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# HPLC Determination of Extractable and Unextractable Proanthocyanidins in Plant Materials

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This study developed a method for the determination of extractable and unextractable proanthocyanidins. Extractable proanthocyanidins were separated according to their degree of polymerization using normal phase HPLC. Unextractable proanthocyanidins were measured after acid-catalyzed depolymerization as flavan-3-ols (terminal units) and benzylthioethers (external units). Electrospray ionization mass spectrometry (ESI-MS) was used for the identification of proanthocyanidins in the samples. Hubaux-Vos detection limits were 0.01−0.15 ng/injection for extractable proanthocyanidins, with recovery rates from 69 to 91%. Detection limits for unextractable proanthocyanidin derivatives were 0.002−0.035 ng/injection with 80% recovery. The developed method was applied to the analysis of several fruit and berry samples. Results showed great variation in the proportion of unextractable proanthocyanidins in total proanthocyanidin content between samples, being highest in the green variety of table grape (63%) and lowest in the apple cultivar 'Valkeakuulas' (4.1%). The method reported herein is reliable and gives valuable information on the nature of proanthocyanidins in plant-derived foods.

**KEYWORDS: Determination; proanthocyanidins; flavan-3-ols; thiolysis; HPLC; ESI-MS**

## INTRODUCTION

Proanthocyanidins (condensed tannins) are oligomers and polymers of flavan-3-ol units widely present in the plant kingdom, for example, in fruits, berries, nuts, seeds, and the bark of pine trees (*1*−*4*). Proanthocyanidins can be divided into several classes based on hydroxylation of the constitutive units and the linkages between them. Their most common constitutive units are (epi)catechins and (epi)gallocatechins, leading to procyanidin and prodelphinidin structures, respectively. Flavan-3-ol units are most frequently linked via B-type bonds, that is, $C_4 \rightarrow C_8$ or $C_4 \rightarrow C_6$ linkages. Occasionally an additional $C_2 \rightarrow O_7$ or $C_2 \rightarrow O_5$ linkage may exist, producing doubly bonded A-type proanthocyanidins (**Figure 1**). Proanthocyanidins mainly occur in free, unbound form, although there are some well-known exceptions to this rule: esters with gallic acid have been commonly found in green tea and grapes (*4*−*6*).

The physical, chemical, and biological features of proanthocyanidins depend largely on their structure and particularly on their degree of polymerization (DP) (*7*−*9*). Proanthocyanidins are powerful antioxidants due to their polyphenolic structure, but they have also been reported to demonstrate antibacterial, antiviral, anticarcinogenic, anti-inflammatory, and vasodilatory activities (*7*−*15*). The presence of proanthocyanidins in foods produces a sensation of astringency arising from the precipitation of oral proteins and mucopolysaccharides. Their propensity to form complexes with other compounds (proteins, carbohydrates, alkaloids) is believed to be responsible for many of their interactions with biological systems (*14, 15*).

The current shortage of reliable quantitative data on proanthocyanidins in plants and plant products is in large part due to the lack of appropriate analytical methodology and commercially available standards. Various chemical methods based on the formation of colored products have been developed (e.g., acid−butanol assay, vanillin assay), but their results are considerably affected by the type of proanthocyanidin analyzed and the conditions employed (*16, 17*).

Determination of proanthocyanidins by high-performance liquid chromatography (HPLC) is problematic because proanthocyanidins often occur as complex mixtures, which makes their separation difficult. In reversed phase HPLC the presence of many isomers results in overlapping retention times between different proanthocyanidins, and the separation of large polymers (DP > 4) is not possible (*17, 18*). Proanthocyanidins are, therefore, often depolymerized before HPLC. Acid-catalyzed depolymerization of proanthocyanidins in the presence of suitable nucleophilic reagent, that is, benzylmercaptan or phloroglucinol, converts flavan-3-ol extender units into corresponding thioethers (or phloroglucinol adducts), whereas the terminal units are released as monomeric flavan-3-ols (*19*). This makes it possible to determine the nature and proportion of the constitutive units of proanthocyanidins. It also enables the calculation of their average degree of polymerization. However, no information of the proportions of individual proanthocyanidins can be derived with this method.

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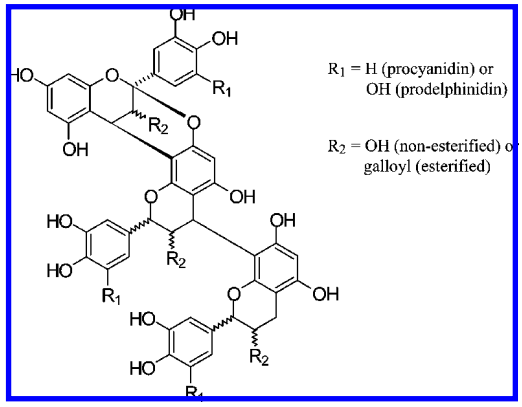


**Figure 1.** Structure of a trimeric proanthocyanidin with A- and B-type linkages.

Proanthocyanidin oligomers can be separated by normal phase HPLC according to their degrees of polymerization up to decamers (*18, 20*). In addition, the use of a sharp gradient elution in the end of the HPLC run makes it feasible to determine proanthocyanidin polymers with a high degree of polymerization (DP > 10) as a single group (*20*). Hence, it is possible to obtain rather detailed information on proanthocyanidin profiles in the samples by normal phase HPLC. However, the extraction of proanthocyanidins in their native form (i.e., without hydrolysis) is usually incomplete due to their tendency to form strong complexes with the insoluble polymeric plant material (e.g., cell wall polysaccharides) (*3, 17, 21*). Thus, total proanthocyanidin content is easily underestimated if only extractable proanthocyanidins are determined.

This study describes and validates a procedure for the determination of both extractable and unextractable proanthocyanidins in plant foods by HPLC. Normal phase HPLC is used for the assay of extractable proanthocyanidins having different degrees of polymerization. Unextractable proanthocyanidins are analyzed from the extracted residue by reversed phase HPLC after thioacidolysis.

## MATERIALS AND METHODS

**Chemicals.** (−)-Epicatechin, (−)-epicatechin gallate, and (−)-epigallocatechin were purchased from Sigma (Sigma-Aldrich Chemie Inc., Steinheim, Germany), and (+)-catechin was purchased from Cayman Chemical Co. (Ann Arbor, MI). Procyanidin dimers B1 [epicatechin-(4$\beta$→8)-catechin] and B2 [epicatechin-(4$\beta$→8)-epicatechin] were obtained from PlantChem Co. (Sandnes, Norway). Procyanidin oligomers with different degrees of polymerization were isolated from Saskatoon berry by MTT Agrifood Research Finland (*22*). Benzylmercaptan (α-toluolthiol) came from Fluka (Fluka Sigma-Aldrich Chemie Inc., Buchs, Switzerland). Twenty-five percent ammonium hydroxide, concentrated hydrochloric acid (37−38%), acetone, methanol, dichloromethane, acetic acid, and phosphoric acid (85%) were purchased from J. T. Baker (Mallinckrodt Baker Inc., Utrecht, The Netherlands). Formic acid (99%) was from Acros Organics Fischer Scientific Inc. (Geel, Belgium), *N*,*N*-dimethylformamide from Rathburn Chemicals Ltd. (Walkerburn, Scotland), and sodium hydroxide from Merck Sharp & Dohme GmbH (Haar, Germany). All chromatographic solvents were of HPLC grade.

**Standard Solutions.** For extractable proanthocyanidins, standard solutions for calibration curves were prepared for each procyanidin oligomer class at five concentration levels (20−2000 $\mu$g/mL) in methanol. Additionally, five standard solutions at low concentration levels (5−50 $\mu$g/mL) were used to determine the Hubaux−Vos detection limits (*23*). For unextractable proanthocyanidins, standard solutions of catechins and dimeric procyanidins (B1, B2) were prepared at five concentration levels (5−150 $\mu$g/mL).

**Plant Materials.** Roasted pine bark powder obtained from the inner bark of Scots pine (*Pinus sylvestris*) was kindly provided by Finnpettu Oy (Ranua, Lappland, Finland). Apples (*Malus domestica*, four cultivars, 'Valkeakuulas', 'Lobo', 'Red Delicious', and 'Granny Smith') and grapes (*Vitis vinifera* L., red and green varieties) were purchased from local supermarkets in Finland. Saskatoon berries (*Amelanchier alnifolia*) were kindly provided by Marjakka Ky (Niittylahti, North Carelia, Finland), and lingonberries (*Vaccinium vitis-idaea*) were purchased from a wholesaler in Kuopio, Finland. Seeds were manually removed from the grapes before analyses. Apples were cored with a cork borer; part of them were peeled, and also the peels were analyzed. Prior to extraction of proanthocyanidins, apples, apple peels, grapes, and berries were freeze-dried and ground.

**Extraction and Purification of Proanthocyanidins.** Extraction efficiencies between three commonly used mixtures of water and organic solvent were compared: (1) 80% aqueous acetone; (2) 70% acetone in aqueous 0.5% acetic acid; (3) acetone/methanol/water (2:2:1). Furthermore, the effect of added ascorbic acid (5 g/L of extraction solvent) on proanthocyanidin recovery was tested. The extraction solvent selected for the analyses consisted of acetone, methanol, and water in proportions of 2:2:1, respectively, with no added ascorbic acid. Freeze-dried and ground samples (0.5−2 g) were homogenized with the extraction solvent (10−30 mL), followed by ultrasound-assisted extraction. After 10 min of sonication, the solution was centrifuged at 3000 rpm for 4 min and the supernatant was removed. The extraction procedure was repeated twice, and all three extracts were combined. Organic solvents in the extract were evaporated under vacuum (35 °C), and the remaining aliquot was purified with solid phase extraction using prepacked Supelco Discovery DPA-6S 1 g polyamide cartridges (Sigma-Aldrich Chemie Inc.), previously activated with methanol followed by water. Sugars and most of the interfering phenolics were washed from the cartridges by 20% methanol (10 mL), after which the proanthocyanidins were eluted with 85% *N*,*N*-dimethylformamide (5 mL) and analyzed by HPLC as extractable proanthocyanidins. After the above extraction procedure, the residual sediment in the extraction vessel was collected quantitatively with the aid of rinsewater, freeze-dried, and weighed to perform thioacidolysis before HPLC quantification of unextractable proanthocyanidins.

**Thioacidolysis.** After solvent extraction of proanthocyanidins, the thioacidolysis method described by Guyot et al. (*19*) with slight modifications was utilized for the residual sediment. Briefly, a freeze-dried sample (10−20 $\mu$g) was dissolved in a 5% solution (1 mL) of benzylmercaptan in methanol containing 1.1% HCl. The vials were sealed and incubated at 40 °C for 30 min, after which they were cooled in an ice bath prior to HPLC analysis.

**HPLC Analysis of Extractable Proanthocyanidins.** A modification of the normal phase HPLC method of Gu et al. (*20*), as described elsewhere (*22*), was used for extractable proanthocyanidin determination. Separation was performed on a 250 × 4.6 mm i.d., 5 $\mu$m, Silica Luna column (Phenomenex Inc., Darmstadt, Germany) and monitored by UV detection (UVD; $\lambda$ = 280 nm) and fluorescence detection (FLD; $\lambda_{ex}$ = 280 nm, $\lambda_{em}$ = 323 nm). An external standard consisting of procyanidin oligomers isolated from Saskatoon berries along with commercially available monomeric and dimeric procyanidins was used for quantification. Quantification was based on FLD using flat baseline integration as proposed by Gu et al. (*20*).

**HPLC Analysis of Unextractable Proanthocyanidins.** Reversed phase HPLC, using conditions described elsewhere (*22*), was utilized to analyze the thioacidolyzed samples with some modifications in gradient elution. The column was a 150 × 4.0 mm i.d., 3 $\mu$m, Inertsil ODS-3 (GL Sciences Inc., Torrance, CA). The mobile phase consisted of (A) 50 mM phosphoric acid (aqueous), pH 2.5, adjusted by NaOH and (B) acetonitrile. Elution was started isocratically with a constant flow of 5% B in A, 5 min; followed by 5−27.5% B in A, 5−30 min; 27.5−50% B in A, 30−32 min; and 50% B in A, 32−38 min. Separation was monitored by diode array detection (DAD; $\lambda_1$ = 270 nm, $\lambda_2$ = 280 nm). External standards derived from authentic compounds were used for the quantification of terminal units ($\lambda$ = 280 nm, catechin, epicatechin, epicatechin gallate; $\lambda$ = 270 nm, epigallocatechin). The standard curve for epicatechin benzylthioether ($\lambda$ = 280 nm) was obtained by thioacidolysis of procyanidin B2. Other extension units were quantified against epicatechin benzylthioether using the response factor ratios reported by Vivas et al. (*4*).

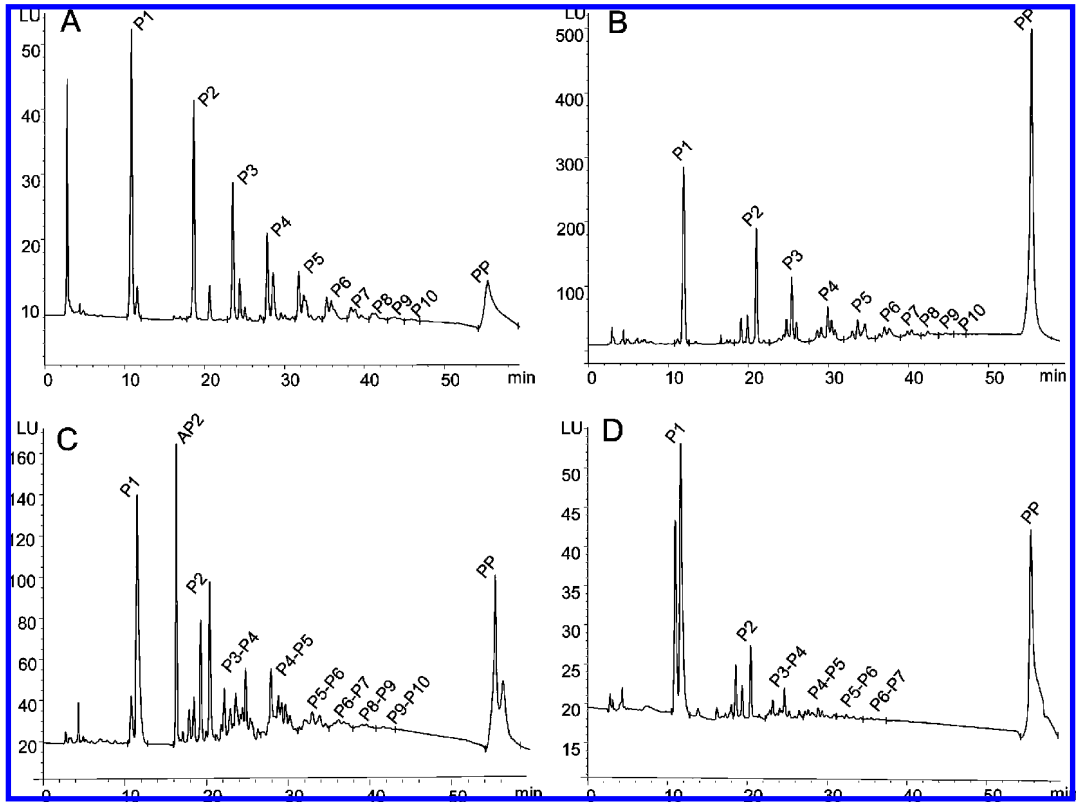


**Figure 2.** Normal phase HPLC-FLD trace of proanthocyanidins from (**A**) apple, (**B**) pine bark powder, (**C**) lingonberry, and (**D**) grape. Labels P1−P10 indicate the degrees of polymerization (DP) of proanthocyanidins in the peaks. The single peak labeled AP2 indicates an A-type dimeric proanthocyanidin. Polymeric proanthocyanidins (PP) with DP > 10 appeared as a single peak at the end of the chromatogram.

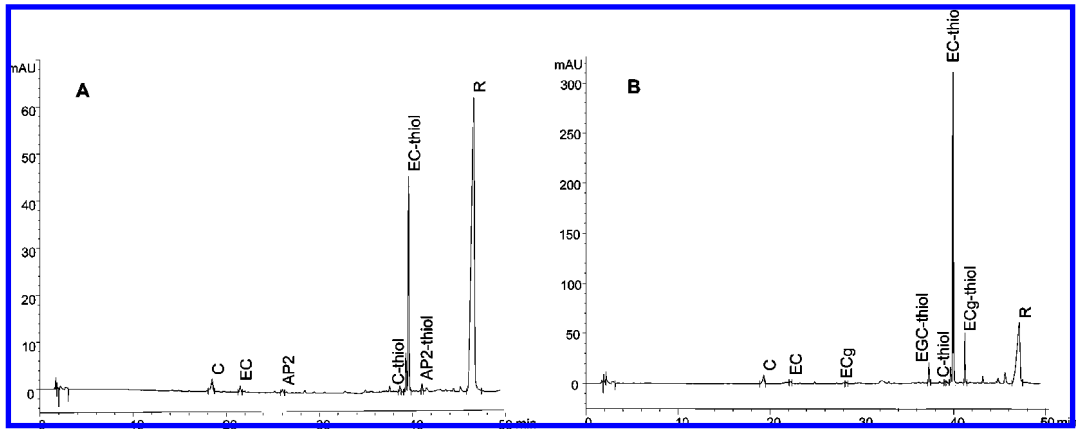


**Figure 3.** Reversed phase HPLC-UVD trace of proanthocyanidin derivatives from (**A**) lingonberry and (**B**) grape after thiolytic degradation. Catechin, epicatechin, epigallocatechin, epicatechin gallate, and an A-type dimeric procyanidin have been labeled C, EC, EGC, ECg, and AP2, respectively. Benzylthioethers of them have been indicated with an incidental "thiol"-label. R is thiolytic reagent (benzylmercaptan).

**HPLC-MS.** All samples were analyzed by HPLC-MS to confirm their proanthocyanidin structures. A Thermo Finnigan Surveyor HPLC with DAD was connected to a Finnigan MAT ion trap mass spectrometer. An ESI interface in negative ionization mode was used under full scan (*m*/*z* 200−2000). The operating conditions were spray voltage at 4.50 kV and capillary at 270 °C. The column for extractable proanthocyanidins was a 250 × 2.0 mm i.d., 5 $\mu$m, Silica Luna 100A (Phenomenex Inc.) with a precolumn filled with the same sorbent. The gradient was the same as in quantitative HPLC analysis, with a flow rate of 0.3 mL/min. Ten millimolar ammonium acetate in methanol was used as an ionization reagent prior to MS and added via a tee in the eluant stream with an auxiliary Suprex Modifier Pump MPA-1 at a flow rate of 0.04 mL/min.

The structures of reaction products after thiolytic degradation were confirmed with the same HPLC-MS apparatus as extractable proanthocyanidins, but with a 150 × 3.0 mm i.d., 5 $\mu$m, Gemini C18 column (Phenomenex Inc.). The gradient was the same as in quantitative HPLC with the exception that phosphoric acid was replaced by 0.5% formic acid and the pH was adjusted to 2.50 with $NH_4OH$. The flow rate was 0.3 mL/min. The same ESI-MS parameters were used as in the characterization of extractable proanthocyanidins.

**Validation.** Laboratory validation of the method for extractable proanthocyanidins was performed by analyzing spiked samples. Recoveries were studied by adding known amounts of procyanidin oligomers to freeze-dried food samples with no detectable proanthocyanidins (potato and rice), after which the samples were analyzed as described above. The decision and detection limits (LOC and LOD, respectively) for each oligomeric class were derived from low-concentration calibration curves as proposed by Hubaux and Vos (*23*). Briefly, the LOC value was defined as the corresponding concentration of the detector response caused by the analytes with 95% probability, that is, the possibility of false positives is 5%, and the LOD value as

**Table 1.** Proanthocyanidin Contents in Samples

| sample | Latin name | DM (%) | extractable proanthocyanidins (mg/100 g of FW) ± SD deviation ($n = 3$) | | | | | | unextractable | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | P1 | P2 | P3 | P4−P6 | P7−P10 | >P10 | total | DP | total |
| Saskatoon berry | *Amelanhcier alnifolia* | 12.8 | 21.4 ± 0.4 | 20.2 ± 0.7 | 21.7 ± 1.3 | 46.1 ± 3.7 | 21.0 ± 1.9 | 44.6 ± 4.1 | 175 ± 12 | 101 ± 9 | 8.2 ± 0.1 | 276 ± 21 |
| apple, 'Lobo' | *Malus domestica* | 12.2 | 6.7 ± 0.3 | 5.5 ± 0.3 | 5.1 ± 0.3 | 11.6 ± 1.1 | 4.3 ± 0.4 | 5.2 ± 0.5 | 38.4 ± 2.9 | 4.9 ± 0.2 | 9.1 ± 0.1 | 43.3 ± 3.1 |
| apple peel, 'Lobo' | *M. domestica* | 14.9 | 16.5 ± 1.1 | 9.5 ± 0.6 | 9.2 ± 0.2 | 17.7 ± 0.4 | 10.8 ± 0.5 | 9.9 ± 0.9 | 73.6 ± 3.7 | 13.3 ± 0.6 | 9.6 ± 0.5 | 86.9 ± 4.3 |
| apple, 'Valkeakuulas' | *M. domestica* | 10.5 | 7.6 ± 0.4 | 10.5 ± 0.9 | 9.4 ± 0.7 | 18.8 ± 1.3 | 7.1 ± 0.5 | 37.7 ± 3.3 | 91.1 ± 7.1 | 4.1 ± 0.1 | 7.8 ± 0.1 | 95.2 ± 7.2 |
| apple peel, 'Valkeakuulas' | *M. domestica* | 12.3 | 14.2 ± 0.6 | 15.6 ± 1.3 | 14.4 ± 1.3 | 27.3 ± 2.3 | 15.5 ± 1.1 | 72.6 ± 4.4 | 160 ± 11 | 21.5 ± 1.7 | 9.1 ± 0.4 | 182 ± 13 |
| apple, 'Red Delicious' | *M. domestica* | 13.7 | 19.5 ± 1.0 | 25.5 ± 1.3 | 23.2 ± 1.2 | 49.5 ± 4.6 | 15.1 ± 1.3 | 21.6 ± 0.8 | 154 ± 10 | 7.4 ± 0.4 | 6.0 ± 0.3 | 162 ± 10 |
| apple peel, 'Red Delicious' | *M. domestica* | 19.5 | 21.8 ± 1.3 | 27.5 ± 1.3 | 28.7 ± 1.8 | 61.3 ± 3.7 | 22.1 ± 1.6 | 50.9 ± 3.8 | 212 ± 14 | 14.7 ± 1.4 | 6.9 ± 0.3 | 227 ± 15 |
| apple, 'Granny Smith' | *M. domestica* | 15.1 | 7.6 ± 0.3 | 13.3 ± 0.5 | 11.1 ± 0.3 | 22.3 ± 0.5 | 9.1 ± 0.5 | 8.6 ± 0.4 | 72.0 ± 2.5 | 7.0 ± 0.7 | 7.7 ± 0.3 | 79.0 ± 3.2 |
| apple peel, 'Granny Smith' | *M. domestica* | 18.2 | 13.7 ± 1.3 | 24.5 ± 2.4 | 26.9 ± 2.4 | 41.6 ± 3.5 | 49.7 ± 4.4 | 7.6 ± 0.7 | 164 ± 15 | 37.1 ± 3.3 | 9.5 ± 0.3 | 201 ± 18 |
| pine bark powder | *Pinus sylvestris* | 94.4 | 300 ± 19 | 342 ± 17 | 353 ± 19 | 807 ± 43 | 516 ± 21 | 2980 ± 140 | 5300 ± 260 | 231 ± 3 | 7.6 ± 0.1 | 5530 ± 260 |
| lingonberry | *Vaccinium vitis-idaea* | 14.1 | 14.4 ± 0.5 | 29.2 ± 1.1 | 27.2 ± 1.7 | 46.4 ± 2.9 | 10.3 ± 1.0 | 14.1 ± 1.3 | 141 ± 9 | 16.7 ± 1.1 | 8.2 ± 0.1 | 158 ± 10 |
| grape, red | *Vitis vinifera* L. | 14.8 | 2.6 ± 0.1 | 2.9 ± 0.1 | 2.1 ± 0.1 | 3.6 ± 0.3 | traces | 19.3 ± 1.4 | 30.5 ± 2.0 | 23.5 ± 1.8 | 12.3 ± 0.8 | 54.0 ± 4.2 |
| grape, green | *V. vinifera* L. | 15.2 | 1.8 ± 0.1 | 2.3 ± 0.1 | 1.6 ± 0.1 | 2.7 ± 0.2 | traces | 3.8 ± 0.2 | 12.2 ± 0.8 | 20.4 ± 0.9 | 19.4 ± 1.1 | 32.6 ± 1.7 |

the concentration which had 95% probability that the response signal was not under LOC, that is, the possibility of false negatives is 5%. Ninety-five percent confidence intervals for calibration curves were calculated by GraphPad Prism 5 (GraphPad Prism Software Inc., San Diego, CA). The precision of sample preparation was tested by preparing nine samples of pine bark flour, analyzing them on three different days, and then calculating the relative standard deviation for the analyzed proanthocyanidin levels. Recovery of thiolysis was studied with commercial dimeric procyanidin standards.

## RESULTS AND DISCUSSION

**Extraction of Proanthocyanidins.** There is much evidence suggesting that aqueous acetone is the most effective overall extraction solvent for proanthocyanidins. Acetone is a strong hydrogen bond breaker, and its superiority over alcoholic solvents is obvious, especially in the extraction of polymeric proanthocyanidins (*17, 21*). However, methanol has shown great extraction efficiency toward catechins (*24*) and small proanthocyanidin oligomers (*25, 26*), and some studies have recommended the use of aqueous methanol instead of acetone for the extraction of flavan-3-ols (*27*). It has further been opined that methanol can prevent oxidative loss of flavan-3-ols by inactivating the oxidative enzymes (*25, 26*). In the present study, replacing half of the acetone with methanol in the extraction solvent (acetone/methanol/water, 2:2:1) showed enhanced extraction power for apple proanthocyanidins, with 117 ± 3.5% ($p < 0.001$, paired $t$ test, $n = 5$) recovery compared to 80% acetone, whereas the effect on pine bark proanthocyanidins was negligible (101 ± 3.1%; $p = 0.46$). Acidification of the extraction solvents is often recommended for phenolic compounds including flavan-3-ols (*20*), but this may lead to partial hydrolysis of proanthocyanidins due to the labile nature of interflavanoid bonds toward acids (*17*). In the present study, acidification of the extraction solvent with acetic acid had no effect on extraction efficiency ($p = 0.48$, $n = 5$).

The effect of added ascorbic acid in the extraction solvent was studied only in freeze-dried apple. Proanthocyanidin recovery was 79 ± 4.8% ($p < 0.001$, $n = 4$) compared to the extraction solvent without added ascorbic acid, indicating significantly impaired extraction efficiency. It has been previously shown that in the presence of oxygen and transition metal ions, ascorbic acid may act as a prooxidant rather than an antioxidant through the Fenton and Haber-Weiss reactions (*29, 30*). Earlier studies have shown that added ascorbic acid can accelerate the oxidative degradation of catechins and other polyphenols (*31*−*33*) and may decrease the recovery of proanthocyanidins (*33*). On the basis of these results, the mixture of aqueous acetone and methanol with no added ascorbic acid was chosen as the most suitable extraction solvent for proanthocyanidins. The volume of extraction solvent was approximately 15 times the amount of sample. A sonication time of 10 min was used at each extraction step. Overall recovery of proanthocyanidins with two extraction steps was 93.4 ± 3.6% ($p < 0.01$, $n = 4$) as compared to three steps, and with four steps it was 102.2 ± 2.4% ($p = 0.189$) as compared to three. Three extraction steps were, thus, considered to be optimal.

**Purification of Sample Extracts.** Because the crude proanthocyanidin extract contained sugars and other impurities that might contaminate the HPLC column, a further purification step was needed. Common practice for the purification of phenolic extracts has been solid phase extraction (SPE) with octadecasilane (C18) columns (*34, 35*). However, our preliminary trials indicated that the capacity of C18 to adsorb proanthocyanidins can be seriously limited in the presence of other phenolic compounds (unpublished data), and so we chose polyamide

columns with high adsorption efficiency toward proanthocyanidins for SPE purification. The SPE column was washed with 20% methanol, which removed sugars and most of the nontannic phenolics but did not cause any loss of proanthocyanidins. Proanthocyanidins were strongly retained onto polyamide, and an elution solvent with great efficiency toward polyphenols was, therefore, necessary. The selected eluent was 85% *N*,*N*-dimethylformamide (DMF) on the basis of its use in a previous study by Papagiannopoulos et al. (*36*). A drawback was that the sample could not be concentrated after SPE due to the low vapor pressure of DMF.

**Determination of Unextractable Proanthocyanidins.** The freeze-dried extraction residue was acid-hydrolyzed with benzylmercaptan for the determination of unextractable proanthocyanidins. Analyses of procyanidin B2 after thiolysis indicated partial epimerization of epicatechin in the applied reaction conditions. Approximately 5% of epicatechin was converted into catechin after 30 min of incubation at 40 °C, and the converted amount increased steadily to 15% during incubation at room temperature for 35 h. Standards were continuously added to each sample sequence to monitor the epimerization process during HPLC analysis. The gradient elution employed earlier (*26*) was modified to improve the separation of benzylthioethers corresponding to the extension units. A sufficient resolution of compounds was obtained under the adapted HPLC conditions.

**Validation.** LOC values for extractable proanthocyanidins varied from 0.5 to 8.6 $\mu$g/mL and LOD values from 1.0 to 15 $\mu$g/mL for P1 and PP, respectively. The sensitivity of the method decreased as the polymerization of proanthocyanidins increased, due to weaker detector responses and wider chromatogram peaks for higher oligomers. Recovery rates varied from 69% (P10) to 91% (P1, P2) and deviated significantly from 100% in every oligomer class. Highest losses of proanthocyanidin oligomers presumably occurred during SPE purification due to incomplete desorption from the polyamide columns. Nevertheless, recovery rates were at the same level as in previous studies (*20, 37*) and, hence, were considered to be acceptable. To evaluate the precision of the method, the pine bark powder sample was analyzed nine times on three different days (three replicates on each day). Percentual standard deviations (RSD) for the determined proanthocyanidin contents varied from 2.2 to 5.8% within one day and from 4.0 to 7.8% between days for different oligomer classes, being 4.0% for total proanthocyanidin content in the samples. The results indicate acceptable precision and reproducibility for the method.

LOC and LOD values for the constituent units of unextractable proanthocyanidins varied from 0.1 to 3.2 $\mu$g/mL and from 0.2 to 3.5 $\mu$g/mL, respectively. The recovery of thiolysis was approximately 80% for the dimeric procyanidin standards. It has been previously shown that the recovery does not depend on the polymerization state under current reaction conditions (*19*).

**Proanthocyanidins in the Samples.** The suitability of the developed method was tested by analyzing several plant materials, namely, Saskatoon berry, lingonberry, pine bark powder, two samples of table grapes (red and green varieties), and four cultivars of apples ('Valkeakuulas', 'Lobo', 'Red Delicious', and 'Granny Smith'). Normal phase HPLC chromatograms of the extracted proanthocyanidins and reversed phase HPLC chromatograms of the thiolyzed proanthocyanidins are shown in **Figures 2** and **3**, respectively. **Table 1** presents the determined contents of the extractable and unextractable proanthocyanidins.

Analyses of apple extracts and pine bark powder extract proved that proanthocyanidins of different molecular sizes can be nicely separated by normal phase HPLC, as long as their structural units are relatively homogeneous (**Figure 2A**,**B**). HPLC-MS analyses of these extracts showed that the peaks in the chromatogram comprise a series of compounds with molecular ions $[M - 1]^-$ at $m/z$ $[289 + 288(n - 1)]$ ($n$ = degree of polymerization), typical of procyanidins with B-type linkages. Thiolytic cleavage of unextractable proanthocyanidins produced only catechin, epicatechin, and epicatechin benzylthioether, confirming that proanthocyanidins in apple and pine bark powder are essentially polymers of (epi)catechin. This result is in agreement with previous studies (*1, 3, 19*). Epicatechin was the only extension unit in both samples, but the terminal unit was predominantly catechin in pine bark powder procyanidins and epicatechin in apple procyanidins. Saskatoon berry proanthocyanidins, which were characterized already in our previous study (*22*), were very similar to apple procyanidins.

Normal phase HPLC analyses of lingonberry and table grape extracts (**Figure 2C**,**D**) did not result in distinct separation between different proanthocyanidin classes, indicating higher complexity in their proanthocyanidin structures. Mass spectrometric data of extracted lingonberry proanthocyanidins indicated the presence of B-type procyanidins, but, additionally, ions of 2 Da less at $m/z$ $[289 + 286(n - 1)]$ were highly abundant, suggesting A-type linkages between flavan-3-ol units as well. This is in accordance with previous studies of lingonberry proanthocyanidins (*38, 39*). Most of the A-type procyanidins in lingonberry contained only one A-type linkage; nevertheless, among pentamers and heptamers procyanidins with two A-type linkages were tentatively identified. Thiolytic degradation of unextractable lingonberry proanthocyanidins produced catechin, epicatechin, and epicatechin benzylthioether as the major products, but some minor compounds were also detected (**Figure 3A**). Approximately one minute before epicatechin benzylthioether another compound with a similar mass spectrum ($m/z$ 411 with a fragment at $m/z$ 287) was eluted and consequently identified as catechin benzylthioether. A-type linkages are known to resist thiolytic degradation (*1*), resulting in the release of A-type proanthocyanidins (terminal units) and A-type proanthocyanidin benzylthioethers (extension units). As expected from the MS data of lingonberry extract, a dimeric procyanidin with an A-type linkage ($m/z$ 575) and an A-type dimer benzylthioether ($m/z$ 697 with a fragment at 573) were identified as minor terminal and extension units, respectively (**Figure 3A**).

HPLC-MS analyses of grape extracts indicated not only the presence of simple B-type procyanidins but also the presence of heterogenic proanthocyanidins (prodelphinidins) constituted of (epi)catechin and (epi)gallocatechin units with molecular ions at $m/z$ $[289 + 288(n - 1) + 16\ g]$ [$n$ = degree of polymerization, $g$ = number of (epi)gallocatechin units] In addition, molecular ions at $m/z$ $[289 + 288(n - 1) + 152]$ indicated that part of the procyanidins were esterified to gallic acid. Only monogalloylated procyanidins were detected, and galloylation was never observed in proanthocyanidins having a prodelphinidin structure. Thiolysis of unextractable proanthocyanidins in grapes produced catechin, epicatechin, and epicatechin gallate from terminal units and catechin benzylthioether and epicatechin benzylthioether from extension units (**Figure 3B**). Mass spectrometric data additionally indicated that (epi)gallocatechin benzylthioether ($m/z$ 427 with a fragment at $m/z$ 303) and (epi)catechin gallate benzylthioether ($m/z$ 563 with a fragment at $m/z$ 439) were also derived from extension units. The

stereochemistry of these compounds could not be confirmed due to a lack of authentic standards, but evidence from previous studies strongly suggests that these were, actually, derivatives of epigallocatechin and epicatechin gallate (*1, 4*).

Concentrations of extracted proanthocyanidins (**Table 1**) were quantified against the calibration curves derived from B-type procyanidins; that is, the results are given as procyanidin equivalents. All apple cultivars had a similar uncomplicated proanthocyanidin pattern, but the concentrations varied significantly. The highest total concentration was found in 'Red Delicious' (227 mg/100 g of fw) and the lowest in 'Lobo' (43.3 mg/100 g of fw). The proanthocyanidin contents determined in 'Red Delicious' and 'Granny Smith' were in good agreement with previously published results for the same cultivars (*2, 40, 41*). The Finnish cultivar 'Valkeakuulas' had a much higher total proanthocyanidin content in this study than reported earlier (64 mg/100 g of dw) by Kähkönen et al. (*27*). The difference can be explained by a disparity between analytical methods. Kähkönen et al. (*27*) applied reversed phase HPLC for the determination of extracted native flavan-3-ols, and presumably the contents of polymeric proanthocyanidins were underestimated. 'Lobo', another Finnish apple cultivar, was analyzed here for the first time. The proportion of unextractable proanthocyanidins in the apples varied from 4.3% ('Valkeakuulas') to 11% ('Lobo'). Apple peels always had clearly higher concentrations of proanthocyanidins than whole apples, which is consistent with previous studies (*41*−*44*).

Pine bark powder had a remarkably high content of proanthocyanidins (5.5 g/100 g), most of which (>95%) were extracted into the extraction solvent (**Table 1**). It has been reported that proanthocyanidins in aged bark tissues can be largely unextractable (*3*). The pine bark powder used in the present study, however, was prepared from fresh inner bark tissue, in which proanthocyanidins are not so strongly bound to cell wall materials. The majority of extracted proanthocyanidins (56%) in pine bark powder were highly polymerized (DP > 10). The results reported herein are consistent with those published earlier on proanthocyanidins in Scots pine bark (*3*).

Proanthocyanidin content in Saskatoon berry was 276 mg/100 g; over one-third of these compounds were determined to be unextractable (**Table 1**). Hosseinian et al. (*45*) quite recently measured the content of extractable proanthocyanidins in Manitoba berries, including Saskatoon. The result reported therein (369.37 mg/100 g) is somewhat higher than that in the present study. Different cultivars and climatic conditions as well as methodological differences may explain the divergence.

Total proanthocyanidin content in lingonberry was 158 mg/100 g, almost 90% of which was extracted into the extraction solvent (**Table 1**). The extracted proanthocyanidins were predominantly small oligomers with a significant proportion of procyanidins with A-type linkages. Total proanthocyanidins have been determined once before in lingonberry by Kähkönen et al. (*27*). The present results are in agreement with their study, although the concentrations reported herein are slightly higher.

Grapes can be divided into winery and table grapes, and grapes with high proanthocyanidin concentrations are generally not used as table grapes due to their strong astringent taste. Of the two analyzed varieties of table grapes, the red variety, had higher total proanthocyanidin content than the green one (**Table 1**). Most of the proanthocyanidins in the green variety were determined as unextractable with a high average degree of polymerization. Proanthocyanidins in the red variety were more efficiently extracted, but still a considerable proportion was measured as unextractable. However, because the extracted proanthocyanidins were quantified with FLD against a procyanidin standard curve. it is possible that their contents were somewhat underestimated. Proanthocyanidins with (epi)gallocatechin structure and/or gallolyated moieties have only weak fluorescence compared to nongallolyated (epi)catechin polymers (*18*). Artés-Hernández et al. (*46*) determined the contents of proanthocyanidin in several samples of the 'Autumn Seedless' table grape cultivar, obtaining similar results (13.42−27.50 mg/100 g) as in the present study. Cantos et al. (*47*), on the other hand, reported slightly lower proanthocyanidin concentrations (1.83−10.91 mg/100 g) in table grapes and Gu et al. (*2*) somewhat higher concentrations (61.0−81.5 mg/100 g). It is well-known that the proanthocyanidin contents in grapes can vary greatly depending on the cultivars and growing conditions (*5, 47*).

In summary, a procedure for the determination of extractable and unextractable proanthocyanidins has been validated. Normal phase HPLC enables determination of proanthocyanidins as individual groups according to their degree of polymerization. However, the complexity of proanthocyanidins impairs the efficiency of separation, making the qualification and quantification of individual oligomers more difficult. Utilization of HPLC-MS is, therefore, required for a reliable identification of the compounds. Because a part of the proanthocyanidins always remains insoluble, these unextractable proanthocyanidins were determined after thiolytic degradation by reversed phase HPLC. This method also gives further information on the structures of proanthocyanidins in the samples. Unextractable proanthocyanidins contribute to the organoleptic quality of foods and beverages and presumably also to the shelf life of food products, due to their antioxidant ability. A large portion of proanthocyanidins may be unextractable, which has to be reckoned in considering a sample's total proanthocyanidin content. Our results showed that proanthocyanidins in Saskatoon berry, lingonberry, apple, and pine bark powder are essentially procyanidin type, whereas in grape they are mixtures of procyanidins, partly esterified with gallic acid, and prodelphinidins. A remarkably high concentration of proanthocyanidins was determined in the pine bark powder.

## ABBREVIATIONS USED

DM, dry matter; ESI-MS, electrospray ionization mass spectrometry; FW, fresh weight; *m/z*, mass to charge ratio; HPLC, high-performance liquid chromatography; DP, degree of polymerization; P*n*, proanthocyanidin with *n* degree of polymerization; SD, standard deviation.

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