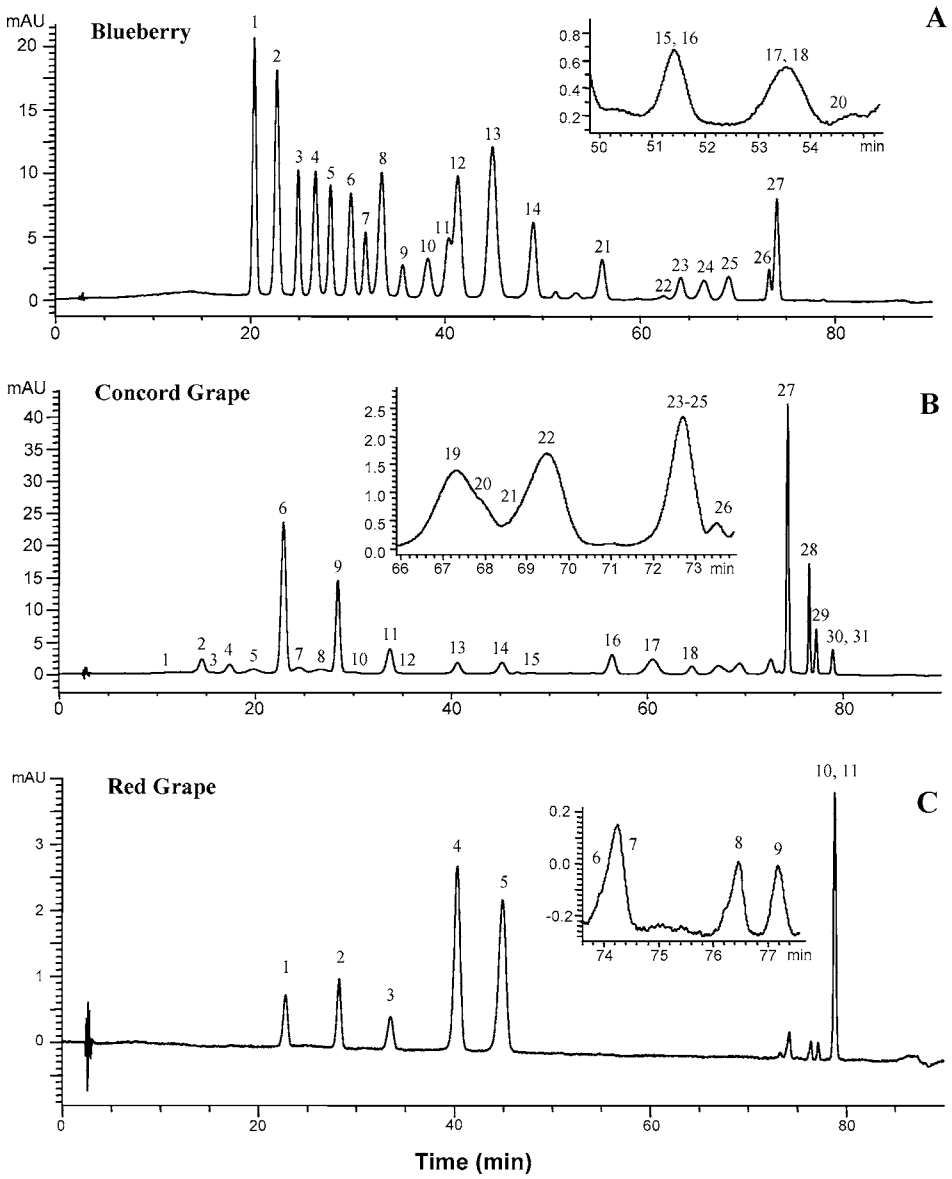

Figure 2. Reverse-phase HPLC chromatograms of anthocyanin profiles of blueberry (**A**), Concord grape (**B**), and red grape (**C**). Elution gradient III was used to separate anthocyanins. Refer to **Table 1** for the identification of each numbered peak.

Table 3. Identification of Anthocyanins in Black Raspberry–ORBC (Gradient II)[a]

peak	t_R (min)	$[M]^+$ (*m*/*z*)	MS/MS (*m*/*z*)	anthocyanin
1	21.3	581	287	cyanidin 3-sambubioside
2	21.7	449	287	cyanidin 3-glucoside
3[b]	22.4	727	581/433/287	cyanidin 3-sambubioside-5-rhamnoside
4	23.9	595	449/287	cyanidin 3-rutinoside
5	25.7	433	271	pelargonidin 3-glucoside
6	28.1	579	433/271	pelargonidin 3-rutinoside
7	30.2	609	463/301	peonidin 3-rutinoside

[a] Sample provided by the Oregon Raspberry and Blackberry Commission. [b] Anthocyanin identified in black raspberry for the first time.

represents the first time that petunidin has been identified in strawberries.

Marionberry. Marionberries are a cross between the Chehalem and Olallieberry blackberries and are grown exclusively in Oregon. Marionberries were found containing four anthocyanins: cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-(6″-malonoyl)glucoside, and cyanidin 3-(6″-coumaroyl)glucoside (*1*, *26*). However, in this study, seven anthocyanins were detected in marionberry (**Figure 3D**; **Table 2**). Peaks 1 and 2 were identified as cyanidin 3-glucoside and cyanidin 3-rutinoside, respectively. Peak 3 was identified as pelargonidin 3-glucoside by comparing its MS spectral data and retention time with those of a standard. Peak 4 had a molecular ion *m*/*z* 609 and two fragment ions *m*/*z* 463 and *m*/*z* 301. The difference of the molecular ion and the aglycon ion is *m*/*z* 308, which would indicate a rutinose residue. This anthocyanin was identified as peonidin 3-rutinoside. Peak 5 was identified as a pentoside of cyanidin ($[M]^+$ *m*/*z* 419, MS/MS *m*/*z* 287). The retention time of peak 5 was 39.4 min, which is much longer than that of cyanidin 3-arabinoside in blueberry. Thus, this anthocyanin was identified as cyanidin 3-xyloside. Peak 6 was identified as cyanidin 3-(6″-malonoyl)glucoside by comparing its mass spectral data and retention time with those of the same anthocyanin in blueberry. Peak 7 was identified as cyanidin 3-dioxaloylglucoside by comparing its mass spectral data (MS *m*/*z* 593, MS/MS *m*/*z* 287) and retention time (t_R = 45.8 min)

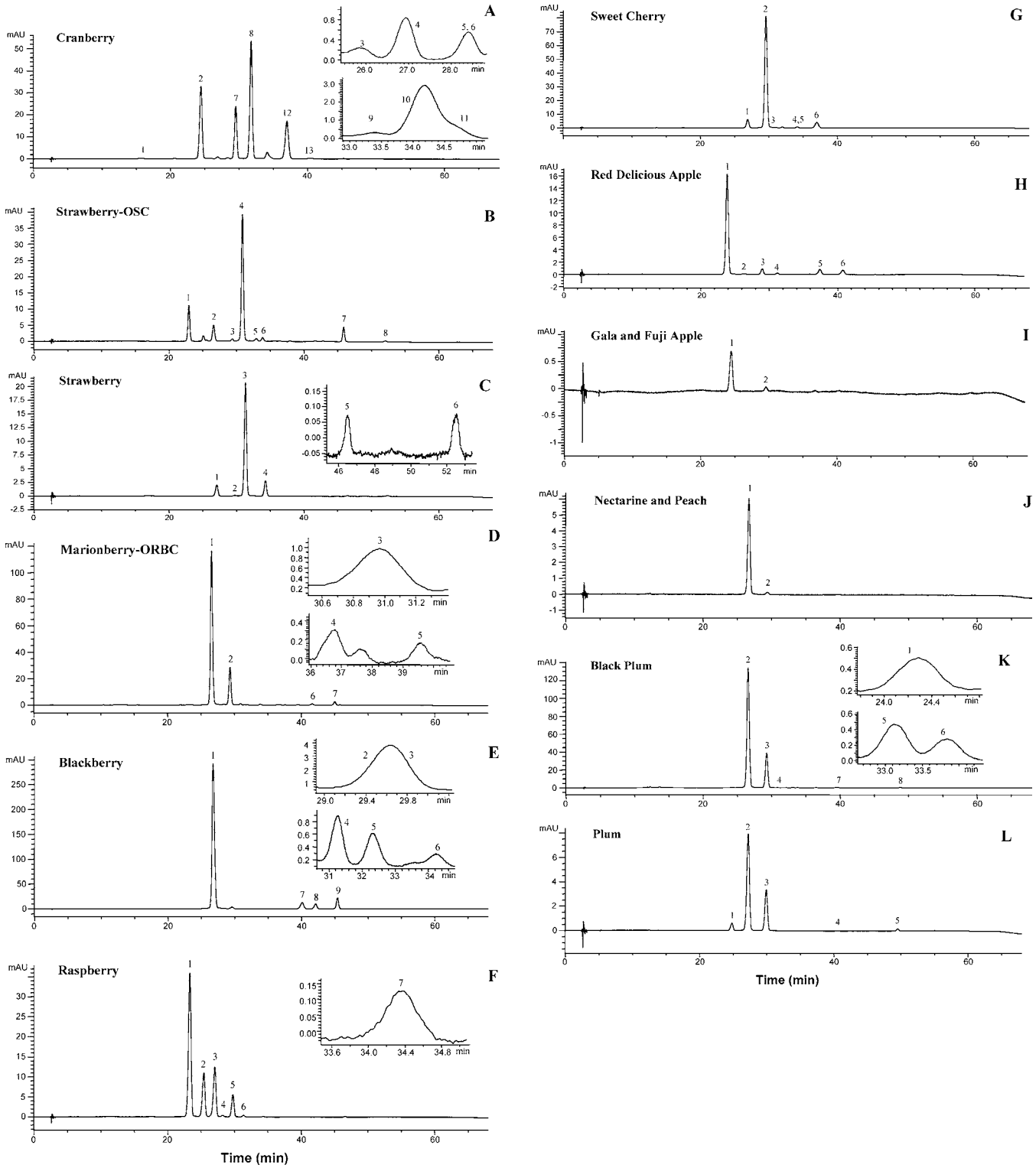

Figure 3. Reverse-phase HPLC chromatograms of anthocyanin profiles of cranberry (**A**), strawberry–OSC (**B**), strawberry (**C**), marionberry–ORBC (**D**), blackberry (**E**), raspberry (**F**), sweet cherry (**G**), Red Delicious apple (**H**), Fuji and Gala apples (**I**), peach and nectarine (**J**), black plum (**K**), and plum (**L**). Elution gradient I was used to separate anthocyanins. Refer to **Table 2** for the identification of each numbered peak.

with previous data (*27*). Pelargonidin and peonidin were identified in marionberries for the first time.

Blackberry. Nine anthocyanins were detected in blackberry (**Figure 3E**; **Table 2**); seven of them were identical with those reported previously (*27*−*29*). They are peak 1, cyanidin 3-glucoside; peak 2, cyanidin 3-arabinoside; peak 3, cyanidin 3-rutinoside; peak 4, pelargonidin 3-glucoside; peak 7, cyanidin 3-xyloside; peak 8, cyanidin 3-(6″-malonoyl)glucoside; and peak 9, cyanidin 3-dioxaloylglucoside; respectively. The major anthocyanin is peak 1, cyanidin 3-glucoside. Peak 5 had the same mass spectral data as cyanidin 3-(6″-malonoyl)glucoside ($[M]^+$ *m*/*z* 535, MS/MS *m*/*z* 287), but its retention time was much shorter (32.1 vs 41.8 min for cyanidin 3-malonoylglucoside). From a comparison of its mass spectral data and retention time with a published paper (*30*), this anthocyanin was tentatively identified as cyanidin 3-(3″-malonoyl)glucoside. Peak 6 was

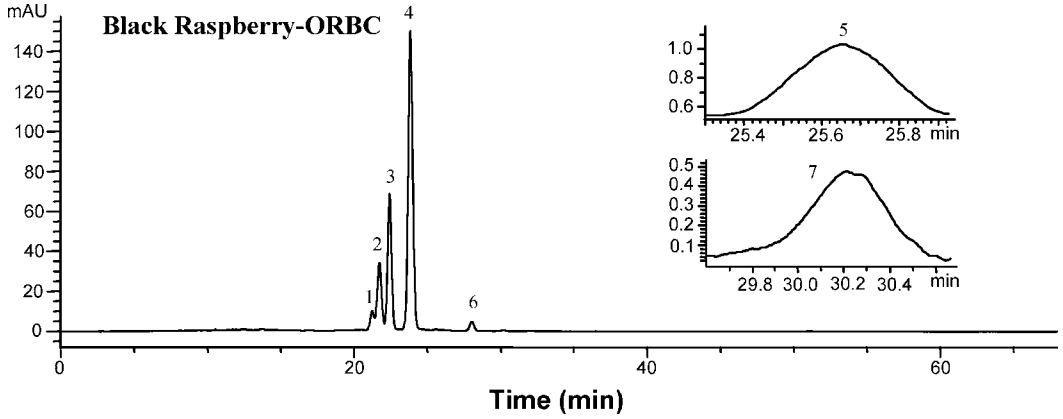

Figure 4. Reverse-phase HPLC chromatograms of anthocyanin profiles of black raspberry–ORBC. Elution gradient II was used to separate anthocyanins. Refer to **Table 3** for the identification of each numbered peak.

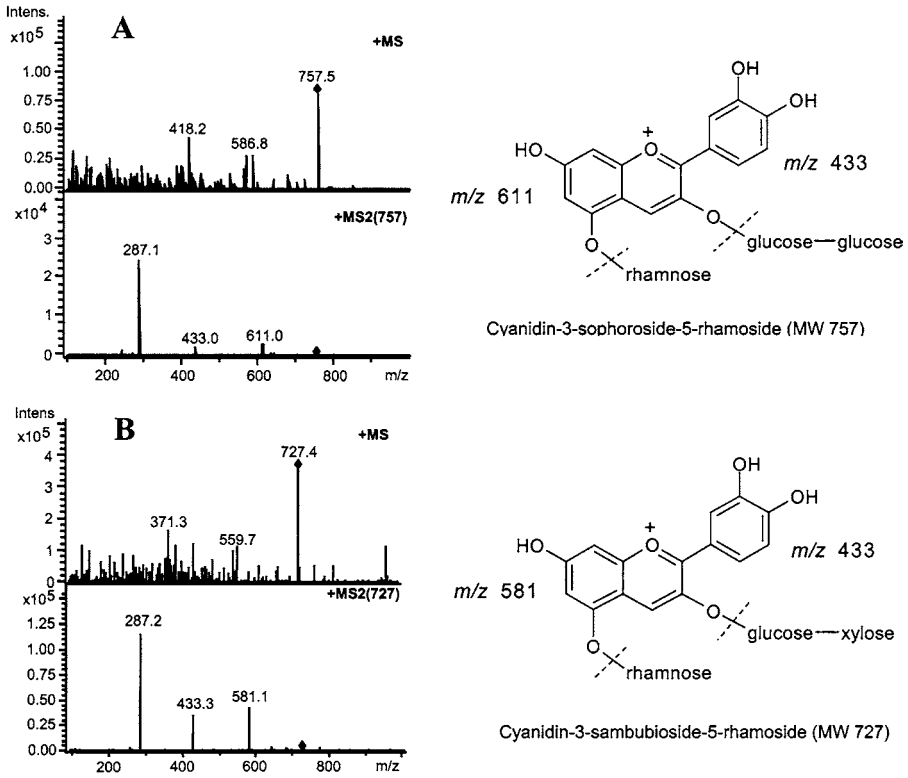

Figure 5. MS and MS/MS spectra of peak 2 in raspberry (**A**) and peak 3 in black raspberry (also peak 4 in raspberry) (**B**). These two anthocyanins were previously identified as cyanidin 3-(2^G-glucosylrutinoside) and cyanidin 3-xylosylrutinoside. A fragment ion of *m*/*z* 433 was found in the MS/MS spectra of these two anthocyanins.

identified as peonidin 3-glucoside by comparing its MS data and retention time with those of standards. Peonidin was found in blackberry for the first time.

Raspberry and Black Raspberry. A similar number of anthocyanins were detected in raspberry and black raspberry, but their profiles were quite different (**Figures 3F** and **4**). In raspberry, by comparison of our data with standards and published data (*1*, *31*−*33*), five of the seven anthocyanins were identified as peak 1, cyanidin 3-sophoroside; peak 3, cyanidin 3-glucoside; peak 5, cyanidin 3-rutinoside; peak 6, pelargonidin 3-glucoside; and peak 7, pelargonidin 3-rutinoside (**Table 2**). In black raspberry, six of the seven anthocyanins were similarly identified. They are peak 1, cyanidin 3-sambubioside; peak 2, cyanidin 3-glucoside; peak 4, cyanidin 3-rutinoside; peak 5, pelargonidin 3-glucoside; peak 6, pelargonidin 3-rutinoside; and peak 7, peonidin 3-rutinoside (**Table 3**). Remarkably, two trisaccharide glycosides were found in raspberry and/or in black raspberry, which were identified previously as cyanidin 3-(2^G-glucosylrutinoside) of peak 2 in raspberry and as cyanidin 3-xylosylrutinoside in both raspberry (peak 4) and black raspberry (peak 3) (*1*, *32*). From the MS/MS spectra (**Figure 5**), except for the $[M]^+ - 146$ peaks (with a loss of a rhamnosyl group), we see a fragment ion peak *m*/*z* 433 that appeared in both of them. Considering the molecular weights of the aglycon and different sugars, this fragment should be cyanidin plus a rhamnose. If the structures above were correct, one would not likely see this peak because rhamnose was not directly linked to cyanidin. According to the MS data, we tentatively modified their structures as cyanidin 3-sophoroside-5-rhamnoside of peak 2 in raspberry and as cyanidin 3-sambubioside-5-rhamnoside

Table 4. Distribution of Anthocyanins in Common Fruits and Berries[a]

	aglycon (anthocyanidin)						sugar moiety									acylated groups				
food	Dp	Cy	Pt	Pg	Pn	Mv	Glc	Gal	Ara	Xyl	Rha	Rut	Sam	Sop	Unk	Ace	Cou	Myl	Mal	Oxa
blueberry	+	+	+		+	+	+	+	+						+	+			±[b]	
Concord grape	+	+	+	+	+	+	+								+	+	+			
red grape	+	+	+		+	+	+									+	+			
cranberry	+	+	+	+	+	+	+	+	+											
strawberry–OSC		+	+	+			+					+		+		+			+	
strawberry		+		+			+					+				+			+	
marionberry–ORBC		+		+	+		+			+		+							+	+
blackberry		+		+	+		+		+	+		+							+	+
raspberry		+		+			+				+	+		+						
black raspberry–ORBC		+		+	+		+				+	+	+							
sweet cherry		+		+	+		+					+								
Red Delicious apple		+			+		+	+	+	+						+				
Fuji apple		+						+	+											
Gala apple		+						+	+											
peach		+					+					+								
nectarine		+					+					+								
black plum		+		+	+		+	+		+		+				+		+		
plum		+					+	+		+		+				+				

[a] Abbreviations: (for anthocyanidins) Dp, delphinidin; Cy, cyanidin; Pt, petunidin; Pg, pelargonidin; Pn, peonidin; Mv, malvidin; (for sugar moieties) Glc, glucose; Gal, galactose; Ara, arabinose; Xyl, xylose; Rha, rhamnose; Rut, rutinose; Sam, sambubiose; Sop, sophorose; Unk, unknown sugars; (for acylated groups) Ace, acetoyl; Cou, coumaroyl; Myl, maloyl; Mal, malonoyl; Oxa, oxaloyl. [b] ±, not observed in all samples.

of peak 4 in raspberry and of peak 3 in black raspberry, respectively. However, this modification needs NMR for further verification. In black raspberry, peonidin was found for the first time.

Sweet Cherry. Six anthocyanins were found in sweet cherry (**Figure 3G**; **Table 2**). They were identified as 3-glucoside and 3-rutinoside of cyanidin, pelargonidin, and peonidin by comparing their MS data to standards and previous data (*34*, *35*).

Apple. Three different varieties of apple, Red Delicious, Fuji, and Gala, were studied and found to contain six, two, and two anthocyanins, respectively (**Figure 3H**,**I**; **Table 2**). In Red Delicious apple, by comparison of their MS data to published data (*1*, *36*, *37*), five of them were identified as cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside, cyanidin 3-xyloside, and cyanidin 7-arabinoside. The anthocyanin that was not found before was identified as peonidin 3-glucoside by comparison of its MS data and retention time to those of six mixed standards. This is the first time that peonidin was found in Red Delicious apple. Cyanidin 3-galactoside and cyanidin 3-arabinoside were found to be the major anthocyanins in Fuji and Gala varieties of apple.

Peach and Nectarine. Peach and nectarine have identical anthocyanin profiles (**Figure 3J**). Cyanidin 3-glucoside and cyanidin 3-rutinoside were identified from these two fruits by comparison of their MS data and retention time data (*38*) (**Table 2**).

Plum and Black Plum. Plum and black plum shared a similar anthocyanin profile. Former studies (*1*, *12*, *38*) showed that at least three anthocyanins were found as major anthocyanins in plum; they were cyanidin 3-glucoside, cyanidin 3-rutinoside, and 3-(6″-acetoyl)glucoside. Eight anthocyanins were identified in black plum (**Figure 3K**), and five were identified in plum (**Figure 3L**) in this study. By comparison of their MS data and retention times to those of standards, blueberry, and marionberry, seven of the eight anthocyanins were identified in black plum. They were cyanidin 3-galactoside (peak 1), cyanidin 3-glucoside (peak 2), cyanidin 3-rutinoside (peak 3), pelargonidin 3-glucoside (peak 4), peonidin 3-glucoside (peak 6), cyanidin 3-xyloside (peak 7), and cyanidin 3-(6″-acetoyl)glucoside (peak 8). Peak 5, which has a molecular ion of *m*/*z* 565, a fragment ion of *m*/*z* 287, and a retention time of 33.2 min, should be an acylated anthocyanin with a high-polar acylated substitute, which was tentatively identified as cyanidin 3-(maloyl)glucoside (**Table 2**). This is the first time that pelargonidin and a maloyl group (a malic acid derivative) have been identified in plum. Plum shared a similar anthocyanin profile with black plum, and five anthocyanins were identified in plum. They were cyanidin 3-galactoside (peak 1), cyanidin 3-glucoside (peak 2), cyanidin 3-rutinoside (peak 3), cyanidin 3-xyloside (peak 4), and cyanidin 3-(6″-acetoyl)glucoside (peak 5) (**Table 2**), respectively. Galactoside and xyloside were found in the plum for the first time.

Distribution of Anthocyanins in Common Fruits and Berries. The distribution of anthocyanins in common fruits and berries is shown in **Tables 1**−**3** and is summarized in **Table 4**. Among all six widely distributed anthocyanidins, cyanidin was found in all samples that were analyzed and was a major anthocyanidin in all except strawberry. Only Concord grape and cranberry were found to contain all six common anthocyanidins. Glucose was the dominant monosaccharide, and rutinose was the most common disaccharide, which was linked to aglycons (anthocyanidins) to form anthocyanins. Half of all samples were found to contain acylated anthocyanins. Of them, the most predominant acylated substitute was the acetyl group.

Identification and Characterization of Anthocyanins. With the advancement of analytical technology, analysis and identification of anthocyanins have varied from thin-layer chromatography (TLC) and paper chromatography (PC) in early times to HPLC with photodiode array detector (PDA; or diode array detector, DAD), and, then, HPLC or CE with mass spectrometry or with tandem mass spectrometry (*39*−*42*). In recent years, HPLC coupled with mass spectrometry has become the standard and most powerful method for routine anthocyanin analysis. Several different MS technologies have been used for anthocyanin identification, including electron impact mass spectrometer (EI-MS), electrospray ionization mass spectrometer (ESI-MS), atmospheric pressure chemical ionization mass spectrometer (APCI-MS), and matrix-assisted laser desorption/ionization mass spectrometer (MALDI-MS) (*42*). Among them, ESI-MS has been preferred by the majority of investigators because of its unique advantages (*43*, *44*). HPLC with tandem ESI-MS provides the intact molecular ion as well as fragment ions by collision-induced decomposition (CID) technology in one run

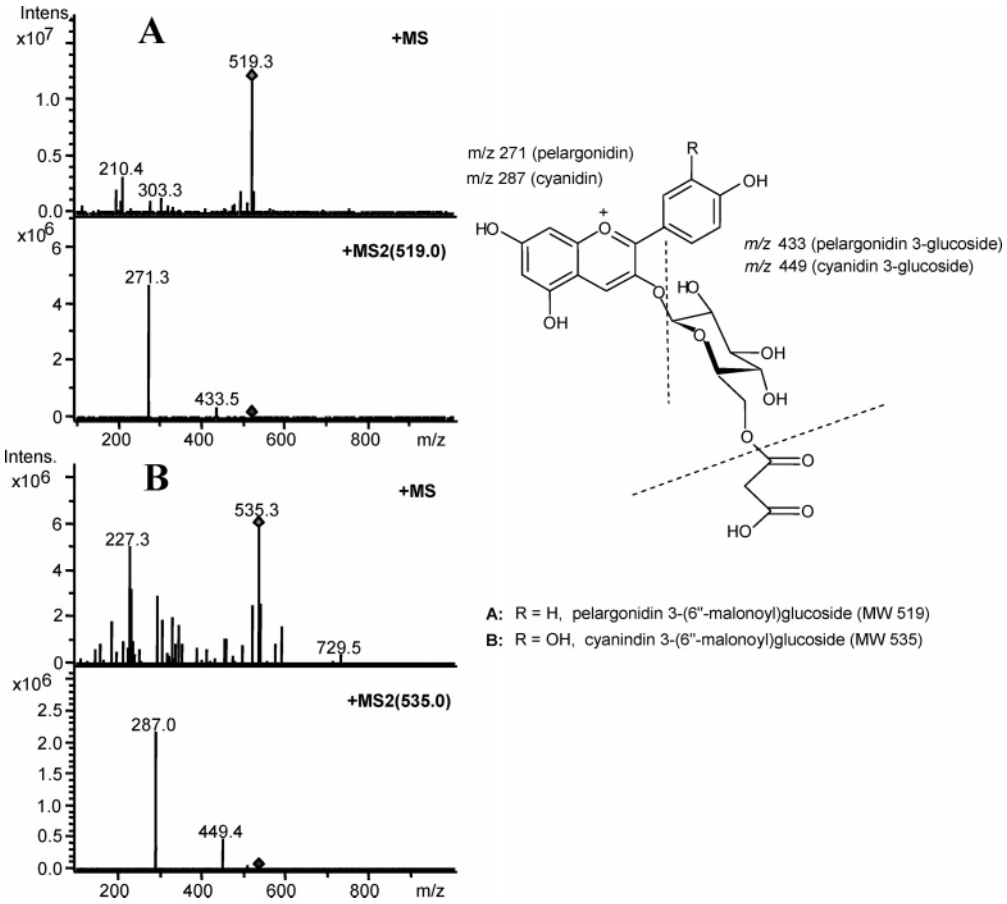

Figure 6. MS and MS/MS spectra of cyanidin 3-(6″-malonoyl)glucoside of cyanidin in marionberry (**A**) and pelargonidin 3-(6″-malonoyl)glucoside in strawberry (**B**). In the MS/MS spectra, except for the aglycon peak (*m*/*z* 287 for cyanidin and *m*/*z* 271 for pelargonidin), anthocyanidin glucosides (*m*/*z* 449 for cyanidin glucoside and *m*/*z* 433 for pelargonidin glucoside) were also observed.

(*45*). Use of mass spectrometry can reduce the reliance on retention time and UV−visible spectra and provide more useful structural information regarding molecular weight and fragmentation. However, mass spectra alone are not 100% effective because MS cannot provide complete structural information. For different anthocyanins with the same mass spectra, we have to combine other useful information that we can obtain for peak identification. Uniquely, MS data can distinguish coeluting peaks, which are common in samples with complex anthocyanin compositions. For example, in Concord grape, peaks 23−25 appeared as one peak in the HPLC chromatogram. However, from the MS data, it is clear that there are three anthocyanins in this single peak.

Proper column selection is critical for any HPLC separation. For anthocyanin analysis, the most important aspect of this issue is the column's stability in a low pH mobile phase. From our experiences, Zorbax Stablebond C18 column is quite stable for a mobile phase containing 5% formic acid. The column efficacy does not show significant decreases even after several years of usage. Cleaning and storing the column with a neutral solvent immediately after anthocyanin analysis are highly recommended to prolong the life of column.

Regularities in Anthocyanin Identification. Because identification of anthocyanins requires a combination of several pieces of information, we have developed several guidelines that will help to identify anthocyanins from an unknown sample from our data.

Retention Time. Retention time (t_R) is very important for the determination of anthocyanins even with MS data. Thus, theoretical calculation and regularities of retention times were studied (*46*, *47*). With a complex anthocyanin composition, elution order may be different for those with very close retention times under different conditions. A simple elution order (retention time from short to long) for some common anthocyanidin glycosides using reverse-phase HPLC seems to fit most experimental conditions: (a) for the six common anthocyanidins, delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin; and (b) for different glycosides and/or acylated groups with the same anthocyanidin (cyanidin), cyanidin 3,5-diglucoside, cyanidin 3-diglucoside, cyanidin 3-galactoside, cyanidin 3-sambubioside, cyanidin 3-glucoside, cyanidin 3-arabinoside, cyanidin 3-rutinoside, cyanidin 3-(maloyl)glucoside, cyanidin 3-xyloside, cyanidin 3-(6″-malonoyl)-glucoside, cyanidin 3-(6″-acetoyl)-glucoside, and cyanidin 3-(6″-coumaroyl)-glucoside.

Mass Spectrum. Giusti et al. (*20*) reported that MS/MS resulted in cleavage of glycosidic bonds only between the flavylium ring and sugars directly attached to it. The only exception to this rule was with the rutinoside. From our data, we agree with this conclusion in most cases, but it may not always be true. For instance, we found the MS/MS data from cyanidin 3-(6″-malonoyl)glucoside of cyanidin in marionberry or pelargonidin 3-(6″-malonoyl)glucoside in strawberry showed two fragment peaks: relative anthocyanidin 3-glucoside and anthocyanidin, respectively (**Figure 6A**,**B**). This meant that rutinoside is not the only exception, and cleavage does not necessarily just occur between the anthocyanidin and glycosides in all other cases. We assume that whether the cleavage happens or not depends on the groups linked to the anthocyanidins. If an unstable acylated group like malonyl is linked to a glycoside, the cleavage could also happen. Acknowledging this may help

us to identify anthocyanins when we observe a peak with a "strange" fragmentation pattern.

UV−Vis Spectrometry. The UV−vis spectra of anthocyanins have been studied extensively and have been valuable analytical tools for anthocyanin identification (*48*). However, the structural information they provide largely overlap with the mass spectral data. With more and more use of the mass spectrometer, the use of UV−vis data is limited, although it still may be useful in some cases.

Distribution. Investigation of the distribution of anthocyanins could also help identify anthocyanins. From our data, we found in these common fruits that there are two distribution patterns of anthocyanins. One includes blueberry, Concord grape, grape, cranberry, and sweet cherry. We called this group the "sugar-determined group". In this group, different anthocyanidins have the same sugar patterns. If one sugar was found to be linked with one anthocyanidin, it most likely will be found to be linked to all other anthocyanidins in this given food, although the concentrations may be low. The second group is termed the "anthocyanidin-determined group," which includes all other fruits in this study. In this group, only one anthocyanidin is dominant, such as cyanidin in blackberry and pelargonidin in strawberry. In addition to the dominant anthocyanidin, one or two other minor anthocyanidins may also exist in relatively low concentration. However, the minor anthocyanidins may not share the same glycoside pattern as that of the dominant anthocyanidin. During analysis and identification of anthocyanins from unknown samples, assigning them to these different categories can help to identify them. For instance, if we find one sample falls in the first group, we would expect to see different anthocyanidins having the same sugar pattern.

Purification of Anthocyanins. Solid-phase extraction (SPE) is the most widely used purification method for anthocyanin extraction prior to instrumental analysis (*42*, *49*). It has been used to remove undesirable products such as sugars, acids, amino acids, and proteins that were thought to interfere with the analysis of anthocyanins. In addition to SPE, several other cleanup procedures have been adopted (*20*, *42*, *50*). From our data, we did not see a significant difference between extractions using cleanup and those not using any cleanup (data not shown). Actually, the products that really interfere with anthocyanins are those with similar polarities and chemical characterizations, such as other phenolic compounds or flavonoids. Regular cleanup procedures do not work very well to remove these compounds. However, we can try to reduce interference by modifying the elution gradient. If we lengthen the retention time such that the peak of cyanidin 3-glucoside appears at 25−30 min, we see that most peaks at 280 and 320 nm will concentrate in the area from 2 to 20 min. In this way, we can see clear mass spectral data even for anthocyanin peaks with very low concentrations. On the other hand, adding one step of purification may cause possible loss of anthocyanins, which would be a problem when we want to perform qualification and quantification in the same run. Hence, we suggest eliminating the purification procedure for routine analysis of anthocyanins and modifying the elution gradient to achieve good separation and good MS data.

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