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LC-PDA-ESI/MS^n Identification of New Anthocyanins in Purple Bordeaux Radish (*Raphanus sativus* L. Variety)

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ABSTRACT: An LC-PDA-ESI/MS^n profiling method was used to identify the anthocyanins of purple Bordeaux radish and led to the assignment of 60 anthocyanins: 14 acylated cyanidin 3-(glucosylacyl)acylsophoroside-5-diglucosides, 24 acylated cyanidin 3-sophoroside-5-diglucosides, and 22 acylated cyanidin 3-sophoroside-5-glucosides. The identifications were supported by the presence of 3-sophoroside-5-diglucoside and 3-sophroside-5-glucoside of cyanidin in the alkaline-hydrolyzed extract. A reliable method to identify the anthocyanins containing 3-(glucosylacyl)acylsophorosyl functions is described. The tentative identifications were obtained from tandem mass data analysis and confirmed by high-resolution mass measurements. Further assignments were made for some anthocyanins from a comparison of the mass and UV−vis data and elution order with those of the anthocyanins in the authors' polyphenol database and from consideration of the structural characteristics of the anthocyanins from similar plants and similar anthocyanins in the literature. The presence of 38 acylated cyanidin 3-sophoroside-5-diglucosides and around 10 acylated cyanidin 3-sophoroside-5-malonylglucosides in plants is reported here for the first time.

KEYWORDS: Purple Bordeaux radish, *Raphanus sativus* L. variety, acelyted cyanidin 3-sophroside-3-diglucosides, acylated cyanidin 3-sophroside-3-glucosides, LC-DAD-ESI/MS^n analysis

INTRODUCTION

Anthocyanins are one of the major flavonoid classes and natural pigments responsible for the blue, purple, violet, and red colors of flowers. They are widely distributed in many plant-derived foods, such as berries, colored fruits, vegetables, and grains. Most anthocyanins are formed from the six common anthocyanidins, glycosylated (mainly mono- or disaccharides) at the 3- or 3,5-positions and with aliphatic and aromatic acyl groups attached to the glycosyl functions. To date, more than 670 anthocyanins have been isolated from the flowers and other colored parts of the plants in around 445 genera, and their structures were determined primarily by nuclear magnetic resonance (NMR) analysis.[1−15]

Anthocyanins are the most consumed of the flavonoids. Their consumption has been linked to resistance to cancer, diabetes, infections, inflammation, neurological, and other diseases related to the aging process.[5,6,16,17] Thus, the reliable identification and accurate quantification of anthocyanins in plant-derived foods and their products are needed to determine anthocyanin dietary intake and to evaluate their potential benefit to human health.

Liquid chromatography−photodiode array−tandem mass spectrometry with electrospray ionization (LC-DAD-ESI/MS^n) has been shown to be a powerful tool for the online identification of plant polyphenols, including anthocyanins. Nearly all of the common anthocyanin-containing foods have been studied,[17−30] many repeatedly, with the continuing advancements of analytical technology, such as ultraperformance liquid chromatography (UPLC) and high-resolution mass spectrometry (HRMS).[18,20−24,29] Peak absorbance at 520 nm is commonly used for the quantification. In addition, selective ion monitoring (SIM and HRSIM) and selected reaction monitoring (SRM, also known as multiple reaction monitoring, MRM) are also used to identify coeluting polyphenols. HRSIM or accurate mass detection has been used to quantify anthocyanins at very low concentrations in samples.[18,20,23,27,29]

Radishes, the roots of *Raphanus sativus* L. and its varieties and cultivars (Cruciferae), are common vegetables found worldwide with a wide variety of colors, shapes, and sizes. Red radish and two Chinese colored radishes, Beijing Hongxin (Beijing red heart radish, referred to as Chinese red radish in the text below) and Benikanmi (referred to as Chinese purple radish), have been studied extensively. These studies have led to the isolation and structural determination of more than 30 acylated 3-sophoroside-5-glucosides of pelargonidin or cyanidin, and 3 acylated pelargonidin 3-sophoroside-5-(4-glucosyl)glucosides.[1−4,7−13,21] Thirty-four anthocyanins, including 21 acylated pelargonin 3-diglucoside-5-glucosides, were identified in red radish by LC-DAD-MS^n analysis.[22]

A new purple radish cultivar that has recently been released commercially is the Bordeaux radish imported from Korea. Unlike the small, ball-shaped red radish, with a red peel and white interior, the purple Bordeaux radish is much bigger, with a purple exterior and purple streaked interior (Figure 1). So far, there has not been a detailed study of the anthocyanins of this purple radish.

As part of our project to systematically identify polyphenols in foods, over 200 flavonoid standards and 400 food samples, including the red and purple radishes, have been screened using a standardized LC-DAD-ESI/MS method. More than 300 anthocyanins and 700 other polyphenols have been identified and stored in our food polyphenol database and used as references to provide reliable identification of the compounds in the analyzed samples.[31,32] Using this identification strategy and

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Figure 1. Red and purple Bordeaux radishes. The small red ones, including cut pieces, are red radishes, and the big purple one and the two big cut pieces are purple Bordeaux radishes.

LC-DAD-ESI/MS^n with ion trap or Orbitrap mass spectrometers, 60 anthocyanins were identified in the purple Bordeaux radish. Of these, at least 48 are reported in plants for the first time.

MATERIALS AND METHODS

Chemicals. HPLC grade methanol, acetonitrile, formic acid, acetic acid, and NaOH were purchased from VWR International, Inc. (Clarksburg, MD). HPLC water was prepared from distilled water using a Milli-Q system (Millipore Laboratory, Bedford, MA).

Plant Materials and Extraction. Red and purple Bordeaux radishes (*R. sativus* L. varieties) and red cabbage (*Brassica oleracea* var. *capitata*) were purchased in local food stores in Maryland. The leaves of purple sweet potato (*Ipomoea batatas*) and red kale (*B. oleracea* var. *sabellica* cultivar) were collected in Brookside Garden in Maryland. Heavenly blue morning glory flower (*Ipomoea tricolor*) was obtained from the USDA anthocyanin sample collection in this laboratory. All of the fresh samples were freeze-dried and ground to a fine powder.

Each powdered sample (500 mg) was extracted with 5.00 mL of methanol/water/HCl (60:40−0.03, v/v) using sonication for 60 min at room temperature. The slurry mixture was centrifuged at 2500 rpm for 15 min (IEC Clinical Centrifuge, Damon/IEC Division, Needham, MA). The supernatant was filtered through a 17 mm (0.45 μm) PVDF syringe filter (VWR Scientific, Seattle, WA), and 10 μL of the extract was used for each HPLC injection.[31]

Alkaline-Hydrolyzed Extracts. Each of the filtered extracts (1.00 mL) was concentrated to dryness at 40 °C under vacuum. The residue was mixed with 0.30 mL of 2 N NaOH under a N_2 atmosphere and kept at room temperature for 20 min. Then, 0.10 mL of HCl (37%) was added to the reaction mixture. This mixture was passed through a Waters OASIS HLB cartridge and washed with water (2 mL × 3) to take the salts off and then with methanol−1% HCl (2 mL × 2) to remove the parent anthocyanins. The methanol portion was concentrated to dryness, and the residue was dissolved in 1.0 mL of the extraction solvent. Samples were filtered prior to injection into the HPLC.[31]

LC-PDA-ESI/MS^n Conditions. LC analyses were performed on an Agilent 1100 HPLC instrument coupled to a binary pump, a photodiode array detector (PDA), an autosampler, and a column compartment. A column (150 mm × 2.1 mm i.d., 3.5 μm, Symmetry 100 RP-18), with a 10 mm × 2.1 mm i.d. guard column of the same material (Waters Corp., Milford, MA) at flow rate of 0.2 mL/min, was used at a flow rate of 0.2 mL/min. The mobile phase consisted of a combination of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The linear gradient was from 4 to 20% B (v/v) at 40 min, to 35% B at 60 min, and to 100% B at 61 min and held at 100% B to 65 min. The column temperature was set at 25 °C, and the PDA was set at 520, 530, 350, and 280 nm to record the peak intensities; UV−vis spectra were recorded from 200 to 700 nm. A Finnigan LCQ Deca XPplus ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) was connected to the Agilent HPLC instrument via an ESI interface, and positive ionization mode was used. The acquisition parameters were set as follows: ion spray voltage, 4.5 kV; sheath gas (N_2), 80 arbitrary units; auxiliary gas (N_2), 10 arbitrary units; capillary temperature, 250 °C; capillary voltage, 15 V; tube lens offset voltage, 30 V; and mass range, from m/z 150 to 2000. Data-dependent scans were performed at a collision energy of 35% to obtain the MS^2 and MS^3 spectra of the screened anthocyanins. The main anthocyanins containing glucosylacyl function were identified using an MS^4 scan, too. Selective ion monitoring (SIM) at m/z 1095.30 with a width of 1.00 was used to check whether cyanidin 3-triglucoside-5-diglucoside was in the alkaline-hydrolyzed extracts. Both HPLC and MS systems were controlled by Xcalibur software (Thermo Finnigan).

Accurate Mass Measurements. The UPLC-HRMS system used consisted of a Thermo LTQ Orbitrap XL mass spectrometer with an Accela 1250 binary pump, an autosampler, a PDA detector, and an Agilent column compartment (G1316A). The separation was carried on a column (200 mm × 2.1 mm i.d., 1.9 mm, Thermo Hypersil Gold AQ RP- C18) with an HPLC/UHPLC precolumn filter (UltraShield Analytical Scientific Instruments, Richmond, CA) at a flow rate of 0.3 mL/min, using the same mobile phase and gradient as mentioned above. The positive ionization mode was used, and the conditions were set as follows: sheath gas, 70 arbitrary units; auxiliary and sweep gas, 15 arbitrary units; spray voltage, 4.8 kV; capillary temperature, 300 °C; capillary voltage, 15 V; and tube lens, 70 V. The mass range was from m/z 200 to 2000 with a resolution of 15000, FT MS AGC target at 2e5, FT-MS/MS AGC target at 1e5, isolation width of 1.5 amu, and maximum ion injection time of 500 ms. The most intense ion was selected for the data-dependent scan to offer their MS^2 fragments with a normalization collision energy at 35%.

RESULTS AND DISCUSSION

General Strategy for Anthocyanin Identification. Sixty acylated cyanidin 3,5-diglycosides were detected in purple Bordeaux radish, a new foodstuff found in some Asian food stores in Maryland, and were reliably identified using the same strategy previously employed in this laboratory.[33] Each of the identifications was confirmed using HRMS.

In general, tandem MS cannot easily determine the identity of the glycosyl, that is, glucosyl from galactosyl or sophorosyl (2-glucosylglucosyl) from gentiobiosyl (6-glucosylglucosyl), and the linkage positions of the aglycone for the glycosyls. A positive identification of the anthocyanin glycosylation pattern can be achieved only by direct comparison with reference compounds. Thus, all of the acylated anthocyanins of the purple radish were converted into their parent anthocyanin(s) through alkaline hydrolysis, and the parent compound(s) were were identified by comparison to reference compounds.[31−33]

It has been observed in this laboratory and in the literature,[18−30] in positive ionization mode, that MS^2 and MS^3 product ions can be used to determine acylated glycosyl groups at the 3- and 5-positions. Anthocyanins, such as cyanidin 3-acylsophoroside-5-malonylglucoside, produce two main MS^2 product ions for losses of the glycosylated groups at the 3- and 5-positions. The main MS^3 product ion was usually the aglycone ion ($[A]^+$) formed by the loss of a glycosyl from either of the MS^2 precursor ions. If the main MS^3 product ion was glysoylated (at the 3-position), an additional MS^4 experiment was conducted to produce the $[A]^+$ ion. Minor MS^2 or MS^3 product ions were used to identify the acyl groups as presented in Table 1.

Table 1. LC-DAD-MS^n Data and Tentative Identification of Anthocyanins of Purple Bordeaux Radish

peak	t_R (min)	$[M]^+$ weight	$[M]^+$ formula	error (ppm)	major and important MS^2 ions (m/z) (%)	MS^3 ions (m/z) (%)	MS^4 ions (m/z) (%)	UV−vis λ_{max} (nm)	tentative identification
1	15.39	1021.2681	$C_{42}H_{53}O_{29}$	1.34	697 (100), 611 (9)	287 (100)	nt[a]	nd[b]	Cy[c] 3-malonylsophoroside-5-diglucoside or isomer
2	15.57	859.2144	$C_{36}H_{43}O_{24}$	1.09	611 (4), 535 (100)	287 (100)	nt	nd	Cy 3-sophoroside-5-malonylglucoside
3	16.22	859.2144	$C_{36}H_{43}O_{24}$	1.09	611 (24), 535 (100)	287 (100)	nt	nd	Cy 3-soporoside-5-malonyglucoside[d]
4	17.60	1097.2983	$C_{48}H_{57}O_{29}$	0.31	773 (100), 611 (53)	287 (100)	nt	328, 523	Cy 3-caffeoylsophoroside-5-diglucoside
5	17.74	935.2463	$C_{42}H_{47}O_{24}$	0.56	773 (54), 449 (62), 287 (100)	287 (100)	nt	nd	Cy 3-caffeoylsophoroside-5-glucoside[e]
6	20.91	1183.2997	$C_{51}H_{59}O_{32}$	1.09	773 (13), 697 (100)	287 (100)	nt	nd	Cy 3-caffeoylsophoroside-5-malonyldiglucoside
7	21.04	1111.3149	$C_{49}H_{59}O_{29}$	1.23	787 (100), 611 (62)	287 (100)	nt	nd	Cy 3-feruloylsophoroside-5-diglucoside
8	21.57	949.2625	$C_{43}H_{49}O_{24}$	1.71	787 (79), 449 (100), 287 (100)	287 (100)	nt	328, 521	Cy 3-feruloylsophoroside-5-glucoside[e]
9	22.64	1453.3981	$C_{64}H_{75}O_{37}$	−0.05	1273 (3), 1111 (100), 949 (2), 611 (7)	949 (100)	nt	289, 320, 525	Cy 3-(glucosylcaffeoyl)feruloylsophoroside-5-diglucoside or isomer
10	23.10	1183.2986	$C_{51}H_{59}O_{32}$	0.16	773 (19), 697 (100)	287 (100)	nt	286, 320, 532	Cy 3-caffeoylsophoroside-5-malonyldiglucoside
11	23.52	1697.4650	$C_{74}H_{89}O_{45}$	−1.22	1287 (100), 697 (26), 1535 (10), 1373 (2)	1125 (100)	nd	nd	Cy 3-(glucosyl-*p*-coumaroyl)(glucosylsinapoyl)sophoroside-5-malonyldiglucoside or isomer
12	24.31	1435.3981	$C_{64}H_{75}O_{37}$	−0.14	1273 (2), 1111 (100), 949 (2), 611 (3)	949 (100)	nd	290, 320, 525	Cy 3-(glucosylcaffeoyl)feruloylsophoroside-5-diglucoside or isomer
13	24.64	1197.3154	$C_{52}H_{61}O_{32}$	0.70	787 (89), 697 (100)	287 (100)	nt	282, 324, 528	Cy 3-feruloylsophoroside-5-malonyldiglucoside
14	24.75	1419.4027	$C_{64}H_{75}O_{36}$	−0.38	1095 (100), 611 (8)	933 (100)	287 (100)	297, 322, 525	Cy 3-(4-glucosyl-*p*-coumaroyl)feruloylsophoroside-5-diglucoside or isomer
15	25.15	1491.3881	$C_{66}H_{75}O_{39}$	−0.33	1081 (100), 697 (56), 1329 (2), 919 (2)	919 (100)	287 (100)	288, 322, 530	Cy 3-(glucosyl-*p*-coumaroyl)caffeoylsophoroside-5-malonyldiglucoside or isomer
16	25.27	1449.4128	$C_{65}H_{77}O_{37}$	−0.68	1125 (100), 611 (13), 1287 (2), 933 (2)	963 (100)	nd	288, 322, 530	Cy 3-(glucosyl-*p*-coumaroyl)sinapoylsophoroside-5-diglucoside or isomer
17	25.67	1287.3624	$C_{59}H_{67}O_{32}$	1.11	1125 (100), 449 (4)	963 (100)	287 (100)	286, 320, 532	Cy 3-(glucosylsinapoyl)-*p*-coumaroylsophoroside-5-glucoside[e]
18	27.32	1521.3981	$C_{67}H_{77}O_{40}$	−0.68	1111 (100), 697 (69), 1359 (2), 949 (2)	949 (100)	287 (100)	288, 322, 530	Cy 3-(glucosylcaffeoyl)feruloylsophoroside-5-malonyldiglucoside or isomer
19	27.95	1505.4033	$C_{67}H_{77}O_{39}$	−0.58	1095 (100), 697 (62), 1343 (2), 933 (2)	963 (100)	nd	284, 320, 530	Cy 3-(glucosyl-*p*-coumaroyl)feruloylsophoroside-5-malonyldiglucoside or isomer
20	28.02	1565.4220	$C_{69}H_{81}O_{41}$	−2.13	1155 (100), 697 (51), 1403 (2), 993 (2)	993 (100)	287 (100)	286, 320, 532	Cy 3-(glucosylferuloyl)sinapoylsophoroside-5-malonyldiglucoside or isomer
21	28.06	1521.3972	$C_{67}H_{77}O_{40}$	−1.24	1111 (100), 697 (82), 1359 (2), 949 (2)	949 (100)	287(100)	286, 320, 532	Cy 3-(glucosylcaffeoyl)feruloylsophoroside-5-malonyldiglucoside or isomer
22	28.13	1183.2998	$C_{51}H_{59}O_{32}$	1.19	935 (89), 535 (100), 773 (2)	287 (100)	nt	293, 320, 530	Cy 3-(glucosylcaffeoyl)sophoroside-5-malonylglucoside
23	28.62	1535.4147	$C_{68}H_{79}O_{40}$	−0.06	1125 (100), 697 (61), 1329 (2)	963 (100)	287 (100)	286, 320, 532	Cy 3-(glucosyl-*p*-coumaroyl)sinapolysophoroside-5-malonylglucoside or isomer

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Table 1. Continued

peak	t_R (min)	$[M]^+$ weight	$[M]^+$ formula	error (ppm)	major and important MS^2 ions (m/z) (%)	MS^3 ions (m/z) (%)	MS^4 ions (m/z) (%)	UV−vis λ_{max} (nm)	tentative identification
24	28.68	1505.4033	$C_{67}H_{77}O_{39}$	−0.58	1095 (100), 697 (62), 1343 (2), 933 (2)	963 (100)	287 (100)	286, 320, 532	Cy 3-(glucosyl-*p*-coumaroyl)feruloylsophoroside-5-malonyldiglucoside or isomer
25	28.75	1021.2468	$C_{45}H_{49}O_{27}$	0.70	977 (100), 773 (33), 535 (18)	287 (100)	nt	286, 320, 532	Cy 3-caffeoylsophoroside-5-malonyldiglucoside[d]
26	29.70	1373.3621	$C_{62}H_{69}O_{35}$	0.09	1125 (96), 535 (100), 1211 (19), 1167 (2)	963 (100)	287	294, 324, 532	Cy 3-(glucosyl-*p*-coumaroyl)sinapoylsophoroside-5-malonylglucoside
27	30.64	1273.3464	$C_{58}H_{65}O_{32}$	1.09	949 (100), 611 (12)	287 (100)	nd	286, 320, 532	Cy 3-caffeoylsophoroside-5-diglucoside
28	31.25	1111.2939	$C_{52}H_{55}O_{27}$	1.42	949 (100), 449 (9)	287 (100)	nt	288, 328, 534	Cy 3-caffeoylsophoroside-5-glucoside[e]
29	31.86	1345.3308	$C_{60}H_{65}O_{35}$	0.13	935 (100), 697 (91)	287 (100)	nt	294, 320, 532	Cy 3-dicaffeoylsophoroside-5-malonylglucoside
30	33.19	1505.4044	$C_{67}H_{77}O_{39}$	0.75	1095 (100), 697 (72), 1343 (2), 933 (2)	933 (100)	287 (100)	296, 318, 529	Cy 3-(4-glucosyl-*p*-coumaroyl)feruloylsophoroside-5-malonylglucoside or isomer
31	33.60	1167.3050	$C_{51}H_{59}O_{31}$	0.57	757 (67), 697 (100)	nd	nt	296, 325, 529	Cy 3-*p*-coumaroylsophoroside-5-malonylglucoside
32	33.67	1389.3569	$C_{62}H_{69}O_{36}$	0.06	979 (100), 697 (89)	287 (100)	nt	286, 320, 532	Cy 3-caffeoylsinapoylsophoroside-5-malonyldiglucoside
33	33.75	1535.4146	$C_{68}H_{79}O_{40}$	−0.14	1125 (100), 697 (71), 1373 (8), 1197 (2), 963 (7)	963 (100)	287 (100)	280, 320, 532	Cy 3-(grucosylferuloyl)feruloylsophoroside-5-malonyldiglucoside
34	34.68	1197.2933	$C_{55}H_{57}O_{30}$	−0.11	949 (45), 535 (100)	287 (100)	nt	282, 328, 532	Cy 3-caffeoylferuloylsophoroside-5-malonylglucoside
35	34.73	1359.3472	$C_{61}H_{67}O_{35}$	0.65	949 (96), 697 (100)	287 (100)	nt	284, 328, 534	Cy 3- caffeoylsophoroside-5-malonyldiglucoside
36	35.20	1329.3358	$C_{60}H_{65}O_{34}$	0.07	919 (95), 697 (100)	287 (100)	nt	284, 321, 534	Cy 3-*p*-coumaroylcaffeoylsophoroside-5-malonyldiglucoside
37	35.35	1257.3510	$C_{58}H_{65}O_{31}$	−0.02	933 (100), 611 (11)	287 (100)	nt	296, 321, 534	Cy 3-*p*-coumaroylferuloylsophoroside-5-diglucoside
38	35.70	1197.2942	$C_{55}H_{57}O_{30}$	0.61	949 (100), 535 (78)	287 (100)	nt	282, 328, 532	Cy 3-caffeoylferuloylsophoroside-5-malonylgrucoside
39	36.06	1287.3615	$C_{59}H_{67}O_{32}$	−0.07	963 (100), 611 (9)	287 (100)	nt	288, 327, 529	Cy 3-*p*-coumaroylsinapoylsophoroside-5-diglucoside or isomer
40	36.58	1035.2625	$C_{46}H_{51}O_{27}$	0.6	787 (65), 535 (100)	287 (100)	nt	293, 323, 533	Cy 3-feruloylsophoroside-5-malonylglucoside[d]
41	36.83	1125.3097	$C_{53}H_{57}O_{27}$	1.52	963 (80), 449 (100)	287 (100)	nt	293, 323, 533	Cy 3- diferuloylsophoroside-5- glucoside [d]
42	37.70	1329.3356	$C_{60}H_{65}O_{34}$	−0.12	919 (83), 697 (100)	287 (100)	nt	294, 326, 532	Cy 3-*p*-coumaroylcaffeoylsophoroside-5-malonyldiglucoside
43	37.85	1035.2634	$C_{46}H_{51}O_{27}$	0.60	787 (76), 535 (100)	287 (100)	nt	293, 321, 533	Cy 3-feruloylsophoroside-5-malonylglucoside
44	38.04	1359.3466	$C_{61}H_{67}O_{35}$	0.20	949 (94), 697 (100)	287 (100)	nt	294, 326, 532	Cy 3-caffeoylferuloylsophoroside-5-malonyldiglucoside
45	39.24	1403.3711	$C_{63}H_{71}O_{36}$	−1.00	993 (100), 697 (73)	287 (100)	nt	291, 323, 533	Cy 3-feruloylsinapoylsophoroside-5-malonyldiglucoside
46	39.51	1197.2939	$C_{55}H_{57}O_{30}$	0.41	949 (45), 535 (100)	287 (100)	nt	294, 326, 532	Cy 3-caffeoylferuloylsophoroside-5-malonylglucoside
47	39.92	1343.3517	$C_{61}H_{67}O_{34}$	0.23	933 (100), 697 (94)	287 (100)	nt	294, 326, 532	Cy 3-*p*-coumaroylferuloylsophoroside-5-malonyldiglucoside
48	40.25	1373.3611	$C_{62}H_{69}O_{35}$	−0.63	963 (100), 697 (73)	287 (100)	nt	294, 326, 532	Cy 3-*p*-coumaroylsinapoylsophoroside-5-malonyldiglucoside or isomer
49	40.64	1343.3517	$C_{61}H_{67}O_{34}$	0.23	933 (100), 697 (85)	287 (100)	nt	294, 326, 532	Cy 3-*p*-coumaroylferuloylsophoroside-5-malonyldiglucoside
50	40.64	1241.3201	$C_{57}H_{61}O_{31}$	0.32	993 (100), 535 (87)	287 (100)	nt	293, 321, 532	Cy 3-feruloylsinapoylsophoroside- 5-malonylglucoside[d]
51	41.09	1181.2990	$C_{55}H_{57}O_{29}$	0.54	933 (91), 535 (100)	287 (100)	nt	295, 325, 537	Cy 3-*p*-coumaroylferuloylsophoroside-5-malonylglucoside[d]
52	41.24	1241.3193	$C_{57}H_{61}O_{31}$	0.21	993 (100), 535 (58)	287 (100)	nt	294, 323, 532	Cy 3-feruloylsinapoylsophoroside-5-malonylglucoside
53	41.49	1181.2990	$C_{55}H_{57}O_{29}$	0.34	933 (64), 535 (100)	287 (100)	nt	290, 324, 532	Cy 3-*p*-coumaroylferuloylsophoroside-5-malonylglucoside
54	41.70	1211.3093	$C_{56}H_{59}O_{30}$	0.18	963 (68), 535 (100), 1035 (2)	287 (100)	nt	288, 328, 532	Cy 3-diferuloylsophoroside-5-malonylglucoside
55	42.16	1373.3617	$C_{62}H_{69}O_{35}$	−0.18	963 (100), 697 (59)	287 (100)	nt	293, 322, 532	Cy 3-*p*-coumaroylsinapoylsophoroside-5-malonyldiglucoside or isomfer

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Table 1. Continued

peak	t_R (min)	$[M]^+$ weight	$[M]^+$ formula	error (ppm)	major and important MS^2 ions (m/z) (%)	MS^3 ions (m/z) (%)	MS^4 ions (m/z) (%)	UV−vis λ_{max} (nm)	tentative identification
56	42.20	1373.3611	$C_{62}H_{69}O_{35}$	−0.63	963 (93), 697 (100), 1227 (1)	287 (100)	nt	288, 322, 531	Cy 3-*p*-coumaroylsinapoylsophoroside-5-malonyldiglucoside
57	43.37	1373.3608	$C_{62}H_{69}O_{35}$	−0.59	963 (100), 697 (86), 1197 (1)	287 (100)	nt	288, 325, 531	Cy 3-diferuloylsophoroside-5-malonyldiglucoside
58	43.50	1211.3097	$C_{56}H_{59}O_{30}$	1.12	963 (78), 535 (100)	287 (100)	nt	283, 320, 525	Cy 3-*p*-coumaroylsinapoylsophoroside-5-malonylglucoside[d]
59	43.56	1549.4083	$C_{72}H_{77}O_{38}$	−0.4	1301 (100), 535 (40)	1139 (100)	nd	nd	Cy 3-(feruloylglucosylferuloyl)feruloylsophoroside-5-malonylglucoside
60	44.06	1301.3480	$C_{70}H_{61}O_{25}$	−1.25	1257 (19), 1215 (4), 891 (86), 697 (100), 653 (5)	nd	nd	nd	Cy 3-diacylglycoside-5-malonyldiglucoside
parent anthocyanins in alkaline hydrolyzed extract									
AH-1	11.56	773.2132	$C_{33}H_{41}O_{21}$	0.14	611 (43), 449 (76), 287 (100)	287 (100)		278, 514	Cy 3-sophoroside-5-glucoside[e]
AH-2	12.44	935.2670	$C_{39}H_{51}O_{26}$	0.72	611 (100), 287 (95)	287 (100)		282, 514	Cy 3-sophoroside-5-diglucoside

[a] nt, no test was made. [b] nd, not detected. [c] Cy, cyanidin. [d] Known anthocyanin. [e] Identified with standard or reference compound.

In some cases, further assignments were made after consideration of biogenic pathways described in the literature and by comparison to mass and UV−vis spectra and elution orders in the database. Even so, without reference compound for close comparison, identifications based on the tandem mass data should be considered as tentative. The stereochemistry of the acyl(s) and their exact linkage position of the glycosyl cannot be determined conclusively from the mass data analysis.

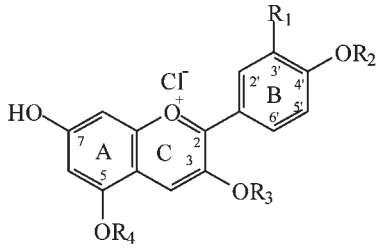
Cyanidin 3-(Glucosylacyl)acylsophoroside-5-diglucosides and Their 5-Malonates. The structures of colored radish anthocyanins are shown in Figure 2. LC and HRMS chromatograms (520 nm and TIC) of the purple Bordeaux radish extract are shown in Figure 3. Table 1 summarizes the retention times (t_R), HRMS weights $[M]^+$, molecular formulas, error (ppm) between the theoretical and measured values, major and important MS^2, MS^3, and MS^4 fragments, UV−vis λ_{max}, and the anthocyanin tentative identification. In this paper, we will use the notation $MS^n\{P\}\rightarrow X, Y, Z$, where n represents the normal notation for the step in the ionization process, P is the precursor ion, and X, Y, and Z are the product ions. Peak percentages are provided for each as they are not always listed in descending order of abundance.

Among the 60 purple Bordeaux radish anthocyanins, 14 were acylated cyanidin 3-(glucosylacyl)-sophoroside-5-diglucosides and 24 were acylated cyanidin 3-sophoroside-5-diglucosides. Alkaline hydrolysis was used to remove the acyl and glucosylacyl groups and convert them into the parent anthocyanin. The parent anthocyanin (peak AH-2) was confirmed to be cyandin 3-dihexoside-5-dihexoside by its UV−vis (λ_{max} at 278 and 512 nm), MS (935), MS^2 (611 and 287), and MS^3 (287) product ions, and t_R (12.44 min), as shown in Table 1.

All of the positively identified acylated anthocyanidin 3-diglucoside-5-glycosides for colored radishes reported in the literature had a sophorosyl group at the 3-position and a glucosyl or (4″-glucosyl)glucosyl group at the 5-position. All nine anthocyanins formed with cyanidin were isolated from Chinese purple radish, a variety with purple skin, and all three anthocyanins containing 5-diglucosyl were isolated from Chinese red radish, the variety having a red interior. This structural similarity strongly suggested that all of them were formed from the same biosynthetic pathway and the variety of anthocyanin was related to the color.[1−4,6−13] Thus, the acylated cyandin 3-sophoroside-5-diglucosides and -5-glucoside might be formed in purple Bordeaux radish, a variety with purple color in the skin and interior (Figure 1).

No trace amount of cyanidin 3-triglucoside-5-diglucoside was detected (by SIM detection) in the alkaline-hydrolyzed extract. This suggested that all 14 anthocyanins have 3 glucosyls at the 3-position and should be cyanidin 3-(glucosylacyl)acylsophoroside-5-diglucosides or their 5-malonates.

The analytical process can be illustrated using peak 33 as an example. Peak 33 has UV−vis λ_{max} at 280, 320, and 532 nm, a typical spectrum for acylated cyanidin 3,5-diglycosides.[7,14,15,21−28] Figure 4 shows typical MS^2, MS^3, and MS^4 profiles. The m/z 287 product ions from $MS^3\{697\}$ and $MS^4\{963\}$ identify the anthocyanidin as cyanidin (Cy). Two main $MS^2\{1535\}$ product ions at m/z 1125 (100%, [Cy 3-acylated glycosyl]$^+$) and m/z 697 (71%, [Cy 5-malonyldiglucosyl]$^+$) suggested 3 glucosyl groups and 2 acyl groups at the 3-position and 2 glucosyls and 1 malonyl group at the 5-position. This suggestion was supported by the fact that all of the acylated anthocyanidin 3,5-diglyocsides have their larger glycosyl at the 3-position and the smaller glycosyl at the 5-position, respectively.[1,4,7−13]



Anthocyanidins

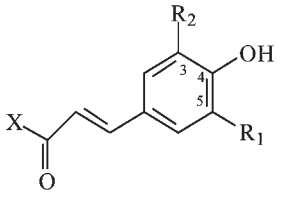
Cyanidin: R_1=OH, R_2=H, $[A]^+$ (287 Da)

Pelargonidin: R_1=R_2=H, $[A]^+$ (271 Da)

Radish anthocyanins:

R_3=sophorosyl, with acyls,

R_4= glucosyl, diglucosyl, with malonyl



Hydroxycinnamoyls (the mass data):

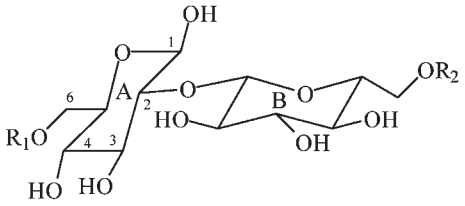
p-Coumaroyl: R_1=R_2=H (146 Da)

Caffeoyl: R_1=OH, R_2=H (162 Da)

Feruloyl R_1=OMe, R_2=H (176 Da)

Sinapoyl: R_1=R_2=OMe (206 Da)

X=sophorosyl



Sophorose: R_1= R_2 =H (324 Da)

Figure 2. Structures of the anthocyanins from purple Bordeaux and colored radishes.

This anthocyanin also had two important minor $MS^2\{1535\}$ product ions at *m/z* 1373 (8%, $[M]^+$-glc) and *m/z* 963 (7%, 1125 − 162), indicating the loss of a glucosyl from the molecular ion and the main $MS^2\{1535\}$ product ion [Cy 3-acyl-glycosyl]$^+$. The $MS^3\{1125\}$ product ion at *m/z* 963 and an $MS^4\{963\}$ ion at *m/z* 287 (from *m/z* 1125 to 963, 100%) formed by the loss of a glucosyl from the major $MS^2\{1535\}$ product ion at *m/z* 1125 and the whole glycosyl from the MS^3 {1125} product ion at *m/z* 963. This set of four fragments confirmed that this anthocyanin has a glucosylacyl function at its 3-position.

An additional minor $MS^2\{1535\}$ product ion at *m/z* 1197 (2%, $[M]^+$ − 162 − 176) indicated the existence of a feruloyl. Combined with the presence of 3 glucosyls at the 3-position, this suggested the presence of a second feruloyl at the 3-position (176 = 838 − (3 × 162) − 176), too. These data rule out the presence of a *p*-coumaroyl (146 Da) or a sinapoyl (206 Da). Thus, this anthocyanin was tentatively identified as cyanidin 3-(glucosylferuloyl)feruloylsophoroside-5-malonyldiglucoside. Considering that the glucosylacyl function of structurally similar anthocyanin analogues are connected to the first glucosyl (the glucosyl connected directly to the 3-position) and that the glucosyl has been found to be connected to the 4-position of the four common acyls (except caffeoyl, which has an extra 3-position),[1−4,7−15,30,34] this anthocyanin might be identified further as cyanidin 3-[2″-(6‴-feruloylglucosyl)-6″-(4-glucosylferuloyl)]glucoside-5-(6″-malonyl-4″-glucosyl)glucoside. Finally, the accurate HRMS measurement of 1535.4146 confirmed the identification as it is perfectly matched to $C_{68}H_{79}O_{40}$, the formula of this anthocyanin (−0.14 ppm).

The anthocyanin in peak 33 also had other $MS^2\{1535\}$ product ions, such as *m/z* 1491 (2%, $[M]^+$ − CO_2), 1449 (5%, $[M]^+$ − mal), 653 (5%, [Cy 5-glycosyl]$^+$ − CO_2), 491 (2%, [Cy 5-glycosyl]$^+$ − CO_2 − glc), that supported a malonyl at the 5-position (Figure 3A). These ions were not listed in Table 1 because the existence of malonyl has been fully confirmed by other listed ions.

In the same manner, peak 23, an isomer of peak 33, had the same molecular ion and $MS^2\{1535\}$ product ions, but a minor $MS^2\{1535\}$ product ion at *m/z* 1329 (2%, $[M]^+$ − 206). This suggested the existence of a sinapoyl (206 Da) with no connection to the extra glucosyl group. The difference in mass between the sinapoyl and the feruloyl was offset by the presence of *p*-coumaroyl rather than feruloyl. Thus, on the basis of the same consideration, this isomer might be identified further as cyanidin 3-[2″-(6‴-sinapoylglucosyl)-6″-(4-glucosyl-*p*-coumaroyl)]glucoside-5-(6″-malonyl-4″-glucosyl)glucoside. For conciseness, similar discussions regarding such assignment will not be given to the remaining anthocyanins.

Peaks 18 and 21 are isomers with identical MS data (Table 1). These peaks were identified as cyanidin 3-(glucosylcaffeoyl)feruloylsophoroside-5-malonyldiglucoside or its isomer with a 3-(glucosylferuloyl)caffeoylsophorosyl group. Similarly, peaks 19, 24, and 30, with identical MS, MS^2, MS^3, and MS^4 product ions, were identified as cyanidin 3-(glucosyl-*p*-coumaroyl)feruloylsophoroside-5-malonyldiglucoside, or its isomer with 3-(glucosyl-feruloyl)-*p*-coumaroylsophorosyl, and another isomer with a different position for the acyl group, respectively. Peak 20 was identified as cyanidin 3-(glucosylferuloyl)sinapoyl-sophoroside-5-malonyldiglucoside or its isomer having a 3-(glucosylsinapoyl)feruloyl-sophorosyl group. Peak 15 was identified as cyanidin 3-(glucosyl-*p*-coumaroyl)caffeoyl-sophoroside-5-manoyldiglucoside or its isomer having a 3-(glucosylcaffeoyl)-*p*-coumaroylsophorosyl group.

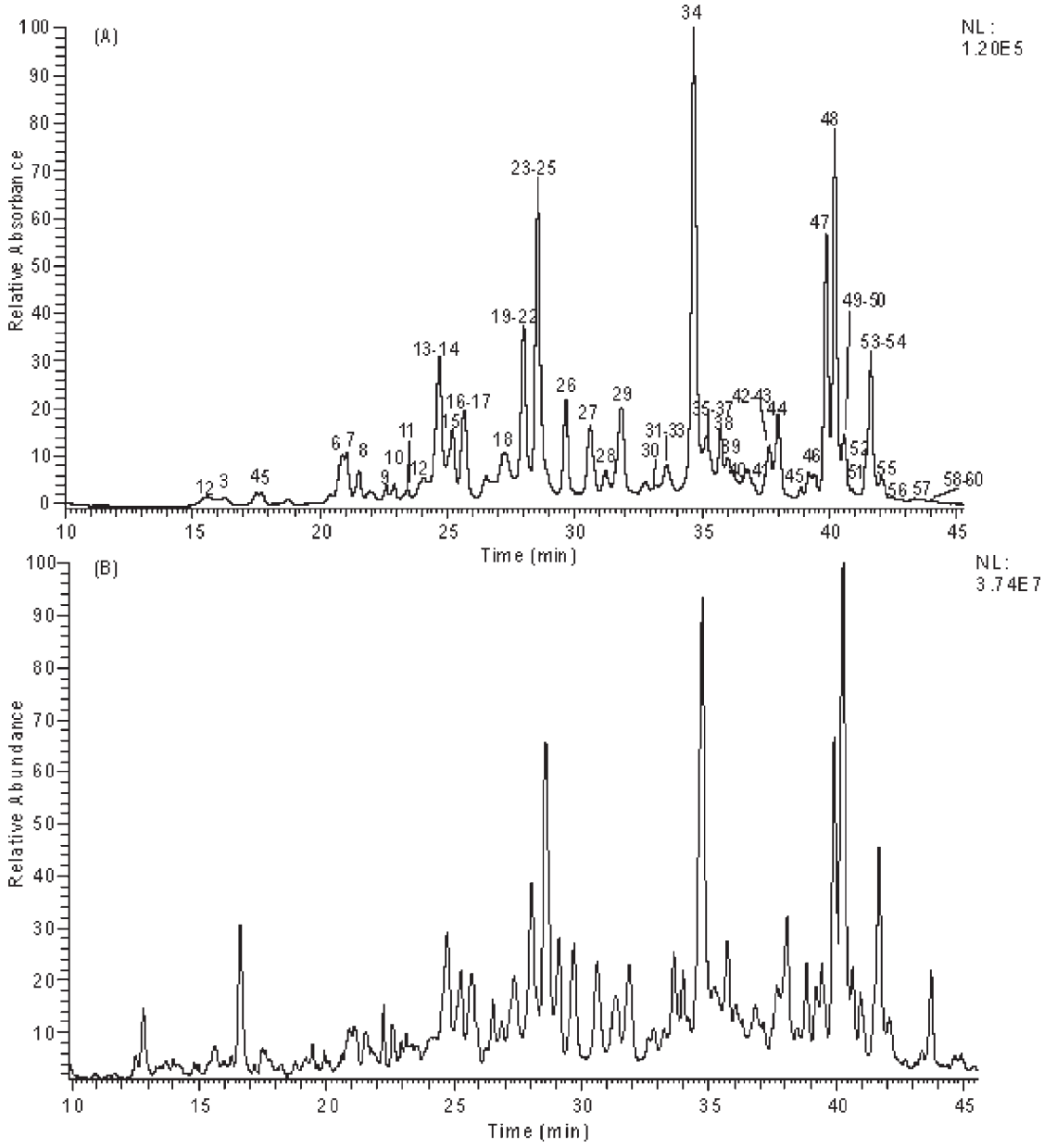


Figure 3. Chromatograms of purple Bordeaux radish extracts: (A) absorbance at 520 nm; (B) MS TIC.

Four other anthocyanins were found to have no malonyl at the 5-position with MS^2 product ions at m/z 611. The similar MS^2, MS^3, and MS^4 product ions indicate the existence of the 3-glucosylacyl. Thus, peaks 9 and 12 were isomers and identified as cyanidin 3-(4-glucosylcaffeoyl)feruloylsophoroside-5-diglucoside and cyanidin 3-(4-glucosylferuloyl)caffeoylsophoroside-5-diglucoside, respectively. Peak 14 was identified as cyanidin 3-(4-glucosyl-*p*-coumaroyl)feruloylsophoroside-5-diglucoside or the isomer with 3-(4-glucosylferuloyl)-*p*-coumaroylsophoroside group. Peak 16 was identified as cyanidin 3-(glucosyl-*p*-coumaroyl)sinapoylsophoroside-5-diglucoside or its isomers with a 3-(glucosylsinapoyl)-*p*-coumaroylsophorosyl group or with two feruloyls.

Finally, the mass data for peak 11 suggested two glucosylacyls at the 3-position. Thus, this anthocyanin was identified as cyanidin 3-(glucosyl-*p*-coumaroyl)(glucosylsinapoyl) sophoroside-5-malonyldiglucoside or its isomer with two glucosylferuloyl groups.

Acylated anthocyanins containing a glucosylacyl function have been isolated from plants from other genera, such as *Brassica*, *Ipomoea*, *Orychophragonus*, *Lobularia*, *Iberis*, and *Arabidopsis*.[1−4,14,15,21−26,30] However, only a few of them were studied with LC-MS^n analysis.[21−26,30] These compounds have been identified in red cabbage from their acylated 3-triglucosyl isomers, but different elution orders for some of the isomers were reported,[23−25] indicating that the identification was not reliable. In this study, two key minor MS^2 product ions were used to determine the existence of a 3-glucosylacyl function. This determination was confirmed by major MS^3 and MS^4 product ions and alkaline hydrolysis. The accuracy of this was confirmed by the correct identification of glucosylacylated anthocyanins in several plants from the *Brassica* and *Ipomoea* genera (Figure 6).

Acylated Cyanidin 3-Sophoside-5-malonyldiglucosides or 5-Diglucosides. Acylated cyanidin 3-sophoroside-5-malonyldiglucosides have no glucosylacyl group at the 3-position, do not have

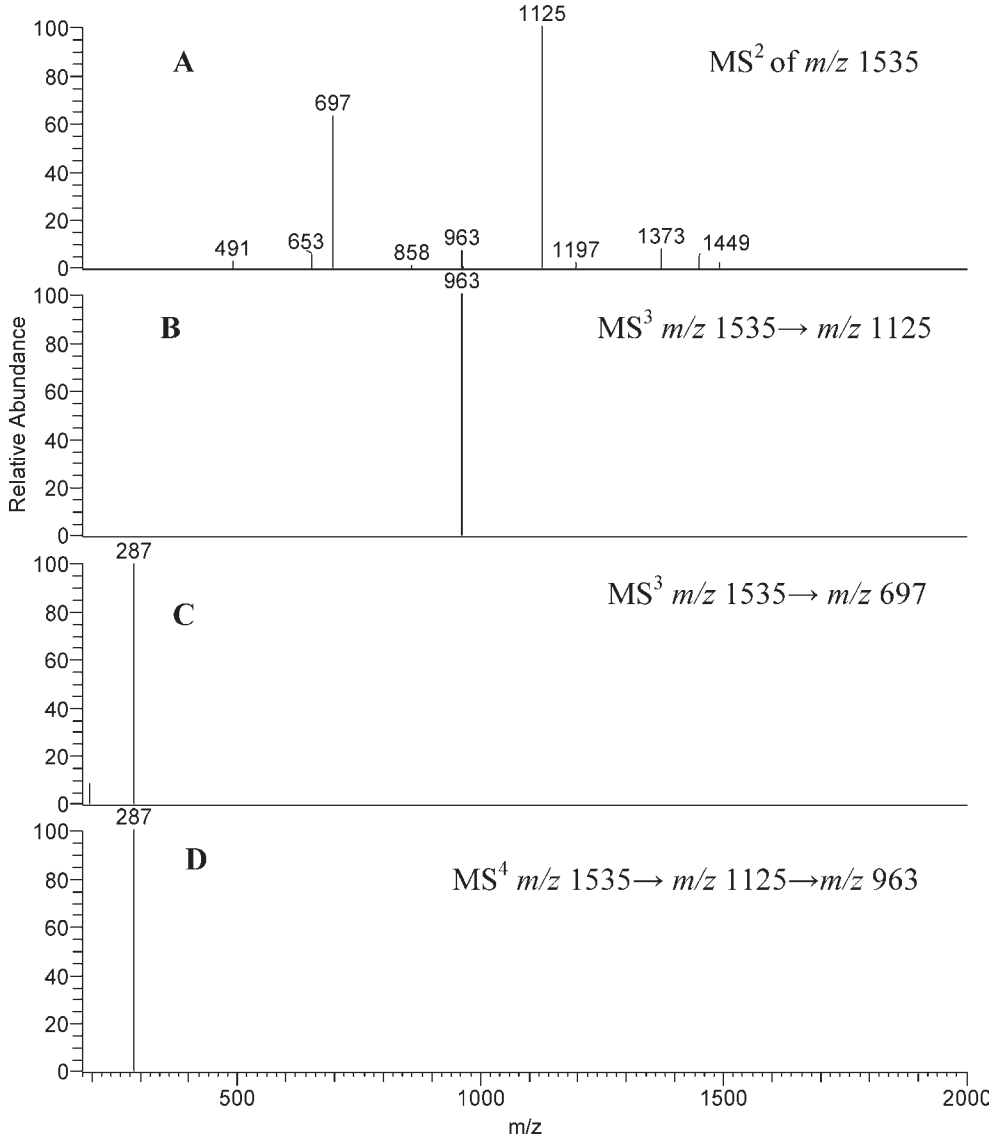


Figure 4. MS^2, MS^3, and MS^3 product ions of cyanidin 3-(glucosylferuloyl)feruloyl-sophororide-5-malonyldiglucoside (peak 33): (A) $S^2\{1535\}\rightarrow$1491 (2%, $[M]^+ - CO_2$), 1449 (5%, $[M]^+ -$ mal), 1373 (8%, $[M]^+ -$ glc), 1197 [2%, $[M]^+ -$ 162 (glu) − 176 (fur)], 1125 (100%, [Cy 3-(glucosylferuloyl)feruloylsophorosyl]$^+$), 963 (7%, 1125 − 162), 697 (63%, [Cy 5-malonyldiglycosyl]$^+$)], 653 (5%, [Cy 5-glycosyl]$^+ - CO_2$), 491 (2%, [Cy 5-glycosyl]$^+ - CO_2 -$ glc); (B) $MS^3\{1125\}\rightarrow$963 (100%, [Cy 3-diferuloylsophorosyl]$^+$); (**C**) MS^3 $\{697\}\rightarrow$287 (100%, [Cy]$^+$); (**D**) $MS^4\{963\}\rightarrow$287 (100%, [Cy]$^+$).

the related minor MS^2 and MS^3 product ions, and do not need an additional MS^4 step to identify the aglycone. Peak 57, for example, has main product ions $MS^2\{1373\}\rightarrow$963 and 697 and $MS^3\{963\}\rightarrow$287, and a minor MS^2 product ion at m/z 1197 (1%) represents the 176 Da loss of a feruloyl from the molecular ion (Figure 5). Thus, it was identified as cyanidin 3-diferuloylsophoroside-5-malonyldiglucoside. This anthocyanin might be further identified as cyanidin 3-[2″-(6‴-feruloyl)glucosyl-6″-feruloyl]glucoside-5-(6″-malonyl-4″-glucosyl)-glucoside after consideration of the fact that all of the anthocyanins isolated from the colored radishes have their first acyl group at the 6-position of the first glucosyl group and two acyls at the 6-positions of both glucosyls of the 3-sophorosyl group.[1,4,7−13] Peaks 48, 55, and 56, isomers of peak 57, have the same molecular ion and the same major MS^2 and MS^3 product ions. Peak 56 has a minor $MS^2\{1373\}$ product ion at m/z 1227 not seen for peaks 48 and 55, which suggested the loss of a *p*-coumaroyl, and was identified as cyanidin 3-*p*-coumaroylsinapoylsophoroside-5-malonyldiglucoside. The other two peaks were identified as the isomers of peaks 56 and 57.

In the same manner, peak 29 was identified as cyanidin 3-dicaffeoylsophoroside-5-malonyldiglucoside.[1,4,7−13] Peaks 45 and 32 were also identified as cyanidin 3-diacylsophoroside-5-malonyldiglucosides with a feruloyl and sinapoyl, and a caffeoyl and sinapoyl, respectively. Three pairs of isomeric anthocyanins, peaks 47 and 49, peaks 35 and 44, and peaks 36 and 42, were identified as cyanidin 3-diacylsophoroside-5-malonyldiglucosides containing a *p*-coumaroyl and feruloyl, a caffeoyl and feruloyl, and a *p*-coumaroyl and caffeoyl, respectively. Peak 60 was cyanidin 3-acylglycoside-5-malonyldiglucoside, but its acyls and disaccharosyl group were not assigned.

Another three anthocyanins with significant MS^2 product ions at m/z 611 showed no malonyl at the 5-position. Peak 27 was identified as cyanidin 3-caffeoylferuloyl-sophoroside-5-diglucoside. Peak 37 was identified as cyanidin 3-*p*-coumaroylferuloyl-sophoroside-5-diglucoside.

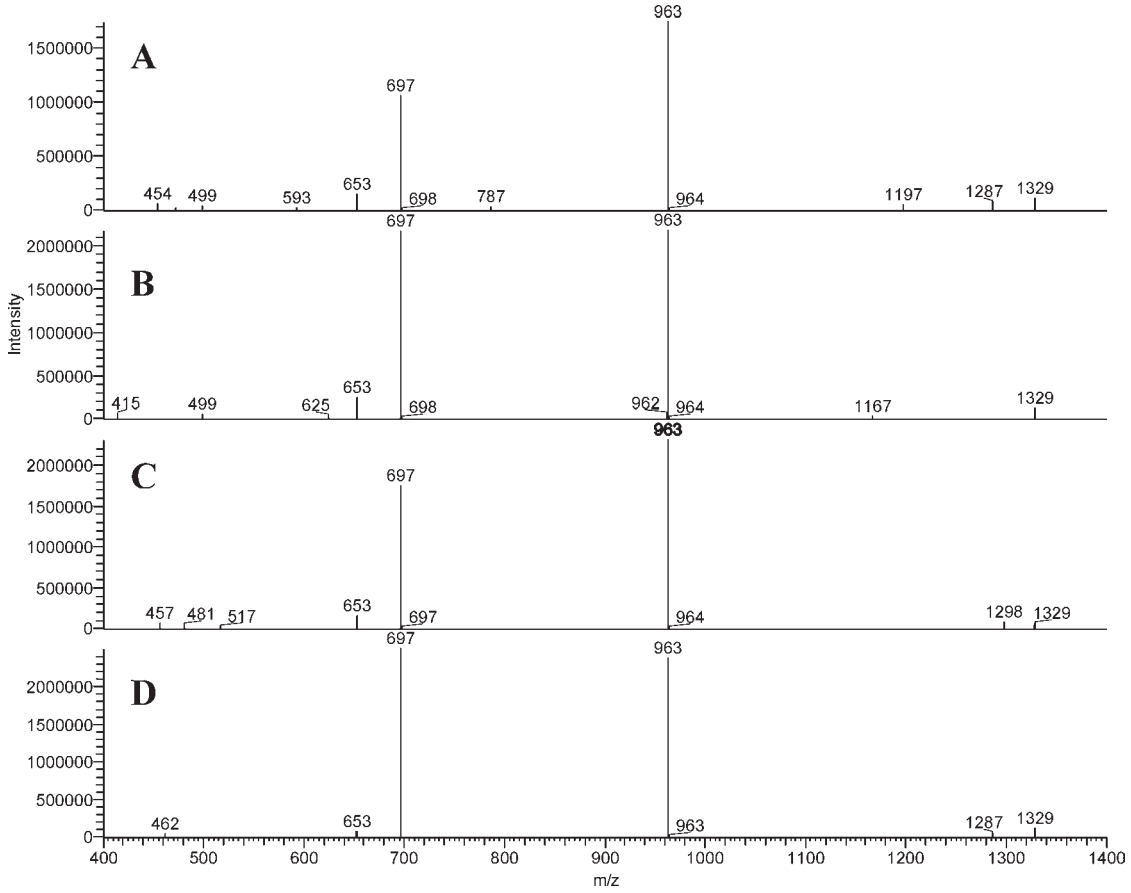


Figure 5. MS^2 spectra of cyanidin 3-[2″-(6‴-feruloyl)glucosyl-6″-feruloyl]glucoside-5-(6″-malonyl-4″-glucosyl)glucoside (A, peak 57) and its three isomers, peaks 56, 55, and 48 (B−D), respectively.

Peak 39 was identified as cyanidin 3-*p*-coumaroylsinapoyl-sophoroside-5-diglucoside or its isomer with two feruloyl groups.

Seven anthocyanins with low weight molecular ions were found to have one acyl group at the 3-position and MS^2 product ions of Cy 3-acylsophorosyl. Peaks 6, 10, 13, and 31 have the same MS^2 product ion at *m/z* 697 of Cy 5-malonyldiglucosyl and $[M]^+$ and mass differences of 410 Da between their molecular ion and their primary MS^2 product ion. They were identified as cyanidin 3-sophoroside-5-malonyldiglucosides containing caffeoyl (peaks 6 and 10), feruloyl (peak 13), and *p*-coumaroyl (peak 31) at the 6-position of the first glucosyl.[1−4,7−13]

Two other anthocyanins (peaks 4 and 7) have no malonyl at the 5-position as indicated by the MS^2 ion at *m/z* 611. Their primary MS^2 product ions at *m/z* 773 (peak 4) and 787 (peak 7) allow them to be identified as cyanidin 3-caffeoyl- and feruloylsophoroside-5-diglucosides, respectively. Peak 1 contains a malonyl as indicated by its primary MS^2 product ion at *m/z* 697 (100%) and can be an isomer with the malonyl at the 3- or 5-position.

To the best of our knowledge, only a few anthocyanidin 3-glycoside-5-dihexosides have been reported. They are the three acylated pelargonidin 3-sophoroside-5-(4-glucosyl)glucosides reported in Chinese red radish[4,11] and the two acylated cyanidin 3-sambubioside-5-sophorosides in *Sinapis alba* (Cruciferae).[1] Thus, the 38 acylated cyanidin 3-sophoroside-5-diglucosides from purple Bordeaux radish and their parent anthocyanins are reported for the first time in the current study.

Acylated Cyanidin 3-Sophoroside-5-glucosides. The LC-PDA-ESI/MS analyses of purple Bordeaux radish extracts also revealed 22 acylated cyanidin 3-dihexoside-5-hexosides. Cyanidin 3-sophoroside-5-glucoside was chosen as a reference on the basis of the fact that it and many of its acylated derivatives from red cabbage and sweet potato have been unequivocally identified by NMR analysis.[1−4] The exact retention times (in spiking tests on two more different columns) and UV−vis and MS data for the parent anthocyanins in the 3 alkaline-hydrolyzed extracts were used to confirm that cyanidin 3-sophoroside-5-glucoside (peak AH-1) was also the parent anthocyanin of the 22 acylated derivatives in purple Bordeaux radish. This conclusion was further supported by the reports that three other colored radishes contained 3-sophoroside-5-glucoside of pelargonidin or cyanidin and their acylated derivatives.[1−4,7−13]

Four of the 22 anthocyanins had one glucosylacyl function and showed the same product ion pattern as the similar compounds described above. Peak 26 has a minor $MS^2\{1373\}$ product ion at *m/z* 1167 (2%), a loss of 206 from the molecular ion indicating the loss of sinapoyl. It also has a $MS^2\{1373\}$ product ion at *m/z* 1211 (19%, loss of 162) and a $MS^3\{1125\}$ product ion at *m/z* 963 (loss of 162). Thus, this peak was identified as cyanidin 3-(glucosyl-*p*-coumaroyl)sinapoylsophoroside-5-malonylglucoside or its isomer with a 3-(glucosylsinapoyl)-*p*-coumaroylsophorosyl group. The latter is the isomer of cyanidin 3-[2″-(2‴-sinapoylglucosyl)-6″-(4-glucosyl)-*p*-coumaroyl]glucoside-5-(6″-malonyl)-glucoside from *Iberis umbellata*.[34] It cannot be determined whether the

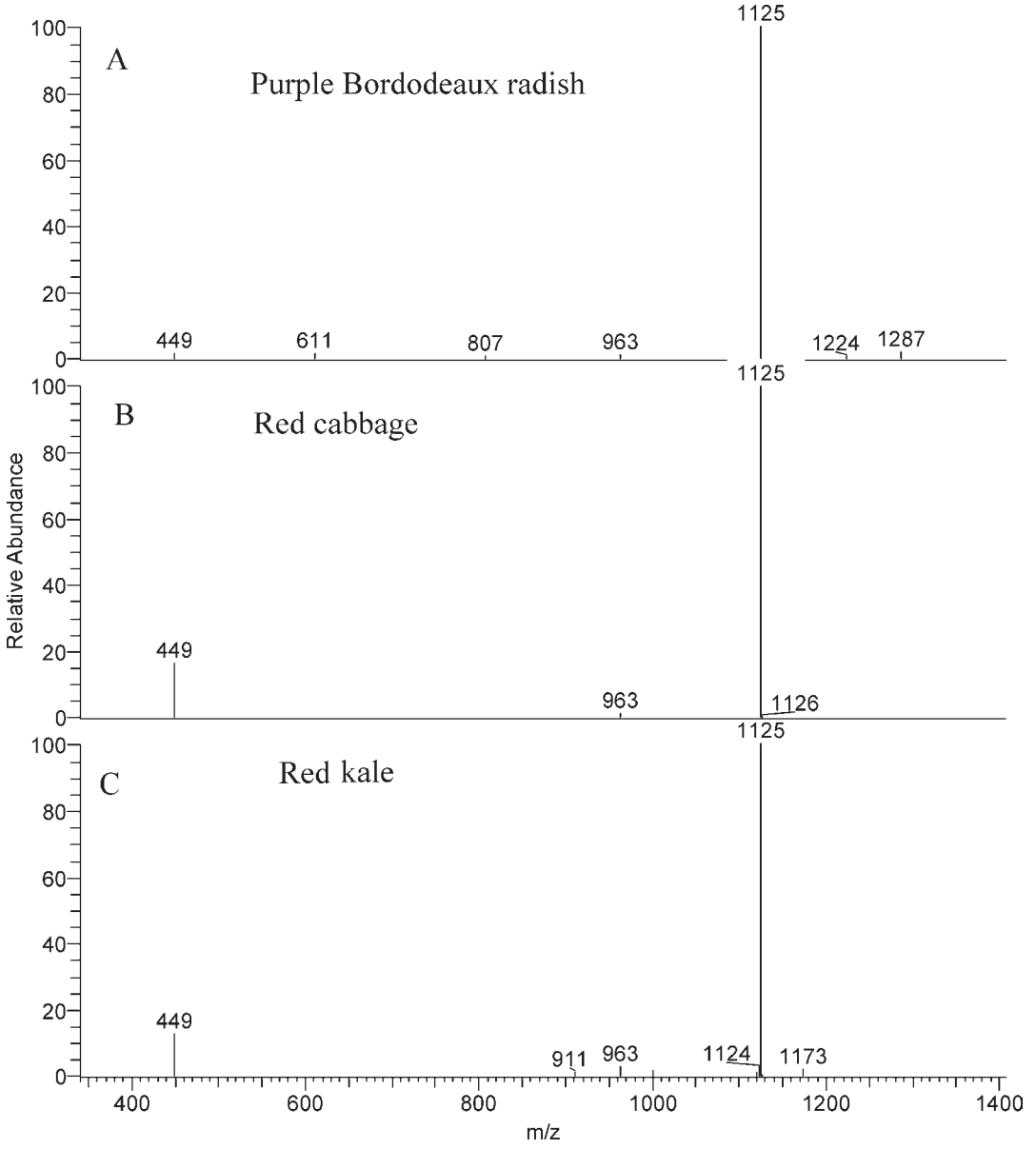


Figure 6. MS^2 spectra of cyanidin 3-(glucosylsinapoyl)-*p*-coumaroylsophoroside-5-glucoside in (A) purple Bordeaux radish (peak 17), (B) red cabbage, and (C) red kale.

sinapoyl is connected to the 2- or 6-position of the second glucosyl on the basis of tandem mass data analysis.

Similarly, peak 22 showed a minor MS^2 product product ion at *m/z* 773 $\{[M]^+ - 535 - 162)\}$ for loss of glucosyl and was assigned as cyanidin 3-(glucosylcaffeoyl)sophoroside-5-malonylglucoside. Peak 59 was identified as cyanidin 3-(feruloylglucosyl-feruloyl)feruloyl-sophoroside-5-malonylglucoside or its isomer with one *p*-coumaroyl and one sinapoyl group to replace two feruloyl groups. The last one, peak 17, has no malonyl at the 5-position and no minor MS^2 product ions for the loss of an acyl group. As shown in Figure 6, this anthocyanin was identified as cyanidin 3-(glucosylsinapoyl)-*p*-coumaroylsophoroside-5-glucoside by direct comparison to this anthocyanin in the database that had previously been identified by LC-PDA-ESI/MS^n in red cabbage.[22]

The remaining 19 acylated cyanidin 3-sophoroside-5-glucosides do not have a glucosylacyl group at the 3-position and can be easily identified. The product ions of peak 54 shows the loss of a feruloyl group, and it was identified as cyanidin 3-diferuloylsophoroside-5-malonylglucoside. This identification might be further refined to cyanidin 3-[2″-(6‴-feruloylglucosyl)-6″-feruloyl]glucoside-5-(6″-malonyl)glucoside on the basis of data in the literature.[1−4,7−15] Similarly, its isomer, peak 58, might be identified further as cyanidin 3-[2″-(6‴-*p*-coumaroylglucosyl)-6″-sinapoyl)glucoside-5-(6″-malonyl)glucoside or its isomer with a 3-[2″-(6‴-sinapoylglucosyl)-6″-*p*-coumaroyl)glucosyl group. The latter compound was reported in *Brassica campestris* var. *chinensis*[35] and is an isomer of cyanidin 3-[2″-(2‴-sinapoylglucosyl)-6″-*p*-coumaroyl)]glucoside-5-(6″-malonyl)glucoside from *I. umbellata*.[34]

Similarly, three isomeric anthocyanins (peaks 34, 38, and 46) and two pairs of isomeric anthocyanins (peaks 50 and 52 and peaks 51 and 53) have MS^2 product ions at *m/z* 949, 993, and 933, respectively, indicating the loss of malonylglucosyls. These peaks were identified as cyanidin 3-diacylsophoroside-5-malonylglucosides with two different acyl groups at the 6-position of both glucosyls.

Among them, one of the peaks at 50 and 52 was tentatively identified as cyanidin 3-(6-feruloyl-2-sinapoylglucosyl)glucoside-5-(6-malonyl)glucoside on the basis of a similar compound detected in *B. campestris* var. *chinensis*[35] and broccoli sprouts.[26] Peak 51 or 53 might be an isomer of cyanidin 3-[2″-(2‴-feruloylglucosyl)-6″-*p*-coumaroyl]glucoside-5-(6″-malonyl)glucoside from *I. umbellata*.[34]

Three anthocyanins have 5-malonylglucosyl at the 5-position (MS^2 product ion at m/z 535) and one acyl group at the 3-position. Peak 25 was identified as cyanidin 3-(2″-glucosyl-6″-caffeoyl)glucoside-5-(6″-malonyl)glucoside, a compound first isolated from Chinese purple radish.[7] The remaining two isomers (peaks 40 and 43) should be cyanidin 3-feruloylsophoroside-5-malonylglucosides. One of them could be cyanidin 3-(2″-glucosyl-6″-feruloyl)glucoside-5-(6″-malonyl)glucoside, isolated from Chinese purple radish[7] and detected in red radish.[22]

Another five anthocyanins contain only a glucosyl function at the 5-position (MS^2 product ions at m/z 449). Peaks 28 and 41 were identified as the 3-caffeoylferuloylsophoside-5-glucoside and the 3-feruloylsophoroside-5-glucoside of cyanidin, respectively. Peak 5 was identified as cyanidin 3-caffeoylsophoroside-5-glucoside. Peak 8 was identified as cyanidin 3-feruloylsophoroside-5-glucoside. These three compounds have also been detected in red cabbage and purple sweet potato.[1−4,21−28] The compounds in peaks 2 and 3 contained 5-malonylglucosyl but no acyl group at the 3-position. They were identified as cyanidin 3-sophoroside-5-malonylglucoside, and their malonyl group was connected to a different position on the 5-glucosyl function. One of them might be malonyl cyanidin 3-sophoroside-5-glucoside, a new anthocyanin identified recently in Chinese purple radish. No location was specified for the malonyl group.[7] It was noted that the anthocyanins containing a malonyl group at the 5-position are more easily ionized (higher ion counts) than the anthocyanins without a malonyl group at this position.

Of the many acylated cyanidin 3-sophoroside-5-glucosides identified in *Brassica*, *Ipomoea*, and *Raphanus*, 12 had malonyl at the 5-position (8 were from 4 isomer pairs),[1−4,7,21−28,35] and 11 others from *I. umbellate* (Cruciferae) had an acyl at the 2-position of the second glucosyl.[34] Thus, around 12 of the purple Bordeaux radish acylated cyanidin sophoroside-5-glucosides might be previously detected in plants (indicated by footnotes *d* and *e* in Table 1), and the uncertainty of the exact number of compounds arises because 12 are isomers.

This study showed that it is possible to make reliable assignments to complex anthocyanins in a plant, using the systematic identification strategy. The strategy can be summarized as (1) identification of the parent anthocyanin with standard, (2) analysis of the LC-DAD-ESI/MS^n data to provide tentative identification, (3) comparison of the LC-DAD-ESI/MS^n data and the MS^n product ions with those of analogous anthocyanidins in the database and literature, and (4) confirmation of the identities with HRMS measurements. However, the positive identification of isomeric structures, including stereochemistry, for the new anthocyanins will still require NMR analysis.[7−15]

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