

## Combined Normal-Phase and Reversed-Phase Liquid Chromatography/ESI-MS as a Tool To Determine the Molecular Diversity of A-type Procyanidins in Peanut Skins

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Peanut skins, a byproduct of the peanut butter industry, are a rich source of proanthocyanidins, which might be used in food supplements. Data on the molecular diversity of proanthocyanidins in peanut skins is limited and conflicting with respect to the ratio of double- (A-type) versus single-linked (B-type) flavan-3-ol units. NP- and RP-HPLC-MS were used as tools to analyze the molecular diversity of proanthocyanidins in a 20% (v/v) methanol extract of peanut skins. NP-HPLC was used to prepurify monomeric to pentameric fractions, which were further separated and characterized by RP-HPLC-MS. With this method, 83 different proanthocyanidin molecular species were characterized and quantified. Furthermore, it was possible to determine that A-type procyanidin oligomers were predominant and represented 95.0% (w/w) of the extract. In addition, the position of the A-linkages in 16 trimers and 27 tetramers could be determined, which in this case appeared to occur at all possible positions. The majority of trimers and tetramers with one or more A-linkage always had an A-linkage at the terminal unit.

**KEYWORDS:** Peanut skins; proanthocyanidins; A-type; quantification; molecular diversity; NP-HPLC; RP-HPLC

### INTRODUCTION

Proanthocyanidins, present in a wide variety of food products and beverages, are the second most abundant class of phenolic compounds in our diet (1). Intervention studies with proanthocyanidin-rich extracts and products such as cocoa and wine suggest protective effects of proanthocyanidins against cardiovascular diseases (2, 3). Peanut skins, a byproduct from the peanut butter industry, might be an excellent source for the production of proanthocyanidin-rich extracts for use in food supplements, like the ones already available from grape seed and pine bark. To explore the full potential of peanut skins as a food supplement, it is important to know the molecular diversity of the proanthocyanidins present.

Proanthocyanidins can be divided into several groups. The most common proanthocyanidins, occurring in food sources such as peanut skins, are the procyanidins (1), exclusively consisting of (epi)catechin units. Besides procyanidins, prodelphinidins, a heterogeneous group consisting of at least one (epi)gallocatechin unit and additional (epi)catechin units, and propelargonidins, a heterogeneous group consisting of at least one (epi)afzelechin unit and additional (epi)catechin units, occur in food sources.

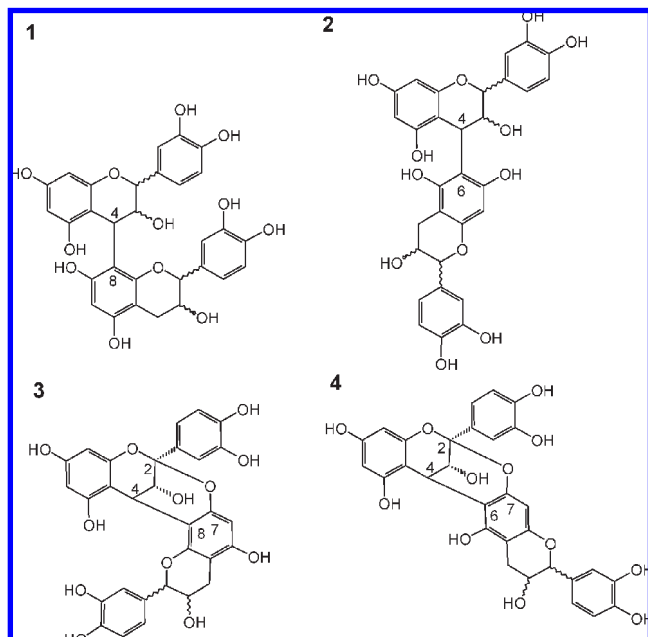
The monomeric units of proanthocyanidins are linked through a C4–C8 or C4–C6 bond (B-type), which can coexist with an

additional C2–O–C7 linkage (A-type) (Figure 1). Each proanthocyanidin oligomer contains one terminal unit of which the C-ring is not connected to another monomeric unit. All other units are called extension units. The most well-known sources of A-type proanthocyanidins, mainly procyanidins, are peanuts, plums, cranberries, and cinnamon (1, 4), but data about the molecular diversity of procyanidins are limited. The position of A-linkages in several tetramers and pentamers from plums and cinnamon has been identified by mass spectrometric (MS) analysis on the basis of their product ions (5). The position of the A-linkage can be source dependent. In plums, it was located at the terminal unit, whereas in cinnamon it was located between the extension units. Similarly, the monomeric composition within prodelphinidins and propelargonidins [(epi)catechin, (epi)gallocatechin, and (epi)afzelechin] and positions of the monomers in the molecule have been determined with MS analysis (5–7).

Peanut skins have a high proanthocyanidin content [17% (w/w)] (8). The degree of polymerization of proanthocyanidins in peanut skins has been reported to range from 2 to 8, whereas only low amounts of monomers are present (9, 10). In the skins, both C4–C8- and C4–C6-linked A- and B-type procyanidins have been identified (8, 11, 12).

Conflicting results have been obtained on the abundance of A- and B-type proanthocyanidins in peanut skins. Lazarus and co-workers (10), who analyzed a peanut procyanidin extract containing monomers up to octamers with NP-HPLC-UV-MS,

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**Figure 1.** Representative structures of proanthocyanidins. The monomeric units can be linked through a single carbon-carbon bond [C4-C8 (1) or C4-C6 (2)] or with an additional ether bond [C4-C8, C2-O-C7 (3) or C4-C6, C2-O-C7 (4)].

reported that A-type procyanidins dominated over B-type procyanidins. In contrast to Lazarus, Karchesy and Hemingway (8) reported a ratio of 1:15 of A- versus B-linkages. These latter observations were based on mass spectrometric data only, without separation of oligomers, which limits both accurate qualification and quantification of the molecular diversity. Besides the conflicting data on the ratio of A- versus B-type linkages, the analytical techniques (NP-HPLC and NMR) that were used might underestimate the molecular diversity, because it poorly separates (NP-HPLC) or does not separate (NMR) oligomers with the same degree of polymerization. Data on the position of the A-linkages in peanut skins are limited and have been identified in only one trimer and one tetramer (12).

In our study, we explored whether a combination of NP- and RP-HPLC-MS can provide a more accurate fingerprint of the molecular proanthocyanidin species in a 20% (v/v) methanol extract of peanut skins, compared to NP-HPLC-MS alone.

NP-HPLC has been used before to separate and characterize peanut procyanidins (10), but we used it as a first step to obtain fractions of monomers to pentamers. Subsequently, each fraction was further separated by RP-HPLC-MS, which enabled us, in contrast to previous studies (8, 10), to determine the molecular diversity of procyanidins in a 20% (v/v) methanol extract of peanut skin, which appeared to be much larger than reported before. Additionally, the combination of NP- and RP-HPLC enabled us to estimate the contribution of each peak to the total extract, on the basis of their UV responses. The most abundant molecular species were determined, and the positions of A-linkages in a large number of trimers and tetramers were established.

## MATERIALS AND METHODS

**Materials.** Peanut skins were kindly provided by Imko—The Nut Company B.V. (Doetinchem, The Netherlands). Organic solvents used for extractions were of analytical grade. Organic solvents used for HPLC analysis were all of HPLC grade. Hexane, dichloromethane, acetonitrile, and acetone were purchased from Sigma-Aldrich (Steinheim, Germany), methanol was from Mallinckrodt Baker B.V. (Deventer, The Netherlands),

and ethyl acetate, sulfuric acid, hydrochloric acid, and glacial acetic acid were from Merck (Darmstadt, Germany). Other chemicals were of analytical grade and purchased from Merck. Milli-Q water from a Millipore system was used.

**Extraction.** Peanut skins (75 g) were defatted with hexane using Soxhlet extraction. The residue was air-dried. The defatted skins (59.6 g) were extracted successively three times with 1 L of 20% (v/v) aqueous methanol, followed by three extractions with 1 L of 70% (v/v) aqueous methanol and three extractions with 1 L of 70% (v/v) aqueous acetone. After each extraction, the suspension was filtered over a 595 1/2 filter (Schleicher & Schuell, Dassel, Germany), after which the retentate was subjected to the next extraction. The three 20% (v/v) methanol fractions were combined, as well as the three 70% (v/v) methanol and the three 70% (v/v) acetone fractions. Subsequently, they were concentrated with a rotary evaporator and lyophilized, resulting in three extracts referred to as 20MeOH, 70MeOH, and 70Acetone. During the extractions and evaporation, light was excluded as much as possible by the use of marquee and foil. The three extracts were subjected to solvent partitioning for further purification. The 20MeOH extract was put in a separation funnel to which 500 mL of water and 500 mL of ethyl acetate were added and placed in an ultrasonic bath for 10–15 min at room temperature. After vigorous mixing, the water and ethyl acetate phases were collected separately. The water phase was re-extracted twice following the same solvent partitioning procedure, resulting in three ethyl acetate phases and one water phase. The ethyl acetate phases were combined and subsequently extracted with water (1:1, v/v) twice. At the end of the partitioning procedure, one ethyl acetate phase and three water phases were obtained. For the 70MeOH and the 70Acetone extracts the same procedure was followed. The ethyl acetate was evaporated with a rotary evaporator, and the material was dissolved in water and lyophilized, resulting in three final extracts referred to as 20MeOH-EA, 70MeOH-EA, and 70Acetone-EA. The solvent partitioning might have resulted in the removal of sugars. Therefore, the water phases of each extract were analyzed for their total neutral saccharide content to follow the purification.

**Analysis of the Total Neutral Saccharide Content.** The total neutral saccharide content was determined according to the Dubois method (13) with some alterations. A stock solution of 150  $\mu\text{g}$  of glucose/mL water was used to make a standard curve: 0, 50, 100, and 200  $\mu\text{L}$  of the glucose stock was filled to 1000  $\mu\text{L}$  with a 2.5% (w/v) phenol (Fluka Chemie GmbH, Buchs, Switzerland) solution in water ( $R^2 = 0.9965$ ). From the water phases, obtained after extraction, 100–200  $\mu\text{L}$  was taken and filled to 1000  $\mu\text{L}$  with the 2.5% (w/v) phenol solution. After the addition of 2.5 mL of sulfuric acid (96% w/w) and cooling, the absorbance was measured at 490 nm in a UV 1601 spectrophotometer (Shimadzu Benelux B.V., 's Hertogenbosch, The Netherlands).

**Determination of Proanthocyanidin Oligomer Composition by NP-HPLC-UV-MS.** The presence of proanthocyanidin oligomers in the 20MeOH-EA, 70MeOH-EA, and 70Acetone-EA extracts was determined by NP-HPLC-UV-MS as described elsewhere (14). In brief, proanthocyanidins were separated on a 250 mm  $\times$  4.6 mm i.d., 5  $\mu\text{m}$ , Luna Silica column with a mobile phase consisting of eluent A (dichloromethane), eluent B (methanol), and eluent C [50% (v/v) aqueous acetic acid], which were mixed into a multistep gradient. The flow rate was 1 mL/min, and UV detection was performed at 280 nm.

An LCQ Classic equipped with an electrospray ionization source (ESI) was coupled to the HPLC system by means of a splitter (Accurate, LC Packings, Amsterdam, The Netherlands), which reduced the flow rate 20 times. The MS detector was controlled by Xcalibur software (Thermo Finnigan, San Jose, CA). Measurements were performed in the negative mode with an ion spray voltage of 4.5 kV, a capillary voltage of  $-5.0$  V, and a capillary temperature of 270  $^{\circ}\text{C}$ . The scan range was set from  $m/z$  100 to 2000. The MS<sup>2</sup> function was performed in the data-dependent mode. The collision energy value was 27%.

**Preparative Isolation of Monomers to Pentamers by NP-HPLC.** Fractionation by degree of polymerization was performed on a 250 mm  $\times$  30 mm i.d., 10  $\mu\text{m}$ , Inertsil PREP-SIL normal-phase column (GL Sciences Inc., Tokyo, Japan). A Waters system equipped with a 2767 sample manager, a 2525 binary gradient module, a 2996 photodiode array detector (PDA), and a UV fraction manager was used (Waters Inc., Etten-Leur, The Netherlands). The binary mobile phase consisted of

eluent A (hexane and eluent B (acetone). The elution gradient was as follows: starting condition, 40% B; 0–30 min, 40–60% B; 30–50 min, isocratic at 60% B; 50–70 min, 60–75% B; 70–80 min, 75–98% B; 80–95 min, isocratic at 98% B; 95–98 min, 98–40% B; and 98–105 min, isocratic at 40% B. The flow rate was 27.2 mL/min, and PDA spectra from 210 to 300 nm were recorded. In each run, 10 mL [ $\sim$ 50 mg/mL acetone/hexane/ethanol (7:3:2) 20MeOH-EA extract, in total five runs, 2654 mg] was applied. Fractions (18 mL) were collected during 90 min and pooled into nine fractions: F1 (13.6–22.3 min, 55 mg), F2 (22.3–28.2 min, 16 mg), F3 (28.2–38.1 min, 447 mg), F4 (38.1–46.8 min, 82 mg), F5 (46.8–62.0 min, 346 mg), F6 (62.0–64.0 min, 59 mg), F7 (64.0–71.2 min, 304 mg), F8 (71.2–77.2, 257 mg), and F9 (77.2–90.0, 651 mg). These fractions [total of 2217 mg = 84% (w/w) recovery] were rotary evaporated, lyophilized, and weighed prior to further analysis.

**Characterization of Proanthocyanidin Oligomers by RP-U/HPLC-UV-MS.** Fractions F1–F4 were analyzed by RP-HPLC-UV-MS as described elsewhere (14). In brief, procyanidins were separated on a 150 mm  $\times$  4.6 mm i.d., 3.5  $\mu$ m, XterraRP dC18 column with a mobile phase consisting of eluent A [water with 0.1% (v/v) acetic acid] and eluent B [acetonitrile with 0.1% (v/v) acetic acid], which were mixed into a multistep gradient. The flow rate was 0.7 mL/min, and UV detection was performed at 280 nm. MS analysis was similar to NP-HPLC-UV-MS.

Fractions F5–F9 were analyzed by RP-UPLC-UV-MS on an Accela system equipped with an Accela pump, column oven (50 °C), autosampler, and PDA detector and controlled by Xcalibur software (Thermo Scientific). Analysis was performed on a 150 mm  $\times$  2.1 mm i.d., 1.7  $\mu$ m, Acquity UPLC-BEHC18 Shield column (Waters). The different oligomeric fractions were dissolved (1 mg/mL) in 0.1% (v/v) aqueous acetic acid, and 5  $\mu$ L was injected. The mobile phase was composed of eluent A [water + 0.1% (v/v) acetic acid] and eluent B [acetonitrile + 0.1% (v/v) acetic acid], which were mixed into a multistep gradient. The flow rate was 0.6 mL/min, and the column oven temperature was set on 50 °C. Detection was performed at 280 nm. The elution gradient was as follows: first 0.25 min, isocratic elution at 9% B; 0.25–18 min, 9–32.5% B; 18–19 min, 32.5–90% B; 19–23 min, isocratic at 90% B; 23–24 min, 90–9% B; and 24–27 min, isocratic at 9% B.

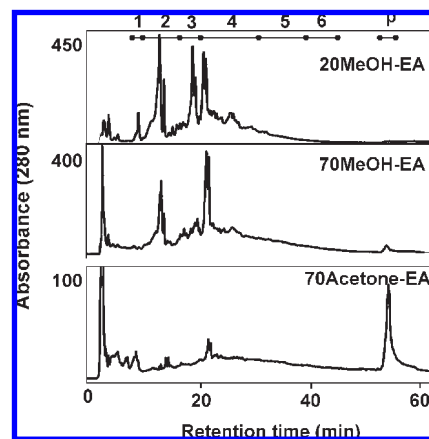
The UPLC was connected to an MS detector by means of a splitter [Analytical Scientific Instruments (ASI), El Sobrante, CA], which reduced the flow rate to 0.3 mL/min. An LTQ XL equipped with an ESI source was used and controlled by Xcalibur software (Thermo Scientific). Measurements were performed in the negative mode with an ion spray voltage of 4.5 kV, a capillary voltage of 5.0 V, and a capillary temperature of 200 °C. Optimal collision energy values (CID) were obtained by infusion of the different oligomeric mixtures and were  $m/z$  575, CID 40%;  $m/z$  863, CID 35%;  $m/z$  1149, 1151, 1439, and 1437, CID 40%; and  $m/z$  1435, CID 45%. For each oligomeric fraction, a full scan was made within the range of  $m/z$  150–2000, and MS<sup>2</sup> scans were recorded for further characterization and to determine the position of the A-linkage.

## RESULTS AND DISCUSSION

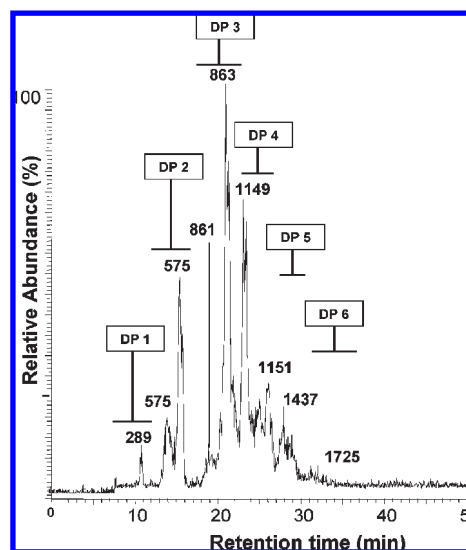
**Oligomeric Composition of the Extracts.** Successive extraction of defatted peanut skins with 20% methanol, 70% methanol, and 70% acetone yielded 15.8, 6.2, and 5.1 g of dry material, respectively. Additionally, ethyl acetate partitioning resulted in a final yield of 2.8 g of 20MeOH-EA and 0.9 g of 70MeOH-EA extract. The ethyl acetate partitioning of these two fractions removed 82–86% (w/w) dry material, including 34–49% (w/w) glucose equivalents. For the 70Acetone-EA extract 27% (w/w) glucose equivalents were removed by ethyl acetate partitioning, and a final yield of <0.1 g was obtained.

The oligomeric composition of each extract was determined by NP-HPLC-UV-MS. NP-HPLC enables separation by degree of polymerization (15) (Figure 2). The degree of polymerization, depicted as numbers in Figure 2, was assigned on the basis of the  $m/z$  values. As an example, Figure 3 shows the precursor ions present in the 20MeOH-EA extract, which contained a range of monomers ( $m/z$  289) to hexamers ( $m/z$  1725).

On the basis of NP-HPLC-MS, only precursor ions of A-linked procyanidins were present ( $m/z$  575, 861, 863, 1151, 1149, 1437,



**Figure 2.** Normal-phase chromatograms of the extracts 20MeOH-EA, 70MeOH-EA, and 70Acetone-EA. The numbers 1–6 represent the degree of polymerization, determined by MS analysis, and p represents polymeric material.



**Figure 3.** Precursor ions that were detected in the 20MeOH-EA extract, analyzed by NP-HPLC-UV-MS, using identical conditions as in Figure 1. DP represents the degree of polymerization.

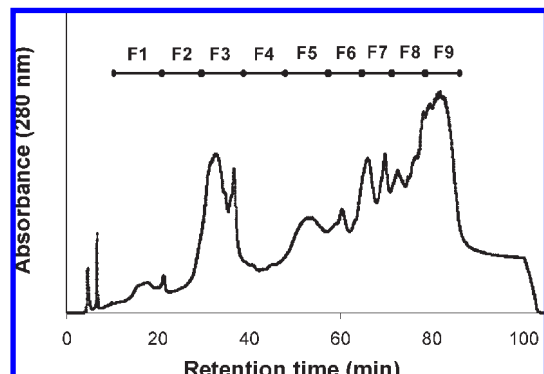
and 1725), which can be distinguished from B-linked oligomers (e.g.,  $m/z$  577, 865, 1153, 1441, and 1729) by the 2 Da difference for each A-linkage present. The 20MeOH-EA extract was richer in small oligomers than the other extracts (Figure 2). The acetone extract contained mainly polymeric material, eluting at the end as one unresolved peak.

The 20MeOH-EA extract was further characterized because of its high yield and its large variety in procyanidins with different degrees of polymerization. Nine fractions, F1–F9 (Figure 4), were obtained by separation, using preparative NP-HPLC.

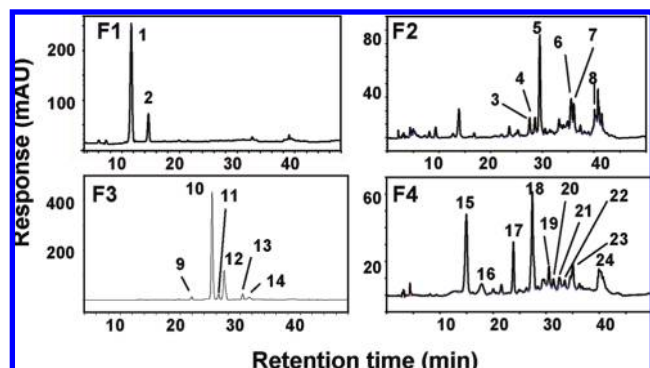
**Molecular Diversity of Procyanidins in the 20% Methanol Extract of Peanut Skins. Monomers and Dimers.** Fractions F1–F4 were characterized with RP-HPLC-UV-MS (Figure 5). By comparison with standards it could be shown that fraction F1 exclusively contained catechin (1) and epicatechin (2) in a molar ratio of 5:1, which is in line with the ratio of 9:1 reported previously (8). Only A-type dimers were detected in fraction F2. The composition of each peak, which was determined by their precursor ions ( $[M - H]^-$ ) and product ions, is given in Table 1. Peaks 3–6 in F2 represented A-type procyanidin dimers

( $m/z$  575). Furthermore, peak 7 ( $m/z$  589) might represent an A-type methylated procyanidin dimer, but no MS<sup>2</sup> data were obtained to confirm this finding. Methylated dimers are not commonly present in food, although methylated A-type proanthocyanidins have been detected in *Cassipourea gummiflua* (16).

Besides procyanidin dimers, fraction F2 also contained an A-type propelargonidin dimer, consisting of one (epi)afzelechin and one (epi)catechin unit (8) ( $m/z$  559). A tentative structure of



**Figure 4.** Separation of the 20MeOH-EA extract with preparative normal-phase HPLC. Nine fractions were collected: F1–F9. Reprinted with permission from ref 14. Copyright 2009 Elsevier.



**Figure 5.** RP-HPLC profiles of fractions F1–F4 (Figure 3). Compositional information on the peaks is given in Tables 1 and 2.

this dimer, with (epi)afzelechin as the extension unit (E) and (epi)catechin as the terminal unit (T), was drawn on the basis of the product ions detected (Figure 6A). The linkage was arbitrarily chosen as the MS data do not give such information.

Three characteristic fragmentation routes have been described for proanthocyanidins: quinone methide fission (QM), retro-Diels–Alder fission (RDA), and heterocyclic ring fission (HRF) (5, 17). The product ions resulting from QM cleavage of the terminal ( $T$ ) and extension ( $E$ ) units,  $[M_T - H]^-$  and  $[M_E - 5H]^-$ , for A-type dimers provided information about the monomeric sequence within propelargonidins and prodelfphinidins (5, 18). The QM product ions of the dimer ( $m/z$  559) were  $m/z$  289  $[M_T - H]^-$  and  $m/z$  269  $[M_E - 5H]^-$  (Figure 6A), representing an (epi)catechin [ $290 \text{ Da} - H = 289$ ]<sup>-</sup> and (epi)afzelechin unit [ $274 \text{ Da} - 5H = 269$ ]<sup>-</sup>, respectively. RDA fragmentation for A-type dimers has been reported to occur mainly at the terminal unit (5). The RDA product ion of  $m/z$  407 indicated a loss of 152 Da, confirming that the terminal unit is an (epi)catechin unit. Also, the RDA  $m/z$  391 ( $407 - 16 \text{ Da}$ ) was present, which has been reported for A-type dimers (19). On the basis of both the QM and RDA product ions, we concluded that (epi)catechin was the terminal unit and (epi)afzelechin the extension unit. Product ions derived from RDA, QM, and HRF fragmentation could be used to determine only the position of (epi)afzelechin and (epi)catechin units, but not to distinguish between the epimers.

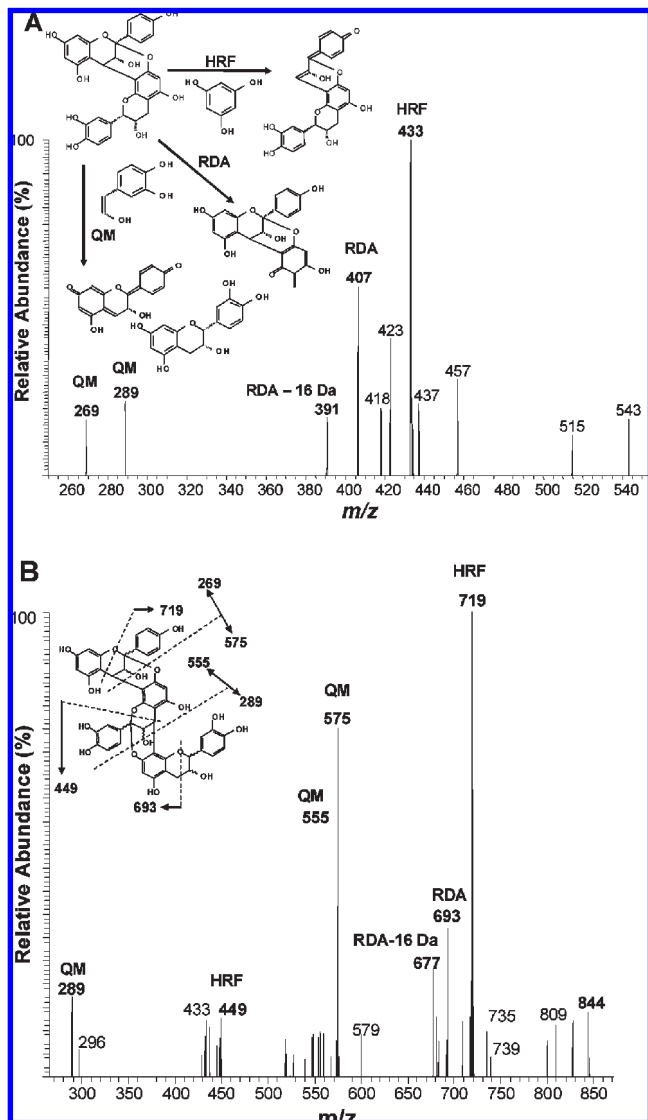
The dimers in fraction F3 have been identified as described in detail elsewhere (14). In brief, F3 contained four A-type procyanidin dimers (peaks 10–13), of which A1 (peak 10) and A2 (peak 12) were most abundant, and two B-type procyanidin dimers (peaks 9 and 14). Fraction F4 contained some B-type procyanidin dimers (peaks 15–17).

**Trimers to Heptamers.** Besides B-type procyanidin dimers, fraction F4 also contained A-type procyanidin trimers with one (peaks 18–20) or two (peaks 21–23) A-type linkages. In addition to the procyanidin trimers present, an A-type propelargonidin trimer was detected (peak 24,  $m/z$  845). In a similar way as described for the propelargonidin dimer (peak 8, Figure 6A), a tentative structure on the positions of the monomeric units is given in Figure 6B on the basis of the product ions detected. The RDA product ion of  $m/z$  693 indicated a loss of 152 Da. Therefore, the terminal unit was annotated (epi)catechin.

**Table 1.** Compositional Information of the Peaks Observed in Figures 4 and 7 Based on MS Analysis

| peaks   | $[M - H]^-$ | product ions                    | no. of A-bonds | DP | composition <sup>a</sup>   | % (w/w) of 20MeOH-EA |
|---|-------------|---------------------------------|----------------|----|----------------------------|----------------------|
| 1   | 289         | 245                             | 0              | 1  | (epi)cat                   | 2.5                  |
| 9, 14–17  | 577         | 451, 425, 407, 289, 287         | 0              | 2  | (epi)cat                   | 2.4                  |
| 8   | 559         | 433, 407, 289, 269              | 1              | 2  | (epi)afz–A–(epi)cat        | <0.1                 |
| 3–6, 10–13                                      | 575         | 449, 423, 407, 289, 285         | 1              | 2  | (epi)cat                   | 19.9                 |
| 7   | 589         |                                 | 1              | 2  | (epi)cat + CH <sub>3</sub> | <0.1                 |
| 57  | 865         | 739, 713, 577, 575              | 0              | 3  | (epi)cat                   | <0.1                 |
| 18–20, 25–37, 39–47, 56, 60                     | 863         | 737, 711, 693, 575, 451, 411    | 1              | 3  | (epi)cat                   | 17.0                 |
| 21–23, 38, 55                                   | 861         | 735, 709, 575, 571              | 2              | 3  | (epi)cat                   | 1.6                  |
| 24  | 845         | 719, 693, 677, 575, 555, 289    | 2              | 3  | (epi)cat + (epi)afz        | 0.5                  |
| 59, 65, 71–75, 77, 79, 80, 82, 89               | 1151        | 1025, 999, 863, 575             | 1              | 4  | (epi)cat                   | 4.3                  |
| 48–51, 53, 58, 61–64, 66–69, 76, 78, 83, 85, 90 | 1149        | 863, 861, 859, 575, 573         | 2              | 4  | (epi)cat                   | 18.8                 |
| 54, 70  | 1147        |                                 | 3              | 4  | (epi)cat                   | 0.1                  |
| 52  | 1133        |                                 | 2              | 4  | (epi)cat + (epi)afz        | <0.1                 |
| 81, 84, 86–94, 97, 99–103, 105–108, 110         | 1437        | 1311, 1285, 1149, 863, 861, 573 | 2              | 5  | (epi)cat                   | 26                   |
| 95, 96  | 1435        |                                 | 2              | 5  | (epi)cat                   | 0.4                  |
| 98, 104   | 1727        |                                 | 1              | 6  | (epi)cat                   | 2.2                  |
| 110–114   | 1723        |                                 | 3              | 6  | (epi)cat                   | 3.5                  |
| 109   | 2011        |                                 | 3              | 7  | (epi)cat                   | 0.8                  |

<sup>a</sup> The A represents a double (A-type) bond, (epi)cat = (epi)catechin, (epi)afz = (epi)afzelechin, CH<sub>3</sub> = an additional methyl group.

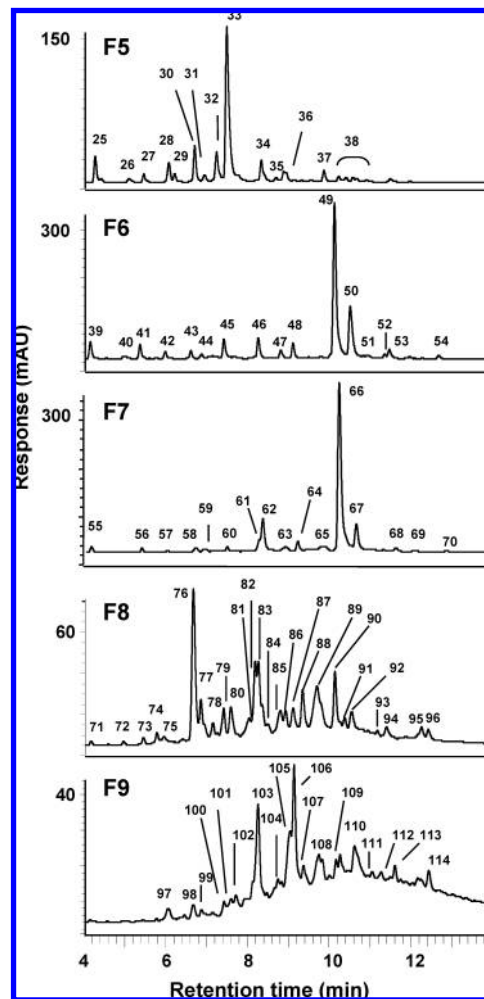


**Figure 6.** Fragmentation pattern of (A) peak 8 ( $m/z$  559) (Figure 4), consistent with a structure composed of (epi)afzelechin (extension unit) and (epi)catechin (terminal unit) and (B) peak 24 ( $m/z$  845) (Figure 4), consistent with a structure composed of one (epi)afzelechin and two (epi)catechin units. Tentative structures are given.

Also, the RDA product ion  $m/z$  677 ( $693 - 16$  Da) was present. The identified QM product ions had  $m/z$  values of 575, 289 [ $M_T - H$ ]<sup>-</sup>, and  $m/z$  555 [ $M_E - 5H$ ]<sup>-</sup> (Figure 6B). On the basis of these QM and RDA product ions, the trimer was characterized as (epi)afzelechin-A-(epi)catechin-A-(epi)catechin, in which A represents an A-linkage.

To improve resolution of the complex, multicomponent chromatograms, fractions F5–F9 were analyzed with RP-UPLC-UV-MS (Figure 7). Fraction F5 exclusively contained A-type procyanidin trimers. Fractions F6 and F7 also contained A-type procyanidin trimers, and one B-type procyanidin trimer ( $m/z$  865) (peak 57) was detected.

A-type procyanidin tetramers with one ( $m/z$  1151), two ( $m/z$  1149), or three ( $m/z$  1147) A-linkages were detected in fractions F6–F8. B-type procyanidin tetramers ( $m/z$  1153) did not occur in any fraction. One peak ( $m/z$  1133) (52) was tentatively identified as a propelargonidin tetramer, consisting of one (epi)afzelechin unit, three (epi)catechin units, and two A-linkages. No MS<sup>2</sup> data could be obtained to confirm this finding.



**Figure 7.** RP-UPLC profiles of fractions F5–F9 (Figure 3). Compositional information on the peaks is given in Tables 1 and 2.

Procyanidin pentamers, hexamers, and one heptamer (peak 109), containing up to three A-linkages, were detected in fractions F8 and F9.

In total, 114 peaks were detected. However, peaks with the same  $m/z$  values and retention times that were detected in successive fractions might represent the same proanthocyanidin molecule. For example, peaks 9 (fraction F3) and 17 (fraction F4) both had an  $m/z$  value of 577 and similar retention times. Therefore, these two peaks were considered to represent the same B-type procyanidin dimer. In the same way, all peaks with similar  $m/z$  values and retention times in successive fractions have been compared. In addition, the positions of the A-linkages were accounted for. For example, the A-linkage of the procyanidin trimers in peak 36 (fraction F5) and peak 47 (fraction F6), having the same  $m/z$  value (863) and retention time, occurred at different positions. Thus, peaks 36 and 47 represent different molecules. On the basis of these considerations, the 114 peaks appeared to account for 83 different proanthocyanidin molecular species, which were characterized with respect to the number and composition of subunits, as well as to the number and position of A-linkages.

**Quantification of Procyanidin Oligomers.** Similar molar extinction coefficients (at 280 nm) have been reported for epicatechin ( $\epsilon = 3750$  M<sup>-1</sup> cm<sup>-1</sup>) and catechin ( $\epsilon = 4050$  M<sup>-1</sup> cm<sup>-1</sup>) (20). Because mainly procyanidins were present in the 20% MeOH-EA extract, it was assumed that the oligomers present within one fraction, which are of similar degrees of polymerization, also had

**Table 2.** Position of A-Linkages in Trimers and Tetramers Separated by RP-UPLC (Figure 7) Based on MS Analysis

| peaks                                    | [M - H] <sup>-</sup> | product ions <sup>a</sup> | connection sequence <sup>b</sup>          | % (w/w) of 20MeOH-EA |
|--|----------------------|---------------------------|---|----------------------|
| 26, 27, 34, 35, 40, 41, 46, 47           | 863                  | 573, 289                  | (epi)cat-A-(epi)cat-(epi)cat              | 1.9                  |
| 25, 28-33, 36, 37, 39, 42-45             | 863                  | 573, 287                  | (epi)cat-(epi)cat-A-(epi)cat              | 13.6                 |
| 38, 55                                   | 861                  | 575, 571, 289, 285        | (epi)cat-A-(epi)cat-A-(epi)cat            | 1.1                  |
| 73, 75                                   | 1151                 | 861, 577, 573             | (epi)cat-A-(epi)cat-(epi)cat-(epi)cat     | 0.3                  |
|  | 1151                 | 863, 861                  | (epi)cat-(epi)cat-A-(epi)cat-(epi)cat     |                      |
| 59, 65, 77, 80, 82, 89                   | 1151                 | 863, 575                  | (epi)cat-(epi)cat-(epi)cat-A-(epi)cat     | 3.4                  |
| 78                                       | 1149                 | 859                       | (epi)cat-A-(epi)cat-A-(epi)cat-(epi)cat   | 0.2                  |
| 58, 61, 63, 69, 83                       | 1149                 | 861                       | (epi)cat-(epi)cat-A-(epi)cat-A-(epi)cat   | 1.7                  |
| 48-50, 53, 62, 64, 66-69, 76, 83, 85, 90 | 1149                 | 575, 573                  | (epi)cat-A-(epi)cat-(epi)cat-A-(epi)cat   | 17.3                 |
| 54, 70                                   | 1147                 |                           | (epi)cat-A-(epi)cat-A-(epi)cat-A-(epi)cat | 0.1                  |

<sup>a</sup> Product ions derived from quinine methide fission. <sup>b</sup> The monomeric unit on the right side represents the terminal unit. **A** denotes the position of the A-linkage.

a similar molar extinction coefficients. For each peak, its relative contribution to the total peak area of that fraction was determined. For example, F1 (Figure 5) contained peaks 1 and 2, representing 83.1 and 16.9% of the total area, respectively. Subsequently, the relative mass contribution of each peak to the 20MeOH-EA extract was determined. For example, 55 mg of F1 was obtained after preparative NP-HPLC, which represents 2.5% (w/w) of the total amount of 20MeOH-EA extract (2217 mg). Therefore, peak 1 was calculated to represent (2.5 × 0.831) 2.1% (w/w) of the 20MeOH-EA extract. Similar calculations were performed for each peak in fractions F2-F9 (Tables 1 and 2). If a peak represented, for example, two different structures, on the basis of MS data, the area was divided by 2.

On the basis of these calculations the A-type oligomers represented 95.0% (w/w) of the 20MeOH-EA extract (Table 1), which supports the data of Lazarus and co-workers (10). Only 2.4% (w/w) B-type procyanidin dimers and < 0.1% (w/w) B-type procyanidin trimers were present. No B-type tetramers to heptamers were detected. The tentatively identified A-type propeprlargonidins (peaks 8, 24, and 52) represented < 0.7% (w/w) of the total amount of A-type oligomers [95.0% (w/w)].

Procyanidin trimers with one A-linkage (*m/z* 863) represented 17.0% (w/w), whereas procyanidin trimers with two A-linkages (*m/z* 861) represented only 1.6% (w/w) (Table 1). Of all the tetramers [23.3% (w/w) of the 20MeOH-EA extract], those with two A-linkages (*m/z* 1149) were the most abundant and accounted for 18.8% (w/w) of the 20MeOH-EA extract (Table 1). The main peaks were detected in fractions F6 (peaks 49 and 50), F7 (peaks 61, 62, 66, and 67), and F8 (peak 76). Most of the pentamers, hexamers, and heptamers contained two or more A-linkages (Table 1).

**Position of the A-Linkages in Trimers and Tetramers from Peanut Skins.** The position of the A-linkages of several trimers and tetramers (Table 2) could be determined via the product ions derived from QM fission (Supporting Information Figure 1). In a similar way different positions of the A-linkages for several procyanidin trimers to pentamers have been determined in plum and cinnamon (5). However, the molecular diversity was not addressed in that study and a precursor ion corresponding to a procyanidin trimer with one A-linkage (*m/z* 863) could represent many different structures. For peanut procyanidins only the positions of the A-linkages for a purified trimer and tetramer have been reported (12).

Our results show that the position of the A-linkage within trimers was present at both possible positions: between the internal and terminal units and between the two extension units. When tetramers contained one A-linkage (*m/z* 1151), this linkage was not located between the two internal units. When tetramers contained two A-linkages (*m/z* 1149), these linkages were present at all possible positions. Using the same considerations for

preventing overestimation of the number of compounds as described for the determination of molecular diversity, we were able to determine the positions of A-linkages in 16 trimers and 27 tetramers. The positions of the A-linkages within larger oligomers could not be determined, because insufficient amounts of product ions were obtained to draw conclusions.

Separation by RP-HPLC prior to MS analysis enabled quantification of the main peaks. These results showed that the position of the A-linkage in the majority of the procyanidin trimers with one A-linkage [13.6% (w/w) with 30, 32, and 33 as main peaks of a total of 17.0% (w/w)] was between the internal and terminal units (Table 2). Similarly as with the trimers, tetramers that contained at least one A-linkage between the internal and terminal units predominated and represented 22.5% (w/w) of the 20MeOH-EA extract from a total of 23.0% (w/w) of A-type tetramers in which the A-linkage position was determined (Table 2).

In plums, the A-linkage within tetramers is also located at the terminal unit (5, 21). On the other hand, in cinnamon the A-linkage was detected between the extension units, but not at the terminal unit (5, 22). Therefore, our data provide further support for the concept that the location of the A-linkages seems to be source-dependent.

In conclusion, by combining NP- and RP-HPLC, we were able to demonstrate the presence of a large molecular diversity of proanthocyanidins in a 20% MeOH extract of peanut skins. Furthermore, this method enables estimation of the most abundant molecular proanthocyanidin species that were present. These data may contribute to the evaluation of the health potential of peanut skin based supplements.

**Supporting Information Available:** Supplemental Figure 1, schematic representation of QM fragmentation (*m/z* values) of trimers and tetramers. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## LITERATURE CITED

- Gu, L. W.; Kelm, M. A.; Hammerstone, J. F.; Beecher, G.; Holden, J.; Haytowitz, D.; Gebhardt, S.; Prior, R. L. Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *J. Nutr.* **2004**, *134*, 613-617.
- Hooper, L.; Kroon, P. A.; Rimm, E. B.; Cohn, J. S.; Harvey, I.; Le Cornu, K. A.; Ryder, J. J.; Hall, W. L.; Cassidy, A. Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials. *Am. J. Clin. Nutr.* **2008**, *88*, 38-50.
- Williamson, G.; Manach, C. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am. J. Clin. Nutr.* **2005**, *81*, 243S-255S.
- Gu, L. W.; Kelm, M. A.; Hammerstone, J. F.; Beecher, G.; Holden, J.; Haytowitz, D.; Prior, R. L. Screening of foods containing proanthocyanidins and their structural characterization using

- LC-MS/MS and thiolytic degradation. *J. Agric. Food Chem.* **2003**, *51*, 7513–7521.
- (5) 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.
- (6) Li, H. J.; Deinzer, M. L. Tandem mass spectrometry for sequencing proanthocyanidins. *Anal. Chem.* **2007**, *79*, 1739–1748.
- (7) Karchesy, J. J.; Hemingway, R. W.; Foo, Y. L.; Barofsky, E.; Barofsky, D. F. Sequencing procyanidin oligomers by fast-atom-bombardment mass-spectrometry. *Anal. Chem.* **1986**, *58*, 2563–2567.
- (8) Karchesy, J. J.; Hemingway, R. W. Condensed tannins—(4 $\beta$ -8, 2 $\beta$ -O-7)-linked procyanidins in *Arachis hypogaea* L. *J. Agric. Food Chem.* **1986**, *34*, 966–970.
- (9) Yu, J. M.; Ahmedna, M.; Goktepe, I.; Dai, J. A. Peanut skin procyanidins: Composition and antioxidant activities as affected by processing. *J. Food Compos. Anal.* **2006**, *19*, 364–371.
- (10) Lazarus, S. A.; Adamson, G. E.; Hammerstone, J. F.; Schmitz, H. H. High-performance liquid chromatography/mass spectrometry analysis of proanthocyanidins in foods and beverages. *J. Agric. Food Chem.* **1999**, *47*, 3693–3701.
- (11) Lou, H. X.; Yamazaki, Y.; Sasaki, T.; Uchida, M.; Tanaka, H.; Oka, S. A-type proanthocyanidins from peanut skins. *Phytochemistry* **1999**, *51*, 297–308.
- (12) Lou, H. X.; Yuan, H. Q.; Ma, B.; Ren, D. M.; Ji, M.; Oka, S. Polyphenols from peanut skins and their free radical-scavenging effects. *Phytochemistry* **2004**, *65*, 2391–2399.
- (13) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356.
- (14) Appeldoorn, M. M.; Sanders, M.; Vincken, J.-P.; Cheynier, V.; Le Guernevé, C.; Hollman, P. C. H.; Gruppen, H. Efficient isolation of major procyanidin A-type dimers from peanut skin and B-type dimers from grape seeds. *Food Chem.* **2009**, doi: 10.1016/j.foodchem.2009.04.047.
- (15) Rigaud, J.; Escribanobailon, M. T.; Prieur, C.; Souquet, J. M.; Cheynier, V. Normal-phase high-performance liquid-chromatographic separation of procyanidins from cacao beans and grape seeds. *J. Chromatogr., A* **1993**, *654*, 255–260.
- (16) Drewes, S. E.; Taylor, C. W. Methylated A-type proanthocyanidins and related metabolites from *Cassipourea gummiflua*. *Phytochemistry* **1994**, *37*, 551–555.
- (17) Hellstrom, J.; Sinkkonen, J.; Karonen, M.; Mattila, P. Isolation and structure elucidation of procyanidin oligomers from saskatoon berries (*Amelanchier alnifolia*). *J. Agric. Food Chem.* **2007**, *55*, 157–164.
- (18) Friedrich, W.; Eberhardt, A.; Galensa, R. Investigation of proanthocyanidins by HPLC with electrospray ionization mass spectrometry. *Eur. Food Res. Technol.* **2000**, *211*, 56–64.
- (19) Zhu, Q. Y.; Hammerstone, J. F.; Lazarus, S. A.; Schmitz, H. H.; Keen, C. L. Stabilizing effect of ascorbic acid on flavan-3-ols and dimeric procyanidins from cocoa. *J. Agric. Food Chem.* **2003**, *51*, 828–833.
- (20) Whiting, G. C.; Coggins, R. A. Estimation of monomeric phenolics of ciders. *J. Sci. Food Agric.* **1975**, *26*, 1833–1838.
- (21) Nunes, C.; Guyot, S.; Marnet, N.; Barros, A. S.; Saraiva, J. A.; Renard, C.; Coimbra, M. A. Characterization of plum procyanidins by thiolytic depolymerization. *J. Agric. Food Chem.* **2008**, *56*, 5188–5196.
- (22) Nonaka, G.; Morimoto, S.; Nishioka, I. Tannins and related-compounds 13. Isolation and structures of trimeric, tetrameric, and pentameric proanthocyanidins from cinnamon. *J. Chem. Soc., Perkin Trans. 1* **1983**, 2139–2145.

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