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UHPLC-PDA-ESI/HRMS^n Profiling Method To Identify and Quantify Oligomeric Proanthocyanidins in Plant Products

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ABSTRACT: Oligomeric proanthocyanidins were successfully identified by UHPLC-PDA-HRMS^n in a selection of plant-derived materials (jujube fruit, Fuji apple, fruit pericarps of litchi and mangosteen, dark chocolate, and grape seed and cranberry extracts). The identities of 247 proanthocyanidins were theoretically predicted by computing high-accuracy masses based on the degree of polymerization, flavan-3-ol components, and the number of A type linkages and galloyls. MS^n fragments allowed characterization on flavan-3-ol based on the monomer, connectivity, and location of A-type bonds. Identification of doubly or triply charged ions of 50 PAs was made on the basis of theoretical calculations. A single catechin standard and molar relative response factors (MRRFs) were used to quantify the well-separated PAs. The ratios of the SIM peak counts were used to quantify each of the unseparated isomers. This is the first report of direct determination of each of the proanthocyanidins in plant-derived foods and proanthocyanidins containing an epifisetinidol unit in grape seeds.

KEYWORDS: *oligomeric proanthocyanidins, identification, quantification, plant products, UHPLC-PDA-ESI/HRMS^n profiling method*

INTRODUCTION

Proanthocyanidins (PAs) are various length polymers of flavanols (catechins and their enantiomers) linked through a single C_4-C_8 or C_4-C_6 bond (B-type PAs) or with an additional C_2-O-C_7 or C_2-O-C_5 bond (A-type PAs) as shown in Figure 1. There are a variety of different classes of PAs, depending on the substitution pattern of the monomeric flavan-3-ols (mainly epicatechins, epigallocatechins, and epiafzelechins to form procyanidins, propelargonidins, and prodelphinidins), acyls (usually galloyl), glycosyls, and other substituents.[1−4] The highly polymerized PAs are reported to have molecular weights up to 30000 Da. However, these PAs may not be efficiently extracted from plant materials.[1−3]

PAs are the main polyphenolic components in many different plant-derived foods, such as grains, berries, fruits, nuts, and teas, and are reported to have a variety of health-promoting benefits.[1−7] As the degree of polymerization increases, the compounds become less soluble in aqueous solution and less bioavailable in the intestine. Fermentation in the colon, however, leads to absorption of many of the metabolic products. The most absorbed PAs in the intestine have a degree of polymerization (DP) less than or equal to 4 (DP ≤ 4).[1−7] Accurate analytical methods for the separation, identification, and quantification of individual oligomeric PAs in foods are necessary to establish the relationship between dietary intake of polyphenols and health outcomes from biological, epidemiological, and clinical studies.

PAs have a high structural diversity with many regioisomeric (order of linkage for the flavan-3-ols) and stereoisomeric (physical structure of individual flavan-3-ols) forms, which makes identification and quantification difficult tasks. In general, analytical methods have focused on each oligomer as a class and have been unable to identify the PAs within each class. Matrix-assisted laser desorption ionization−time-of-flight-mass spectrometry (MALDI-TOF-MS) has been used to detect PA metal adducts and to determine the types and DP values of the compounds.[1,8−12] ESI-MS^n has also been used to identify PA molecular ions and their fragments.[8,13−23] However, neither of these methods can identify the PA isomers.[1,7−16]

Normal and reverse phase HPLC methods have been used to separate PA oligomers and tandem MS has been used to characterize the PAs for DP ≤ 6 (typically m/z 50−2000).[1,12,19−23] Doubly and triply charged negative molecular ions of some higher oligomers (DP > 6) have been detected using negative ionization.[1,4,14−19] Reverse phase HPLC-PDS-MS analysis of thiolytically degraded products of PAs has been used to identify the PA terminal (with the C_8 connection) and extension units (with the C_4 connection) and to determine the mean DP value (mDP).[1−4,11−17] Both ^{1}H and ^{13}C NMR analyses have been used to identify PA flavan-3-ols and the *cis* or *trans* stereochemistries of PAs.[10,11] Until now, however, there has been only limited application of UHPLC-HRMS^n to the study of oligomeric PAs.[20−23]

Total PA concentration has been estimated using colorimetric methods. In addition, total concentrations for each oligomeric class (DP = 2−10) have been estimated using fluorescence detection and relative response factors (based on mass) following separation by normal phase chromatography.[2−4,6] HPLC-PDA-MS analysis of PA thiolytic degraded

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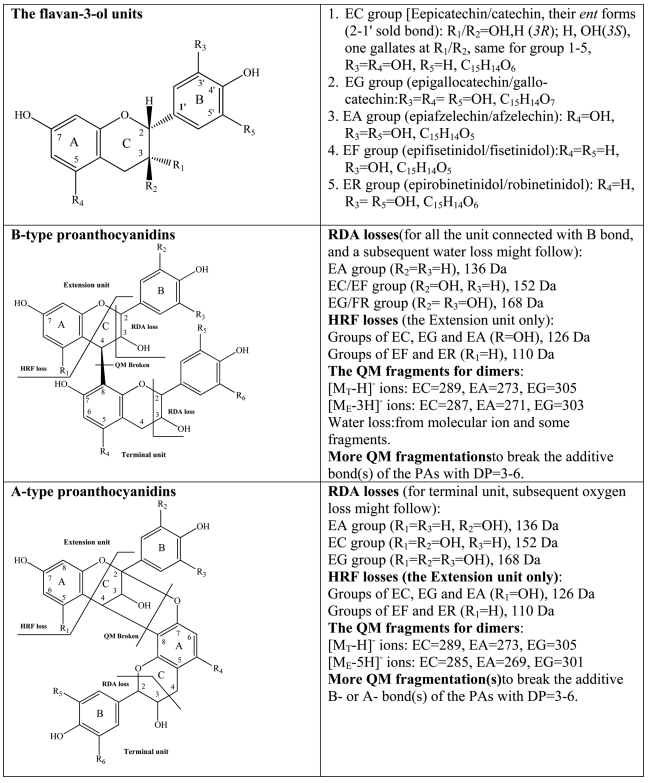

Figure 1. Structures of flavan-3-ol units and common fragmentation patterns for proanthocyanidins.

mixtures has also been used for quantification of PAs.[1−4,6,10−12] However, direct quantification of the different PAs comprising each oligomeric class is still problematic due to the difficulty of separation and the lack of standards.[1−4]

As a part of a project to systematically identify and quantify food phenolic compounds, a standardized HPLC-PDA-ESI/MS method was developed for the identification and quantification of food polyphenols, including some PAs.[24] Quantification was based on UV absorbance and molar relative response factors (MRRFs).[25] This method has been upgraded and now uses ultrahigh-performance liquid chromatography−photodiode array detection−high-resolution mass spectrometry operated in the tandem mode (UHPLC-PDA-ESI/HRMS^n).[26] In the current study, this method was employed to identify nearly 300 oligomeric PAs in selected plants (fruit pericarps of litchi and mangosteen), extracts (from grape seed and cranberry), and food samples (jujube, Fuji apple, and chocolate) and to quantify PAs in grape seed extract. The main PAs in each of the oligomeric classes were quantified.

MATERIALS AND METHODS

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

Standards. (+)-Catechin, (−)-epicatechin, (−)-gallocatechin-3-*O*-gallate, (−)-epigallocatechin-3-*O*-gallate, procyanidin B_1, procyanidin B_2, procyanidin C_1, and procyanidin A_2 were obtained from Chromadex, Inc. (Irvine, CA, USA). The standards were vacuum-dried using a vacuum drying box (National Appliance Co., Portland, OR, USA) at 110 °C until a constant weight was reached (about 24 h). These dried standards were used to determine the MRRF that were used for calibration.[25]

Plant Materials and Extraction. Fresh fruits of jujube (*Ziziphus jujuba* Mill), Fuji apple (*Malus domestica* Borkh cv. Fuji), litchi (*Litchi chinensis* Sonn.), and mangosteen (*Garcinia mangostana* Linn.) were purchased from local food stores. Dark chocolate was purchased from a local Trader Joes store in Maryland, USA. The extracts of grape seed and cranberry were kindly supplied by Triarco Industries, Inc. (Paterson, NJ, USA). The fruit pericarps of litchi and mangosteen and the skins of fresh jujubes and apples were lyophilized, and the dried materials were powdered.[24−26]

Each of the powdered fruit samples (250 mg) was extracted with 5.000 mL of a methanol/water (60:40, v/v) solvent using sonication for 60 min at room temperature. The slurry mixture was centrifuged at 2500 rpm for 15 min. The supernatant (4.000 mL) was taken from the tube and filtered through a 17 mm (0.45 μm) PVDF syringe filter (VWR Scientific, Seattle, WA, USA) for injections.[24−26] A second extraction using acetone/methanol/water (2:2:1, v/v/v, 4.000 mL) was treated in the same way to check the extraction efficiency of the general extraction method. The result showed that >95% of the mass for each main compound was extracted from the plant material by the first extraction.

Powdered chocolate samples (2000 mg) were extracted with 40 mL of the same aqueous methanol and treated as described above, and the supernatant was taken to dryness under vacuum at 40 °C. The approximately 30 mg of the residue was dissolved in water (1 mL) and passed through Sep-PakVac RC (500 mg) C_{18} cartridge (Waters Corp., Milford, MA, USA). After washing with water (5 mL), the PAs were eluted with methanol (5 mL) and again taken to dryness under vacuum. The residue was dissolved in 1.000 mL of the methanol/water solvent and filtered for injection.

The grape seed (10.80 mg) and cranberry (10.20 mg) extracts were dissolved in the same aqueous methanol (1.0 mL) and filtered. Triplicate injections (1 μL) of each solution were used to determine the average concentration and the relative standard deviation for each of the PAs in the extract. Dried catechin was used as the external calibration standard; 4 mg was placed in a 10 mL volumetric flask, dissolved in the methanol/water (60:40, v/v) solvent, and brought to volume. This stock solution was diluted 1:4 and 1:16. The stock and each dilution were injected onto the column three times and used to construct a calibration curve.

UHPLC-PDA-ESI/HRMS^n Conditions. The UHPLC-HRMS system used consisted of an LTQ Orbitrap XL mass spectrometer with an Accela 1250 binary pump, a PAL HTC Accela TMO autosampler, a PDA detector (ThermoScientific, San Jose, CA, USA), and a G1316A column compartment (Agilent, Palo Alto, CA, USA). The separation was carried out on a U-HPLC column (200 mm × 2.1 mm i.d., 1.9 μm, Hypersil Gold AQ RP-C_{18}) (Thermo- Scientific) with an HPLC/UHPLC precolumn filter (UltraShield Analytical Scientific Instruments, Richmond, CA, USA) at a flow rate of 0.3 mL/min. The mobile phase consisted of a combination of A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile, v/v). 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 PDA recorded spectra from 200 to 700 nm and provided real-time monitoring at 280 and 330 nm.[26]

The HRMS was operated in the negative ionization mode using the following conditions: sheath gas at 70 (arbitrary units), aux and sweep gas at 15 (arbitrary units), spray voltage at 4.8 kV, capillary temperature at 300 °C, capillary voltage at 15 V, and tube lens at 70 V. The mass range was from m/z 50 to 2000 with a resolution of 15000, FTMS 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 provide MS^2 to MS^5 product ions with a normalized collision energy at 35%.[26] The selective ion monitoring (SIM) mode was used to select the molecular ions of the isomers from each of the PA groups in grape seed extract for their quantification.

Table 1. Computed High-Resolution Mass, Molecular Weight, Molecular Ions, and Composition of Common Oligomeric PAs[a]

DP	proanthocyanidin	HRMW (Da)	HR $[M - H]^-$ (m/z)	HR $[M - 2H]^{2-}$ (m/z)	HR $[M - 3H]^{3-}$ (m/z)	C	H	O	EA/EF	EG	galloyl	A-bond
dimers	B-type propelargonidin	546.1518	545.1440			30	26	10	2	0	0	0
	B-type proanthocyanidin	560.1311	559.1233			30	24	11	1	0	0	1
	B-type proanthocyanidin	562.1467	561.1389			30	26	11	1	0	0	0
	B-type procyanidin	578.1416	577.1338			30	26	12	1	1	0	0
	A-type procyanidin	576.1260	575.1182			30	24	12	0	0	0	1
	B-type procyanidin	**578.1416**	**577.1338**			**30**	**26**	**12**	**0**	**0**	**0**	**0**
	galloylated procyanidin	730.1524	729.1446			37	30	16	0	0	1	0
	galloylated procyanidin	882.1632	881.1554			44	34	20	0	0	2	0
	B-type proanthocyanidin	592.1209	591.1131			30	24	13	0	1	0	1
	B-type proanthocyanidin	594.1365	593.1287			30	26	13	0	1	0	0
	B-type prodelphinidin	610.1314	609.1236			30	26	14	0	2	0	0
	galloylated proanthocyanidin	746.1473	745.1395			37	30	17	0	1	1	0
	galloylated prodelphinidin	914.1530	913.1452			44	34	22	0	2	2	0
trimers	B-type propelargonidin	818.2199	817.2121			45	38	15	3	0	0	0
	A-type proanthocyanidin	832.1992	831.1914			45	36	16	2	0	0	1
	B-type proanthocyanidin	834.2148	833.2070			45	38	16	2	0	0	0
	A-type proanthocyanidin	848.1941	847.1863			45	36	17	1	0	0	1
	B-type proanthocyanidin	850.2097	849.2019			45	38	17	1	0	0	0
	galloylated proanthocyanidin	986.2256	985.2178			52	42	20	2	0	1	0
	A-type procyanidin	862.1734	861.1656			45	34	18	0	0	0	2
	A-type procyanidin	864.1890	863.1812			45	36	18	0	0	0	1
	B-type procyanidin	**866.2046**	**865.1968**			**45**	**38**	**18**	**0**	**0**	**0**	**0**
	galloylated procyanidin	1018.2154	1017.2076			52	42	22	0	0	1	0
	galloylated procyanidin	1170.2262	1169.2184			59	46	26	0	0	2	0
	B-type proanthocyanidin	882.1995	881.1917			45	38	19	0	1	0	0
	B-type proanthocyanidin	898.1944	897.1866			45	38	20	0	2	0	0
	B-type prodelphinidin	914.1893	913.1815			45	38	21	0	3	0	0
	galloylated prodelphinidin	1034.2103	1033.2025			52	42	23	0	1	1	0
tetramers	A-type proanthocyanidin	1120.2622	1119.2544			60	48	22	2	0	0	1
	B-type proanthocyanidin	1122.2778	1121.2700			60	50	22	2	0	0	0
	A-type proanthocyanidin	1136.2571	1135.2493			60	48	23	1	0	0	1
	B-type proanthocyanidin	1138.2727	1137.2649			60	50	23	1	0	0	0
	A-type procyanidin	1148.2208	1147.2130			60	44	24	0	0	0	3
	A-type procyanidin	1150.2364	1149.2286			60	46	24	0	0	0	2
	A-type procyanidin	1152.2520	1151.2442			60	48	24	0	0	0	1
	B-type procyanidin	**1154.2676**	**1153.2598**			**60**	**50**	**24**	**0**	**0**	**0**	**0**
	galloylated procyanidin	1306.2784	1305.2706			67	54	28	0	0	1	0
	galloylated procyanidin	1458.2892	1457.2814			74	58	32	0	0	2	0
	A-type proanthocyanidin	1168.2469	1167.2391			60	48	25	0	1	0	1
	B-type proanthocyanidin	1170.2625	1169.2547			60	50	25	0	1	0	0
pentamers	A-type proanthocyanidin	1392.3303	1391.3225	695.1574		75	60	27	3	0	0	1
	B-type proanthocyanidin	1410.3408	1409.3330	704.1626		75	62	28	2	0	0	0
	A-type proanthocyanidin	1424.3201	1423.3123	711.1523		75	60	29	1	0	0	1
	B-type proanthocyanidin	1426.3357	1425.3279	712.1601		75	62	29	1	0	0	0
	A-type procyanidin	1436.2838	1435.2760	717.1341		75	56	30	0	0	0	3
	A-type procyanidin	1438.2994	1437.2916	718.1419		75	58	30	0	0	0	2
	A-type procyanidin	1440.3150	1439.3072	719.1497		75	60	30	0	0	0	1
	B-type procyanidin	**1442.3306**	**1441.3228**	**720.1575**		**75**	**62**	**30**	**0**	**0**	**0**	**0**
	galloylated procyanidin	1594.3414	1593.3336	796.1629		82	66	34	0	0	1	0
	B-type proanthocyanidin	1458.3255	1457.3177	728.1550		75	62	31	0	1	0	0
hexamers	B-type proanthocyanidin	1682.4089	1681.4011	840.1967		90	74	33	3	0	0	0
	A-type proanthocyanidin	1696.3882	1695.3804	847.1863		90	72	34	2	0	0	1

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Table 1. continued

DP	proanthocyanidin	HRMW (Da)	HR $[M - H]^-$ (m/z)	HR $[M - 2H]^{2-}$ (m/z)	HR $[M - 3H]^{3-}$ (m/z)	C	H	O	EA/EF	EG	galloyl	A-bond
	B-type proanthocyanidin	1698.4038	1697.3960	848.1941		90	74	34	2	0	0	0
	A-type proanthocyanidin	1710.3675	1709.3597	854.1760		90	70	35	1	0	0	2
	A-type proanthocyanidin	1712.3831	1711.3753	855.1838		90	72	35	1	0	0	1
	B-type proanthocyanidin	1714.3987	1713.3909	856.1916	570.4584	90	74	35	1	0	0	0
	A-type procyanidin	1724.3468	1723.3390	861.1656	573.7745	90	68	36	0	0	0	3
	A-type procyanidin	1726.3624	1725.3546	862.1734	574.4463	90	70	36	0	0	0	2
	A-type procyanidin	1728.3780	1727.3702	863.1812	575.1182	90	72	36	0	0	0	1
	B-type procyanidin	**1730.3936**	**1729.3858**	**864.1890**	**575.7901**	**90**	**74**	**36**	**0**	**0**	**0**	**0**
	galloylated procyanidin	1882.4044	1881.3966	940.1944	626.4603	97	78	40	0	0	1	0
	B-type proanthocyanidin	1746.3885	1745.3807	872.1865	581.1217	90	74	37	0	1	0	0
	galloylated proanthocyanidin	1898.3993	1897.3915	948.1919	631.7920	97	78	41	0	1	1	0
heptamers	A-type proanthocyanidin	1980.4200	1979.4122	989.2022	659.1322	105	80	40	2	0	0	3
	A-type proanthocyanidin	1996.4149	1995.4071	997.1997	664.4638	105	80	41	1	0	0	3
	B-type proanthocyanidin	2002.4617	2001.4539	1000.2231	666.4794	105	86	41	1	0	0	0
	A-type procyanidin	2012.4098	2011.4020	1005.1971	669.7955	105	80	42	0	0	0	3
	A-type procyanidin	2014.4254	2013.4176	1006.2049	670.4673	105	82	42	0	0	0	2
	A-type procyanidin	2016.4410	2015.4332	1007.2127	671.1392	105	84	42	0	0	0	1
	B-type procyanidin	**2018.4566**	**2017.4488**	**1008.2205**	**671.8111**	**105**	**86**	**42**	**0**	**0**	**0**	**0**
	galloylared procyanidin	2170.4674	2169.4596	1084.2259	722.4813	112	90	46	0	0	1	0
	A-type proanthocyanidin	2032.4359	2031.4281	1015.2102	676.4708	105	84	43	0	1	0	1
	B-type proanthocyanidin	2034.4515	2033.4437	1016.2180	677.1427	105	86	43	0	1	0	0
octamers	B-type proanthocyanidin	2274.5298	2273.5220	1136.2571	757.1688	120	98	46	2	0	0	0
	B-type proanthocyanidin	2290.5247	2289.5169	1144.2546	762.5004	120	98	47	1	0	0	0
	A-type procynidin	2302.4884	2301.4806	1150.2364	766.4883	120	94	48	0	0	0	2
	A-type procynidin	2304.5040	2303.4962	1151.2442	767.1602	120	96	48	0	0	0	1
	B-type procynidin	**2306.5196**	**2305.5118**	**1152.2520**	**767.8321**	**120**	**98**	**48**	**0**	**0**	**0**	**0**
	galloylated procyanidin	2454.4992	2453.4914	1226.2418	817.1586	127	98	52	0	0	1	2
	B-type proanthocyanidin	2322.5145	2321.5067	1160.2495	773.1637	120	98	49	0	1	0	0
nonamers	B-type proanthocyanidin	2562.5928	2561.5850	1280.2886	853.1898	135	110	52	2	0	0	0
	B-type proanthocyanidin	2578.5877	2577.5799	1288.2861	858.5214	135	110	53	1	0	0	0
	A-type procyanidin	2592.5670	2591.5592	1295.2757	863.1812	135	108	54	0	0	0	1
	B-type procyanidin	**2594.5826**	**2593.5748**	**1296.2835**	**863.8531**	**135**	**110**	**54**	**0**	**0**	**0**	**0**
	galloylated procyanidin	2742.5622	2741.5544	1370.2733	913.1796	142	110	58	0	0	1	2
	B-type proanthocyanidin	2610.5775	2609.5697	1304.2810	869.1847	135	110	55	0	1	0	0
decamers	B-type proanthocyanidin	2850.6558	2849.6480	1424.3201	949.2108	150	122	58	2	0	0	0
	B-type proanthocyanidin	2866.6507	2865.6429	1432.3176	954.5424	150	122	59	1	0	0	0
	A-type procyanidin	2878.6144	2877.6066	1438.2994	958.5303	150	118	60	0	0	0	2
	B-type procyanidin	**2882.6456**	**2881.6378**	**1440.3150**	**959.8741**	**150**	**122**	**60**	**0**	**0**	**0**	**0**
	galloylated procyanidin	**3030.6252**	**3029.6174**	1514.3048	1009.2006	157	122	64	0	0	1	2
	B-type proanthocyanidin	2898.6405	2897.6327	1448.3125	965.2057	150	122	61	0	1	0	0
	galloylated proanthocyanidin	**3050.6513**	**3049.6435**	1524.3179	1015.8760	157	126	65	0	1	1	0

[a]Composition is used for the numbers of the atoms of carbon, hydrogen, and oxygen of the molecular formula and the numbers of the flavan-3-ol units, A-type bonds, and galloyls. Abbreviations: DP, degree of polymerization; G, galloyl; EC, EA, EG, epicatechin, epiafzelechin, and epigallocatechin, respectively; C, H, O, carbon, hydrogen, and oxygen.

RESULTS AND DISCUSSION

Exact Masses and Molecular Formula for Proanthocyanidins. Chemically, each flavan-3-ol unit of a PA has two stereogenic (or chiral) centers (Figure 1), which can result in four (or 2^2) stereoisomers, that is, (2*R*,3*S*)-catechin or (+)-C, (2*R*,3*R*)-epicatechin or (+)-EC, (2*S*,3*R*)-catechin, or (−)-C, and (2*S*,3*S*)-epicatechin or (−)-EC. In this paper, EC will be used to represent all four isomers in the text, tables, and figures. Similarly, the abbreviations for epiafzelechin (EA), epigallocatechin (EG), epifisetinidol (EF), and robinetinidol (ER) will be used to represent their isomers in PAs. In this paper, the PAs formed with only EA, EC, or EG units are called propelargonidin, procyanidin, or prodelpeinidin, whereas those formed from two different units are called proanthocyanidins.

The B-type PA dimers have two flavan-3-ol units (i.e., four chiral centers) and an additional asymmetric center at C4.

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Table 2. Proanthocyanidins Found in Seven Samples

DP	proanthocyanidin	plant source[a]	HR [M − H]$^-$ (m/z)	mol formula	major MS^2 ions (m/z)(%)
monomer	epiazfelechin	L, C	273.0761	$C_{15}H_{13}O_5$	167(100)
	catechin	ALL	289.0710	$C_{15}H_{13}O_6$	245(100), 205(35), 179(12)
	epicatechin	ALL	289.0714	$C_{15}H_{13}O_6$	245(100), 205(33), 179(11)
	epigallocatechin	standard	305.0665	$C_{13}H_{13}O_7$	305(100), 221(19), 219(29), 179(20)
	epicatechin-3-gallate	G	441.0827	$C_{22}H_{17}O_{10}$	331(16), 289(100), 271(9), 169(20)
	catechin-3-gallate	G, M	441.0827	$C_{22}H_{17}O_{10}$	331(19), 289(100), 271(10), 193(6), 169(21)
	gallocatechin-3-gallate	standard	457.0775	$C_{22}H_{17}O_{11}$	331(67), 305(36), 287(10), 193(10), 169(100)
	epigallocatechin-3-gallate	M	457.0779	$C_{22}H_{17}O_{10}$	331(53), 305(38), 287(9), 269(7), 193(9), 169(100)
dimer	EA→EC(1)	M(2)	561.1393	$C_{30}H_{25}O_{11}$	543(34), 435(50), 425(19), 407(19), 289(100), 271(13), 245(7)
	EC→EA(1)	M(1)	561.1380	$C_{30}H_{25}O_{11}$	543(9)435(100), 409(64), 391(7), 299(44), 287(50), 273(57), 161(8)
	EF→EC(1)	G(2)	561.1382	$C_{30}H_{25}O_{11}$	451(40), 435(89), 423(100), 409(49), 325(17), 289(13), 271(26)
	EF→EC(2)	G(3)	561.1383	$C_{30}H_{25}O_{11}$	451(100), 435(78), 423(91), 409(56), 397(17), 299(25), 289(15), 271(48)
	EF→EC(3)	G(5)	561.1389	$C_{30}H_{25}O_{11}$	451(42), 435(100), 423(100), 409(39), 325(13), 289(15), 271(21)
	EC→A→EC(1)	A(2)	575.1190	$C_{30}H_{23}O_{12}$	539(23), 449(82), 423(100), 411(13), 407(19), 289(26), 285(18)
	EC→A→EC(2)	C(11), D(1), L(8), M(5)	575.1181	$C_{30}H_{23}O_{12}$	557(15), 539(30), 453(20), 452(16), 449(100), 447(20), 423(30), 407(20), 289(26), 287(16), 285(27)
	EC→A→EC(3)	C(1)	575.1179	$C_{30}H_{23}O_{12}$	449(27), 413(13), 395(88), 377(100), 333(21)
	EC→A→EC(4)	C(1)	575.1202	$C_{30}H_{23}O_{12}$	535(22), 509(47), 391(29), 347(100), 329(84), 285(22)
	EC→EC(1)	B(1), B(2), G(10), M(3), A(3)	577.1345	$C_{30}H_{25}O_{12}$	559(17), 451(37), 425(100), 407(53), 299(8), 289(26), 287(8)
	EC→EC(2)	G(3)	577.1335	$C_{30}H_{25}O_{12}$	559(57), 467(20), 451(100), 425(86), 407(59), 289(65)
	EC→EC(3)	D(1), M(1)	577.1340	$C_{30}H_{25}O_{12}$	559(75), 533(46), 451(29), 439(67), 425(75), 407(20), 393(100), 289(29), 269(35)
	EC→EC(4)	D(1), G(1), M(1)	577.1335	$C_{30}H_{25}O_{12}$	559(100), 533(31), 451(21), 439(34), 425(32), 407(18), 393(35)
	EC→EG(1)	G(1)	593.1279	$C_{30}H_{25}O_{13}$	575(13), 525(6), 467(24), 441(100), 427(6), 423(12), 305(16)
	(EC→EC)g(1)	G(5)	729.1434	$C_{37}H_{29}O_{16}$	603(14), 577(100), 559(46), 451(13), 425(20), 407(50)
	(EC→EC)g(2)	G(2)	729.1435	$C_{37}H_{29}O_{16}$	711(23), 603(45), 577(99), 559(88), 451(46), 441(42), 407(100), 289(19)
	(EC→EC)g(3)	G(1)	729.1437	$C_{37}H_{29}O_{16}$	711(35), 619(29), 603(100), 577(80), 559(51), 451(31), 441(29), 433(18), 407(28), 289(15), 245(17)
	(EC→EC)2g	G(2)	881.1541	$C_{44}H_{33}O_{20}$	729(100), 711(26), 559(20), 407(23)
trimer	EA→EA→EC(1)	M(1)	833.2083	$C_{45}H_{37}O_{16}$	816(23), 707(81), 561(91), 543(100), 435(23), 289(35)
	EA→A→EC→EC (1)	M(1), L(1)	847.1853	$C_{45}H_{37}O_{17}$	711(30), 693(12), 557(34), 435(37), 411(100), 289(13)
	EA→EC→EC(1)	M(5)	849.2026	$C_{45}H_{37}O_{17}$	723(31), 697(31), 577(100), 571(15), 559(51), 451(17), 425(28), 407(23),289(9), 287(15)
	EA→EC→EC(2)	G (4)	849.2014	$C_{45}H_{37}O_{17}$	831(94), 723(69), 697(26), 679(79), 561(100)
	EA→EC→EC(3)	M (2)	849.2017	$C_{45}H_{37}O_{17}$	831(45), 723(68), 697(16), 679(70), 561(38), 559(100), 433(36), 407(50), 289(19)
	EA→EC→EC(4)	M (2)	849.2204	$C_{45}H_{37}O_{17}$	723(100), 697(37), 679(49), 577(51), 571(39), 561(39), 451(43), 425(24), 407(37), 289(32)
	EA→EC→EC(5)	G(2)	849.2010	$C_{45}H_{37}O_{17}$	831(16), 723(30), 697(100), 679(71), 561(14), 545(12)
	EA→EC—EC(6)	G(2)	849.2018	$C_{45}H_{37}O_{17}$	831(100), 723(61), 679(35), 561(41)
	EF→EC→EC(7)	G(2)	849.2010	$C_{45}H_{37}O_{17}$	739(21), 697(100), 679(59), 559(67), 545(26), 527(16), 451(12), 407(11), 397(17), 289(13)
	EC→EC→A→EC (1)	L(3), M(6), C(1)	863.1814	$C_{45}H_{35}O_{18}$	737(72), 711(62), 693(42), 591(69), 575(100), 573(58), 449(34), 439(32), 289(89), 287(67)
	EC→EC→A→EC (2)	M(2)	863.1823	$C_{45}H_{35}O_{18}$	845(13), 737(19), 711(100), 693(41), 575(94), 573(15), 451(23), 411(17)
	EC→A→EC→EC (1)	C(2)	863.1804	$C_{45}H_{35}O_{18}$	737(8), 711(100), 693(8), 575(9), 573(41), 559(7), 531(10), 451(47), 411(43), 299(6), 289(19), 285(7)
	EC-(4β-8)-EC-(4β-8)-EC(2)	C(1), A(4)	865.1971	$C_{45}H_{37}O_{18}$	847(18), 749(48), 695(100), 577(68), 575(31), 425(27), 407(30)
	EC-(4β-8)-EC-(4β-8)-EC(2)	M(12), G(10), J(7), D(3), L(2)	865.1971	$C_{45}H_{37}O_{18}$	847(18), 749(48), 695(100), 577(68), 575(31), 425(27), 407(30)
	EC→EC→EC(3)	M(4), G(1), A(2), D (2)	865.1961	$C_{45}H_{37}O_{18}$	847(40), 779(51), 739(56), 713(57), 695(68), 577(89), 575(100), 449(22), 407(35), 289(27), 287(24)
	EC→EC→EC(4)	M(1)	865.1939	$C_{45}H_{37}O_{18}$	801(41), 789(49), 779(100), 720(70), 695(51), 577(74), 575(55)
	EC→EC→EC(5)	J(2), D(2)	865.1955	$C_{45}H_{37}O_{18}$	847(38), 739(100), 713(58), 695(87), 577(64), 575(35), 451(37), 449(26), 407(30), 287(29)

Table 2. continued

DP	proanthocyanidin	plant source[a]	HR [M − H]$^-$ (m/z)	mol formula	major MS^2 ions (m/z)(%)
	(EC→EC→EC)g(1)	G(4)	1017.2069	$C_{52}H_{41}O_{22}$	999(31), 891(47), 865(40), 847(57), 739(19), 729(100), 727(23), 695(28), 677(32), 575(20)
	(EC→EC→EC)g(2)	G(1)	1017.2054	$C_{52}H_{41}O_{22}$	999(100), 891(48), 865(50), 847(62), 729(40), 695(39), 677(25)
	(EC→EC→EC)g(3)	G(2)	1017.2054	$C_{52}H_{41}O_{22}$	999(19), 891(54), 865(33), 847(100), 729(83)
	(EC→EC→EC)g(4)	G(3)	1017.2056	$C_{52}H_{41}O_{22}$	999(20), 891(24), 865(100), 847(53), 727(24), 695(24)
	(EC→EC→EC)→2g (1)	G(1)	1169.2184	$C_{59}H_{45}O_{26}$	not recorded
tetramer	EC→EA→A→EC →EC(1)	L(1)	1135.2472	$C_{60}H_{47}O_{23}$	983(36), 965(22), 847(100), 845(30), 829(11), 693(26), 557(22), 411(15)
	EA→EC→EC→EC (1)	M(3)	1137.2649	$C_{60}H_{49}O_{23}$	1119(42), 1011(59), 865(100), 847(51), 739(26), 577(46), 559(33), 407(26)
	EA→EC→EC→EC (2)	M(3)	1137.2666	$C_{60}H_{49}O_{23}$	1119(42), 1011(67), 985(35), 967(85), 849(87), 847(100), 723(36), 575(32), 561(32)
	HA→EC→HC→EC (3)	M(1)	1137.2651	$C_{60}H_{49}O_{23}$	1119(32), 1011(56), 985(41), 967(62), 849(74), 847(88), 577(100), 559(58), 407(50)
	EC→A→EC→E-C→A→EC(1)	L(2), C(1)	1149.2268	$C_{60}H_{45}O_{24}$	997(58), 997(19), 979(34), 845(43), 737(22), 575(100), 573(85), 411(85)
	EC→A→EC→E-C→A→EC(2)	L(1)	1149.2285	$C_{60}H_{45}O_{24}$	1131(20), 997(75), 979(35), 845(24), 737(17), 575(80), 573(75), 411(100)
	EC→EC→A→E-C→EC(1)	L(3)	1151.2423	$C_{60}H_{47}O_{24}$	1133(14), 1025(41), 999(45), 981(87), 863(100), 861(45), 711(32), 573(41), 411(34)
	EC→A→EC→E-C→EC(2)	L(2), C(1)	1151.2419	$C_{60}H_{47}O_{24}$	1005(32), 999(48),981(48), 861(100), 739(68), 573(58), 611(61), 407(35)
	EC→A→EC→E-C→EC(3)	L(1)	1151.2419	$C_{60}H_{47}O_{24}$	1133(32), 999(81), 981(70), 863(43), 861(84), 739(100), 699(38), 577(72), 573(49), 411(39), 407(43)
	EC→A→EC→E-C→EC(3)	L(1)	1151.2421	$C_{60}H_{47}O_{24}$	1133(45), 999(100), 981(48), 863(35), 861(90), 739(83), 587(39), 577(45), 573(59), 411(87), 407(38)
	EC→EC→E-C→A→EC(4)	L(1)	1151.2416	$C_{60}H_{4}9O_{24}$	999(78), 981(100), 863(86), 861(76), 739(70), 709(35), 577(38), 573(57), 531(38), 451(30),411(38)
	EC→EC→EC→. A→EC(5)	L(1)	1151.2419	$C_{60}H_{47}O_{24}$	1133(33), 1067(12), 1025(49), 999(21), 981(100), 863(57), 739(12), 737(15), 711(30), 575(48)
	EC→EC→EC→EC (1)	J(12), G(3), M(3), A (1), D(2)	1153.2571	$C_{60}H_{49}O_{24}$	1135(54), 1027(74), 1002(42), 983(100), 907(21), 865(63), 863(62), 739(35), 695(32), 577(40), 407(21)
	EC→EC→EC→EC (2)	D(2), J(2), M(2), G (2)	1153.2565	$C_{60}H_{49}O_{24}$	1135(55), 1027(52), 1027(23), 1001(59), 983(96), 865(100), 863(48) , 695(21), 577(46), 575(54)
	EC→EC→EC→EC (3)	J(2)	1153.2582	$C_{60}H_{49}O_{24}$	1135(53), 1027(41), 1001(100), 984(71), 865(79), 863(94), 847(26), 739(50), 701(35), 577(44), 575(65)
	EC→EC→EC→EC (4)	J(2)	1153.2577	$C_{60}H_{49}O_{24}$	1135(55), 1027(64), 1001(50), 983(77), 907(41), 865(50), 863(100), 701(32), 577(55), 575(73), 407(27)
	EC→EC→EC→EC (5)	J(1)	1153.2577	$C_{60}H_{49}O_{24}$	1135(89), 1027(78), 1001(56), 983(44), 907(44), 865(44), 863(44), 739(67), 701(67), 577(33), 575(100)
	EC→EC→EC→EC (6)	J(2), A(1), D(1)	1153.2590	$C_{60}H_{49}O_{24}$	1135(48),1027(100), 1001(30), 983(83), 965(16), 908(36), 865(52), 739(55), 695(26), 575(31)
	EC→EC→EC→EC (7)	A(1)	1153.2590	$C_{60}H_{49}O_{24}$	1135(100), 1028(72), 983(50), 865(50), 739(39), 737(33)
pentamer	EC→EC→EC→E-C→A→EC(1)	L(2)	1439.3058	$C_{75}H_{59}O_{30}$	1421(50), 1313(50), 1295(50), 1149(90), 1007(50), 863(100), 861(50)
	EC→EC→EC→E-C→EC(2)	L(1)	1439.3058	$C_{75}H_{59}O_{30}$	1421(67), 1287(100), 1151(67), 1113(33), 863(100), 753(100), 711(67), 637(33), 411(33)
	EC→EC→EC→E-C→EC(3)	L(1)	1439.3058	$C_{75}H_{59}O_{30}$	1379(100), 1353(75), 1313(75), 1269(50), 1131(50), 1111(50), 863(75), 857(50), 751 (25)
	EC→EC→EC→E-C→EC(4)	L(1)	1439.3058	$C_{75}H_{59}O_{30}$	1421(100), 1089(33), 1013(67), 997(33), 863(67), 711(33), 589(33), 587(67), 575(33), 531(67)
	EC→EC→EC→E-C→A→EC(5)	L(1)	1439.3058	$C_{75}H_{59}O_{30}$	1421(33), 1395(33), 1285(33), 981(100), 863(50), 665 (50), 445(33)
	EC→EC→EC→E-C→A→EC(6)	L(2)	1439.3058	$C_{75}H_{59}O_{30}$	1314(17), 1269(100), 1149(17), 1117(33), 863(67), 817(17), 737(17) , 709(17), 575(17), 453(33)
	EC→EC→EC→E-C→EC(7)	L(1)	1439.3058	$C_{75}H_{59}O_{30}$	1441(14), 1421(43), 1269(100), 1151(14), 1107(14), 957(14), 955(29), 863(43), 829(14), 573(14), 531(14)
	EC→EC→A→E-C→EC→EC(S)	L(1)	1439.3058	$C_{75}H_{59}O_{30}$	1371(50), 1269(50), 1143(50), 987(50), 861(50), 711(100), 671(50), 585(50), 575(50), 573(90), 411(50)
	EC→EC→EC→E-C→EC(1)	M(1)	1441.3229	$C_{75}H_{61}O_{30}$	1421(100), 1315(64), 1271(67), 1153(74), 1153(33), 1151(48), 1027(38), 865(86), 863(43),739(36), 575(36)

[a]Abbreviations: A, apple; C, cranberry extract; D, dark chocolate; G, grape seed extract; J, jujube; L, litchi; M, mangosteen; AL, all tested plants (the number of similar peaks in the sample is listed in parentheses); B_1, B_2, A_2, and C_1, procyanidin B-type dimers B_1, B_2, A-type dimers A_2, and trimer C_1;

Table 2. continued

DP, degree of polymerization; g, galloyl; EC, EA, EG, and EF, epicatechin, epiafzelechin, epigallocatechin, and epifisetinidol, respectively. The signal $unit_1 \rightarrow unit_2$ or $unit_1 \rightarrow A \rightarrow unit_2$ expresses the units bonded by B-type (4,8- or 4,6-) bond or A-type (plus additional C_2-O-C_7- or C_2-O-C_5-) bond, respectively.

Thus, it is possible for them to be connected in two ways, through a C_4-C_8 or C_4-C_6 linkage, providing 64 (i.e., 2^6) possible isomers.[1−4,10−23] The formation of B-type trimers and tetramers leads to an exponential increase in the possible number of isomers, which makes the separation and quantification of such complex PA isomers an enormous challenge.[1−4]

UHPLC columns (with 1.9 μm or smaller particles) provide much better separation of the PA isomers than HPLC columns.[1,20−23] In addition, the molecular ions detected by HRMS provide high-resolution molecular weight (HRMW) and exact molecular formula (MF). The HRMW, MF, and singly and multiply charged ions for different PAs are related to the DP, the flavan-3-ols (EC, EA, EF, EG, and ER) in the oligomer, the number of galloyls, and the number of type A bonds as described in the equations

$$\begin{aligned} \mathrm{HRMW} = {} & 12.0000 \times (15n + 7c) + 1.0078 \\ & \times (12n + 2 + 4c - 2d) + 15.9949 \\ & \times (6n + 4b - a + b) \end{aligned} \quad (1)$$

$$\mathrm{MF} = C_{15n+7c}H_{12n+2+4c-2d}O_{6n-a+b+4c} \quad (2)$$

$$\begin{aligned} [\mathrm{M} - \mathrm{H}]^- = {} & 12.0000 \times (15n + 7c) + 1.0078 \\ & \times (12n + 1 + 4c - 2d) + 15.9949 \\ & \times (6n + 4c - a + b) \end{aligned} \quad (3)$$

$$[\mathrm{M} - 2\mathrm{H}]^{2-} = (\mathrm{HRMW} - 2 \times 1.0078)/2 \quad (4)$$

$$[\mathrm{M} - 3\mathrm{H}]^{3-} = (\mathrm{HRMW} - 3 \times 1.0078)/3 \quad (5)$$

where n = degree of polymerization, a = number of ECs that were replaced by EAs or EFs (as regioisomers), b = number of ECs replaced by EGs, c = number of galloyls, and d = number of A-type bonds; 12.0000, 1.0078, and 15.9949 are the accurate masses of carbon, hydrogen, and oxygen, and 15, 12, and 6, and 7, 4, and 4 are the numbers of carbon, hydrogen, and oxygen atoms for each EC (or its regioisomer ER) and galloyl unit, respectively. The equations can be easily modified to accommodate the PAs that contain acyl, glycosyl, or phloroglucinol adducts.[1,19]

Low-resolution ions are usually expressed to two decimal places in most cases and can be obtained directly from the high-resolution $[M - H]^-$ values. Formulas have been described for computing the PA molecular ion metal adduct values (in low resolution) from MALDI-TOF-MS, but the PA mass values can be obtained only after the mass of the metal has been subtracted.[11−13,20] Thus, eqs 1−5, for PA mass, are easier to use.

Table 1 presents the HRMW and deprotonated molecule ($[M - H]^-$) (m/z) for most of the PAs (DP = 2−10) detected in common foods in this laboratory and described in the literature.[1,4] For each oligomer, the nongalloylated B-type procyanidins (in bold) have the simplest formulas ($a = b = c = d = 0$), indicating that the PAs have no EC units replaced by EA, EF, or EG and do not contain any galloyls and A-type bonds. To be as systematic as possible, for each oligomer class, the related propelargonidins and proanthocyanidin-containing EA units are listed above the procyanidins, whereas the related prodelphinidins containing EG units are listed below the procyanidins. Similarly, all of the A-type PAs for each oligomer are listed above the B-type PAs and the galloylated PAs are listed below.

The data in Table 1, calculated from eqs 1−5, were found to agree well with experimentally determined $[M - H]^-$ values with an error of <3 ppm in most cases. Consequently, Table 1 can be used to provide the PA structure based on experimental high-resolution $[M - H]^-$ values. For example, trace ions recorded in grape seed extract were easily identified as galloylated procyanidin tetramers (1305.2698, error < 3 ppm), pentamers (1441.3250), hexamers (1729.3898), and their gallates (1593.3354 and 1881.4033). Thus, a detailed analysis of plant PAs can be achieved easily without using purified PA or PA-enriched samples.

The data in Table 1 permit a detailed PA oligomeric profile of a sample to be obtained from a single chromatogram using HRMS. Although identification of specific PAs based on the recorded HR $[M - H]^-$ values is putative, they are all correctly identified as PAs. It should be noted that nominal MS (typically masses to two decimal places) cannot positively identify them as PAs. The data in Table 1 also provide the opportunity to fully identify interesting or minor PAs (i.e., to specify the flavan-3-ol units and their connectivity) by selecting specific ions for fragmentation as described below.

Identification of Proanthocyanidins in Foods. The UHPLC-PDA-ESI-HRMS^n profiling method provides retention time, UV, $[M - H]^-$, and MS^{2-5} product ions for the PAs. Consideration of the product ions, especially MS^2 ions, permits easy putative identification of PAs. Table 2 lists 247 proanthocyanidins in 90 isomeric groups from 7 food materials, their plant sources, single-parent ions, formulas, and diagnostic and main MS^2 productions. The number of the isomers identified in each sample is in parentheses following the plant name. Approximately 130 of the PAs were detected in the grape seed and mangosteen extracts, and the rest were detected in the other five samples (Fuji apples, cranberry extract, dark chocolate, jujube, and litchi). Many of the PAs were detected in these plants for the first time.

It was noted that catechin and epicatechin showed the same product ions and very similar ratios at MS^2 [245 (100%), 205 (35%), and 179 (11−12%)], MS^3 [227 (28−30%), 203 (100), and 187 (20−25%)], and even at MS^4 [185, (20−37%), 175 (100%), 161 (28−42%)]. Similarly, dimeric procyanidins B_1 (EC-4β-8-C) and B_2 (EC-4β-8-EC) have very similar MS^2 [451 (27−37%), 425 (100%), 407 (41−47%), 289 (17−26%), and 287 (7−8%)], MS^3 [407 (100%) and 273 (6−8%)], MS^4 [285 (100%), 283 (36−43%), 389 (29−36%), 297 (27−37%), and 255 (17−27%)] and MS^5 [257 (100%) and 213 (4−7%)] fragments.

These data indicate that the slight differences in the relative ratio among the fragments might be caused by the stereochemistry of the monomers. However, there are insufficient data to predict the effect of the linkages and the positions of the PA flavan-3-ol units on product ion formation and relative abundance. At present, LC-MS^n methods are not able to

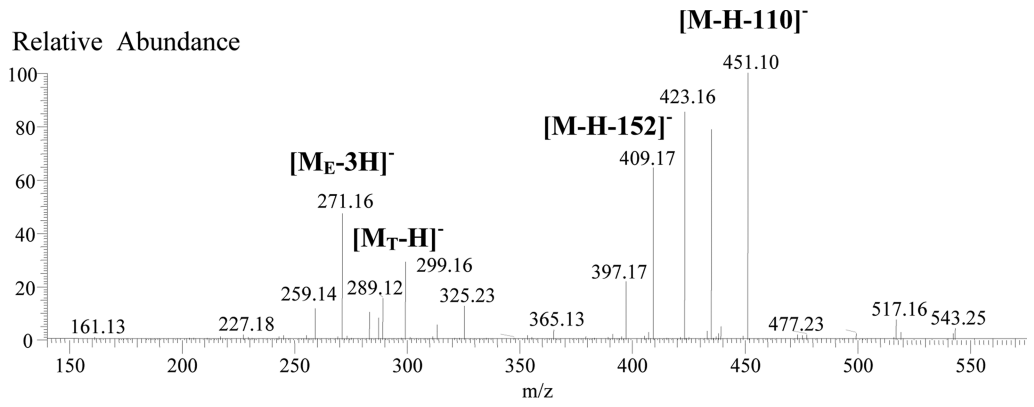

Figure 2. MS^2 spectrum of the EF−EC dimer with retention time at 26.98 min.

discriminate between the regioisomeric forms of the PAs or the related stereoisomeric forms.

As shown in Figure 1, the most important MS^2 fragments of B- and A-type PA dimers are formed by quinone methide (QM) fission, that is, breaking of the interflavan bond between the monomers to form $[M_T - H]^-$ and $[M_E - 3H]^-$ ions for B-type PAs and $[M_E - 5H]^-$ ions for A-type PAs, where E = extension unit and T = terminal unit. Other typical PA fragments were formed by retro-Diels−Alder (RDA) fission (loss of the whole B-ring with C_2−C_3 part of the C-ring, i.e., loss of 152, 136, and 168 Da for EC, EA, and EG, respectively) and by heterocyclic ring fission (HRF) of the extension unit (loss of the A-ring, i.e., loss of 126 Da for EC, EA, or EG and loss of 110 Da for EF or ER). Product ions formed by the loss of water (−18 Da), O (−16 Da), CO (−28 Da), HC≡CH (−26 Da), HC≡COH (−42 Da), and HC≡CH—CO (−54 Da) were also observed.[4,13,14,19−23,27] For PAs with DP ≥ 3, further fragmentation can occur from repeated QM breaks of interflavan bonds connecting the flavan-3-ols of the extension units. These product ions were also frequently used to identify the PAs. Information obtained from the analysis of thiolytic degradation products of the PAs from similar plants has proven useful for the identification of PAs.[1,4,13,14,19−23,27−30]

In this study, 247 PAs were identified in 7 tested materials (Table 2) using only the most intense ions among the coeluted ions (each peak) as the target ions. However, the identified PAs can be enhanced by selecting more target ions, such as the second and third most intense ions of each peak. The PAs are denoted as combinations of EC, EA, EG, and EF, and A-type bonds are designated by placing an "-A-" between the flavan-3-ols. Although there are numerous isomers in each oligomeric class, only one isomer was selected to represent all of the remaining isomers (each having the same MS^2 base and main fragments). There was no correlation between the PAs found in the different samples. Positive identification was achieved for only some of the PAs in Table 2 based on direct comparison to reference PAs (procyanidins B_1, B_2, C_1, A_2) or PAs positively identified in other studies.[1,4]

Procyanidins with DP = 2−5 have been previously reported in common foods.[4,19−23,27] Consequently, special attention was paid to PAs containing different flavan-3-ol units or A-type bonds because these features lead to more regioisomers. For example, 13 PA dimers ($[M - H]^-$ at 561.1388) contained two different flavan-3-ols. One of the three detected in mangosteen had MS^2 fragments at m/z 435 (−126 Da, HRF from EA or EC), 409 (−152 Da, RDA from EA or EC), 287 ($[M_E - 3H]^-$), and 273 ($[M_T - H]^-$), suggesting it to be EC-EA, a PA dimer containing an EA unit as the terminal unit. The other two in mangosteen had MS^2 fragments at m/z 289 ($[M_T - H]^-$, 100%), 435 (−126 Da), 425 (−136 Da), 407 [−(136 + 18) Da), and 271 ($[M_E - 3H]^-$), corresponding to EA-EC, the isomers containing EA as extension unit (Figure 1).

Another 10 interesting dimers were detected in grape seed extract. Three had MS^2 fragments at m/z 451 (HRF loss of 110 Da, $C_6H_6O_2$ for the deoxy-A-ring of EF or ER, 100%), 423 [−(110 +28), 91−98%], 409 (−152 Da, RDA loss, 29−60%), 289 ($[M_T - H]^-$, 12−18%), and 271($[M_E - 3H]^-$, 20−48%) (Figure 2). Five had the same MS^2 fragments but with different intensities; one at m/z 423 (100%), 451 (around 50%), 409 (28−64%), 289, and 271. The remaining two had MS^2 fragments at m/z 435 (100) and 451 (40−50%). These fragments suggested that all might be proanthocyanidin dimeric isomers (EF-EC). To date, the PAs containing an EF unit have only been reported to exist in plant woods, such as quebracho (*Schinopsis balansae* var. *chaqueno*) wood, but rarely in common foods, such as grapes.[1,28−31]

A PA dimer ($[M - H]^-$ at 593.1279 Da) detected in grape seed extract had mass fragments at m/z 441 (−152, RDA) and 305 ($[M_T - H]^-$), suggesting it was an EC-EG isomer. A PA trimer containing two EAs ($[M - H]^-$ at m/z 833.2083) was found in mangosteen with MS^2 fragments at m/z 543 (100%, $[M_E - 3H]^-$), 707, and 289 ($[M_T - H]^-$) and MS^3 fragments at m/z 271 (100%, the second $[M_E - 3H]^-$), 417, and 407, indicating that two EA units were formed the extension units and that the EC was the terminal unit. Two PA trimers found in mangostenn and litchi with $[M - H]^-$ of 847.1860 Da had one A-type bond, MS^2 fragments at m/z 557, 411, and 289, and a MS^3 base fragment at m/z 285. This suggested that EA was the extension unit with the A-type bond connected to one of the two ECs and that the remaining EC was the terminal unit.

Nineteen PA trimers (9 in mangosteen and 10 in grape seed extract) had $[M - H]^-$ fragments at 849.2030 Da indicating EA or its constitutional isomer EF. Of these, 5 had MS^2 fragments at 577 (−272 Da, 100%) and 559 (−290 Da around 40−50%), 1 had MS^2 fragments at m/z 561 (−288 Da), and the others had MS^2 fragments at 559 (−290 Da), 723 (−126 Da), 697 (−152 Da), and 831 (−18 Da). All of these PAs might be EA-EC-EC isomers. The two detected in grape seed extract (expressed as EF-EC-EF in the last line for this oligomeric class) had a MS^2 fragment at m/z 739 (−110 Da, ∼20%) and might have EF instead of EA as the extension unit.

Fourteen procyanidin trimers ($[M - H]^-$ at 863.1800 Da) contained one A-type bond. Ten of them (6 from mangosteen, 3 from litchi, and 1 from cranberry) had MS^2 fragments at m/z

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Table 3. Doubly and Triply Charged Proanthocyanidins Found in Mangosteen and Litchi

proanthocyanidin	HRMS (Da)	HR $[M - 2H]^{2-}$ (m/z)	^{12}C isotope (m/z)	^{13}C isotope (m/z)	plant source[a] (no. of PAs)
A-type procyanidin pentamers with two A-bonds	1438.2994	718.1419	718.1417	718.6428	L(2)
A-type procyanidin pentamers with one A-bond	1440.3150	719.1497	719.1494	719.6509	M(1), L(1)
B-type procyanidin pentamers	1442.3306	720.1575	720.1566	720.6591	M(5)
B-type proanthocyanidin hexamers with two EA units	1698.4038	848.1941	848.1951	848.6995	M(2)
B-type proanthocyanidin hexamers with one EA unit	1714.3987	856.1916	856.1921	856.6930	M(6)
A-type procyanidin hexamers with two A-bond	1726.3624	862.1734	862.1740	862.6748	L(2)
B-type procyanidin hexamers	1730.3936	864.1890	864.1893	864.6890	M(3)
B-type proanthocyanidin heptamers with one EA units	2002.4617	1000.2231	1000.2235	1000.7247	M(1)
A-type procyanidin heptamers with two A-bond	2014.4254	1006.2049	1006.0000	1006.7057	L(1)
A-type procyanidin heptamers with one A-bond	2016.4410	1007.2127	1007.2120	1007.7230	M(1), L(1)
B-type procyanidin heptamers	2018.4566	1008.2205	1008.2223	1008.7228	M(6)
B-type proanthocyanidin octamers with two EA units	2274.5298	1136.2571	1136.2540	1136.7643	M(2)
B-type procynidin octamers	2306.5196	1152.2520	1152.2527	1152.7537	M(7)
B-type proanthocyanidin nonamers with one EA unit	2578.5877	1288.2861	1288.2840	1288.7855	M(1)
B-type procyanidin nonamers	2594.5826	1296.2835	1296.2828	1296.7859	M(1)
B-type proanthocyanidin decamers with two EA units	2850.6558	1424.3201	1424.3212	1424.8262	M(1)
B-type proanthocyanidin decaamers	2866.6507	1432.3176	1432.3185	1432.8220	M(1)
A-type procyanidin decamers with two A-bond	2878.6144	1438.2994	1438.2985	1438.7967	M(1)
B-type procyanidin decamers	2882.6456	1440.3150	1440.3169	1440.8147	M(1)
	HR $[M - 3H]^{3-}$ m/z		^{12}C isotope m/z	^{13}C isotope m/z	
B-type procyanidn decamers	959.8741		959.8727	960.2103	M(4)

[a]Abbreviations: L, litchi; M, mangosteen (number of similar peaks in the sample is listed in parentheses). The value was taken from one of the PA and close to those of the remaining ones.

575 (−288 Da, 21−100%), 711 (42−100%), and 289 (20−89%), indicating they were EC-EC-A-EC isomers. Others (two from litchi and two from cranberry) had MS^2 fragments at m/z 573 (−290 Da, 35−62%), 411 (43−100%), and 711 (91−100%) to suggest they were EC-A-EC-EC isomers.

One A-type PA tetramer in litchi ($[M - H]^-$ at 1135.2472) contained one EA and one A-type bond and had MS^2 fragments at m/z 847 (−288 Da, 100%), 983 (−152 Da, 36%), 845 (−290 Da, −30%), 693 [−(152 + 290) Da, 26%], and 557 [−(288 + 290) Da, 22%]. This suggested it might be an EC-EA-A-EC-EC or EC-EC-A-EA-EC isomer.

Seven PA tetramers in mangosteen ($[M - H]^-$ at 1137.2450) contained one EA. Three had MS^2 fragments at m/z 865 (−272 Da, 100%), 847 (−290 Da, 30%), and 577 [−(288 + 272) Da, 46%]. Another three had MS^2 fragments at m/z 847 (−290 Da, 100%) and 575 [−(288 + 274 or 290 + 272) Da]. The remaining tetramer had MS^2 fragments at m/z 1011, 985, 967, 849 (−288 Da), 847, and 577 [−(290 + 274) Da]. These fragments indicated EA was a part of the extension unit with two ECs and might be the final extension unit.

Four procyanidin tetramers (three from Litchi and one from cranberry) with $[M - H]^-$ at 1149.2280 had two A-type bonds and MS^2 fragment at m/z 575 (80−100%) {−(288 + 286) Da for $[M_T - H]^-$} and 573 (75−85%) {−(2 +286 × 2) Da for $[M_E - 3H]^-$}, indicating that the A-type bonds were between the first and second and between the third and fourth flavan-3-ols. Ten procyanidin tetramers (9 from Litchi and 1 from cranberry) with $[M - H]^-$ at 1151.2415 had one A-type bond. Three (group 1) had MS^2 fragments at m/z 863 (100%) (−288 Da for $[M_E - 3H]^-$) and 573 (41%) indicating the A-type bond was between the second and third flavan-3-ols. Five (groups 2−4) had MS^2 fragments at m/z 861 (84−100%) (−290 Da for $[M_T - H]^-$) and 573 (49−59%) {−(2 +286 × 2) Da for $[M_E - 3H]^-$} indicating an A-type bond between the first and second flavan-3-ols. Two (groups 4 and 5) had MS^2 fragments at m/z 863 (57−86%), 575 (48%) {−(288 + 286) Da for $[M_T - H]^-$}, or 577 (38%) and 573 (57%) indicating an A-type bond between the third and fourth flavan-3-ols.[14,19] Similarly, the PA pentamers in eight groups (1−8) have one A-type bond, and the PAs of the first six groups (1−6) showed main fragment at m/z 863 (50−100%), indicating the A-type bond between the fourth and fifth flavan-3-ols. The PS of the last group (8) showed the main fragment at m/z 861 (50%), indicating the A-type bond between second and third flavan-3-ols, whereas the remaining one in group 7 showed fragments at m/z 863 and 573 to suggest that this compound might have its A-type bond between the third and fourth flavan-3-ols.[14]

Ten galloylated dimers and 11 trimers were detected in grape seed extract. The existence of a galloyl connected to a PA with DP ≥ 2 provides the possibility of forming regioisomers; for example, EC-ECg and ECg-EC and EC-EC-ECg, EC-ECg-EC, and ECg- EC-EC. Unfortunately, the ECg position cannot be deduced from the mass fragments because gallate was very easy to lose. Thus, they were expressed as (EC-EC)g or (EC-EC-EC)g, respectively.

Jujube fruit was analyzed because PAs (DP = 2, 3, 5, and 7) consisting of both EA and EG have been isolated from jujube leaves and bark.[32] These PAs have the same molecular weight and formula as those of their related procyanidins, but can be easily distinguished from the procyanidins by the noticeable difference in their fragments. For example, the dimers of EA and EG will have QM (271 and 305 Da for EA-EG or 303 and 273 Da for EG-EA) and RDA fragments formed by the loss of 136 Da from EA and 168 Da from EG, whereas the related procyanidin dimers should have QM (289 and 287 Da) and RDA fragments formed by the loss of 152 Da. A careful check confirmed that all 30 of the detected PAs in jujube consisted of EC units only.

Identification of Highly Polymerized PAs Based on the Doubly and Triply Charged Molecular Ions. Negative

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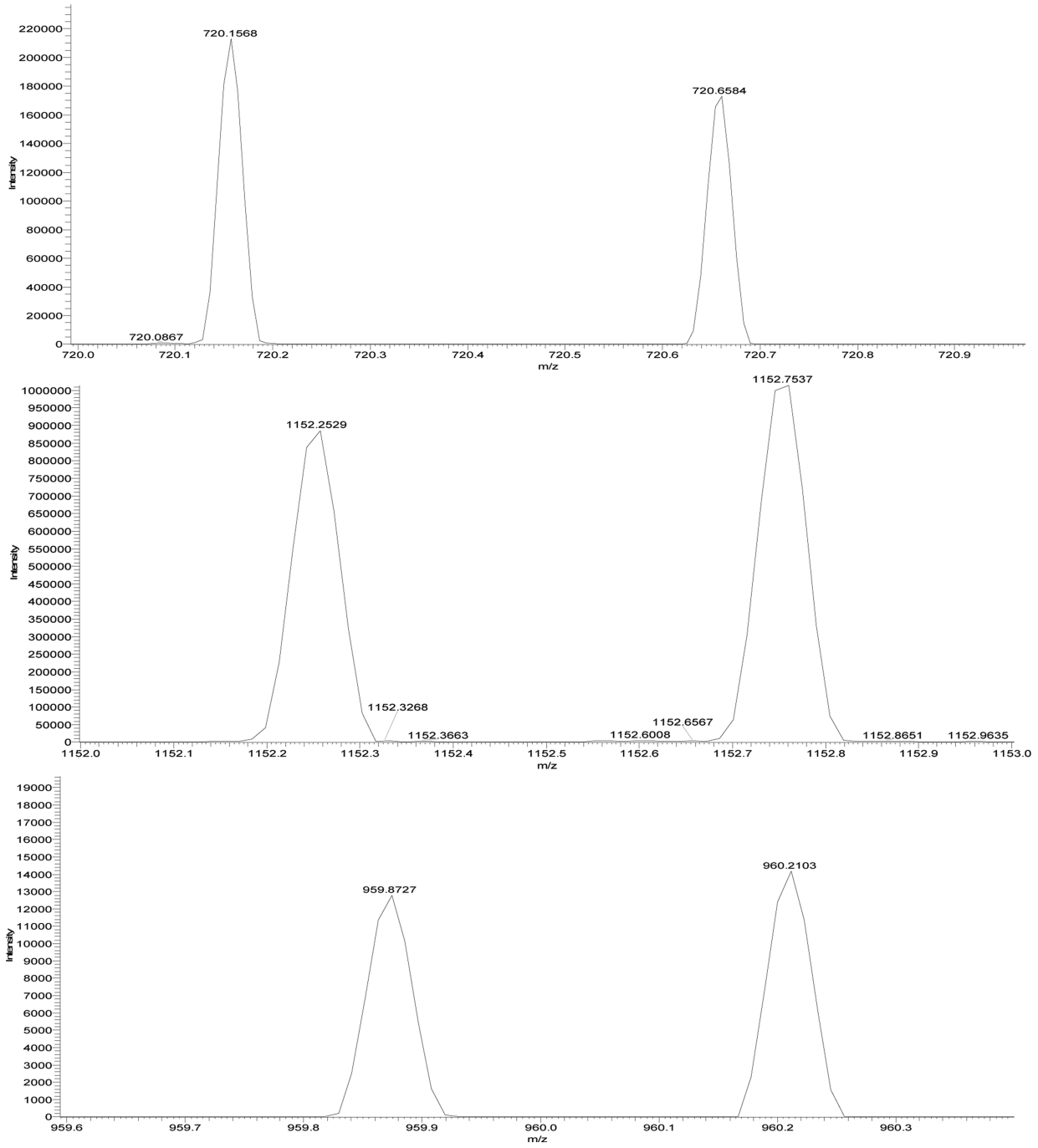

Figure 3. Accurate ^{12}C and ^{13}C isotope ion peaks for $[M - 2H]^{2-}$ of m/z 720 and 1152 and for $[M - 3H]^{3-}$ of m/z 960.

ionization of many highly polymerized PAs (DP ≥ 5) produces multiply (mainly doubly and triply) charged molecular ions. To date, several dozen multiply charged molecular ions have been reported and used to identify PAs with DP = 7−25.[1,4,13−19] With nominal resolution MS, these ions were recognized as doubly or triply charged molecules on the basis of the distance between the ^{12}C and ^{13}C isotope ions. As the charge increases from 1 to 2 to 3, the distance between the isotopes will decrease from 1 to 0.5 to 0.33 amu.[17,18] It was noted that the ion masses for PA isotopes were always slightly different.[1,4,13−20] This was attributed to the differences in the relative abundances of the ^{12}C and ^{13}C isotopes.

Table 1 contains the accurate $[M - 2H]^{2-}$ and $[M - 3H]^{3-}$ values for PAs with DP = 5−10, which matched the $[M - 2H]^{2-}$ or $[M - 3H]^{3-}$ of around 50 proanthocyanidins detected in mangosteen and litchi extracts (Table 3). The ^{12}C and ^{13}C isotope ions of each proanthocyanidin were easily found by examining the distance between the two isotopic ion peaks. For example, in mangosteen the main $[M - 2H]^{2-}$ ions were found at m/z 720.1566, 856.1928, 864.1863, 1000.2235, 1008.7217, and 1152.7537 (Figure 3; Tables 1 and 3). The first four values were taken from the ^{12}C isotope ion and perfectly matched (error < 3 ppm) the listed $[M - 2H]^{2-}$ data in Table 1 for the B-type procyanidin pentamer and hexamer, the B-type propelargonidin hexamer containing one EA unit, and the B-type propelargonidin heptamer containing two EAs. The values of the ^{13}C isotope were m/z 0.500 more than that from ^{12}C isotope (Table 3). However, the last two values, m/z 1008.7217

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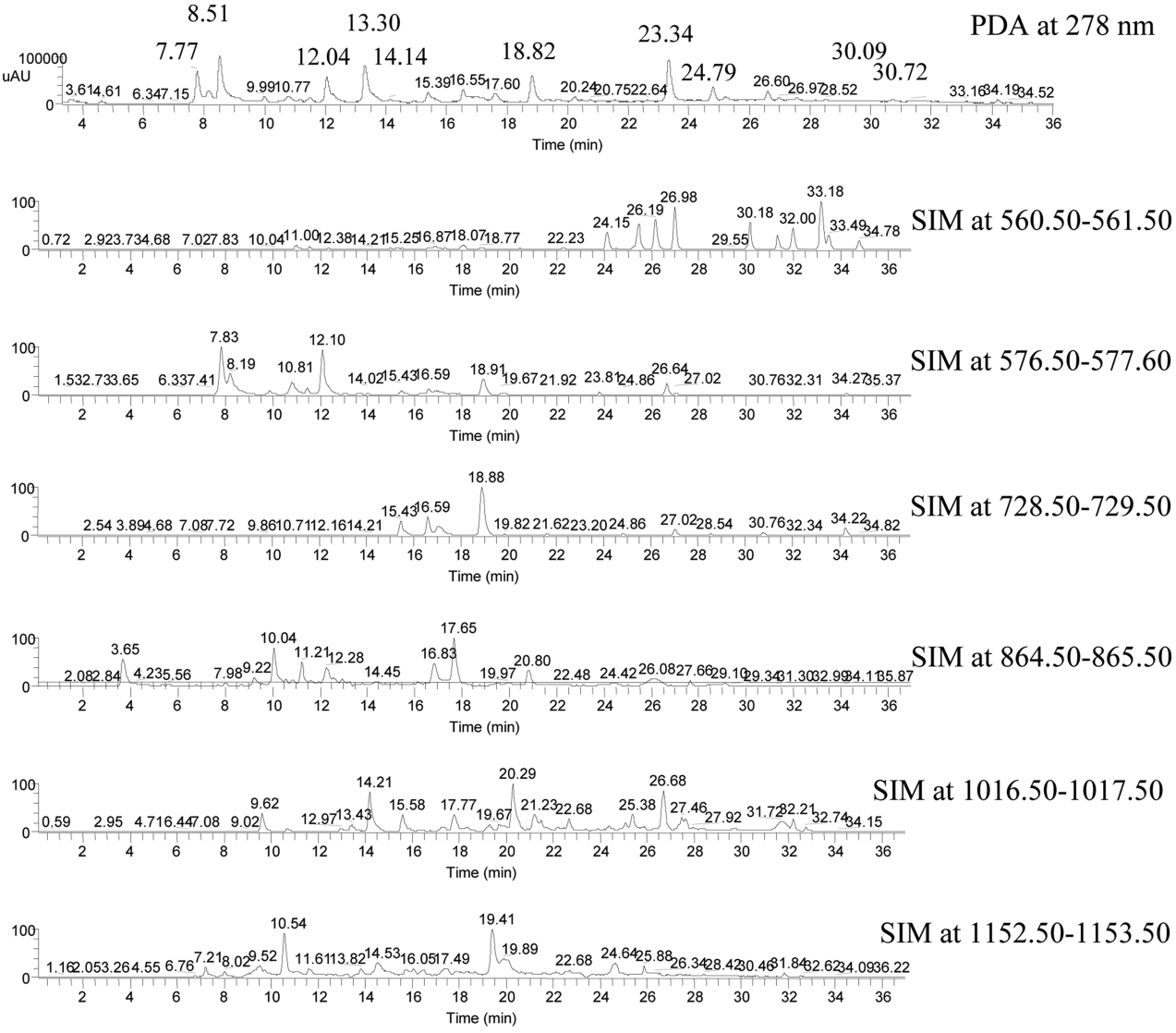

Figure 4. PDA (at 278 nm) and SIM chromatograms of grape seed extract.

and 1152.7537, were taken from the ^{13}C isotope ions of B-type procyanidin heptamer and octamer, respectively, so these masses were larger than the listed $[M - 2H]^{2-}$ values for the ^{12}C isotope ion by 0.50 amu.

Checking the distance between isotopes led to the detection of several minor PA ions in the TIC chromatogram of mangosteen extract. For example, the ions at m/z 1296.2828, 1007.2120, 1136.2540, and 1288.2840 were close matches to the listed values for doubly charged B-type procyanidin octamers, A-type procyanidin heptamers with one A bond, B-type propelargonidin octamers with two EA, and B-type propelargonidin nonamers with one EA (Tables 1 and 3), respectively.

Similarly, checking for ^{12}C and ^{13}C isotopes with a 0.33 amu distance led to the discovery of several $[M - 3H]^{3-}$ ions. However, only one of them (in mangosteen) was for a PA with a DP $\leq$ 10. As shown in Table 3 and Figure 3, the HRMS values for this PA for the ^{12}C and ^{13}C isotope ions were 959.8727 and 960.2103 (Figure 3), respectively. To date, only five multiply changed ions have been reported in the pericarps of mangosteen.[12] This is the first report to use the high-resolution isotope ion values for accurate identification of multiply charged PAs based on the use of ^{12}C and ^{13}C isotope ions.

Quantification of Proanthocyanidin Oligomers. The extraction efficiency of the standardized method for PAs in plant materials was determined by a follow-up extraction using acetone/methanol/water (2:2:1, v/v/v), a solvent frequently used for PA extraction in other studies.[2−4,21,22] No additional material was found in the follow-up extractions as determined by the lack of detectable peaks. This indicated that the general extraction method was suitable for the quantification of PAs in jujube, Fuji apple, litchi, and mangosteen.

The UV absorbance of phenolic compounds is widely used for the quantification of PAs.[2−4,7,19,20,22] The MRRF of flavan-3-ol (catechin and epicatechin) monomers, dimeric procyanidin B_1, B_2, and A_2, and trimeric procyanidin C_1 at 274−280 nm were found to be proportional to the DP number in our previous study.[25] This established that, in molar units, the response of the monomers was additive. The MRRF values for catechin, gallocatechin, and gallic acid were determined to be 1.00, 0.31, and 2.8.[25] Thus, the MRRF for EC-EC is 2.0, that for EC-EC-A-EC is 3.0, and that for EC-EC-ECg is 4.8. There were no commercial standards for afzelechin or fisetinodol, so an

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Table 4. Retention Time, Molecular Weight, MRRF Value, and Concentration for the Main PAs in Grape Seed Extract

compound (or code) (min)	t_R (UV) (min)	t_R(SIM) (min)	MW_x	MRRF	content (%, w/w on dry basis), av ± SD
catechin	8.51		290	1.0	6.55 ± 0.26
epicatechin	13.30		290	1.0	7.58 ± 0.30
epicatechin-gallate	23.34		442	3.8	2.34 ± 0.09
catechin-gallate		25.25	442	3.8	0.16 ± 0.01
monomer concentration					**16.63 ± 0.67**
proanthocyanins					
EF-EC-5	30.09	30.18	562	2.0	0.07 ± 0
EF-EC-1		24.15	562	2.0	0.05 ± 0
EF-EC-2		25.05	562	2.0	0.08 ± 0
EF-EC-3		2619	562	2.0	0.08 ± 0
EF-EC-4		26.98	562	2.0	0.11 ± 0
EF-EC-6		31.34	562	2.0	0.03 ± 0
EF-EC-7		32.00	562	2.0	0.05 ± 0
EF-EC-8		33.18	562	2.0	0.11 ± 0
EF-EC-9		33.46	562	2 0	0 05 ± 0
EF-EC-10		34.78	562	2.0	0.03 ± 0
EC-EC-1	7.76	7.83	578	2.0	2.53 ± 0.10
EC-EC-2		8 19	578	2.0	1.88 ± 0.08
EC-EC-3		9.86	578	2.0	0.22 ± 0.01
EC-EC-4		10.81	578	2.0	0.87 ± 0.03
EC-EC-S		11.46	578	2 0	0.19 ± 0.01
EC-EC-6		12.10	578	2.0	2.41 ± 0.10
EC-EC-7		16.59	578	2.0	1.60 ± 0.06
EC-EC-S		18.91	578	2.0	1.07 ± 0.04
EC-EC-9		26.64	578	2.0	1.18 ± 0.05
EC-EG		6.41	594	2.0	0.01 ± 0
(EC-EC)g-4	18.83	18.88	730	4.8	1.61 ± 0.06
(EC-EC)g-1		15.43	730	4.8	0.53 ± 0.02
(EC-EQg-2		16.59	730	4.8	1.13 ± 0
(EC-EC)g-3		17.40	730	4.8	0.54 ± 0.02
(EC-EC)g-5		27.04	730	4.8	0.23 ± 0.01
(EC-EC)g-6		34.23	730	4.8	0.21 ± 0.01
$(EC\text{-}EC)_2$g-1	24.79	24.86	882	7.6	0.57 ± 0
dimer concentration					**17.44 ± 0.70**
EA-EC-EC-7	30.72	30.76	850	3.0	0.04 ± 0
EF-EC-EC-1		18.55	850	3.0	0.03 ± 07
EA-EC-EC-2		19.67	850	3.0	0.04 ± 0
EF-EC-EC-3		22.80	850	3.0	0.03 ± 0
EA-EC-EC-4		23.67	850	3.0	0.04 ± 0
EA-EC-EC-5		28.04	850	3.0	0.03 ± 0
EA-EC-EC-6		30.03	850	3.0	0.02 ± 0
EC-EC-EC-4	12.04	12.28	866	3.0	2.25 ± 0.09
EC-EC-EC-1		3.65	866	3 0	1.95 ± 0.08
EC-EC-EC-2		10.04	866	3.0	1.92 ± 0.08
EC-EC-EC-3		11.21	866	3.0	0.78 ± 0.03
EC-EC-EC-5		16.83	866	3.0	1.35 ± 0.05
EC-EC-EC-6		17.65	866	3.0	2.34 ± 0.09
EC-EC-EC-7		20.76	866	3.0	0.89 ± 0.04
EC-EC-EC-8		26.08	866	3.0	1.73 ± 0.07
(EC-EC-EC)g-2	14.14	14.21	1018	5.8	0.14 ± 0.01
(EC-EC-EC)g-1		9.62	1018	5.8	0 06 ± 0
(EC-EC-EC)g-3		13.43	1018	5.8	0.06 ± 0
(EC-EC-EC)g-4		20.29	1018	5.8	0.16 ± 0.01
(EC-EC-EC)g-5		25.38	1018	5 8	0.07 ± 0
(EC-EC-EC)g-6		26.68	1018	5.8	0.12 ± 0
(EC-EC-EC)g-7		27.46	1018	5.8	0.08 ± 0
(EC-EC-EC)g-8		32.21	1018	5 8	0.10 ± 0
$(EC\text{-}EC\text{-}EC)_2$g-1		28.23	1170	8.6	trace
trimer concentration					**14.24 ± 0.57**

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Table 4. continued

compound (or code) (min)	t_R (UV) (min)	t_R(SIM) (min)	MW_x	MRRF	content (%, w/w on dry basis), av ± SD
EC-EC-EC-EC-5	19.34	19.40	1154	4.0	0.23 ± 0.01
EC-EC-EC-EC-1		9.62	1154	4.0	0.06 ± 0
EC-EC-EC-EC-2		10.63	1154	4.0	0.09 ± 0
EC-EC-EC -EC-3		14.81	1154	4.0	0.06 ± 0
EC-EC-EC-EC-4		17.31	1154	4.0	0.03 ± 0
EC-EC-EC-EC-6		24.64	1154	4.0	0 04 ± 0
EC-EC-EC-EC-7		25.88	1154	4.0	0.03 ± 0
tetramer concentration					**0.47 ± 0.20**
total catechin and PA concentration					**48.79 ± 1.95**

MRRF value of 1.00 was assigned to each. The additivity of the molar absorption coefficient makes it possible to quantify most of the PAs using (+)-catechin as a standard with the MRRF values listed above.

Unfortunately, even with UHPLC, only a few PAs were well separated and could be quantified on the basis of their UV peak area. Most PAs, when viewed with UV or TIC, had peaks that overlapped (coeluted) with other PAs. Selected ion monitoring (SIM) and multiple reaction monitoring (MRM) are the only methods that allow deconvolution of the overlapping peaks, that is, isolation of the ions of interest.[33,34] Consequently, concentrations had to be computed on the basis of ion counts obtained from SIM or MRM as reported in previous studies[19,22] The few well-separated absorbance peaks were used to equate the peak area in absorbance to integrated counts of specific ions. In other words, MRRF values based on absorbance were converted to MRRF values based on integrated ion counts. This approach allowed catechin and the MRRF values reported above to be used for computing PA concentrations.

Use of MRRFs based on ion counts assumes constant ionization efficiency for all PAs. Unlike absorbance, the ion count of a PA isomer can be expected to be dependent on its structural ionization sensitivity and the mobile phase. The SIM peak intensity might change with the solvent ratio at different retention times, the isomer concentration, and the presence of coeluting PAs (Figure 4). Tests performed with flavan-3-ol monomers and procyanidins B_1 and B_2 showed the variation in ionization efficiency to be <±10%. Further testing with PAs with DP = 3−5, A-type bond, or galloyls is needed but must wait on the availability of suitable standards.

The PA concentrations in dry weight percent (%) and milligrams per 100 g of dry plant material were calculated using the formulas

$$\mathrm{C\ (\%,\ w/w)} = [100A_x \times MW_x \times V_s \times M_s \times W_s] / [A_s \times MW_s \times W_x \times V_x \times \mathrm{MRRF}] \quad (6)$$

$$\mathrm{C\ (mg/100\ g)} = [1000A_x \times MW_x \times W_s \times V_s] / [A_s \times MW_S \times V_x \times W_x \times \mathrm{MRRF}] \quad (7)$$

where A_x, MW_x, W_x, and V_x and A_s, MW_s, W_s, and V_s are the peak area, molecular weight, sample weight, and volume of the extract for the sample and standard, respectively.

As shown in Table 4 for grape seed extract, at least one PA in each of the oligomers was found to have a well-separated peak (no coeluting compounds) that could be used to equate absorbance with ion counts from SIM. The concentrations of monomers, dimers, trimers, and tetramers as percent dry weight were 16.63 ± 0.67, 17.44 ± 0.70, 14.24 ± 0.57, and 0.47 ± 0.20%, respectively. The concentration for PAs with DP > 4 was negligible. The total concentration of PAs was 48.79 ± 1.95%.

Highly accurate masses can be computed for PAs on the basis of the degree of polymerization, the specific flavan-3-ol components, the number of A-type bonds, and the number of galloyls. PAs can be identified by comparing experimentally obtained high-accuracy masses to the computed masses. Identifications can be further confirmed by the analysis of fragments from tandem MS. Conversion of MRRF values from UV absorbance to ion counts with SIM was used for the quantification of individual PAs. Thus, this standardized UHPLC-PDA-ESI/HRMS^n profiling method was able to offer identification and quantification of oligomeric PAs in plant-derived foods.

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Notes

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