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Antioxidant Oligomeric Proanthocyanidins from Sea Buckthorn (*Hippophaë rhamnoides*) Pomace

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After flavonol glycosides, monomeric flavan-3-ols, and dimeric and trimeric proanthocyanidins were fractionated from an extract of sea buckthorn (*Hippophaë rhamnoides*) pomace by Sephadex LH-20 gel chromatography, oligomeric proanthocyanidins were eluted. The oligomeric fraction accounted for 84% of the total proanthocyanidins and 75% of the total antioxidant activity of the sea buckthorn pomace extract. To elucidate the structure of the oligomeric fraction, it was depolymerized by acid catalysis in the presence of phloroglucinol. The structure of the resulting flavan-3-ol monomers and flavan-3-ol-phloroglucinol adducts was determined by electrospray ionization mass spectrometry (ESI-MS) and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Quantitative high-performance liquid chromatography investigations demonstrated (+)-gallocatechin as the predominating subunit in the oligomeric fraction. This observation was confirmed by ESI-MS, matrix-assisted laser desorption/ionization mass spectrometry, and ^{13}C NMR spectroscopy. The results showed that the majority of the flavan-3-ol subunits possessed a 2,3-trans configuration. The oligomers consisted mainly of prodelphinidin subunits whereas procyanidins were present in smaller amounts, indicating a very uncommon composition of the sea buckthorn proanthocyanidins. The mean degree of polymerization of the oligomeric proanthocyanidins was between 6 and 9.

KEYWORDS: Sea buckthorn; *Hippophaë rhamnoides*; pomace; polyphenols; proanthocyanidins; ESI-MS; MALDI-TOF-MS; ^{13}C NMR; acid catalysis; ESR; Fremy's salt

INTRODUCTION

During recent years, a considerable number of investigations have dealt with qualitative and quantitative analyses of byproducts from plant processing (*1*, *2*). The production of sea buckthorn (*Hippophaë rhamnoides*) juice results in a large amount of pomace, which is suggested to contain substantial amounts of valuable natural antioxidants. The pomace can be used for the separation of the seeds and subsequent production of seed oil (*3*). Regarding the polyphenolic constituents, it has been demonstrated that flavonols are extracted to a larger amount into the juice than proanthocyanidins (*4*). It is suggested in particular that proanthocyanidins of high molecular mass remain in the pomace due to their ability to form strong hydrogen bonds with other cellular components (*5*).

Proanthocyanidins, also known as condensed tannins, are oligomeric or polymeric compounds composed of flavan-3-ol subunits (**Figure 1**). Procyanidins consist of (epi)catechin subunits (3′,4′-dihydroxy substitution on the B ring) whereas prodelphindins are composed of (epi)gallocatechin subunits (3′,4′,5-trhydroxy substitution on the B ring). Proanthocyanidins are mainly linked through C4→C8 or C4→C6 bonds (B type). An additional ether bond can be formed between C2→O7 (A type). The most widely distributed proanthocyanidins in foods

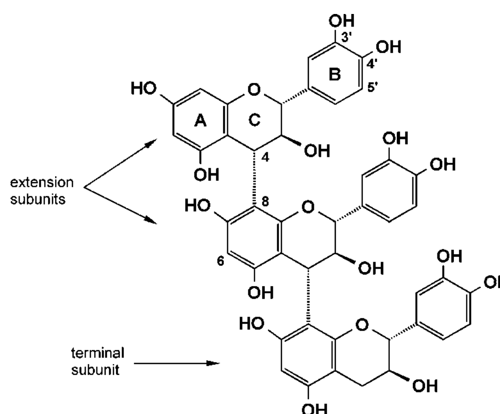

Figure 1. Structure of proanthocyanidins. Representative structure of a (+)-catechin-based trimeric procyanidin.

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contain either exclusively procyanidins or occur as a mixture of procyanidins and prodelphinidins (heterogeneous proanthocyanidins) (*6*, *7*). Their physicochemical properties have been reviewed by Santos-Buelga et al. (*8*). They are reported to possess antioxidant, antimicrobial, antiallergy, and antihypertensive activities.

The complexity of oligomeric and polymeric proanthocyanidins makes their analysis and estimation in food difficult. While normal phase high-performance liquid chromatography (HPLC) methods can separate procyanidins according to their molecular mass up to decamers (*9*), reversed phase HPLC is able to separate oligomeric proanthocyanidins up to a degree of polymerization (DP) of 4 (*10*). Furthermore, reversed phase HPLC can be used for investigations of acid-catalyzed cleavage products of oligomeric or polymeric proanthocyanidins. Under acidic conditions, proanthocyanidins become depolymerized releasing terminal subunits (**Figure 1**) such as flavan-3-ol monomers. Extension subunits can be trapped by nucleophilic reagents such as phloroglucinol (*11*) or benzyl mercaptan (*6*) to generate analyzable adducts. From these results, structural features of proanthocyanidins such as mDP and the concentration of the monomeric subunits within the oligomer or polymer can be determined. Also, other methods including electrospray ionization mass spectrometry (ESI-MS) (*12*), matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF-MS) (*13*), and ^{13}C nuclear magnetic resonance (NMR) spectroscopy (*14*) can be applied for the structural elucidation of proanthocyanidins.

In our previous work (*15*), we identified monomeric gallocatechins and dimeric and trimeric proanthocyanidins from Sephadex LH-20 fractionation of sea buckthorn pomace. The aim of this work was the isolation and structural elucidation of higher molecular mass oligomeric proanthocyanidins from sea buckthorn pomace extract. Our investigations included the determination of the contribution of the oligomeric proanthocyanidin fraction to the total antioxidant activity of the extract.

MATERIALS AND METHODS

Materials. (+)-Catechin, (−)-epicatechin, 2,5-dihydroxybenzoic acid, and phloroglucinol were obtained from Fluka (Taufkirchen, Germany). Potassium nitrosodisulfonate (Fremy's salt) was purchased from Sigma-Aldrich (Steinheim, Germany). Reagents and solvents were purchased from Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany) and were of HPLC or analytical grade quality. HPLC grade water was purified with a deionized water treatment system.

Extraction and Fractionation of Plant Material. Sea buckthorn pomace (*H. rhamnoides* subsp. *rhamnoides* cv. Hergo) was extracted and fractionated on a 60 cm × 4 cm i.d. Sephadex LH-20 column (Pharmacia, Uppsala, Sweden) (*16*). After the column was rinsed with 400 mL of water (discarded), fractions named A−M were collected by increasing the methanol content of the eluent from 0 to 100% (v/v) in increments of 10% (400 mL each) followed by 2 × 400 mL fractions of acetone−water (70:30, v/v). The proanthocyanidin composition of the fractions was monitored using thin-layer chromatography (TLC) system A.

Quantitative Analysis of Proanthocyanidins. The content of proanthocyanidins of Sephadex fractions H−M was determined photometrically after acid depolymerization to the corresponding anthocyanidins as previously described (*17*). In the cases of fractions L and M, acetone was removed by a stream of nitrogen and the sample was redissolved in water before starting the reaction.

Electron Spin Resonance (ESR) Analysis. For measuring the antioxidant activity of Sephadex fractions A−M, adequate dilutions with water were prepared and aliquots (500 μL) were allowed to react with an equal volume of a solution of Fremy's salt (1 mM in phosphate buffer pH 7.4) under the conditions previously described (*17*). In the cases of fractions L and M, acetone was removed by a stream of nitrogen and the sample was redissolved in water before starting the reaction.

Table 1. ^{13}C NMR Spectroscopic Data of **1**, **2**, and **3** in CD_3OD (300 MHz)[a]

carbon	1	2	3	carbon	1	2	3
2	84.6	84.3	77.2	3′	146.7	146.1	145.6
3	73.4	73.4	73.1	4′	134.2	145.4	145.9
4	38.5	38.6	37.0	5′	146.7	116.0	115.9
4a	106.7	106.8	101.5	6′	108.2	121.0	119.2
5	157.4	157–159		1″	107.7	107.7	107.5
6	96.2	94–97		2″	157.4	157–159	
7	158.1	157–159		3″	97.4	94–97	
8	95.4	94–97		4″	157.4	157–159	
8a	158.4	157–159		5″	97.4	94–97	
1′	131.6	132.4	132.7	6″	157.4	157–159	
2′	108.2	115.9	115.2				

[a] Chemical shifts (δ) are in ppm. Assignments were confirmed by HMQC and HMBC correlations, but carbons having almost the same chemical shifts may be reversed. For structures, see **Figure 3**.

Isolation and Purification of Flavan-3-ols and Phloroglucinol-Flavan-3-ol Adducts. Proanthocyanidin oligomers (500 mg) from lyophilized Sephadex LH-20 fraction M were reacted with phloroglucinol (350 mg) in 1% HCl in ethanol (4 mL) for 30 min. After the solution was concentrated to 2 mL under a stream of nitrogen, the solution was fractionated on a 45 cm × 1.5 cm i.d. Sephadex LH-20 column using ethanol as the eluent. The elution profile was monitored by HPLC-diode array detection (DAD) (*17*) and TLC (TLC system A). Fractions containing similar compositions were evaporated and cleaned up by semipreparative HPLC on a 250 mm × 10 mm i.d., 5 μm Fluofix 120E column (NEOS Company Ltd., Kobe, Japan) connected to a 10 mm × 10 mm i.d., 5 μm Hypersil BDS C8 guard column. The samples were fractionated using isocratic elution with water−acetic acid (99.5:0.5, v/v). After chromatographic separation was performed at a flow rate of 5 mL/min using a Gynkothek model 480 HPLC pump (Dionex, Germering, Germany), the flow was split into 0.1 mL/min for detection at 280 nm using a variable wavelength UV/vis detector (Linear Instruments, Reno, Nevada) and 4.9 mL/min for fraction collection. The fraction composition was monitored by HPLC-DAD (*17*), and fractions with a similar composition were combined and lyophilized to yield the following compounds.

*(+)-Gallocatechin-(4α→2)-phloroglucinol (**1**).* White amorphous solid (49.4 mg). R_f 0.43 (A), 0.33 (B), 0.49 (C). ESI-MS, *m/z* (relative intensity): 859 [M + M − H]$^-$ (41), 465 [M + Cl]$^-$ (7), 429 [M − H]$^-$ (100). MS-MS of 429: 303 [M − H − $C_6H_6O_3$]$^-$ (100), 261 [M − H − $C_8H_8O_4$]$^-$ (90). ^{1}H NMR (CD_3OD, 300 MHz) δ (ppm): 4.28 (H-2, d, J = 9.3 Hz), 4.40 (H-4, d, J = 8.0 Hz), 4.46 (H-3, dd, J = 9.3, 8.1 Hz), 5.82 (H-3″, H-5″, d, J = 2.4 Hz), 5.85 (H-6, H-8, d, J = 2.4 Hz), 6.52 (H-2′, H-6′, s). ^{13}C NMR data are shown in **Table 1**.

*(+)-Catechin-(4α→2)-phloroglucinol (**2**)* and *(−)-Epicatechin-(4β→2)-phloroglucinol (**3**).* White amorphous solid (15.4 mg). R_f 0.55 (A), 0.49 (B), 0.52, 0.49 (C). ESI-MS, *m/z* (relative intensity): 827 [M + M − H]$^-$ (18), 449 [M + Cl]$^-$ (7), 413 [M − H]$^-$ (100). MS-MS of 413: 287 [M − H − $C_6H_6O_3$]$^-$ (100), 261 [M − H − $C_8H_8O_3$]$^-$ (90). ^{1}H NMR (CD_3OD, 300 MHz) δ (ppm): major signals assigned to **2** at 4.36 (H-2, d, J = 9.3 Hz), 4.41 (H-4, d, J = 7.9 Hz), 4.47[a] (H-3), 6.75 (H-5′, d, J = 8.1 Hz), 6.81 (H-6′ dd, J = 8.1, 1.9 Hz), 6.94 (H-2′, d, J = 1.9 Hz); minor signals assigned to **3** at 3.93 (H-3, dd, J = 2.0, 0.9 Hz), 4.50[a] (H-4), 5.02 (H-2, s), 6.72 (H-5′, d, J = 8.1 Hz), 6. (H-6′ dd, J = 8.1, 1.9 Hz), 6.88 (H-2′, d, J = 1.8 Hz). [a]Signals overlapped with each other. ^{13}C NMR data are shown in **Table 1**.

Furthermore, monomeric flavan-3-ols (+)-gallocatechin (5.9 mg) and (−)-epigallocatechin (2.9 mg) were isolated after reaction of Sephadex fraction M with phloroglucinol. Spectroscopic data are given elsewhere (*15*).

TLC. Analytical TLC was performed on 20 cm × 20 cm silica gel 60 plates (Merck) using acetone−toluene−formic acid (30:30:10, v/v/v, TLC system A) (*18*), 20 cm × 20 cm cellulose plates (Merck) using

tert-butyl alcohol−acetic acid−water (30:10:10, v/v/v, TLC system B), or acetic acid−water (3:47, v/v/v, TLC system C) (*19*). The spots were visualized with vanillin reagent: 1 g of vanillin was dissolved in 25 mL of ethanol, 25 mL of water, and 25 mL of *o*-phosphoric acid (85%).

NMR Spectroscopy. ^{1}H, ^{13}C, $^{1}H-^{1}H$ COSY, HMQC, and HMBC spectra of isolated compound **1** and the mixture of **2** and **3** dissolved in CD_3OD were recorded using a DPX 300 MHz spectrometer (Bruker, Rheinstetten, Germany). Sephadex fraction M was dissolved in $(CD_3)_2CO$ with a small amount of D_2O and characterized by its ^{13}C NMR spectrum recorded on a DPX 300 MHz spectrometer.

ESI-MS. ESI mass spectra (negative ionization) of isolated compound **1**, the mixture of **2** and **3**, and Sephadex fraction M were recorded using an Agilent 1100 Series LC/MSD trap controlled by LCMSD software (version 4.1). Methanolic solutions (10 mg/L) were injected using a syringe pump (5 mL/min). Nitrogen was used as dry gas (5 L/min, 300 °C) and nebulizer gas (15 psi). The capillary, end plate, and capillary exit voltages were set at 3500, −500, and −120 V, respectively. The full scan mass spectra were measured from *m*/*z* 100 up to *m*/*z* 2000. MS-MS spectra were recorded by isolation and fragmentation of the pseudomolecular ions of interest.

MALDI-TOF-MS. The MALDI-TOF mass spectrum of Sephadex fraction M was recorded on a Kompact MALDI instrument (Kratos Analytical, Shimadzu Group Company, Japan). The method described by Borelli et al. (*20*) was applied, but the matrix used in the present work was 2,5-dihydroxybenzoic acid.

Quantitative Analysis of Reaction Products Resulting from Acid-Catalyzed Cleavage of Oligomeric Proanthocyanidins in the Presence of Phloroglucinol. An ethanolic solution (250 μL) of lyophilized Sephadex fraction M (60 g/L) was added to 250 μL of an ethanolic solution of phloroglucinol (60 g/L). The reaction was started by adding 15 μL of concentrated HCl (corresponding to 1% HCl in the reaction mixture). Before the reaction was started and after a reaction time of 15 min under a nitrogen atmosphere, 50 μL of the solution was evaporated to dryness on a nitrogen stream, dissolved in water, and analyzed by HPLC-DAD-ECD as previously described (*17*) with the exception of the applied gradient between eluent A (water−phosphoric acid, 99.5:0.5, v/v) and eluent B (acetonitrile−water−phosphoric acid, 50:45.5:0.5, v/v/v), which was as follows: 0% B (5 min); 0−15% B in 24 min; 15−100% B in 5 min; 100% B (7 min); 100−0% B in 1 min; 0% B (15 min). The concentration of proanthocyanidin cleavage products was estimated from the peak areas at 280 nm and calibration curves of purified isolated compounds or reference compounds. Using these concentrations, the parameters were calculated as follows: (i) conversion yield (%) = 100 × concentration (mM) of flavan-3-ol-phloroglucinol adducts (**1**, **2**, and **3**) and flavan-3-ol monomers (**4**, **5**, **6**, and **7**)/concentration (mM) of the proanthocyanidin reacted [100 mM, calculated as (+)-catechin]; (ii) mean DP = concentration (mM) of flavan-3-ol-phloroglucinol adducts (**1**, **2**, and **3**) and flavan-3-ol monomers (**4**, **5**, **6**, and **7**)/concentration (mM) of flavan-3-ol monomers (**4**, **5**, **6**, and **7**); (iii) prodelphinidin:procyanidin ratio = concentration (mM) of gallocatechin−phloroglucinol adduct (**1**) and (epi)gallocatechin monomers (**4** and **5**)/concentration (mM) of (epi)catechin-flavan-3-ol adducts (**2** and **3**) and (epi)catechin monomers (**6** and **7**); and (iv) 2,3-trans:2,3-cis ratio = concentration (mM) of flavan-3-ol monomers and flavan-3-ol-phloroglucinol adducts with 2,3-trans stereochemistry (**1**, **2**, **4**, and **6**)/concentration (mM) of flavan-3-ol monomers and flavan-3-ol-phloroglucinol adducts with 2,3-cis stereochemistry (**3**, **5**, and **7**).

RESULTS AND DISCUSSION

The defatted 75% aqueous acetone extract of sea buckthorn pomace extract was fractionated on a Sephadex LH-20 column to yield 13 fractions. Flavonol glycosides (*16*), monomeric gallocatechins, and low molecular mass proanthocyanidins (*15*) were isolated from the fractions and identified. To distinguish which compounds are relevant for the antioxidant activity of the pomace, the Sephadex fractions were investigated by ESR spectroscopy (**Figure 2**). The antioxidant activity of the fractions was expressed as its ability to reduce a synthetic free radical species, Fremy's salt. The antioxidant activity of fractions B−G

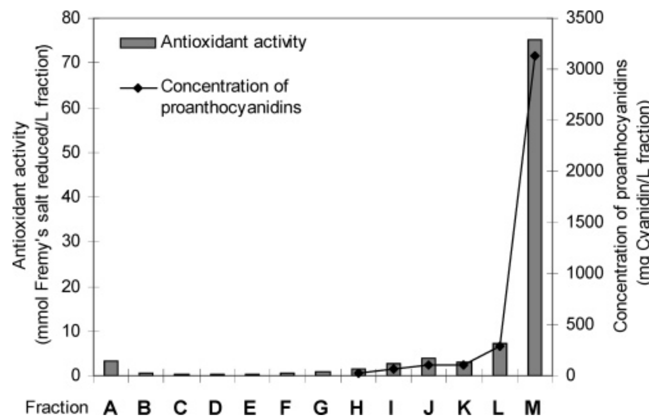

Figure 2. Antioxidant activity of fractions A–M and concentration of proanthocyanidins in fractions H–M resulting from fractionation of sea buckthorn pomace extract by Sephadex LH-20 gel chromatography.

was comparatively small. On one hand, this could possibly be attributed to low concentrations of antioxidants in these fractions. On the other hand, we could demonstrate that these fractions mainly consisted of isorhamnetin 3-glycosides (*16*), which were shown to be poor radical scavengers (*17*). As demonstrated by TLC and HPLC-DAD-ESI-MS investigations, monomeric flavan-3-ols, dimeric, and trimeric proanthocyanidins were eluted in fractions H−L (*15*). Because of the excellent radical scavenging properties of these compounds, the antioxidant activity of fractions H−L was higher than in fractions B−G (**Figure 2**). However, 75% of the total antioxidant activity of the pomace extract was recovered in fraction M. Furthermore, the highest proanthocyanidin concentration was measured in fraction M accounting for 84% of the total proanthocyanidins of the pomace extract. Thus, our objective was to obtain detailed structural information on this oligomeric proanthocyanidin fraction.

Structure Elucidation of Flavan-3-ol-Phloroglucinol Adducts. Sephadex fraction M was reacted on a preparative scale with the nucleophilic reagent phloroglucinol under acid-catalyzed conditions. During this reaction, terminal subunits of proanthocyanidins are released whereas extension subunits are captured as flavan-3-ol-phloroglucinol adducts (*11*, *15*, *19*). As the major product resulting from this reaction, we isolated compound **1**. On ESI-MS analysis (negative mode), **1** showed a pseudomolecular ion peak at *m*/*z* 429 $[M - H]^-$ and further ions at *m*/*z* 465 $[M + Cl]^-$ and *m*/*z* 859 $[M + M - H]^-$, which indicated the presence of an (epi)gallocatechin−phloroglucinol adduct. MS-MS fragmentation of *m*/*z* 429 produced a daughter ion at *m*/*z* 303 $[M - H - C_6H_6O_3]^-$, which was indicative for a loss of phloroglucinol and the retro Diels−Alder (RDA) product at *m*/*z* 261 $[M - H - C_8H_8O_4]^-$. ^{1}H and ^{13}C NMR data (**Table 1**) confirmed the presence of a pyrogallol structure in **1** and allowed us to determine the absolute stereochemistry. As for (+)-gallocatechin (*15*), a large coupling was observed for H-2 (δ 4.28, d, J = 9.3 Hz) in the ^{1}H NMR spectrum of **1**, supporting the presence of a 2,3-trans configuration (*21*). The presence of the phloroglucinol substituent at C-4 should cause a large downfield shift ($\Delta\delta \sim 5$ ppm) to the C-3 resonance and a smaller downfield shift ($\Delta\delta$ 1−2 ppm) to the C-2 resonance in the ^{13}C NMR spectrum (*19*). Signals for C-3 (δ 73.4 ppm) and C-2 (δ 84.6 ppm) of **1** (**Table 1**) appeared downfield ($\Delta\delta$ 4.6 ppm and $\Delta\delta$ 1.7 ppm) to the corresponding signals of (+)-gallocatechin (*15*). By the influence of the phloroglucinol substituent, the signal for H-4 appeared at δ 4.40 ppm as a doublet with a coupling constant of J = 8.0 Hz, which was

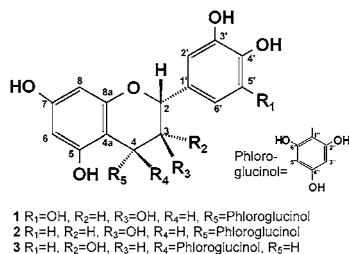

Figure 3. Structures of flavan-3-ol-phloroglucinol adducts identified after acid-catalyzed depolymerization of Sephadex fraction M from sea buckthorn pomace in the presence of phloroglucinol.

consistent with a 3,4-trans orientation (*5*, *22*). In accordance with corresponding ^{13}C NMR data (*23*), the structure (+)-gallocatechin-(4α→2)-phloroglucinol was assigned to **1** (**Figure 3**).

Compounds **2** (major) and **3** (minor) were coeluted from semipreparative HPLC as a mixture. ESI-MS analysis of the mixture showed ions at *m/z* 413 $[M - H]^-$, *m/z* 449 $[M + Cl]^-$, and *m/z* 827 $[M + M - H]^-$, which indicated the presence of (epi)catechin−phloroglucinol adducts. MS-MS fragmentation daughter ions of *m/z* 413 were detected at *m/z* 287 $[M - H - C_6H_6O_3]^-$ (loss of phloroglucinol) and at *m/z* 261 $[M - H - C_8H_8O_3]^-$ (RDA fission). The major compound **2** exhibited a ^{13}C NMR spectrum quite similar to that of **1** but instead of the pyrogallol B ring the chemical shifts suggested the presence of a catechol B ring (**Table 1**). The presence of a 2,3-trans configuration could be deduced from a characteristic shift of H-2 at δ 4.36 (d, $J = 9.3$ Hz) in the ^{1}H NMR spectrum of **2**. Unfortunately, we failed to detect the ^{1}H NMR signals for the A ring H-6 and H-8 as well as the phloroglucinol H-3″ and H-5″, which may be due to a facile H/D exchange (*24*). In accordance with ^{1}H and ^{13}C NMR spectroscopic data and R_f values (*19*, *25*, *26*), we identified **2** as (+)-catechin-(4α→2)-phloroglucinol (**Figure 3**).

Compound **3** exhibited ^{13}C (**Table 1**) and ^{1}H NMR spectra, which also indicated the presence of a catechol B ring. In contrast to **2**, the H-2 of **3** was measured at 5.02 ppm (s) corresponding to a 2,3-cis configuration in **3** (*26*). This was confirmed by the C-2 signal of **3**, which appeared at higher field (δ 77.2 ppm) as compared to the C-2 signal of (−)-epicatechin (δ 79.8 ppm) (data not shown). This upfield shift of $\Delta\delta$ 2.5 ppm in **3** was caused by the normally axial conformation of the C-4 substituent in the 2,3-cis-3,4-trans derivative (*27*), which resulted in 1,3-diaxial interactions ("γ-effect") (*19*). In accordance with the results of Foo et al. (*19*, *25*, *26*), **3** was identified as (−)-epicatechin-(4β→2)-phloroglucinol (**Figure 3**).

Quantitative Analysis of Reaction Products Resulting from Acid-Catalyzed Cleavage of Oligomeric Proanthocyanidins in the Presence of Phloroglucinol. Sephadex fraction M was reacted with phloroglucinol/HCl, and reaction products were analyzed by HPLC-DAD. The chromatogram before starting the depolymerization reaction showed two major signals, which were assigned to phloroglucinol and to the oligomeric proanthocyanidins (**Figure 4A**). After a reaction time of 15 min, the intensity of these two peaks decreased and flavan-3-ol monomers as well as flavan-3-ol-phloroglucinol adducts were formed from the terminal and extension subunits, respectively (**Figure 4B**). The concentration of the cleavage products allowed us to calculate the mDP of Sephadex fraction M, which was 6.3 (**Table 2**). However, it has to be considered that acid-catalyzed cleavage in the presence of phloroglucinol only leads to partial hydrolysis of oligomeric proanthocyanidins (*11*). In the case of

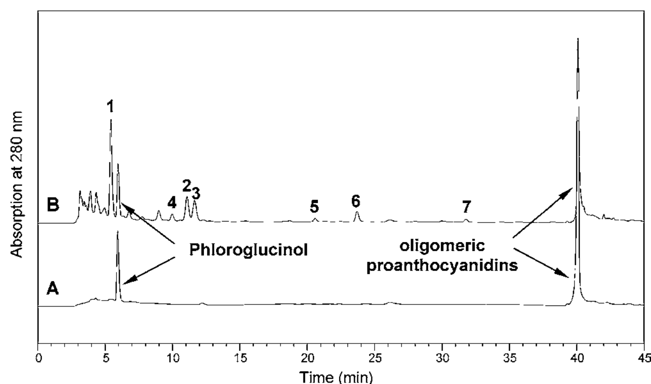

Figure 4. HPLC-DAD plot (absorbance at 280 nm) (**A**) before and (**B**) after acid-catalyzed cleavage of sea buckthorn proanthocyanidins from Sephadex fraction M. See **Table 2** for peak identification.

Table 2. Concentration of Reaction Products after Acid-Catalyzed Cleavage (Determined by HPLC) and Calculated Composition of Sea Buckthorn Proanthocyanidins from Sephadex Fraction M[a]

compound	concentration (mM)[b]
(+)-gallocatechin-(4α→2)-phloroglucinol **1**	18.6 ± 0.50
(+)-catechin-(4α→2)-phloroglucinol **2**	4.63 ± 0.19
(−)-epicatechin-(4β→2)-phloroglucinol **3**	4.46 ± 0.49
(+)-gallocatechin **4**	2.24 ± 0.13
(−)-epigallocatechin **5**	1.31 ± 0.02
(+)-catechin **6**	1.23 ± 0.08
(−)-epicatechin **7**	0.48 ± 0.00
conversion yield (%)	33
mDP[c]	6.3
PD:PC ratio[d]	2.1:1
2,3-trans:2,3-cis ratio	4.3:1

[a] Molar concentration before the reaction was 100 mM [calculated as (+)-catechin]. [b] Mean ± SD of duplicate tests. [c] Mean DP. [d] Prodelphinidin: procyanidin ratio.

the sea buckthorn proanthocyanidins from Sephadex fraction M, the conversion yield was 33% (**Table 2**). The incomplete cleavage might have preferred a higher yield of flavan-3-ol monomers as compared to flavan-3-ol-phloroglucinol adducts. Thus, the real mDP was suggested to be >6.3.

From the molar concentrations of compounds formed from the reaction with phloroglucinol, the ratio between prodelphinidins and procyanidins (PD:PC 2.1:1) and the ratio between subunits with a 2,3-trans and a 2,3-cis configuration (2,3-trans: 2,3-cis 4.3:1) were calculated (**Table 2**). Because of the low conversion yield, these results could not reflect the structure of the whole proanthocyanidin oligomers.

ESI-MS Analysis. The composition of Sephadex fraction M was also investigated by ESI-MS. This soft ionization technique usually gives ion peaks making it useful for molecular mass determination (*12*). Because of the high acidity of the phenolic protons, proanthocyanidins were measured in the negative ion mode. They are more negatively charged as the chain length increases. Consequently, oligomeric proanthocyanidins can be detected as singly charged ions up to DP 8. Beyond this DP, they can only be measured as multiply charged ions (*28*). The ESI-MS data confirmed the occurrence of heterogenic proanthocyanidins in Sephadex fraction M (**Table 3**). For a trimeric prodelphinidin, the expected mass-to-charge value was *m/z* 913.8. An additional (epi)gallocatechin subunit would result in a mass increase of 304.3 Da. We detected trimeric (*m/z* 914.1), tetrameric (*m/z* 1218.1), and pentameric prodelphinidins (*m/z* 1522.0) as singly charged ions $[M - H]^-$ in Sephadex fraction M (**Table 3**). The occurrence of (epi)catechin subunits was

Table 3. Observed $[M - H]^-$ and $[M - 2H]^{2-}/2$ Ions in the ESI Spectrum and $[M + Na]^+$ Ions in the MALDI-TOF Spectrum of Sephadex Fraction M from Sea Buckthorn Pomace

		ESI		MALDI-TOF
DP	PD[a]	$[M - H]^-$	$[M - 2H]^{2-}/2$	$[M + Na]^+$
3	1	882.2		906.6
	2	898.0		923.0
	3	914.1		939.6
4	0	1154.2		1180.2
	1	1170.0		1196.2
	2	1186.0		1212.1
	3	1202.2		1228.7
	4	1218.1		1244.7
5	0	1442.0		1470.2
	1	1458.1		1485.9
	2	1474.0	736.7	1500.9
	3	1490.1	744.8	1517.2
	4	1506.2	753.0	1533.3
	5	1522.0	760.8	1549.3
6	0			1758.4
	1			1773.2
	2		881.2	1789.7
	3			1805.8
	4		897.2	1821.4
	5		905.2	1837.0
	6		913.0	1853.6
7	1			2061.5
	2			2077.1
	3			2094.3
	4			2109.8
	5			2125.9
	6		1057.1	2142.0
	7		1065.2	2157.6
8	0		1153.0	
	1		1161.0	2351.9
	2		1169.0	2364.8
	3		1177.2	2382.3
	4		1185.1	2397.5
	5		1193.3	2413.7
	6		1201.0	2429.8
	7		1209.0	2445.9
	8		1217.0	2462.3
9	3			2672.1
	4		1329.2	2686.1
	5			2702.2
	6			2717.6
	7			2734.1
	8		1361.0	2751.1
	9		1369.0	2766.4
10	0		1441.0	
	2		1457.1	
	3		1465.3	
	4		1473.3	
	5		1481.3	2990.1
	6		1489.3	3004.8
	7		1497.3	3022.3
	8		1505.0	3038.0
	9		1513.0	3053.1
	10		1521.2	3069.6
11	7			3308.5
	8			3323.2
	9			3341.1
	10			3357.6
	11			3369.3
12	8			3610.1
	9			3631.0
	10			3648.1
	11			3661.6
13	11			3947.3
	12			3962.2

[a] PD = number of prodelphinidins.

concluded from the ESI-MS data and is explained for the tetramers as follows. Besides *m/z* 1218.1, proanthocyanidins with *m/z* 1202.2, *m/z* 1186.0, *m/z* 1170.0, and *m/z* 1154.2 were also detected, which are separated by 16 Da (**Table 3**). As in the cases of tetramers and pentamers, the highest intensity was measured for the all-prodelphinidin tetramer (*m/z* 1218.1), which demonstrates (epi)gallocatechin as the predominating subunit.

In the mass range between *m/z* 1153.0 and 1217.0, further signals were detected, which were separated by 8 Da (**Table 3**). These signals were assigned to double charged ions $[M - 2H]^{2-}/2$ of octameric proanthocyanidins. Further double charged ions were detected for pentameric, hexameric, heptameric, nonameric, and decameric proanthocyanidins. In each case, the most abundant proanthocyanidin consisted only of (epi)-gallocatechin subunits.

MALDI-TOF Analysis. MALDI-TOF mass spectra of proanthocyanidins are easier to interpret than ESI mass spectra because of the absence of multiply charged ions (*13*). The ions are predominately detected as sodium adducts $[M + Na]^+$ (*13*, *29*, *30*). A suitable matrix is an important prerequisite for the production of these ions. Several matrixes were tested by Onishi et al. for the analysis of flavan-3-ols of different DP (*31*). The authors concluded that *trans*-3-indoleacrylic acid and 2,5-dihydroxybenzoic acid were most suitable for the ionization of oligomeric proanthocyanidins whereas other matrixes should be applied for investigations of monomeric and dimeric flavan-3-ols. Thus, 2,5-dihydroxybenzoic acid was used for the MALDI-TOF-MS investigation of Sephadex fraction M. The resulting spectrum shows 11 mass clusters (**Figure 5A**) with *m/z* values that were characteristic for the adduct ions $[M + Na]^+$ of proanthocyanidins with DP 3 to DP 13 (**Table 3**). The highest intensities were measured for tetramers, pentamers, hexamers, and heptamers. Because small proanthocyanidins are more readily ionized by MALDI-TOF-MS than larger ones (*13*), the mass spectrum might not reflect the actual proportion of different size oligomers. As in the case of the phloroglucinol degradation study, the results of MALDI-TOF-MS suggested a mDP > 6 for the proanthocyanidins from Sephadex fraction M.

The most abundant ions within the clusters of DP 4, 5, 6, and 7 showed *m/z* values of 1244.7, 1549.3, 1853.6, and 2157.6 (**Table 3**). The mass difference of 304 Da demonstrated that these peaks are separated by an (epi)gallocatechin subunit. The detected ions within these clusters are separated by intervals of 16 Da as demonstrated for the proanthocyanidins of DP 5 (**Figure 5B**). The most abundant peak (*m/z* 1549.3) resulted from the adduct $[M + Na]^+$ of a proanthocyanidin, which consisted of five (epi)gallocatechin subunits. The lowest abundance was observed for the ion at *m/z* 1470.2, which corresponded to the $[M + Na]^+$ of a proanthocyanidin consisting of five (epi)catechin subunits. Consequently, (epi)gallocatechins were measured to be the predominating subunits in Sephadex fraction M.

^{13}C NMR Spectroscopy. All signals in the ^{13}C NMR spectrum of Sephadex fraction M (**Figure 6**) showed chemical shifts, which were characteristic for proanthocyanidins (*14*). The absence of other signals demonstrates that a proanthocyanidin fraction of high purity was isolated. The mDP of a proanthocyanidin mixture could be calculated by the relation between the signal intensity of $\delta \sim 72$ ppm (C-3 of extension subunits) and $\delta \sim 67$ ppm (C-3 of terminal subunits) (*14*). Because of the low signal intensity for the extension subunits, this calculation was difficult. We tentatively determined a mDP of 9 for the proanthocyanidins from Sephadex fraction M (**Figure 6**).

The relative occurrence of subunits with a 2,3-trans and a 2,3-cis conformation can be deduced from the relation between the intensity of their C-2 signals at $\delta \sim 84$ ppm and $\delta \sim 77$ ppm (*14*). In the case of Sephadex fraction M, the broad signal at $\delta \sim 83$ ppm was assigned to the C-2 of 2,3-trans subunits

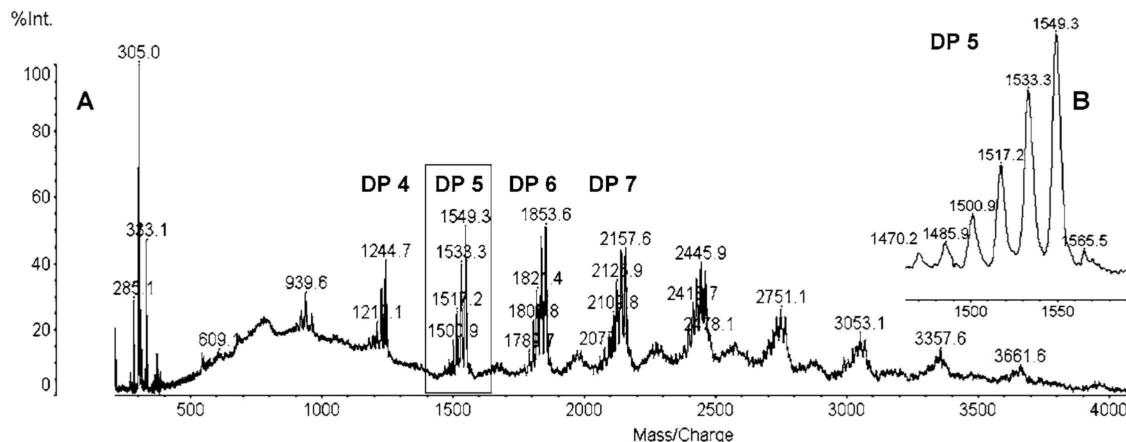

Figure 5. MALDI-TOF mass spectrum of sea buckthorn proanthocyanidins from Sephadex fraction M.

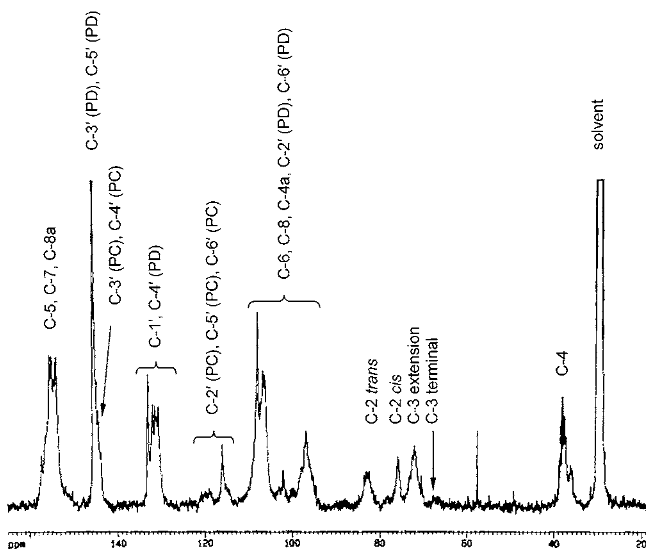

Figure 6. ^{13}C NMR spectrum of sea buckthorn proanthocyanidins from Sephadex fraction M consisting of prodelphinidin (PD) and procyanidin subunits (PC) measured in $(CD_3)_2CO$ with a small amount D_2O. Assignments were made according to the data from Czochanska et al. (*14*).

and the broad signal at $\delta \sim 76$ ppm was assigned to the 2,3-cis subunits (**Figure 6**). The calculated 2,3-trans to 2,3-cis ratio for the sea buckthorn proanthocyanidins from Sephadex fraction M was 1.8:1.

A signal at $\delta \sim 145$ ppm is consistent for the C-3′ and C-4′ in procyanidin subunits whereas an additional signal at $\delta \sim 146$ ppm represents a typical indicator for the C-3′ and C-5′ resonance of prodelphinidins (*14*). The ^{13}C NMR spectrum of Sephadex fraction M exhibits several signals between δ 145 and 146 ppm, which suggested the presence of procyanidins and prodelphinidins (**Figure 6**). However, because of signal overlapping, a quantitative statement was not possible.

In conclusion, our results demonstrate that oligomeric proanthocyanidins accounted for 75% of the total antioxidant activity from sea buckthorn pomace extract. Their composition was elucidated by acid-catalyzed cleavage in the presence of phloroglucinol, ESI-MS, MALDI-TOF-MS, and NMR spectroscopy. Even though every method has its disadvantages, valuable information about the structural properties of the oligomeric proanthocyanidin fraction could be obtained. The mDP was between 6 and 9. Most of the subunits possessed a 2,3-trans configuration and a prodelphinidin structure, i.e., they had a (+)-gallocatechin structure.

Comparing these results with data from the literature, it can be pointed out that a high distribution of (+)-gallocatechin subunits within a mixture of proanthocyanidin oligomers is very uncommon in the plant kingdom (*6*, *7*). A proanthocyanidin composition quite similar to sea buckthorn pomace was observed for the fruits of the poisonous plant *Iris pseudacorus* (*7*). Regarding the DP, similarities were observed to the procyanidin-rich extract from the bark of *Pinus maritima* known as the nutritional supplement Pycnogenol (*6*). To evaluate a potential utilization of proanthocyanidins from sea buckthorn pomace extract as a food supplement, their absorption and biotransformation have to be considered. Because of the complexity of proanthocyanidins, very few studies exist concerning their bioavailability. While monomers, dimers, and trimers are suggested to be absorbed by the human intestinal cell line Caco-2, oligomers (mDP 7) were not absorbed and partially adhered to the cell surface (*32*). However, the procyanidin oligomers were also shown to be degraded by the human colonic microflora into low molecular mass phenolic acids, which might be well-absorbed through the colon.

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