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High Molecular Weight Persimmon (*Diospyros kaki* L.) Proanthocyanidin: A Highly Galloylated, A-Linked Tannin with an Unusual Flavonol Terminal Unit, Myricetin

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MALDI-TOF MS suggested that the high molecular weight proanthocyanidin (condensed tannin) from persimmon (*Diospyros kaki* L.) pulp comprised a heteropolyflavanol series with flavan-3-*O*-galloylated extenders, flavan-3-ol and flavonol terminal units, and A-type interflavan linkages. Thiolysis-HPLC-ESI-MS with DAD, electrochemical, and ESI-MS detection confirmed a previously unreported terminal unit, the flavonol myricetin, in addition to the typical flavan-3-ols catechin and epigallocatechin gallate. The extender units were epicatechin, epigallocatechin, (epi)gallocatechin-3-*O*-gallate, and (epi)catechin-3-*O*-gallate. The crude tannin had a high prodelphinidin content (65%) and a high degree of 3-*O*-galloylation (72%). The material was fractionated on Toyopearl TSK-HW-50-F to yield fractions distinguished by degree of polymerization (DP). Thiolysis suggested that the persimmon tannin was composed of polymers ranging from 7 to 20 kDa (DP 19−47), but sizes estimated by GPC were 50−70% smaller. The crude material was chemically degraded with acid to yield products that were amenable to NMR and ESI-MS analysis, which were used to establish for the first time that persimmon tannin has a mixture of B-type and A-type linkages.

KEYWORDS: Persimmon proanthocyanidin; condensed tannin; myricetin; flavonol; thiolysis-HPLC-ESI/MS; NMR; MALDI-TOF MS; GPC

INTRODUCTION

The Asian persimmon (*Diospyros kaki* L.) is a widely consumed fruit that is characterized by its high proanthocyanidin (condensed tannin) content (*1*−*3*). Persimmon proanthocyanidin has been associated with antioxidant activity, anti-inflammatory activity, and atherosclerosis prevention (*4*). Persimmon condensed tannin is a more potent antioxidant than apple or grape seed proanthocyanidin (*5*, *6*). The biological activities of persimmon tannin are probably a consequence of its chemical structure such as the monomeric flavan-3-ol units, the types of interflavan bonds, and the degree of polymerization (DP).

Only a partial structural characterization has been achieved for persimmon proanthocyanidin, in part because of its high molecular weight. Matsuo and Ito (*7*) reported that *kaki* persimmon tannin consists of catechin, catechin-3-*O*-gallate, gallocatechin, and gallocatechin-3-*O*-gallate residues in a molar ratio of 1:1:2:2, with unknown terminal residues. They reported that the 13.8 kDa polymer belonged to the proanthocyanidin B group with carbon−carbon interflavan linkages between C-4 of one unit and C-8 (or C-6) of the next. Nakatsubo et al. (*8*) examined the proanthocyanidins from fruits of 16 *Diospyros* species and found little variation in subunit composition, with most of the tannins composed of catechin and gallocatechin. In our previous study (*6*), we found that the condensed tannin from Shanxi heart-shaped yellow persimmon (*D. kaki*) consists of (epi)gallocatechin, epigallocatechin-3-*O*-gallate, epicatechin-3-*O*-gallate, an unknown extender unit, and unknown terminal units. Using gel permeation chromatography (GPC) with polyvinyl alcohol standards, we established that the molecular weight distribution was in the range of 11.6−15.4 kDa (*6*).

The structural elucidation of highly polymerized proanthocyanidins such as the persimmon condensed tannin is very challenging due to the heterogeneous character, high molecular weight, and highly sorptive properties of these compounds. As a consequence, little is known about the structure−activity relationships for high molecular weight proanthocyanidins. We proposed to achieve a more complete structural characterization of the high molecular weight proanthocyanidins from Shanxi heart-shaped yellow persimmon fruits using a combination of MALDI-TOF MS (*9*), thiolysis-HPLC (*10*), GPC (*11*), and ^{13}C NMR (*12*) analysis. We were able to elucidate the monomer units, the molecular weight, and the linkage types of the persimmon tannin. Good structural information will serve as the basis for a better understanding of the chemical structure−activity relationships of persimmon tannin, which should allow us to exploit its potential as a beneficial phytonutrient.

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MATERIALS AND METHODS

Chemicals. Analytical or HPLC grade solvents and reagents were used. Trifluoroacetic acid (TFA), phenylmethane thiol (benzyl mercaptan), catechin, epicatechin, cyanidin chloride, delphinidin chloride, cesium trifluoroacetate, 2,5-dihydroxybenzoic acid, eriodictyol, quercetin, myricetin, luteolin, apigenin, kaempferol, and Toyopearl HW50F were from Sigma-Aldrich (St. Louis, MO). Epigallocatechin gallate, epicatechin gallate, and epigallocatechin were provided by Lipton Tea Co. (Newark, NJ). Catechin benzylthioether, epicatechin benzylthioether, epigallocatechin benzylthioether, and gallocatechin benzylthioether were isolated by Moore and Hagerman (personal communication). AB-8 macroporous resin was purchased from Naikai Chemical Plant, Tianjin, China. Dowex 50 × 8-400 cation-exchange resin was from Supelco, Bellefonte, PA.

Characterization of Persimmon Proanthocyanidin. Mature and fully colored fruits of the astringent Shanxi heart-shaped yellow persimmon (*D. kaki*) were harvested in late October from an orchard in Shan'xi (China). After harvest, fruits were held at 100 °C for about 5 min to inactivate polyphenol oxidase and were then stored deep frozen at −20 °C. As we previously reported (*6*), the yield of proanthocyanidin from persimmon is optimized by extraction with acidic methanol, similar to the group II proanthocyanidins from *Sorghum* grain (*13*). We therefore extracted the pulverized fruit with methanol/HCl (1%, v/v) at 90 °C and purified it on AB-8 macroporous resin followed by polysulfone ultrafiltration using a membrane with a molecular weight cutoff of 10 kDa.

MALDI-TOF mass spectra were collected on a Bruker Reflex II MALDI-TOF mass spectrometer (Billerica, MA) equipped with delayed extraction and a N_2 laser (337 nm). Mass spectra were calibrated with bradykinin (M + H 1060.6, monoisotopic) as an external standard. All samples were analyzed in the positive ion reflectron mode to detect either $[M + Na]^+$ or $[M + Cs]^+$ using an acceleration voltage of 25.0 kV and a reflectron voltage of 26.25 kV. All of the sample/matrix mixtures were applied (1 μL) onto a stainless steel target and dried at room temperature.

To prepare the samples, 25 μL of a 30 mg/mL solution of the tannin in methanol was dried under nitrogen. The sample was reconstituted in 25 μL of 100% ethanol immediately before analysis and was then mixed with the matrix solution (50 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 100% ethanol). For detection of $[M + Na]^+$, samples (2 μL) were vortexed with DHB (20 μL), and 300 shots were acquired using a laser attenuation of 68. For detection of $[M + Cs]^+$, samples (2 μL) were vortexed with DHB (16 μL) followed by the addition of Dowex 50W × 8-400 cation-exchange resin (3 μL) in 100% EtOH. The deionized sample/matrix solution (12 μL) was vortexed with cesium trifluoroacetate (0.8 μL of 10 mM), and 500 shots were acquired using a laser attenuation of 69.

Calculated monoisotopic masses are based on the equation $[M + Na]^+ = 290.08 \times CAT + 306.07 \times GCAT + 152.01 \times GALLOYL + 318.04 \times MYR - 2.02 \times B - 4.04 \times A + 22.99$. CAT, GCAT, GALLOYL, and MYR are the numbers of (epi)catechin, (epi)gallocatechin, galloyl esters, and myricetin subunits, respectively. For the average composition, the average molecular weights are substituted for the monoisotopic weights. For the Cs^+ adducts, Cs (132.91 amu) is substituted for Na (22.29 amu).

Thiolysis was carried out on both the crude persimmon proanthocyanidin and the Toyopearl fractions (PT14, PT23, and PT60, see below). About 5 mg of sample was dissolved in 1 mL of methanol, mixed with an equal volume of thiolysis reagent (5% (v/v) benzyl mercaptan in methanol containing 0.2 M HCl), and heated at 60 °C for 2 h (*6*). The products were analyzed with an Agilent series 1100 HPLC (Santa Clara, CA) equipped with a diode array detector in line with a 12 channel CoulArray detector (ESA, Chelmsford, MA). The column used was a 150 mm × 4.6 mm i.d., 5 μm XDB-C8, with a 4 mm × 4 mm i.d. guard column of the same material (Agilent, USA). The flow rate was 0.5 mL/min, and the gradient of 0.13% TFA in H_2O (A) and 0.1% TFA in acetonitrile (B) was as follows: 0−3 min, 15% B; 3−8 min, 15−20% B; 8−10 min, 20−25% B; 10−30 min, 25−35% B; 30−34 min, 35−70% B; 34−44 min, 70% B; 44−47 min, 20% B; 47−52 min, 20−15% B; 52−57 min, re-equilibration with 15% B. Thiolysis products were filtered through a 0.22 μm cellulose acetate spin filter before injection of 10 μL, and the eluant was monitored by DAD from 200 to 800 nm. The electrochemical detector, which provided added sensitivity, was set to monitor over the voltage range from 180 to 840 mV with 60 mV increments between channels. Data collected at 220 nm were integrated (Agilent Chem Station software ver. A.09.03) and were then converted to moles relative to the terminals using standard curves. Composition was calculated as moles relative to moles of the terminal units. For example, the unfractionated tannin yielded 2.2 mol of terminal catechin, 1.97 mol of terminal epigallocatechin gallate, 3.45 mol of terminal myricetin, and 30.1 mol of extender epigallocatechin benzylthiol ether, so the mole ratio was 0.29:0.26:0.45:3.96 for terminal catechin/terminal epigallocatechn gallate/terminal myricetin/extender epigallocatechin.

The average degree of polymerization (mDP) was calculated from the molar ratio of all the extender units plus terminal units to terminal units. Molecular weights were calculated on the basis of the molecular weight of each subunit.

HPLC-ESI-MS analysis was performed on an Agilent 1100 LC interfaced to a Esquire LC ion trap MS (Bruker Daltonics, Billerica, MA). The chromatographic conditions were the same as described above except that solvent A was 0.5% acetic acid in water and solvent B was acetonitrile. A source temperature of 500 °C, negative ion mode, was used with a sprayer needle voltage of 3.5 kV. The capillary temperature was 350 °C. The full scan mass spectra of the eluate from m/z 100 to 700 were measured using 500 ms for the collection time; three microscans were summed.

Direct injection ESI-MS analysis employed the electrospray ionization source (ESI) operated in negative mode. A nominal target mass was set to 1000 before fine-tuning. The capillary, skimmer 1, and trap drive voltages were 3500, −51.8, and 72.9 V, respectively. The ion charge control was on with a target of 30000. The 300 °C nitrogen dry gas flow rate was 4 L/min, and the nebulization gas pressure was 11 psi. A syringe pump was used to infuse the sample at 10 μL/min. Each data point in the spectrum consisted of an average of eight scans over a mass range of m/z 50−2000.

Anthocyanidin monomer analysis was carried out on the products of the acid butanol reaction (*14*) followed by analysis with HPLC on a Hewlett-Packard 1050 (Hewlett-Packard, Waldbronn, Germany) equipped with a diode array detector and controlled with Agilent ChemStation software (A.09.03). The column used was a 30 mm × 2.1 mm i.d., 5 μm, ODS2 C18 with a 4 mm × 4 mm i.d. guard column of the same material (Grace Davison, Deerfield, IL). Separation was achieved with a gradient of 0.13% TFA in H_2O (A) and 0.1% TFA in acetonitrile (B) at 0.5 mL/min in a 45 min program as follows: 0−10 min, 5% B; 10−15 min, increase to 20% B; 20−25 min, increase to 55% B; 25−35 min, 55% B; 35−40 min, decrease to 5% B; 40−45 min, re-equilibrate at 5% B. The samples were filtered through a 0.22 μm cellulose acetate spin filter before injection of 10 μL, and the eluate was monitored at 540 nm. The peaks were identified by comparison of the retention time and spectra with those of the commercial cyanidin and delphinidin standards, and peak areas were calculated with the ChemStation software.

Lyophilized persimmon tannin (0.3 g) was dissolved in about 2 mL of methanol and fractionated by gravity chromatography (30 cm × 500 mm i.d.) on Toyopearl TSK HW-50 (F) using 1000 mL of acetone/methanol (1:4, v/v) to yield fraction PT14, 1000 mL of acetone/methanol (2:3, v/v) to yield fraction PT23, and 250 mL of 60% aqueous acetone to yield fraction PT60. The three fractions were evaporated under reduced pressure at 30 °C, lyophilized, and stored at −20 °C for further analysis.

GPC was carried out on the Agilent series 1050 HPLC equipped with a 300 mm × 7.5 mm i.d., 5.0 μm, Varian PL Gel MIXED-D size exclusion column (Varian, Inc., Amherst, MA). An isocratic mobile phase of 1% water in tetrahydrofuran was delivered at 1 mL/min. The samples were dissolved at 1 mg/mL in tetrahydrofuran containing 1% water and filtered through a 0.22 μm nylon spin filter before injection of 10 μL. The eluent was monitored at 280 nm. Molecular weights were estimated on the basis of a standard curve generated with narrow polydiversity polystyrene standards (Mp 0.162−91.8 kDa).

For ^{13}C NMR, samples were dissolved in 50% H_2O/50% acetone at 300 mg/mL (crude persimmon tannin) or at 65 mg/mL (fraction 4). A Bruker AC-500 MHz NMR spectrometer was employed in the 125 MHz mode using a 5 mm probe at 15 °C with acetone as the reference. For the degradation product the spectrum was collected only between 70 and 170 ppm to obtain sufficient signal/noise.

To improve NMR spectroscopic resolution, tannin was chemically degraded by heating a sample (28 mg/mL) in 6.25% HCl in methanol. After reaction at 70 °C for 2 h, the reaction mixture was cooled and applied onto a column of Toyopearl TSK HW-50 (F) resin (50 cm × 3 cm i.d.) equilibrated with methanol. The column was eluted with methanol at

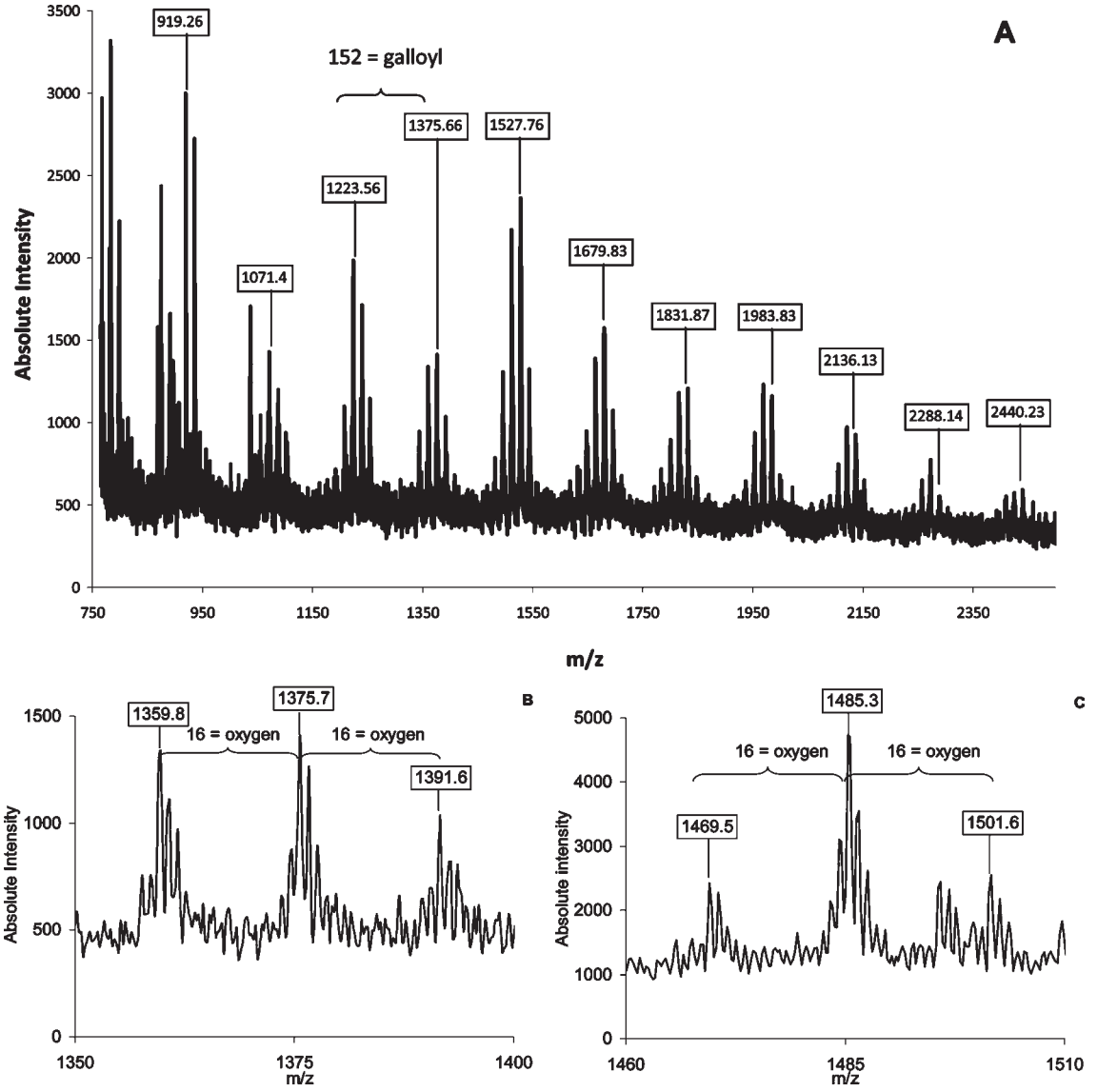

Figure 1. MALDI-TOF positive reflectron mode mass spectra of crude persimmon tannin as the naturally occurring Na^+/K^+ adducts (**A**): expanded scale spectrum of **A** showing the 16 amu repeat (**B**); expanded scale spectrum of the Cs^+ adducts showing the 16 amu repeat (**C**).

1.5 mL/min and monitored at 280 nm. Four fractions were identified and lyophilized for analysis by HPLC, ESI-MS, and ^{13}C NMR.

RESULTS AND DISCUSSION

MALDI-TOF MS is a powerful method for characterizing synthetic and natural polymers such as condensed tannins (*9*, *15*). It has the power to distinguish between molecular weight differences due to procyanidin versus prodelphinidin subunits (Δ 16 amu), due to 3-*O*-galloylation (Δ 152 amu), or due to flavan-3-ol versus flavanone (Δ 2 amu) or flavone (Δ 4 amu) terminal units (*15*). MALDI-TOF MS is particularly useful for revealing the nature of the interflavan bonds (A-type, Δ 2 amu) and has been used to confirm those linkages in mangosteen pericarp tannin (*16*) and cranberry tannin (*17*).

MALDI-TOF mass spectra of the crude persimmon tannin with the natural counterions (Na^+, K^+) (**Figure 1A**) showed a series of polyflavan-3-ols extending from the dimer (*m*/*z* 765) to the octamer (*m*/*z* 2425) with sodium adducts. Proanthocyanidins from dimer (*m*/*z* 875) to the octamer (*m*/*z* 2550) were also noted in samples that had been ion exchanged to the Cs^+ adducts (**Table 1** and Supporting Information) (*15*). The largest *m*/*z* obtained represented a series of octamers, although previous characterization had suggested that the persimmon tannin was much larger (*6*). We did not observe higher polymers by using the linear mode instead of the reflectron mode (data not shown). An inherent problem with MALDI-TOF MS is that lower MW oligomers saturate the detector and make it difficult to detect the larger polymers (*18*). Factors associated with the behavior of high molecular weight polymers themselves, including suppression of ionization, reduction of desorption, and in-source fragmentation leading to formation of noncovalent ion clusters, have been reported to influence the detection of high molecular masses by MALDI-TOF MS (*19*).

The main groups of peaks in the spectra were separated by Δ 152 amu (e.g., *m*/*z* 919, 1071, 1223, 1375, etc.) (**Figure 1A**; **Table 1**), corresponding to the addition of one galloyl group at the heterocyclic C-ring as in (epi)catechin gallate or (epi)gallocatechin gallate. Another strongly repeated pattern within each main set of peaks was signals separated by Δ 16 amu difference (**Figure 1B**). These masses may be produced by prodelphinidin-type flavan-3-ol units, where the third hydroxyl group introduces differences of 15.9949 amu (*15*). However, the atomic mass difference between the monoisotope of Na^+ (22.9900 amu) and the monoisotope of K^+ (39.0980 amu) is 15.9739 amu, so

Table 1. Observed and Calculated Masses of Persimmon Tannin by MALDI-TOF MS

		flavon-3-ols			linkages			$[M + Na]^+$ calcd mass			$[M + Cs]^+$ calcd mass	
DP	flavonol myricetin	(epi) catechin	(epi)gallo catechin	galloyl esters	B	A	obsd mass	av	monoisotopic	obsd mass	av	monoisotopic
2			2	1		1	783.12	763.61	783.11	893.05	893.52	893.02
2			2	1		1		783.61	783.11		893.52	893.02
2	1		1	1	1		797.20	797.59	797.09		907.51	907.01
2		1	1	2		1	919.26	919.72	919.13	1028.83	1029.63	1029.04
2		1	1	2		1		919.72	919.13		1029.63	1029.04
3		1	2		1	1		919.75	919.16		1029.67	1029.08
3		1	2		1	1		919.75	919.16		1029.67	1029.08
2			2	2		1	935.30	935.72	935.12	1045.38	1045.63	1045.03
3			3		1	1		935.75	935.15		1045.67	1045.07
3		2	1	1	1	1	1055.49	1055.86	1055.18	1165.33	1165.78	1165.10
3		2	1	1	1	1		1055.86	1055.18		1165.78	1165.10
3	1	2		1	2		1069.48	1069.85	1069.17	1179.35	1179.76	1179.08
3	1	2		1	2			1069.85	1069.17		1179.76	1179.08
3		1	2	1	1	1	1071.40	1071.86	1071.17	1181.36	1181.78	1181.09
3		1	2	1	1	1		1071.86	1071.17		1181.78	1181.09
3		1	2	1	1	1		1071.86	1071.17		1181.78	1181.09
3	1	1	1	1	2		1085.28	1085.85	1085.16	1195.26	1195.76	1195.07
3	1	1	1	1	2			1085.85	1085.16		1195.76	1195.07
3			3	1	1	1	1087.43	1087.86	1087.16	1197.29	1197.78	1197.08
3			3	1	1	1		1087.86	1087.16		1197.78	1197.08
3	1		2	1	2		1101.41	1101.85	1101.15		1211.76	1211.06
4		2	2		1	2		1205.99	1205.21	1315.50	1315.91	1315.13
4		2	2		1	2		1205.99	1205.21		1315.91	1315.13
4	1	3			3		1205.42	1205.99	1205.22		1315.91	1315.14
3		2	1	2	1	1		1207.97	1207.19	1317.39	1317.89	1317.11
3		2	1	2	1	1		1207.97	1207.19		1317.89	1317.11
3	1	2			1		1221.51	1221.96	1221.18	1331.59	1331.87	1331.09
4		1	3		2	2		1221.99	1221.20		1331.91	1331.12
4		1	3		1	2		1221.99	1221.20		1331.91	1331.12
4	1	2	1	2	3			1221.99	1221.21		1331.91	1331.13
3		1	2	2	1	1	1223.56	1223.97	1223.18	1333.49	1333.89	1333.10
3		1	2	2	1	1		1223.97	1223.18		1333.89	1333.10
3		1	2	2	2	1		1223.97	1223.18		1333.89	1333.10
3	1	1	1		3		1237.47	1237.96	1237.17	1347.54	1347.87	1347.08
4			4		1	2		1237.99	1237.19		1347.91	1347.11
4	1	1	2		3			1237.99	1237.20		1347.91	1347.12
3			3	2	1	1	1239.53	1239.97	1239.17	1349.45	1349.89	1349.09
3			3	2	1	1		1239.97	1239.17		1349.89	1349.09
3	1		2	2	2		1253.54	1253.96	1253.16		1363.87	1363.07
4	1		3		3			1253.99	1253.19		1363.91	1363.11
3		3		3	1	1	1343.53	1344.08	1343.21		1454.00	1453.13
4		3	1	1	2	1		1344.12	1343.25		1454.03	1453.16
4		3	1	1	2	1		1344.12	1343.25		1454.03	1453.16
4		3	1	1	2	1		1344.12	1343.25		1454.03	1453.16
4		3	1	1	2	1		1344.12	1343.25		1454.03	1453.16
3		2	1	3	1	1	1359.79	1360.08	1359.20	1469.54	1470.00	1469.12
3		2	1	3	1	1		1360.08	1359.20		1470.00	1469.12
4		2	2	1	2	1		1360.12	1359.24		1470.03	1469.15
4		2	2	1	2	1		1360.12	1359.24		1470.03	1469.15
4		2	2	1	2	1		1360.12	1359.24		1470.03	1469.15
4		2	2	1	2	1		1360.12	1359.24		1470.03	1469.15
3		1	2	3	1	1	1375.66	1376.08	1375.19	1485.29	1486.00	1485.11
3		1	2	3	1	1		1376.08	1375.19		1486.00	1485.11
4		1	3	1	2	1		1376.12	1375.23		1486.03	1485.14
4		1	3	1	2	1		1376.12	1375.23		1486.03	1485.14
4		1	3	1	2	1		1376.12	1375.23		1486.03	1485.14
4		1	3	1	2	1		1376.12	1375.23		1486.03	1485.14
3			3	3	1	1	1391.63	1392.08	1391.18	1501.62	1502.00	1501.10
4			4	1	2	1		1392.12	1391.22		1502.03	1501.13
4			4	1	2	1		1392.12	1391.22		1502.03	1501.13

prodelphinidin units cannot be determined with certainty in the Na^+/K^+ preparations. MADLI-TOF spectra of samples that were ion exchanged with Cs^+ demonstrate that the Δ 16 amu signals are not an artifact of the natural $[M + Na]^+$ and $[M + K]^+$ mixture, but are derived from prodelphinidin-type monomers (**Figure 1C**).

On the basis of the report by Monagas (*19*), an equation was formulated to calculate the oligomeric proanthocyanidin molecular weights based on (epi)catechin and epi(gallocatechin) subunits with 3-*O*-galloylation. We found that the spectra did not show strong signals at the predicted masses for oligomers based

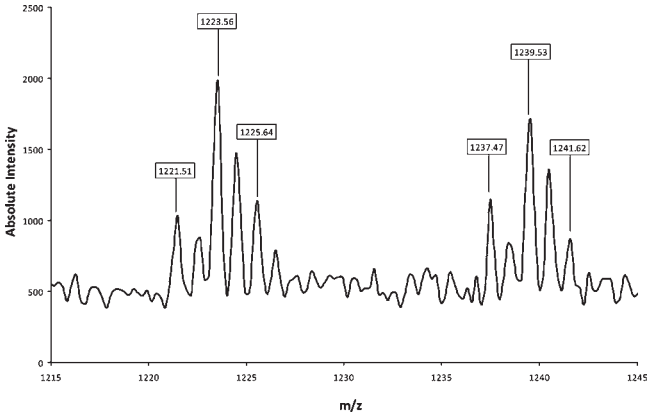

Figure 2. Expanded scale of the MALDI-TOF mass spectrum of Na^+/K^+ adducts of crude persimmon tannin.

on 3-*O*-galloylated (epi)catechin and (epi)gallocatechin with B-type interflavan bonds. For example, the B-linked trimer of (epi)gallocatechin with two 3-*O*-galloyl groups should give a signal in the Na^+/K^+ spectra at *m*/*z* 1241.2. Strong signals were noted instead at *m*/*z* 1239.5 and *m*/*z* 1237.5 (**Figure 2**). The B-linked trimer in which one (epi)gallocatehin is replaced by (epi)catechin has a predicted signal at *m*/*z* 1225.2, but much stronger signals were found at *m*/*z* 1223.6 and 1221.5 (**Figure 2**). A similar pattern, with dominant peaks Δ 2 and Δ 4 amu smaller than the predictions for simple B-linked polyflavan-3-ols, was found throughout the spectra (**Table 1** and Supporting Information).

Quinone methide formation can yield Δ 2 amu signals (*20*), but fragmentation to quinone methides does not occur with the soft ionization used in MALDI-TOF MS (*19*). Proanthocyanidins with A-type interflavan linkages between adjacent flavan-3-ol subunits have characteristic signals Δ 2 amu smaller than B-linked proanthocyanidins, due to loss of two hydrogen atoms with formation of the ether bond. For example, the *m*/*z* 1239.5 peak can be attributed to an (epi)gallocatechin trimer with two 3-*O*-galloyl groups and one A-type linkage as well as one B-type linkage. We postulated that the A-type linkage is an important structural feature of persimmon tannin on the basis of the strength of the Δ 2 amu signal in each cluster of peaks. A-Linked 3-*O*-galloylated proanthocyanidins are rare, but have been reported in bioactive extracts of *Maytenus* leaves (*21*).

In addition to the Δ 2 amu signals attributed to A-type linkages, the MALDI TOF spectra revealed some Δ 4 amu signals. For example, in **Figure 2** the *m*/*z* 1237.5 signal is Δ 4 amu smaller than that of the standard B-linked, flavan-3-ol trimer. Terminal units such as flavanone or flavones can contribute signals that are Δ 2 amu or Δ 4 amu smaller, respectively, than typical oligomers with flavan-3-ol terminals (*15*). Although the MALDI-TOF data are suggestive of unusual terminal units in the persimmon tannin, chemical methods are required to establish the identify of terminal groups.

We used thiolysis-HPLC to evaluate the terminal units of the persimmon tannin (*10*, *22*). The reaction products were identified by comparison of retention times, UV−vis spectra, and mass spectra to those of authentic standards. In the thiolysis reaction, terminal units from proanthocyanidins are released as free flavanoids, with catechin as the most common terminal unit. Because the persimmon tannin is of very high molecular weight, terminal units are present at low concentration relative to the extender units, and consequently the terminal units have not previously been identified (*6*). In the present study, we optimized detection of the terminal units by increasing the concentration of the sample from 1 to 5 mg/mL when performing the thiolysis

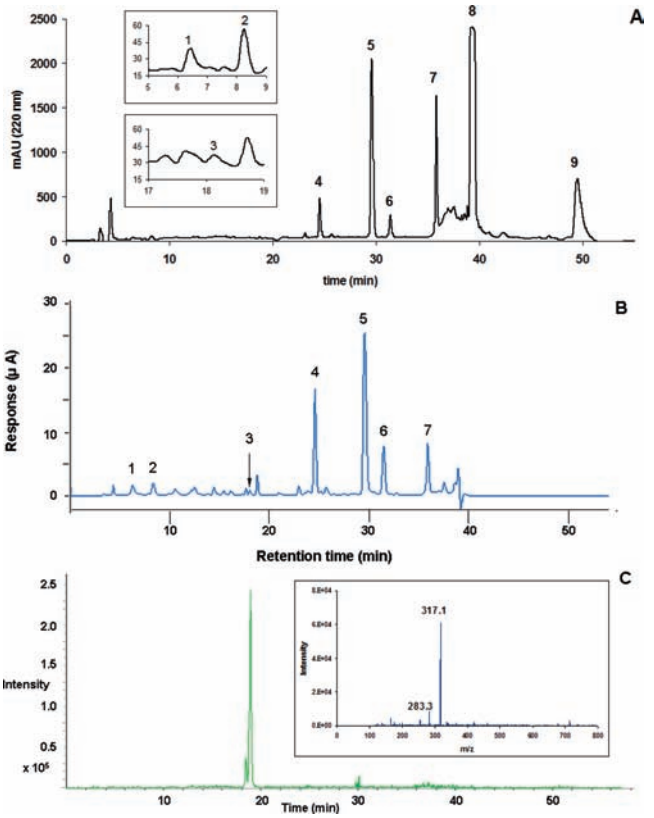

Figure 3. RP-HPLC chromatograms of persimmon tannin thiolysis products with (**A**) detection at 220 nm, (**B**) electrochemical detection at 180 mV, (**C**) MS detection at *m*/*z* 317. The electrochemical detector was in-line immediately after the UV detector, with a lag time of about 0.1 min. **A, B**: 1, catechin; 2, epigallocatechin-3-*O*-gallate; 3, myricetin; 4, epigallocatechin benzylthioether; 5, (epi)gallocatechin-3-*O*-gallate benzylthioether; 6, epicatechin benzylthioether; 7, (epi)catechin-3-*O*-gallate benzylthioether; 8, benzyl mercaptan; 9, thiolysis byproduct. (**C**) Inset is mass spectrum of the main peak.

degradation and by detecting at 220 nm, which is about 10 times more sensitive than detection at 280 nm. Even with these adjustments to the standard protocol, the peaks attributed to the terminal flavan-3-ols (**Figure 3A**, peaks 1 and 2) are very small. We used electrochemical detection, which is about 4 times more sensitive than detection at 220 nm, to confirm the terminal units (**Figure 3B**). The retention time and behavior of peaks 1 and 2 are indistinguishable from those of catechin and epigallocatechin-3-*O*-gallate, respectively, establishing these two flavan-3-ols as terminal units for persimmon tannin.

Because the MALDI-TOF data suggested that persimmon tannin may contain flavanone or flavone terminal units, we examined the UV−vis spectra of all the peaks in the thiolysis reaction products and compared them to the HPLC-DAD spectra of authentic samples of the common flavanones (eriodictyol), flavones (apigenin, luteolin), and flavonols (kaempferol, quercetin, myricetin) (*23*). Only one thiolysis reaction product (t_R 18.2 min) had the spectroscopic properties of a flavonoid (**Figure 4**). That peak (peak 3) was tentatively identified as the flavonol myricetin on the basis of its UV−vis spectrum and its retention time compared to authentic myricetin (**Figure 4**). The flavanone eriodictyol and the flavone apigenin have been established as terminal units in *Sorghum* proanthocynidin (*15*, *24*), but have long retention times (24.8 and 31.9 min, respectively) compared to peak 3. We used HPLC-MS to further authenticate myricetin as a constituent of the persimmon tannin thiolysis products by HPLC

with ESI-MS detection. The extracted ion chromatogram for m/z 317 had a single major peak coincident with myricetin (**Figure 3C**) and had the same mass spectrum as authentic myricetin (**Figure 3C**, inset). Myricetin has been reported as a constituent of persimmon leaves (*25*), but not of the fruit, and neither myricetin nor other flavonols have previously been reported as a proanthocyanidin terminal unit.

The MALDI-TOF data were reanalyzed using a new equation that included myricetin as a possible terminal unit (**Table 1**). As we had hypothesized, the Δ 4 amu signals could be attributed to myricetin terminal units. For example, the 1237.5 signal could represent either a B-linked trimer of myricetin, (epi)catechingallate, and (epi)gallocatechin gallate or a B-linked tetramer of myricetin, (epi)catechin, and two (epi)gallocatechin units. It is typical of MALDI-TOF that different composition oligomers may yield the same signal, so structure assignments from MALDI-TOF are always tentative. However, the combined evidence from MALDI-TOF and thiolysis with HPLC-DAD and HPLC-ESI-MS strongly support our conclusion that the persimmon proanthocyanidin has myricetin terminal units.

The thiolysis products included four benzylthioethers, representing the extender units in the persimmon proanthocyanidin (**Figure 3A**). Peak 4, eluting at 22.9 min, had the same retention time and UV−vis spectra as authentic epigallocatechin benzylthioether. Peak 6, t_R 29.7 min, had the same characteristics as authentic epicatechin benzylthioether. Peak 5, t_R 27.7 min, did not correspond to any of the four standard benzylthioethers. This compound had a molecular ion $[M - H]^-$ at m/z 579 (**Table 2**) with a characteristic MS^2 fragment ion at m/z 455, representing loss of the benzyl thiol, and a major fragment ion at m/z 303, which corresponds to the further loss of galloyl residue. Additional fragment ions at m/z 409 and 169 corresponded to the loss of *O*-galloyl and gallic acid, respectively (**Table 2**). We concluded that peak 5 is the benzylthioether of (epi)gallocatechin-3-*O*-gallate. Peak 7, t_R 34.5 min, also did not correspond to any of the authentic standards. It had a molecular ion $[M - H]^-$ at m/z 563 (**Table 2**), with a MS^2 fragment ion at m/z 439 corresponding to loss of the benzyl thiol. Other fragment ions were at m/z 411, resulting from the loss of galloyl residue from the benzyl thiol, and at m/z 287, corresponding to an additional loss of a galloyl residue. Peak 7 was identified as the benzylthioether of (epi)-catechin-3-*O*-gallate. We could not distinguish between the two epimers for either of these gallates with the methods we used. Our results are consistent with and extend our previous work (*6*).

Peak 8 (**Figure 3A**) was unreacted benzyl mercaptan, and peak 9 (**Figure 3**A) was an unknown compound that was found in the thiolysis reagent blank. Unlike the phenolics and their adducts, neither of these compounds reacts strongly in the electrochemical detector (**Figure 3B**).

As shown in **Table 3**, crude persimmon proanthocyanidin is a heterogeneous polymer consisting of catechin, epigallocatechin gallate, and myrecetin terminal units and epigallocatechin, (epi)gallocatechin gallate, catechin, and (epi)catechin gallate as extension units. Myricetin is slightly more common than the flavan-3-ol terminals. The distribution of monomers between delphinidin type (gallocatechins) and cyanidin type (catechins) is about 58% prodelphinidin in the crude tannin (**Table 3**). We confirmed the high proportion of prodelphinidin subunits by HPLC analysis of the products of the acid butanol reaction (*14*). Cyanidin and delphinidin were the only products of cleavage in acid butanol, and the products were 65% delphinidin. Over 70% of the subunits are galloylated (**Table 3**), consistent with the strong Δ 152 amu pattern in the MALDI-TOF spectra (**Figure 1A**)

The average yield of the thiolysis reaction was 43%, somewhat lower than typical yields for B-linked proanthocyaidins (*26*). Poor reactivity of highly galloylated (*27*) and of A-2 linked (*28*) proanthocyanidins has been reported even for oligomeric forms. Because the A-type linkage is not susceptible to thiolysis, unique thiolysis products representing A-linked dimers are sometimes detected (*26*), but we did not obtain any direct evidence for A-linkages in the thiolysis products from persimmon tannin. Using our reaction conditions we readily identified A-type dimers and A-type dimer thiol from cranberry proanthocyanidin, indicating that our methods were acceptable. We believe that because persimmon tannin is of very high molecular weight, is very highly galloylated, and has A-type linkages, it has significant resistance to thiolysis and A-linked products are not produced in significant quantity. Our results suggest that better methods for chemical degradation need to be developed to further characterize high

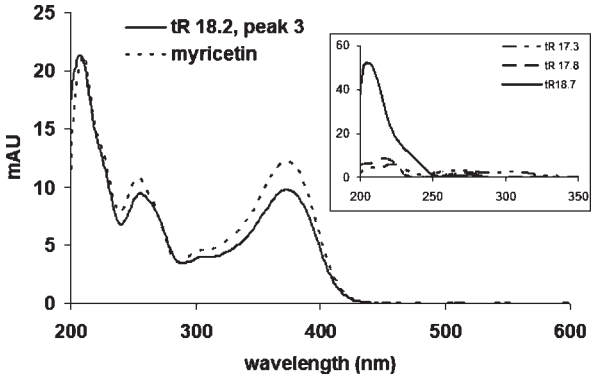

Figure 4. HPLC-DAD spectrum of peak 3 from **Figure 2A**. The spectrum is compared to that of authentic myricetin under identical chromatographic conditions. The inset shows spectra of small peaks just before and just after the peak of interest.

Table 2. HPLC-ESI-MS Analysis of the Products of Thiolysis of Persimmon Proanthocyanidin

peak[a]	compound	$[M - H]^-$ (m/z)	MS^2 ions (m/z)
1	catechin[b]	289	245, 205, 179, 125
2	epigallocatechin-3-*O*-gallate[b]	457	288, 245, 169
3	myricetin[b]	317.1	283.3
4	epigallocatechin benzylthioether[b]	427	303, 124
5	(epi)gallocatechin-3-*O*-gallate benzylthioether	579	455, 409, 303, 169, 124
6	epicatechin benzylthioether[b]	411	287, 124
7	(epi)catechin-3-*O*-gallate benzylthioether	563	439, 411, 287, 124

[a] Peak numbers correspond to the chromatogram in **Figure 1**. [b] Confirmed by comparison to authentic standards.

Table 3. Structural Composition of Persimmon Tannin Determined by Thiolysis-HPLC Analysis

fraction	terminal catechin	terminal epigallocatechin gallate	terminal myricetin	extender epigallocatechin	extender (epi) gallocatechin gallate	extender epicatechin	extender (epi) catechin gallate	mDP
unfractionated persimmon tannin	0.29	0.26	0.45	3.96	11.0	2.78	7.56	26
persimmon fraction PT14	0.32	0.17	0.51	2.04	8.96	1.69	4.81	19
persimmon fraction PT23	0.32	0.42	0.26	3.30	20.3	2.73	9.47	37
persimmon fraction PT60	0.27	0.46	0.27	5.25	25.0	3.84	12.1	47

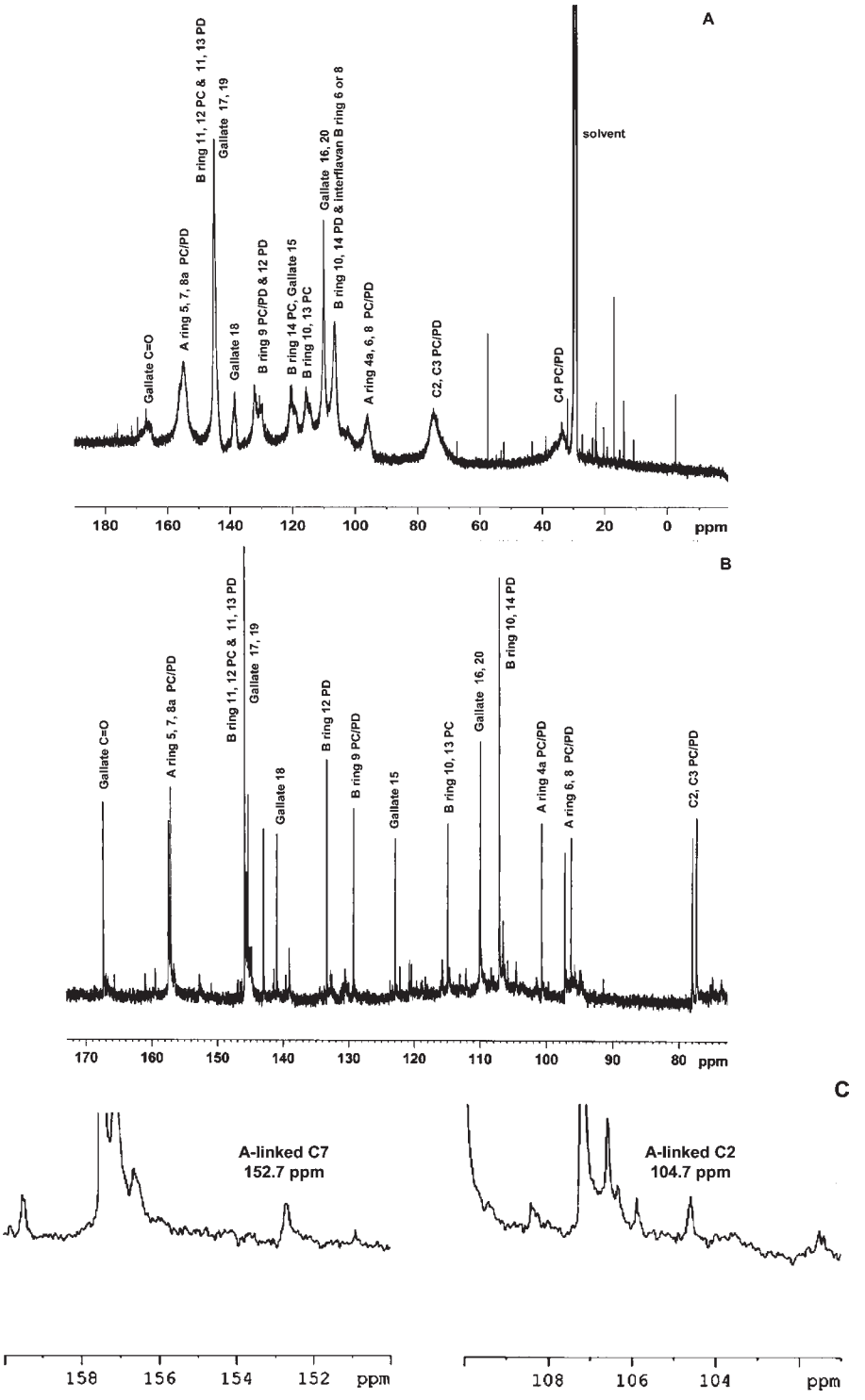

Figure 5. ^{13}C NMR spectra of crude persimmon tannin (**A**) and the fraction 4 product of partial acid degradation 4 (**B**, **C**). Samples were dissolved in 50% H_2O/50% acetone.

molecular weight, highly galloylated, A-type proanthocyanidins such as the persimmon tannin.

We employed NMR in an attempt to obtain additional evidence for A-type interflavan linkages (*29*). The ^{13}C NMR spectrum of the crude persimmon tannin (**Figure 5A**) had the characteristic features of a mixed proanthocyanidin with 3-*O*-galloylation on some subunits (*12*, *30*). Resonances of the gallate carbonyl (165 ppm) and galloyl ring carbons C18 and C16/C20 (138 and 110 ppm, respectively) are unique to galloylated flavan-3-ols (*30*). The characteristic peaks for the A-ring carbons (~95, ~155 ppm) and B-ring carbons (~108, 115, 119, 133, 145 ppm) were consistent with a mixed procyanidin/prodelphinidin polymer, but did not provide specific structural detail (*12*). Theoretically, the mean degree of polymerization could be determined by integrating the C-3 signals of the terminal flavanol unit around 67−68 ppm or the terminal flavonol carbonyl at around 41−42 ppm and the extender unit around 72−73 ppm (*29*). However, signals corresponding to the terminal units were not observed for any samples we ran, and the resonance of the extender units was observed as a wide peak between 70 and 80 ppm.

Table 4. Molecular Weight Estimates for Proanthocyanidins Based on Chemical Degradation (Thiolysis) or Gel Permeation Chromatography (GPC)

		GPC with polystyrene standards[a]		
proanthocyanidin	thiolysis MW	M_n	M_w	polydispersity
persimmon fraction PT14	7679	3071	3812	1.2
persimmon unfractionated	10749	3819	5574	1.5
persimmon fraction PT23	15635	5087	6423	1.3
persimmon fraction PT60	19905	6089	8158	1.2

[a] The regression equation obtained with polystyrene standards was mol weight = $7 \times 10^9\ e^{-1.91x}$, where x is retention time in min; $R^2 = 0.9838$.

This is consistent with the highly polymerized nature of the persimmon proanthocyanidin. Because of the poor resolution of the ^{13}C NMR spectrum of the crude persimmon tannin, we attempted fractionation of the crude tannin.

As reported previously (*6*), reversed phase HPLC does not resolve the persimmon polymeric polyphenol extract into discrete fractions, but yields a single broad peak. Similar results obtained with polyphenolics from other fruits are attributed to the heterogeneous nature of the crude polyphenols, which comprise a variety of isomers and oligomers with different degrees of polymerization (*31*, *32*). Sephadex LH20 eluted with aqueous alcohol or aqueous acetone has been widely used to fractionate proanthocyanidins from plant extracts (*33*). However, we found that the persimmon samples adsorbed to the Sephadex LH20 resin irreversibly. Aparicio-Fernandez et al. (*34*) adopted silica gel to fractionate polymerized polyphenols from the seed coat of Black Jamapa bean. We found that neither silica gel nor polyamide (*35*) could be used to fractionate persimmon tannin, presumably because its high molecular weight and numerous phenolic moieties lead to irreversible sorption on these stationary phases. Chromatography on Toyopearl HW-40-F with alcohol or aqueous acetone mobile phases has been used to fractionate apple proanthocyanidins (*36*). Because we speculated that the persimmon tannin was more highly polymerized than apple procyanidins, we used Toyopearl with a larger pore size (HW-50-F). Although the persimmon tannin adsorbed strongly when methanol was used as the mobile phase, we obtained three fractions (PT14, PT23, and PT60) from the crude persimmon tannin by eluting with acetone/methanol and acetone/water mixtures, increasing acetone in a stepwise fashion. Like the crude persimmon tannin, each of the three fractions produced a broad peak on RP-HPLC but the peaks of PT23 and PT60 were narrower than that of the crude tannin or PT14.

We analyzed the fractions by thiolysis, estimated the mean degree of polymerization (DPn) from the relative moles of products (**Table 3**), and calculated average molecular weights based on the molecular size of each monomer (**Table 4**). The Toyopearl fractions had compositions similar to that of the crude persimmon tannin, with the same terminal units and the same extenders (**Table 3**). Thiolysis indicated that PT60 > PT 23 > PT > PT 14 and provided a molecular weight for the crude persimmon tannin (10.7 kDa) similar to that previously established by GPC in polar solvents with polyvinyl alcohol standards (*6*). We attempted to use a more hydrophobic column with nonpolar solvents and polystyrene standards to assess the polydispersity and relative sizes of the Toyopearl fractions (**Table 4**). The polydispersity of the crude PT is highest, consistent with fractionation to high molecular weight enriched (PT 60) and low molecular weight enriched (PT 14) populations. GPC gave relative molecular weights consistent with the relative sizes of the persimmon tannin preparations as determined by thiolysis, but the calculated M_n and M_w from GPC were 50−70% lower than the values from thiolysis (**Table 4**). We obtained similar very low molecular weight estimates using defined procyanidin oligomers (*37*) as molecular weight standards instead of polystyrene and with acetylated persimmon proanthocyanidins (*11*). Polystyrene is a rigid polymer with no ability to hydrogen bond, but proanthocyanidins are flexible polymers with many hydrogen bond donor and acceptor sites. In nonpolar solvents such as tetrahydrofuran, proanthocyanidins may form secondary structures that decrease their hydrodynamic volume and may also interact with the GPC column via hydrogen bonding, both leading to apparent molecular weights far lower than the actual values (*11*, *38*). We concluded that GPC in polar solvents yields the best estimates of molecular weight for very high molecular weight proanthocyanidins.

Although PT14 was of lower molecular weight than crude persimmon tannin, it did not give the high-resolution NMR data required to confirm A-linkages in the proanthocyanidin. We therefore partially cleaved the crude tannin with 6.25% HCl in methanol, obtained four fractions by Toyopearl chromatography, and characterized those fractions by RP-HPLC-DAD and direct infustion ESI-MS. Fraction 1 was cyanidin and delphinidin (data not shown). HPLC showed that fraction 2 had two main components, one with nondescript spectroscopic features and the other spectroscopically identical to myricetin. Direct infusion ESI-MS revealed a molecular ion typical of myricentin (m/z 316.9) as well as several other species (m/z 163.8, 300.9). Thus, the acid degradation provided supporting evidence for the myricetin terminal units.

Fractions 3 and 4 were postulated to be acid-resistant fragments of the persimmon proanthocyanidin based on A-type linkages, which are generally more acid stable than B-type interflavan bonds (*39*). RP-HPLC suggested that although fraction 3 had two main constituents (t_R 13.7 and 15.4 min), fraction 4 had only one main component (t_R 7.2 min) and two minor components (t_R 16.2 and 24.3 min), making it a better candidate for NMR. None of the components in fraction 3 or 4 had characteristic flavonoid UV−vis spectra, indicating that these fragments did not have myricetin terminal units. The molecular ions in fraction 3 were m/z 879 and 439, and those in fraction 4 were m/z 911 and 455. We deduced that fraction 4 is predominantly the A-linked dimer [(epi)gallocatechin-3-*O*-gallate]$_2$, but also contains some A-linked (epi)catechin$_2$ (**Figure 6**).

The ^{13}C NMR spectrum of fraction 4 (**Figure 4B**) is better resolved than the spectrum of the intact proanthocyanidin (**Figure 4A**) and provides more detailed information on the proanthocyanidin structure. The resonances of the carbonyl carbon and six ring carbons of the 3-*O*-gallate are clearly resolved (**Figure 4B**) (*30*). The cluster of peaks at 146 ppm includes C17 and C19 of the gallate and the B-ring C11 and C13 of the (epi)gallocatechin subunits (**Figure 4B**) (*12*). Like the HPLC and ESI-MS data, the NMR indicates that the (epi)gallocatechin-3-*O*-gallate dimer is contaminated by some (epi)catechin dimers, with B-ring C11, C12 included in the cluster at 146 ppm and the B-ring C10, C13 at 115 ppm (**Figure 4B**) (*12*).

Evidence for an A-type double linkage in the dimer is provided by the signal at 152.7 ppm (**Figure 4C**), which is assigned to the ether-linked carbon 7 (or 5) in the A-ring of the lower subunit (*16*, *29*). The ether-linked carbon 2 in the upper unit has a chemical shift at 104.7 ppm (**Figure 3C**) (*16*, *29*). To our knowledge, this is the first report confirming the existence of the A-type interflavan linkage in persimmon tannin.

The combination of MALDI-TOFMS; analysis of thiolysis reaction products by HPLC coupled to DAD, electrochemical detection, and ESI-MS; GPC; and NMR to characterize the high molecular weight of tannins from persimmon pulp yielded

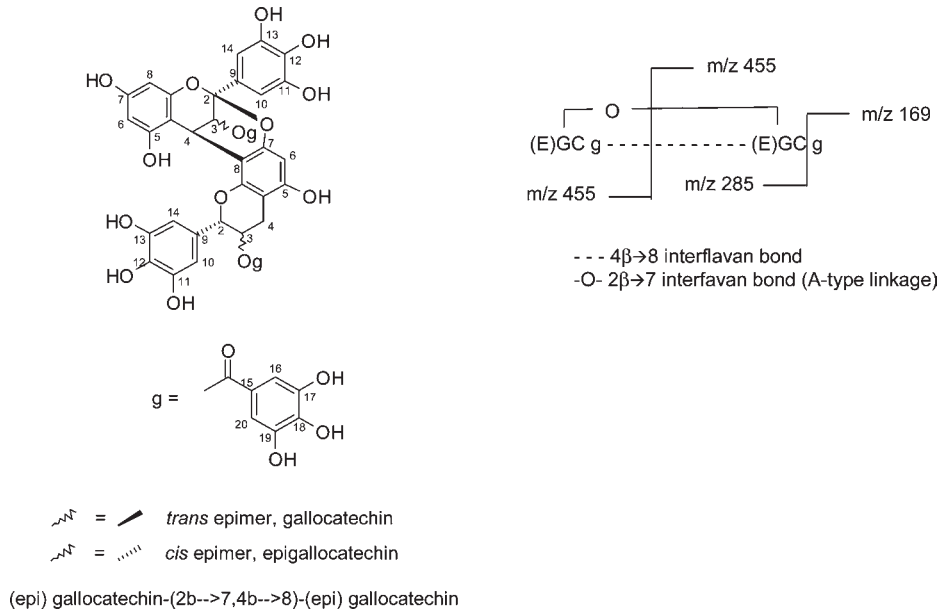

Figure 6. Structural formula of the A-linked dimer [(epi)gallocatechin-3-*O*-gallate]$_2$ and the ESI-MS cleavage pattern.

structural details for the first time. A novel terminal unit comprising the flavonol myricetin was identified along with the more common flavan-3-ols catechin and epigallocatechin-3-*O*-gallate. The extension units were confirmed to be epicatechin, epigallocatechin, (epi)gallocatechin-3-*O*-gallate, and (epi)catechin-3-*O*-gallate. The prodelphinidin content of the persimmon tannin (58−65%) was far higher than that of grape skins (20−30%), a prodelphinidin-rich source of tannins (*40*). The persimmon tannin was highly 3-*O*-galloylated (72%) in comparison to about 13% galloylation in grape seed tannin (*41*). We report for the first time that the persimmon tannin has A-type interflavan linkages in addition to the more common B-type interflavan bonds. The characterization of the structure of persimmon tannin should contribute to a better understanding of the chemical structure−activity relationships of this food-derived polyphenolic. In our previous study (*6*), we found that persimmon proanthocyanidin had more antioxidant activity than grape seed proanthocyanidin, suggesting that persimmon may be a source of therapeutically useful polyphenolics. We propose that the unique structural features of persimmon proanthocyanidin could be exploited to produce high-potency bioactive compounds by the food and drug industry.

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Supporting Information Available: Additional table. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Uchida, S.; Ohta, H.; Niwa, M.; Mori, A.; Nonaka, G.; Nishioka, I.; Ozaki, M. Prolongation of life span of stroke-prone spontaneously hypertensive rats (SHRSP) ingesting persimmon tannin. *Chem. Pharm. Bull.* (*Tokyo*) **1990**, *38*, 1049–1052.

(2) Piretti, M. V. Polyphenol constituents of the *Diospyros kaki* fruit − a review. *Fitoterapia* **1991**, *62*, 3–13.

(3) Achiwa, Y.; Hibasami, H.; Katsuzaki, H.; Imai, K.; Komiya, T. Inhibitory effects of persimmon (*Diospyros kaki*) extract and related polyphenol compounds on growth of human lymphoid leukemia cells. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 1099–1101.

(4) Gorinstein, S.; Kulasek, G. W.; Bartnikowska, E.; Leontowicz, M.; Zemser, M.; Morawiec, M.; Trakhtenberg, S. The effects of diets, supplemented with either whole persimmon or phenol-free persimmon, on rats fed cholesterol. *Food Chem.* **2000**, *70*, 303–308.

(5) Ahn, H. S.; Jeon, T. I.; Lee, J. Y.; Hwang, S. G.; Lim, Y.; Park, D. K. Antioxidative activity of persimmon and grape seed extract: *in vitro* and *in vivo*. *Nutr. Res.* (*N.Y.*) **2002**, *22*, 1265–1273.

(6) Gu, H. F.; Li, C. M.; Xu, Y. J.; Hu, W. F.; Chen, M. H.; Wan, Q. H. Structural features and antioxidant activity of tannin from persimmon pulp. *Food Res. Int.* **2008**, *41*, 208–217.

(7) Matsuo, T.; Ito, S. Mechanisms of removing astringency in persimmon fruits by carbon-dioxide treatment. 2. Chemical structure of kaki-tannin from immature fruit of the persimmon (*Diospyros kaki* L.). *Agric. Biol. Chem.* **1978**, *42*, 1637–1643.

(8) Nakatsubo, F.; Enokita, K.; Murakami, K.; Yonemori, K.; Sugiura, A.; Utsunomiya, N.; Subhadrabandhu, S. Chemical structures of the condensed tannins in the fruits of *Diospyros* species. *J. Wood Sci.* **2002**, *48*, 414–418.

(9) Krueger, C. G.; Dopke, N. C.; Treichel, P. M.; Folt, J.; Reed, J. E. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of polygalloyl polyflavan-3-ols in grape seed extract. *J. Agric. Food Chem.* **2000**, *48*, 1663–1667.

(10) Guyot, S.; Marnet, N.; Sanoner, P.; Drilleau, J. Direct thiolysis on crude apple materials for high-performance liquid chromatography characterization and quantification of polyphenols in cider apple tissues and juices. *Methods Enzymol.* **2001**, *335*, 57–70.

(11) Williams, V. M.; Porter, L. J.; Hemingway, R. W. Molecular weight profiles of proanthocyanidin polymers. *Phytochemistry* **1983**, *22*, 569–572.

(12) Czochanska, Z.; Foo, L. Y.; Newman, R. H.; Porter, L. J. Polymeric proanthocyanidins. Stereochemistry, structural units and molecular weight. *J. Chem. Soc., Perkin Trans. 1* **1980**, 2278–2286.

(13) Asquith, T. A.; Izuno, C. C.; Butler, L. G. Characterization of the condensed tannin (proanthocyanidin) from a group II *Sorghum*. *J. Agric. Food Chem.* **1983**, *31*, 1299–1303.

(14) Porter, L. J.; Hrstich, L. N.; Chan, B. G. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* **1986**, *25*, 223–230.

(15) Krueger, C. G.; Vestling, M. M.; Reed, J. D. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of heteropolyflavan-3-ols and glucosylated heteropolyflavans in sorghum [*Sorghum bicolor* (L.) Moench]. *J. Agric. Food Chem.* **2003**, *51*, 538–543.

(16) Fu, C.; Loo, A. E. K.; Chia, F. P. P.; Huang, D. J. Oligomeric proanthocyanidins from mangosteen pericarps. *J. Agric. Food Chem.* **2007**, *55*, 7689–7694.
(17) Foo, L. Y.; Lu, Y. R.; Howell, A. B.; Vorsa, N. The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic P-fimbriated *Escherichia coli* in vitro. *Phytochemistry* **2000**, *54*, 173–181.
(18) Reed, J. D.; Krueger, C. G.; Vestling, M. M. MALDI-TOF mass spectrometry of oligomeric food polyphenols. *Phytochemistry* **2005**, *66*, 2248–2263.
(19) Monagas, M.; Quintanilla-López, J. E.; Gómez-Cordovés, C.; Bartolomé, B.; Lebrón-Aguilar, R. MALDI-TOF MS analysis of plant proanthocyanidins. *J. Pharm. Biomed. Anal.* **2010**, *51*, 358–372.
(20) Gu, L. W.; Kelm, M. A.; Hammerstone, J. F.; Zhang, Z.; Beecher, G.; Holden, J.; Haytowitz, D.; Prior, R. L. Liquid chromatographic/electrospray ionization mass spectrometric studies of proanthocyanidins in foods. *J. Mass Spectrom.* **2003**, *38*, 1272–1280.
(21) de Souza, L. M.; Cipriani, T. R.; Iacomini, M.; Gorin, P. A. J.; Sassaki, G. L. HPLC/ESI-MS and NMR analysis of flavonoids and tannins in bioactive extract from leaves of *Maytenus ilicifolia*. *J. Pharm. Biomed. Anal.* **2008**, *47*, 59–67.
(22) Prieur, C.; Riguad, J.; Cheynier, V.; Moutounet, M. Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry* **1994**, *36*, 781–784.
(23) Ribereau-Gayon, P. *Plant Phenolics*; Oliver and Boyd: Edinburgh, Scotland, 1972.
(24) Gujer, R.; Magnalato, D.; Self, R. Glucosylated flavonoids and other phenolic compounds from sorghum. *Phytochemistry* **1986**, *25*, 1431–1436.
(25) Chen, G.; Xue, J.; Feng, X. Z. Inhibitory effect of water extract and its main contents of persimmon leaves on stimulus-induced superoxide generation in human neutrophils. *J. Food Biochem.* **2009**, *33*, 113–121.
(26) LeRoux, E.; Doco, T.; Sarni-Manchado, P.; Lozano, Y.; Cheynier, V. A-type proanthocyanidins from pericarp of *Litchi chinensis*. *Phytochemistry* **1998**, *48*, 1251–1258.
(27) Hartisch, C.; Kolodziej, H. Galloylhamameloses and proanthocyanidins from *Hamamelis virginiana*. *Phytochemistry* **1996**, *42*, 191–198.
(28) Koerner, J. L.; Hsu, V. L.; Lee, J.; Kennedy, J. A. Determination of proanthocyanidin A2 content in phenolic polymer isolates by reversed-phase high-performance liquid chromatography. *J. Chromatogr., A* **2009**, *1216*, 1403–1409.
(29) Foo, L. Y.; Lu, Y. R.; Howell, A. B.; Vorsa, N. A-type proanthocyanidin trimers from cranberry that inhibit adherence of uropathogenic P-fimbriated *Escherichia coli*. *J. Nat. Prod.* **2000**, *63*, 1225–1228.
(30) Davis, D. L.; Cai, Y.; Davies, A. P.; Lewis, J. R. ^{1}H and ^{13}C NMR assignments of some green tea polyphenols. *Magn. Reson. Chem.* **1996**, *34*, 887–890.
(31) Gabeta, B.; Fuzzati, N.; Griffini, A.; Lolla, E.; Pace, R.; Ruffilli, T.; Peterlongo, F. Characterization of proanthocyanidins from grape seeds. *Fitoterapia* **2000**, *71*, 162–175.
(32) Peng, Z. K.; Hayasaka, Y.; Iland, P. G.; Sefton, M.; Hoj, P.; Waters, E. J. Quantitative analysis of polymeric procyanidins (tannins) from grape (*Vitis vinifera*) seeds by reverse phase high-performance liquid chromatography. *J. Agric. Food Chem.* **2001**, *49*, 26–31.
(33) Hagerman, A. E.; Butler, L. G. Assay of condensed tannins or flavonoid oligomers and related flavonoids in plants. *Methods Enzymol.* **1994**, *234*, 429–437.
(34) Aparicio-Fernandez, X.; Yousef, G. G.; Loarca-Pina, G.; Mejia, E. D.; Lila, M. A. Characterization of polyphenolics in the seed coat of Black Jamapa bean (*Phaseolus vulgaris* L.). *J. Agric. Food Chem.* **2005**, *53*, 4615–4622.
(35) Duenas, M.; Sun, B.; Hernandez, T.; Estrella, I.; Spranger, M. I. Proanthocyanidin composition in the seed coat of lentils (*Lens culinaris* L.). *J. Agric. Food Chem.* **2003**, *51*, 7999–8004.
(36) Yanagida, A.; Kanda, T.; Shojii, T.; Ohnishi-Kameyama, M.; Nagata, T. Fractionation of apple procyanidins by size-exclusion chromatography. *J. Chromatogr., A* **1999**, *855*, 181–190.
(37) Li, C. M.; Trombley, J. D.; Schmidt, M. A.; Hagerman, A. E. Preparation of an acid butanol standard from fresh apples. *J. Chem. Ecol.* **2010**, *36*, 453–460.
(38) Cadahía, E.; Conde, E.; GarcíaVallejo, M. C.; de Simón, B. F. Gel permeation chromatographic study of the molecular weight distribution of tannins in the wood, bark and leaves of *Eucalyptus* spp. *Chromatographia* **1996**, *42*, 95–100.
(39) Jacques, D.; Haslam, E.; Bedford, G. R.; Greatbanks, D. Plant proanthocyanidins. Part II. Proanthocyanidin-A2 and its derivatives. *J. Chem. Soc., Perkin Trans. 1* **1974**, 2663–2671.
(40) Souquet, J.; Cheynier, V.; Brossaud, F.; Moutounet, M. Polymeric proanthocyanidins from grape skins. *Phytochemistry* **1996**, *43*, 509–512.
(41) Monagas, M.; Gómez-Cordovés, C.; Bartolomé, B.; Laureano, O.; Ricardo-da-Silva, J. M. Monomeric, oligomeric, and polymeric flavan-3-ol composition of wines and grapes from *Vitis vinifera* L. cv. Graciano, Tempranillo, and Cabernet Sauvignon. *J. Agric. Food Chem.* **2003**, *51*, 6475–6481.

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