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Isolation and Structure Elucidation of Procyanidin Oligomers from Saskatoon Berries (*Amelanchier alnifolia*)

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Proanthocyanidin oligomers with different degrees of polymerization were isolated from Saskatoon berries (*Amelanchier alnifolia*) by means of gel adsorption and normal-phase liquid chromatography. The proanthocyanidins were identified using electrospray ionization mass spectrometry, nuclear magnetic resonance spectroscopy, and thiolytic degradation coupled with reversed-phase liquid chromatography. The results established that Saskatoon berries contain proanthocyanidins from dimers through heptamers and higher polymers. Saskatoon proanthocyanidins are essentially of procyanidin type, consisting mainly of epicatechin units linked by B-type bonds. The simple procyanidin profile of Saskatoon berries allowed the procyanidins to be separated precisely according to their degrees of polymerization. In the future they can be used as standard compounds for qualitative and quantitative analysis of procyanidins as well as for elucidation of the biological activities of proanthocyanidins.

KEYWORDS: ***Amelanchier alnifolia;*** **Saskatoon berry; ESI-MS; HPLC; NMR; procyanidins; isolation.**

[3]INTRODUCTION

The Saskatoon berry (*Amelanchier alnifolia*) belongs to the family Rosaceae and is native to the North American prairies. It has been grown commercially in Canada since the mid-1960s (*1*), and the current annual production is several million kilograms (*2*). Interest in the commercial cultivation of Saskatoon berries has increased over the past few years, and cultivars have been exported to various countries in Asia and Europe, including Finland. Traditionally Saskatoon berries have been mostly consumed fresh, baked in pies, or processed into jams and spreads, but recent innovations in processing, freezing, and packaging have greatly increased the potential for these berries to be used in industry, e.g., by cereal, snack food, and ice cream processors (*2*). Saskatoon berries were recently shown to possess strong free-radical scavenging activities (*3*). Flavonoids and other polyphenolic compounds are well-known free-radical scavengers and ubiquitous in the plant kingdom. Anthocyanins, phenolic acids, and flavonols were identified in Saskatoon berries earlier (*1*, *3*), but to the best of our knowledge the presence of proanthocyanidins has not been reported thus far.

Proanthocyanidins (condensed tannins) are oligomers and polymers of flavan-3-ol units (**Figure 1**) and widely present in plants, e.g., in fruits, berries, nuts, seeds, and bark of pine (*4*−*7*). Proanthocyanidins can be divided into several classes on the basis of the hydroxylation patterns of their constitutive units and the linkages between them. The most common constitutive units are (epi)catechins and (epi)gallocatechins, indicating procyanidin and prodelphinidin structures, respectively. Flavan-3-ol units are most frequently linked via B-type bonds, i.e., $C_4 \rightarrow C_8$ or $C_4 \rightarrow C_6$ linkages (*8*). Occasionally an additional $C_2 \rightarrow O_7$ or $C_2 \rightarrow O_5$ linkage may exist, leading to doubly bonded A-type proanthocyanidins. Proanthocyanidins are powerful

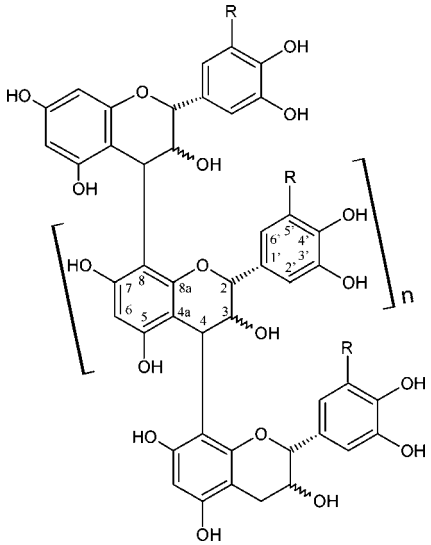

Figure 1. General structure of common B-type proanthocyanidins (procyanidins, R = H; prodelphinidins, R = OH).

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antioxidants, but they have also been reported to demonstrate antibacterial, antiviral, anticarcinogenic, anti-inflammatory, and vasodilatory activities (*9*−*14*). Their physical, chemical, and biological features depend largely on their structure and particularly on their degree of polymerization (*15*−*17*). Quantitative information on the proanthocyanidin profiles in plant products is quite lacking, largely due to the absence of appropriate analytical methodology and commercially available standards for proanthocyanidin oligomers.

The study reported here was undertaken to identify the proanthocyanidins in Saskatoon berries and isolate proanthocyanidin oligomers with different polymerization degrees for future research intents.

MATERIALS AND METHODS

Chemicals. (+)-Catechin was purchased from Cayman Chemical (Cayman Chemical Co., Ann Arbor, MI) and (-)-epicatechin from Sigma (Sigma-Aldrich Chemie Inc., Steinheim, Germany). Procyanidin dimers B1 (epicatechin-(4β→8)-catechin) and B2 (epicatechin-(4β→8)-epicatechin) were obtained from PlantChem (PlantChem Co., Sandnes, Norway) and benzylmercaptan (α-toluolthiol) from Fluka (Fluka Sigma-Aldrich Chemie Inc., Buchs, Switzerland). Deuteriated methanol (CD_3-OD) was from Euriso-Top (Euriso-Top Inc., Saint Aubin, France) and tetramethylsilan from Sigma (Sigma-Aldrich Chemie Inc., Steinheim, Germany). Concentrated hydrochloric acid (37−38%), acetone, methanol, dichloromethane, and acetic acid were purchased from J. T. Baker (Mallinckrodt Baker Inc., Utrecht, Holland). All chromatographic solvents were of HPLC grade.

Plant Material. Ripe Saskatoon berries (*Amelanchier alnifolia*) for the study were kindly provided by Marjakka Co (Niittylahti, Finland). The berries were freeze-dried to facilitate homogenization and prevent enzymatic oxidation of polyphenols.

Isolation of Crude Extract of Proanthocyanidins. Freeze-dried and ground berries (150 g) were homogenized with 80% aqueous methanol (1000 mL), and ultrasound-assisted extraction was used. After 15 min sonication the solution was filtrated, and the sediment was re-extracted three more times with 80% aqueous acetone (1000 mL). All four extracts were combined, and the organic phase was evaporated under vacuum at 35 °C. The aqueous phase (700 mL) was mixed with methanol (300 mL), after which the solution was purified by gel adsorption over Sephadex LH-20 (GE Healthcare Biosciences Inc., Uppsala, Sweden). The gel (50 g) was swollen with 30% aqueous methanol (250 mL) and mixed with the proanthocyanidin solution (250 mL). An excess of 30% methanol (500 mL) was added by vigorous mixing to wash off sugars, phenolic acids, and other impurities. The gel was filtered and recovered, and the washing step was repeated three more times. The proanthocyanidins were released from the gel with 80% aqueous acetone (500 mL) and concentrated under vacuum at 35 °C. The aqueous aliquot was freeze-dried, yielding 3.1 g of crude proanthocyanidin preparation.

Separation of Proanthocyanidin Oligomers. Normal-phase semipreparative HPLC was conducted on an Agilent 1100 liquid chromatograph (Agilent Technologies Inc., Santa Clara, CA) fitted with a 25 × 2.5 cm i.d. 7-μm Tessek Separon SGX (unmodified silica) column (Tessek Ltd., Praha, Czech Republic). The freeze-dried proanthocyanidin preparation was dissolved in 15 mL of methanol prior to HPLC. Previous studies have shown that proanthocyanidins can be separated on a molecular mass basis on a silica column with a mobile phase consisting of dichloromethane/methanol/organic acid/water (*18*−*19*). However, in the present study we used a neutral mobile phase to eliminate the possibility of acid-catalyzed degradation of proanthocyanidins during the subsequent concentration step. The mobile phase consisted of the following: A, dichloromethane/methanol/water (42:7:1, v/v/v); B, dichloromethane/methanol/water (5:44:1, v/v/v). Elution was started with 100% A, followed by 0−13.5% B, 0−75 min; 13.5−29.2% B, 75−235 min; 29.2−100% B, 235−245 min; 100% B, 245−270 min. The injection volume was 5 mL, and the flow rate was 8 mL/min. Fractions of 1.8 min size were collected using a RediFrac fraction collector (GE Healthcare Biosciences Inc., Uppsala, Sweden).

The crude proanthocyanidin extract and fractions obtained by semipreparative HPLC were analyzed by the method of Gu et al. (*19*) with minor modifications. The HPLC equipment consisted of an Agilent 1100 liquid chromatograph (Agilent Technologies Inc., Santa Clara, CA) system equipped with diode array and fluorescence detectors. Normal-phase HPLC with a 250 × 4.6 mm i.d. 5-μm Silica Luna column (Phenomenex Inc., Darmstadt, Germany) was used with the column temperature set at 35 °C. The injection volume was 10 μL. The mobile phase consisted of A, dichloromethane/methanol/water/acetic acid (41:7:1:1, v/v/v), and B, dichloromethane/methanol/water/acetic acid (5:43:1:1, v/v/v). Elution was started with 100% A, followed by 0−13.5% B, 0−20 min; 13.5−29.2% B, 20−50 min; 29.2−100% B, 50−55 min; 100% B, 55−60 min. Separation was monitored using both UV (λ = 280 nm) and fluorescence detection (λ_{ex} = 280 nm, λ_{em} = 323 nm). Fractions showing identical HPLC separation were combined, concentrated, and freeze-dried.

Identity and Purity of Isolates. The identity and purity of the isolated proanthocyanidin extracts were confirmed by ultraviolet (UV) spectroscopy, electrospray ionization tandem mass (ESI-MS/MS) spectrometry, ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectrometry, and thiolytic degradation. UV spectra were measured of samples dissolved in methanol (7−30 μg/mL) with a Perkin-Elmer Lambda 25 UV/vis spectrometer (Perkin-Elmer Inc., Beaconsfield, U.K.). ESI-MS and ESI-MS/MS were conducted with a Finnigan MAT Iontrap Mass Spectrometer (Thermo Electron Corp., Waltham, MA). The freeze-dried samples were dissolved in methanol (2 mg/mL) and analyzed by infusion with a syringe pump at a flow rate of 5−10 μL/min in the negative or positive ionization mode under full scan (*m/z* 200−2000 Da). The ESI operating conditions were as follows: spray voltage at 4.5 kV, capillary temperature at 270 °C, and a sheath gas (N_2) at a flow rate of 10 U (arbitrary units). Capillary voltage was set at −37 V and +37 V in negative and positive ionization mode, respectively. Helium was used as the collision gas for the MS/MS analyses, and the collision energy level was set at 50%. For each spectrum 30−50 repetitive scans of 1.5- (MS) or 2-s (MS/MS) duration were averaged.

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of the samples (30−50 mg) were recorded on a Bruker Avance 400 spectrometer (Bruker BioSpin Inc., Fällanden, Switzerland) equipped with a BBO-5 mm-Zgrad probe with CD_3OD as the solvent (0.5 mL) and tetramethylsilane (TMS) as the internal standard (0.00 ppm). ^{1}H and ^{13}C NMR spectra were recorded at 399.75 and 100.53 MHz, respectively. In addition to basic proton and carbon spectra, 2D techniques such as DQF-COSY and HSQC were also utilized. These spectra were obtained by standard gradient-enhanced pulse programs originally installed by Bruker. NMR analyses were performed at various temperatures (from −60 to +50 °C) to prevent the signal from broadening due to hindered rotation of the interflavanoid bonds.

Thioacidolysis was based on the method described by Guyot et al. (*20*). Briefly, a freeze-dried sample was dissolved in methanol and mixed with a 5% solution of benzylmercaptan in methanol containing 1.1% HCl. The vials were sealed and incubated at 40 °C for 30 min and then cooled in an ice bath. Reversed-phase HPLC analyses were performed with an Agilent 1100 liquid chromatograph (Agilent Technologies Inc., Santa Clara, CA) system equipped with a diode array detector and a 150 mm × 4.0 mm i.d. 3-μm Inertsil ODS-3 column (GL Sciences Inc., Torrance, CA). The HPLC conditions (column temperature, injection volume, flow rate, gradient elution) employed earlier in our laboratory for the flavonoid analyses (*21*) were used for the separation of flavan-3-ols and their counterpart thiol adducts. Separation was monitored by UV (λ = 280 nm) and fluorescence detection (λ_{ex} = 280 nm, λ_{em} = 323 nm). Procyanidin dimers B1 and B2 were used as standards for the quantification of epicatechin benzylthioethers after thioacidolysis. Epicatechin and catechin were quantified using external standards of the authentic compounds.

RESULTS AND DISCUSSION

Freeze-dried berries were used in this study since freeze-drying has been generally considered to be the best way to keep the chemical composition as close as possible to the natural

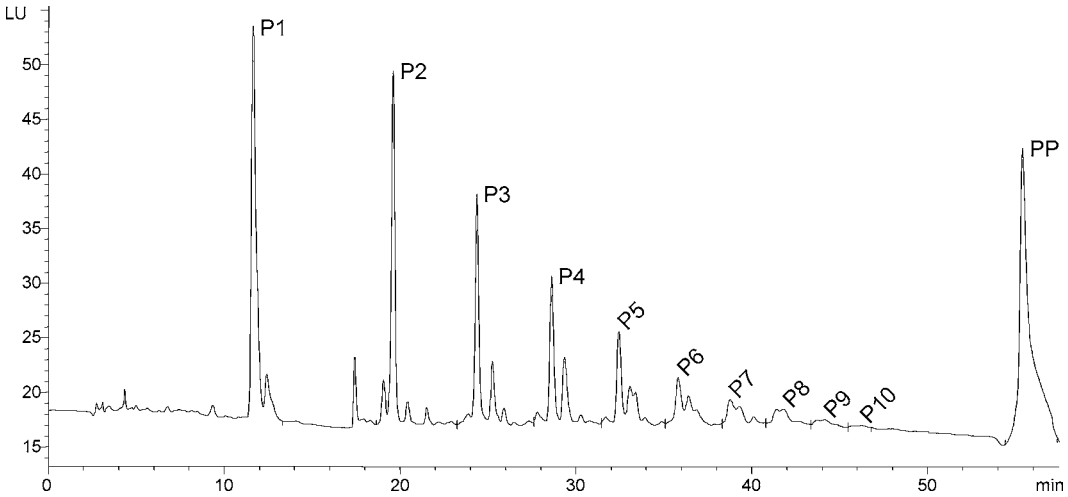

Figure 2. Normal-phase HPLC-FLD trace of proanthocyanidins from Saskatoon berries. Labels P1–P10 indicate the degrees of polymerization (DP) of proanthocyanidins in the peaks. Polymeric proanthocyanidins (PP) with DP > 10 appear as a single peak at the end of the chromatogram. LU, luminescence units.

state. Previous reports have shown that the effect of freeze-drying on the proanthocyanidins is minimal (*22*−*24*). The analytical HPLC profile of the Saskatoon berry extract indicated the presence of proanthocyanidins with different degrees of polymerization (**Figure 2**). The good resolution between each oligomeric class suggested that the Saskatoon proanthocyanidins are quite homogeneous, showing only minor differences in their constituent units and the linkages between them. Proanthocyanidins were isolated from the Saskatoon extract by gel adsorption over Sephadex LH-20 and then fractionated by semipreparative normal-phase HPLC. The procedure allowed the precise separation of proanthocyanidins from dimers (denoted as P2) to heptamers (P7), as could be observed from the spectroscopic and spectrometric data (UV, MS, NMR) of the isolated fractions. HPLC performance of each fraction implied that one isomeric form dominates over each oligomeric class, as could already be seen in the chromatogram of the Saskatoon extract (**Figure 2**). However, few additional peaks detected indicated the presence of some minor isomers as well. Polymeric proanthocyanidins do not resolve by normal-phase HPLC (*19*), and so they were collected as a single fraction (PP) at the end of the analysis. Monomeric flavan-3-ols (P1) were not collected, but two peaks shown in the chromatogram (**Figure 2**) were tentatively identified as (−)-epicatechin (the major form) and (+)-catechin by comparing the retention times and on-line UV spectra with the authentic compounds. For each collected proanthocyanidin fraction the measured UV spectrum was similar, having a pronounced and symmetrical maximum at 280 nm with no band broadening beyond 300 nm (**Figure 3**). This kind of spectrum is typical for homogeneous procyanidin structures (*25*).

ESI-MS and ESI-MS/MS were performed in the negative ionization mode for all fractions since proanthocyanidins are thereby better detected due to the acidity of the phenolic protons (*26*−*27*). ESI-MS full-scan analyses exhibited ions at *m*/*z* 577, 865, 1153, 1441, and 1729 for fractions P2, P3, P4, P5, and P6, respectively, corresponding to the deprotonated molecular ions $[M - 1]^-$ of procyanidin oligomers from dimer to hexamer. For higher oligomers, the molecular ions could not be detected due to the limited scanning range (200−2000 Da) of the mass spectrometer used. However, the most abundant ion of the spectrum for fraction P7 was at *m*/*z* 1008.6 and presumably created by the double-charged ion of heptameric procyanidin. The mass spectrum of fraction P2 exhibited a relatively strong

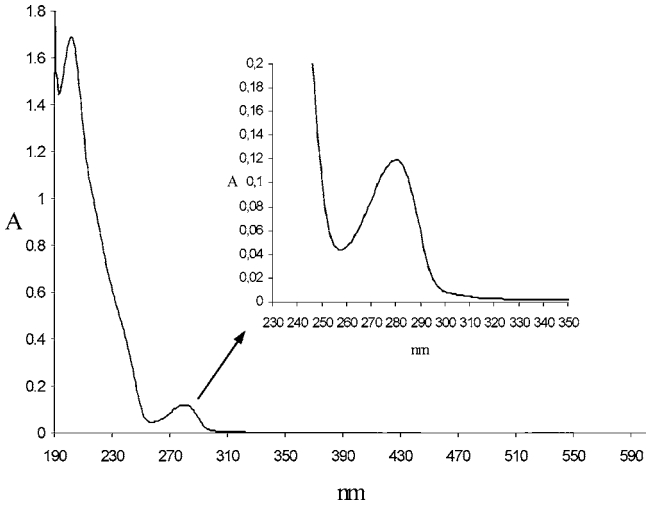

Figure 3. UV spectrum of the fraction P6 dissolved in methanol (7 μg/mL).

signal at *m*/*z* 666 which could not be assigned and was assumed to be produced by some unidentified impurity in the fraction. However, the mass spectrometric data and HPLC separations before and after thioacidolysis suggest that fraction P2 did not contain proanthocyanidins higher than dimers (**Figure 4**). MS/MS spectra of deprotonated molecular ions gave several product ions characteristic to procyanidins (**Table 1**). The main fragmentation pathways have been described before (*28*−*30*), and they are shown in **Figure 5**. Procyanidin sequence ions $[M - 288n - 1]^-$ were largely observed, especially in the MS/MS spectra of higher oligomers (**Table 1**). There are two possible mechanisms for production of these ions: first, direct cleavage of the interflavanoid bonds, and second, quinone methide (QM) cleavage of the interflavanoid bonds (*29*, *30*). The latter mechanism also explains the presence of fragment ions $[M - 288n - 3]^-$ in the MS/MS spectra of the fractions. Retro Diels−Alder (RDA) fission of the heterocyclic ring system of the flavan-3-ol subunits and the fragmentation mechanism designated by Gu et al. (*30*) as heterocyclic ring fission (HRF) are distinctive for fragmentation of proanthocyanidins (*28*−*30*). The RDA product ions $[M - 288n - 153]^-$ and the HRF product ions $[M - 288n - 127]^-$ detected in the samples (**Table 1**) are characteristic for the homogeneous (epi)catechin polymers. RDA products of the top unit were the most detected fragment ions

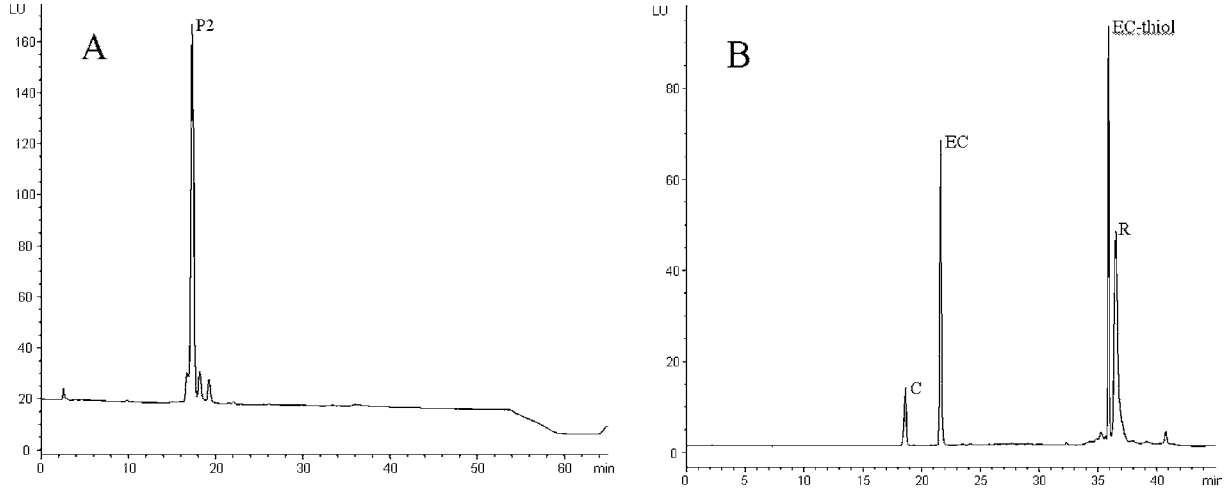

Figure 4. (A) Normal-phase HPLC chromatogram of the P2 fraction and (B) reversed-phase HPLC chromatogram of the P2 fraction after thiolytic degradation. P2 = dimeric procyanidin; C = catechin; EC = epicatechin; EC-thiol = epicatechin benzylthioether; R = thiolytic reagent (benzylmercaptan). LU, luminescence units.

for dimeric and trimeric procyanidins, but for the higher oligomers fragments generated through HRF or cleavage of the interflavanoid bonds were more abundant (**Table 1**). Several additional fragments were usually also observed, e.g., nonspecific elimination of water was common leading to loss of 18 Da of the ions. Generally the fragmentation pattern of isolated proanthocyanidins was more complicated with the increase in the degree of polymerization, but the characteristic product ions were always detectable. The MS/MS spectrum of hexameric procyanidin (P6) is shown in **Figure 6** as a representative example. For P6 the most abundant fragments originated from cleavage of interflavanoid bonds, giving product ions at *m*/*z* 1441, 1423 (after water elimination), 1153, 1135 (after water elimination), 865, 863 (QM), and 575 (QM). RDA fission of the top unit produced the ions at *m*/*z* 1577 and 1559 (after water elimination). Fragment ions shown in the spectrum at *m*/*z* 1603, 1315, 1027, and 739 (**Figure 6**) correspond to the HRF products of procyanidins. All MS and MS/MS data received suggest that the fractions (P2−P7) isolated from Saskatoon berries contained B-type procyanidin oligomers from dimers to heptamers.

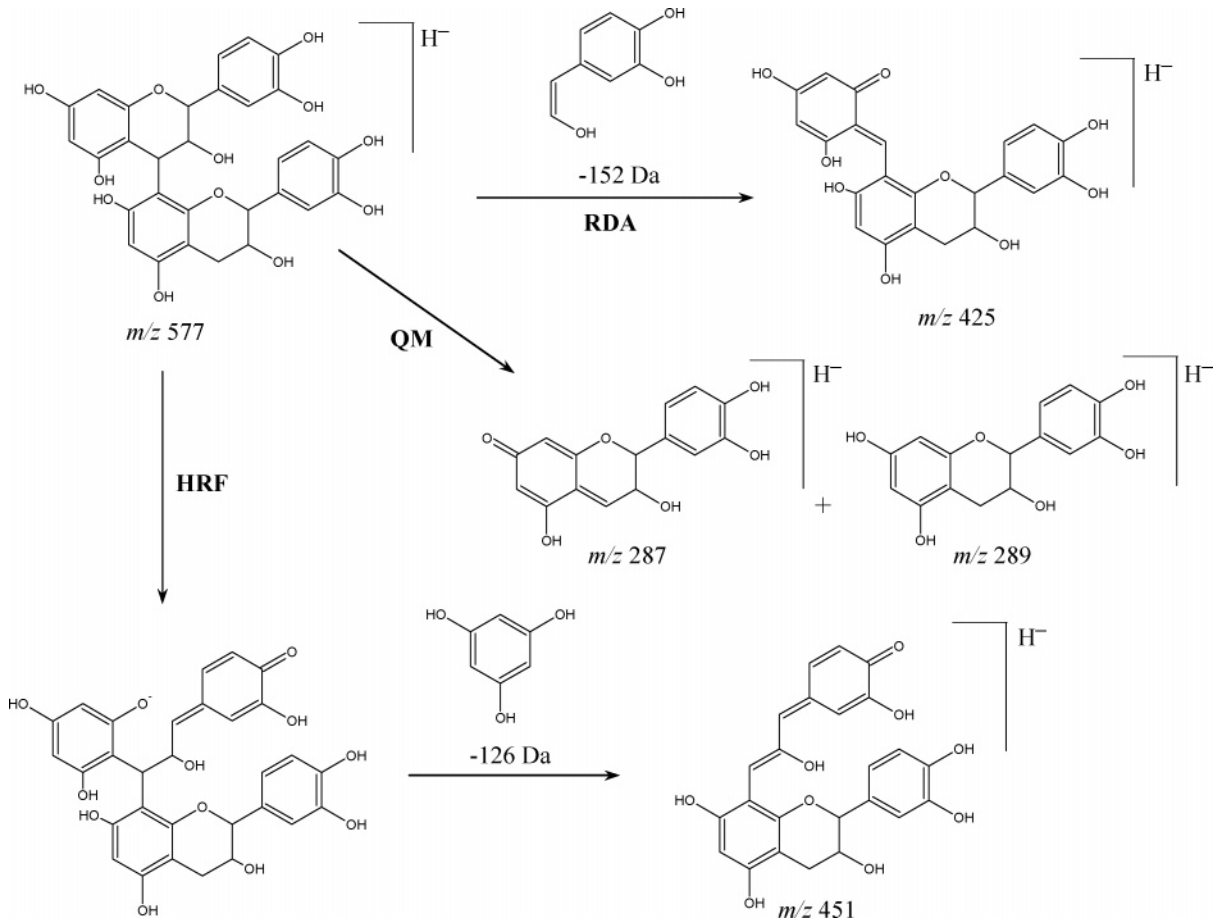

Figure 5. Main fragmentation pathways of dimeric procyanidin as an example of fragmentation of Saskatoon proanthocyanidins (adapted and modified from source (*30*)). The fragment mechanisms are RDA (retro-Diels–Alder), HRF (heterocyclic ring fission), and QM (quinone methide).

Table 1. Molecular Ions and Characteristic Fragment Ions Determined in the Mass Spectra of the Isolated Proanthocyanidins

sample	molecular ions (*m*/*z*)	fragment ions (*m*/*z*)
P2	577	451, 425,[a] 407, 289, 287
P3	865	739, 713,[a] 695, 577, 575, 289
P4	1153	1027,[a] 1001, 983, 865, 863, 739, 577
P5	1441	1315,[a] 1289, 1271, 1153, 1151, 1027, 865, 863, 575
P6	1729	1603, 1577, 1559, 1441, 1315, 1153,[a] 1135, 1027, 865, 863, 739, 575
P7	1009[b]	1729, 1441, 1151, 863,[a] 575

[a] Most abundant fragment ion. [b] Double-charged molecular ion.

Since the NMR studies indicated the presence of some anthocyanin-type impurity in fraction P3 but the previous MS experiments gave no evidence of it, fraction P3 was also analyzed in positive ionization mode which should fit better for anthocyanins due to their tendency to form flavylium cations (*31*). With the positive mode the molecular ion of trimeric procyanidin at *m*/*z* 867 $[M + 1]^+$ was discovered as expected, together with two other major ions at *m*/*z* 449 and 287. MS/MS analyses of the parent ion *m*/*z* 449 produced only one fragment ion at *m*/*z* 287 corresponding to the cyanidin aglycone and showing loss of *m*/*z* 162 corresponding to the loss of hexoside. These results suggest that fraction P3 was contaminated by cyanidinmonohexoside. This is in agreement with the previous studies which identified two cyanidinmonohexosides, cyanidin-3-galactoside and cyanidin-3-glucoside, as the major anthocyanins in Saskatoon berries (*1*, *3*).

Thiolytic degradation of Saskatoon proanthocyanidins released catechin and epicatechin as the only free flavan-3-ols and epicatechin benzylthioether as the only flavan-3-ol adduct, suggesting that these proanthocyanidins essentially consist of (−)-epicatechin units with (+)-catechin present only in a low proportion as a terminal unit. It is well known that under thiolytic conditions part of the (−)-epicatechin can be transformed to (−)-catechin by epimerization (*19*, *32*−*33*). (−)-Catechin could not be separated from (+)-catechin in the current HPLC system, and so part of the (+)-catechin peak area in the chromatogram may correspond to (−)-catechin. The degree of epimerization was estimated through thioacidolysis of procyanidin B2. Conversion of (−)-epicatechin into (−)-catechin was 5.5% analyzed immediately after 30 min incubation at 40 °C, increasing steadily and reaching 15% after incubation in the HPLC vial at ambient temperature for 35 h. Assuming that the proanthocyanidins in the isolated fractions show similar behavior, we conclude that the proportion of (+)-catechin is less than 2% of the terminal units of the procyanidins. By the results of thioacidolysis it was also evident that the fractions from P4 to PP were almost solely procyanidins with no other compounds involved. Procyanidins comprised also over 90% of fraction P3, but fraction P2 was clearly contaminated, containing less than 50% of procyanidins, and thus omitted from NMR studies. The average degree of polymerization (DP) for each fraction was obtained by calculating the molar ratio of all flavan-3-ol units (extension and terminal) to the sum of the terminal units. Molar amounts of the units were calculated using the calibration curves generated by dimeric procyanidins, which release equal molar amounts of terminal units and extension units (as benzylthioethers) under thiolytic conditions. The calculated average DPs (mean ± SD, $n = 3$) were 2.0 ± 0.03, 3.0 ± 0.04, 3.9 ± 0.10, 4.8 ± 0.08, 5.8 ± 0.08, 6.7 ± 0.08, and 19 ± 0.7 for fractions P2, P3, P4, P5, P6, P7, and PP, respectively. This was in good agreement with the mass spectrometric data.

The fractions from P3 to PP were further studied by NMR spectroscopy. A typical feature of proanthocyanidins is the remarkable signal broadening due to restricted rotation of the interflavan bonds (*34*−*36*). There are two common procedures to overcome this problem in an NMR study of proanthocyanidins. The first is acetylation of all hydroxyl groups of the molecule, which has been found to halt rotation (*35*). The second option is to measure the spectra at variable temperatures (*36*). As the temperature is lowered, rotation can be slowed down, resulting in sharper signals. Similarly, if the temperature is raised, rotation can be accelerated and sharper averaged signals observed. Since we wanted to save our isolated oligomers for

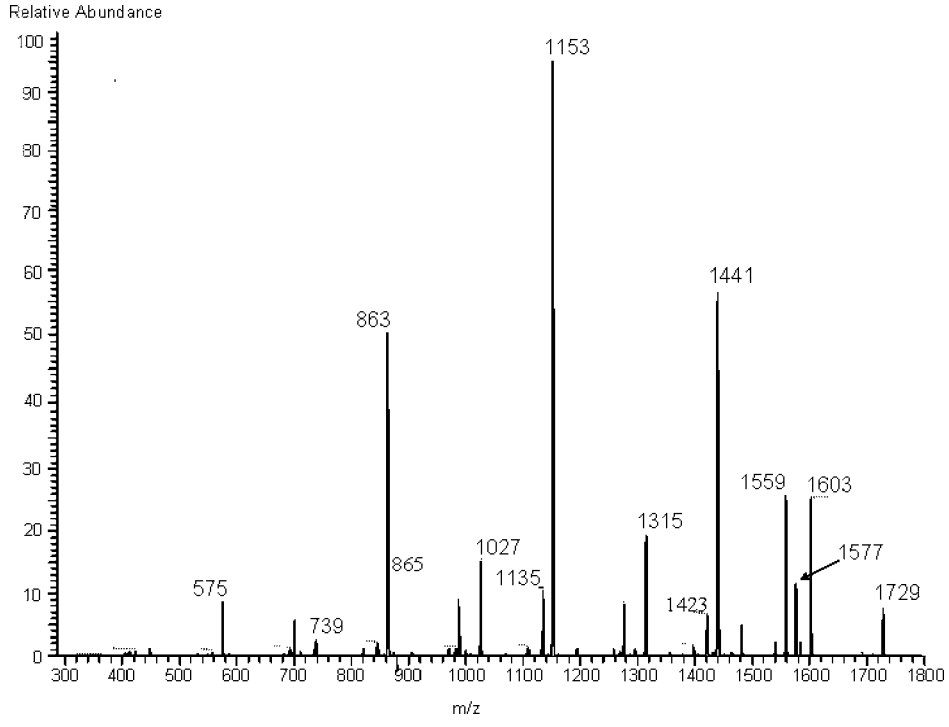

Figure 6. Product ion spectrum of hexameric procyanidin isolated from Saskatoon.

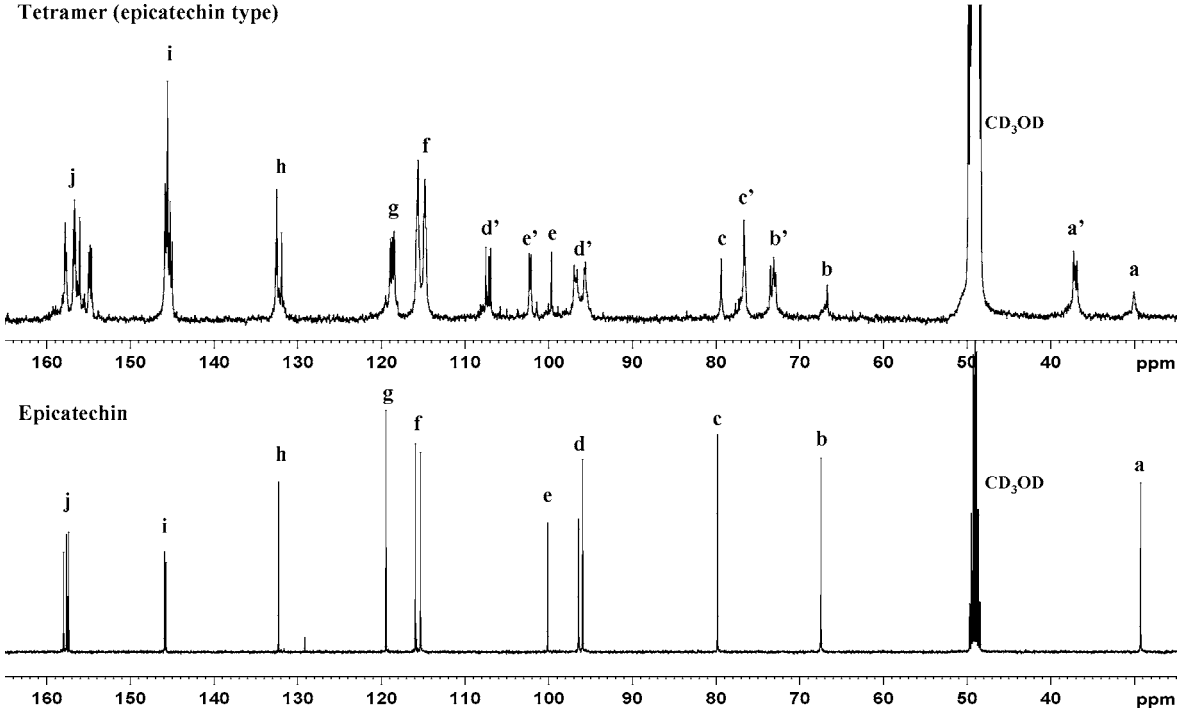

Figure 7. ^{13}C NMR spectra for epicatechin (standard sample) at 25 °C and tetramer at −40 °C in methanol-d_6 (referenced for TMS at 0.00 ppm). Assignments (abbreviations m = monomer, i.e., epicatechin, t = terminal unit, e = extension unit): a, C-4 (m,t); a′, C-4 (e); b, C-3 (m,t); b′, C-3 (e); c, C-2 (m,t); c′, C-2 (e); d, C-6 and C-8 (m); d′, at 96–98 ppm C-6 (e,t) and C-8 (from the last extension unit), at 107–108 ppm C-8 (t and from the first two extension units); e, C-4a (m,t); e′, C-4a (e); f, C-2′ and C-5′ (m,t,e); g, C-6′ (m,t,e); h, C-1′ (m,t,e); i, C-3′ and C-4′ (m,t,e); j, C-5, C-7, and C-8a (m,t,e).

future studies, we did not acetylate them but measured NMR at low and high temperatures in CD_3OD solutions.

At room temperature all compounds from P3 to P7 showed broadened signals as expected. When the temperature was lowered the signals sharpened at first, and optimal sharpness was obtained at approximately −40 °C. Then, as the temperature was decreased further, the signals again began to broaden. This was most likely due to some other dynamic processes which slow down on an NMR time scale, such as rotation of the aryl groups. A temperature increase from room temperature also sharpened the signals. Relatively sharp signals were observed at 50 °C. We did not try higher temperatures than this since we wanted to avoid decomposition of the compounds.

NMR studies of the fractions from P3 to PP indicated the presence of procyanidin structures, mainly consisting of epicatechin units. A typical ^{13}C NMR spectrum of an oligomeric epicatechin-type procyanidin (P4) is presented in **Figure 7**. Although each signal of the spectrum is not assignable individually, it is possible to assign all signals in clusters. The same assignment is applied for all the samples from P3 to PP. The spectrum of epicatechin is shown in **Figure 7** merely for the sake of clarity as it nicely demonstrates how the signals for terminal and extension units differ from each other and how they compare with those for epicatechin. The procyanidin type can be identified based on the signals from the aromatic region. For procyanidins, the nonquaternary carbon signals from the aryl group, i.e., those signals that are visible on the HSQC spectrum, were found at 115−116 (C-2′ and C-5′) and 119−120 ppm (C-6′) (**Figure 7**). Only these were observed for the nonquaternary carbons of the aryl groups in all samples, indicating the presence of procyanidin-type proanthocyanidins. For example, for prodelphinidins the galloyl group produces CH-type signals symmetrically at 107−108 ppm (*37*). Epicatechin units can be identified based on the low (<5 Hz) values of the *J*-coupling constants for protons H-2, H-3, and H-4 (in extension units). Therefore, in the proton spectra of the oligomer samples all signals for H-2, H-3, and H-4 (extension units) are broadened singlets. For catechin units, for example, $J_{H-2,H-3}$ is approximately 8 Hz and the signal for H-2 would be clearly duplet. (The above values of the coupling constants are based on the spectra measured from catechin and epicatechin standard samples.) Thus, the NMR results are well in accordance with the results obtained by thiolysis; that is, they indicate the presence of epicatechin-type procyanidins.

Signals implying the presence of compounds other than procyanidins were not observed, except in the NMR spectra for fraction P3. There the ^{1}H and ^{13}C spectra gave a set of impurity signals. The sugar moiety could be easily identified from these signals. However, the signals for impurity did not correspond to those for procyanidin glycoside found previously in rose hips (*38*). The impurity compound was tentatively identified as anthocyanidin glycoside.

The spectroscopic data for P3 and P4 are well in agreement with the data for structures with the interflavanoid bond 4β→8 reported by Shoji et al. (*36*). Differences in the NMR results for 4β→8- and 4β→6-type oligomeric procyanidins are very small. The position of the interflavan bond can, in principle, be determined by HMBC data, but this requires first assigning ^{1}H and ^{13}C signals to each monomeric unit. It can be done for trimer (*36*) but not for longer oligomers due to overlapping of several signals. Nevertheless, based on the results of Shoji et al. (*36*), there is a minor observable difference in the ^{1}H chemical shifts between 4β→8- and 4β→6-type trimeric procyanidins. In the terminal unit, the H-8 signal for 4β→6 type resonates at 6.1 ppm whereas all other H-6 and H-8 signals for both types resonate at 5.9−6.0 ppm (*36*). On the basis of this small shift effect, all our oligomers can be tentatively deduced to be mainly of 4β→8 type since the overlapping broadened signal containing

Table 2. Degrees of the Polymerization Determined from NMR Measurements by Integrating H-2′, H-5′, and H-6′ Proton Signals (for comparison, results from the integrals of C-2 carbon signals (the most similar magnetic environment) are also given)

sample	^{1}H (–40 °C)	^{1}H (50 °C)	^{13}C (–40 °C)	^{13}C(50 °C)
P3	3.1	3.3	3.1	3.0
P4	4.1	4.1	4.5	4.2
P5	5.5	5.3	5.7	6.0
P6	6.6	6.3	7.6	6.1
P7	7.5	7.8	8.1	7.3
PP	25.5 (at 25 °C)		26.0 (at 25 °C)	

all the H-6 and H-8 protons was, in the case of each oligomer, limited to the region 5.9−6.0 ppm.

The average polymerization degrees of the isolated proanthocyanidins were calculated on the basis of the aromatic ^{1}H signals (at approximately 6.5−7.2 ppm) induced by H-2′, H-5′, and H-6′. In integration, a value of 2 was set as a reference for the signal (at approximately 2.7−3.0 ppm) for H-4 protons from the terminal unit. The integral of these protons should be three times the DP of procyanidin. Consequently, the calculated DPs were 3.1 (3.3), 4.1 (4.1), 5.5 (5.3), 6.6 (6.3), and 7.5 (7.8) for fractions P3, P4, P5, P6, and P7 at −40 °C (+50 °C), respectively (**Table 2**). Integration of the carbon signals for C-2 and C-3 from the terminal and extension units gave quite similar DP values (**Table 2**). However, these values were slightly higher than expected by the results of thiolysis and mass spectrometry. We also determined the average DP for the polymeric sample PP by NMR at room temperature from the proton and carbon integrals. The problem in determining the DP for long polymers is that the signals for the terminal units are very small and their integration is suggestive rather than exact (*39*). Integration of H-2′, H-5′, and H-6′ (referenced to the signal for H-4 from the terminal unit) gave a DP of 25.5, and integration of C-2 signals from the terminal and extension units gave a value of 26.0 (**Table 2**). These are rather well in accordance with the values obtained by thiolysis.

To summarize, we are the first to report the isolation and identification of proanthocyanidins in the Saskatoon berry (*Amelanchier alnifolia*). This study demonstrates that Saskatoon proanthocyanidins are essentially of procyanidin type and consist mainly of epicatechin units with B-type linkages. A similar uncomplicated procyanidin distribution has previously been found in cocoa (*Theobroma cacao*) and apple (*Malus domestica*) (*40*, *41*). The simple procyanidin profile of Saskatoon berries allows for precise separation of procyanidins according to their degrees of polymerization by means of normal-phase chromatography, thereby generating highly pure procyanidin oligomers. They can be utilized in the future as standard compounds for qualitative and quantitative analysis of proanthocyanidins as well as for elucidation of their biological activities.

ABBREVIATIONS USED

DQF-COSY, double-quantum filtered-correlation spectroscopy; HSQC, heteronuclear single quantum correlation; HMBC, heteronuclear multiple bond correlation; DP, degree of polymerization; P*n*, proanthocyanidin with *n* degree of polymerization.

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