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Effective Separation of Potent Antiproliferation and Antiadhesion Components from Wild Blueberry (*Vaccinium angustifolium* Ait.) Fruits

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Extracts from wild blueberry (*Vaccinium angustifolium* Ait.) were separated into proanthocyanidin-rich fractions using liquid vacuum and open column chromatography on Toyopearl and Sephadex LH-20, respectively. Fractions were characterized using analytical tools including mass spectrometry and NMR spectroscopy; fraction composition was correlated with bioactivity using antiproliferation and antiadhesion in vitro assays. There was a significant positive correlation between proanthocyanidin content of different fractions and biological activity in both the antiproliferation and antiadhesion assays. Two fractions containing primarily 4→8-linked oligomeric proanthocyanidins with average degrees of polymerization (DPn) of 3.25 and 5.65 inhibited adhesion of *Escherichia coli* responsible for urinary tract infections. Only the fraction with a DPn of 5.65 had significant antiproliferation activity against human prostate and mouse liver cancer cell lines. These findings suggest both antiadhesion and antiproliferation activity are associated with high molecular weight proanthocyanidin oligomers found in wild blueberry fruits.

KEYWORDS: ***Vaccinium angustifolium* Ait.; blueberry; antiproliferation; antiadhesion; proanthocyanidin; condensed tannins**

Wild blueberries (*Vaccinium angustifolium* Ait.) and related *Vaccinium* species are rich sources of dietary flavonoids including anthocyanins and proanthocyanidins (condensed tannins) (*1*−*3*). *Vaccinium* fruits are recognized for their high antioxidant capacities that rival those of ascorbic acid (vitamin C) and butylated hydroxytoluene (BHT) (*4*). Consuming foods high in antioxidants may decrease the risk of cardiovascular disease and various forms of cancer (*5*). Recent research has suggested a range of other biological mechanisms in addition to antioxidant properties that flavonoids provide to the human consumer. Manthey (*6*) found that flavonoids decrease inflammatory responses by reducing prostaglandin production through enzyme inhibition including cyclooxygenase (COX), lipoxygenase (LOX), and phospholipase. Inflammation is a contributing factor in heart disease and cancer. In a study by Lindenmeyer et al. (*7*), the flavonoid apigenin was found to reduce protease-dependent invasiveness of tumor cell lines, reducing proliferation and other associated processes. Flavonoids in *Gingko biloba* have been shown to be nitric oxide (NO) production regulators. They reduce overproduction of NO in monocytes and macrophages by inhibiting inducible nitric oxide synthase (iNOS) and scavenging excess NO (*8*). *G. biloba* extracts and the flavonoid quercetin suppressed in vivo production of NO when laboratory animals were challenged with administration of bacterial lipopolysaccharide (LPS) (*8*).

Flavonoids in wild blueberries are chemoprotective enzyme modulators as shown by Smith et al. (*3*) and Bomser et al. (*9*, *10*). Research suggests flavonoids have a role in neuroprotection, reducing the incidence of age-related neuronal−behavioral decrements (*11*) and potentially reducing the risk of Alzheimer's disease (*12*). Blueberries have also been shown to reduce in vitro P-fimbriated *E. coli* adhesion of bacterial strains responsible for urinary tract infections (*13*).

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Proanthocyanidins, commonly called condensed tannins, have been a subject of recent studies evaluating the health benefits of *Vaccinium* species (*2*, *3*, *14*−*16*). Proanthocyanidins are highly concentrated in wild blueberries (*3*, *9*) and other *Vaccinium* fruits (*2*, *14*, *17*). These multiunit flavan-3-ols are far more difficult to isolate and characterize than simple polyphenols due to their complex nature. Proanthocyanidins are made up of catechin or epicatechin monomeric units with either A-type or B-type linkages (*18*, *19*). The type of linkage seems to have an effect on bioactivity (*15*, *20*). Additionally, the degree of polymerization may determine the type and level of bioactivity. Lower molecular weight proanthocyanidins (dimers and trimers) had mild activity, and higher molecular weight proanthocyanidins (tetramers through higher polymers) were more potent in bacterial antiadhesion assays (*16*, *20*, *21*).

Cancer and infection are serious health concerns worldwide that may be partially preventable by consuming a diet rich in fruits and vegetables. This study was undertaken to determine the role of proanthocyanidins in the bioactivity of blueberries, to establish a foundation for future studies on the in vivo activity of wild blueberry proanthocyanidins, and to provide methods for the isolation of high molecular weight proanthocyanidins from berries and other plant materials.

MATERIALS AND METHODS

Plant Material. Whole individually quick-frozen wild blueberry fruits were obtained from the Wild Blueberry Association of North America (WBANA), Bar Harbor, ME. The blueberries were a composite of fruit from different growing regions including Prince Edward Island, New Brunswick, Nova Scotia, and Maine. The composite was made in fall 2001, frozen by Cherryfield Foods, Inc. (Cherryfield, ME), and stored at −15 °C. The fruit was shipped frozen on dry ice to the University of Illinois at Urbana−Champaign, where it was stored at −80 °C until use.

Preparation of Crude Extract. Frozen wild blueberries (1.0 kg) were homogenized in a Waring blender with 1000 mL of 70% acetone for 5 min. The resulting slurry was filtered through multiple layers of cheesecloth. The pulp and seeds were rehomogenized with 500 mL of 70% acetone and filtered through cheesecloth. This procedure was repeated one more time, removing all purple pigment from the solids. The crude extract was concentrated by rotary evaporation at 40 °C until all acetone was removed, ∼2 h. A portion of the crude extract was lyophilized and reserved for bioactivity testing. The remaining extract was frozen at −20 °C until fractionation was performed.

Fractionation Procedures. Crude extract (250 mL) was placed on a 160 mm × 70 mm column of 75 g Toyopearl HW 40F (Tosohaas, Bioseparation Specialists, Montgomeryville, PA) (bed volume of approximately 3.1×10^5 mm^3) and fractionated by vacuum (40 psi) liquid chromatography using a series of solvents as follows: fraction 1, 500 mL of water; fraction 2, 500 mL of 50% aqueous methanol; fraction 3, 500 mL of methanol; fraction 4, 500 mL of acetone; and fraction 5, 500 mL of 50% aqueous acetone. After the fifth fraction, all colored material was removed from the column. Analysis of solvents (aqueous acetone and aqueous ethanol) used to clean the column after fraction 5 by thin-layer chromatography (TLC) using vanillin−HCl and dichromate spray reagents plus UV analysis showed no compounds similar to fractions 2−5. Fractions 2−5 were reduced in volume by rotary evaporation at 40 °C. Fraction 1 was discarded as waste. After fractions were concentrated, they were lyophilized and stored at −20 °C until bioassay.

On the basis of bioassay results and preliminary NMR, fraction 5 (F5) was selected for subfractionation. High-performance liquid chromatography (HPLC) using a Hewlett-Packard 1090 instrument with diode array detection (DAD) and a C18 column was first employed to separate proanthocyanidins. Following published protocols for HPLC separation of proanthocyanidins (*22*, *23*), acetonitrile and water (20:80 v/v) were initially used as the mobile phase with a flow rate of 0.5 mL/min. Numerous method variations were attempted including different mobile phase solvents (methanol, acetone, acidified water), flow rates (1.0, 0.75, 0.25 mL/min), and run times (5−85 min).

In addition, F5 was subfractionated using open column chromatography on a 150 mm × 18 mm Sephadex LH20 column (bed volume of 1.9×10^4 mm^3). A wash of 35% ethanol was used to prime the column. F5 (250 mg) was dissolved in 35% ethanol and poured onto the column. The first subfraction (F5.1) was eluted with 100 mL of 35% ethanol. The second subfraction (F5.2) was eluted with 100 mL of 70% acetone, removing all colored material from the column. Both subfractions were reduced in volume using a rotary evaporator at 40 °C, lyophilized, and stored at −20 °C until bioassay and identification procedures were performed.

TLC of Blueberry Fractions. TLC was performed on all fractions and subfractions, using 200 μm thick, 2−25 μm, 60 Å aluminum backed silica gel plates (Sigma-Aldrich, Steinheim, Germany) developed with a solvent ratio of ethyl acetate/MeOH/water (79:11:10). The chromatograms were visualized separately with two spray reagents (vanillin−HCl and dichromate cleaning solution) followed by heating at 100 °C for 10 min. These sprays allow for visual monitoring of proanthocyanidins and sugars in fractions.

Vanillin Proanthocyanidin Quantification Assay. Quantification of proanthocyanidins involves the chemical conversion of proanthocyanidins to red products, which can be measured spectrophotometrically. Condensation of vanillin with the proanthocyanidin phloroglucinol ring, catalyzed by sulfuric acid, results in the formation of a carbonium ion with a red color (*24*). The vanillin reagent was prepared by adding a 50 mL aliquot of 70% sulfuric acid solution to 0.5 g of vanillin. The reagent was cooled in an ice bath to 20 °C. Blueberry fractions were dissolved in 1 mL of MeOH at a concentration of 1.25 mg/mL and vortexed. Aliquots (250 μL) of the blueberry methanol fractions were added to 1.75 mL of vanillin reagent, the tubes were incubated in a water bath at 20 °C for exactly 15 min, and 100 μL was added (in triplicate) to a 96-well plate. Optical densities were recorded at 490 nm against MeOH. The concentration of proanthocyanidin in each blueberry fraction was determined from a standard curve generated from optical density readings of isolated cranberry proanthocyanidin at 490 nm. This standard is composed of oligomeric proanthocyanidins, which have been shown in the vanillin assay to give a more accurate representation of the proanthocyanidin content of unknown samples, as compared to using catechin as a standard (*25*). Blanks (blueberry fraction without vanillin reagent) were run on each blueberry fraction prior to the addition of vanillin reagent. The absorbance for each was subtracted from the sample plus reagent number to get a final absorbance at 490 nm. Due to the production of colored product in the vanillin assay, any interference by anthocyanins in the unreacted sample can be eliminated using a blank of the unreacted sample (*26*). An approximate concentration of proanthocyanidin in each sample was determined from the absorbance values using a standard curve.

Mass Spectrometric Analysis. Electrospray ionization (ESI) mass spectrometric analyses were made with a Micromass Quattro I quadrupole−hexapole−quadrupole (QHQ) mass spectrometer (Waters, Milford, MA) equipped with an electrospray ion source. Samples were introduced into the instrument by loop injection. Data acquisition and processing were controlled by the Micromass MassLynx data system. Samples dissolved in a mixture of aqueous methanol, acetone, and ammonia were run in negative mode with ion optics as follows: capillary voltage, 3.5 kV; and cone voltage, 25 V. The source block temperature was 65 °C, and inlet flow was adjusted to 15 μL/min. Continuous mass spectra were recorded over the range *m*/*z* 100−2000 with a scan time of 9.9 s and an interscan delay of 0.1 s.

Matrix-assisted laser desorption ionization (MALDI) mass spectra were acquired using an Applied Biosystems Voyager-DE STR, a high-performance matrix-assisted laser desorption time-of-flight mass spectrometer (MALDI-TOFMS) with a mass range of 500−300000 Da in the positive ion detection mode using 2,5-dihydroxybenzoic acid as the calibration matrix. It was equipped with a nitrogen laser (337 nm, 3 ns pulse, 20 Hz maximum firing rate) and was run in the linear detection mode for better resolution of larger molecules. Data acquisition and processing were controlled by the Applied Biosystems software.

NMR Analysis. Proton NMR spectra were obtained using a Varian Inova 500 spectrometer, at an operating frequency of 500.078 MHz, using a 5 mm probe at either 20 or 50 °C. Spectra were acquired over a sweep width of 8 kHz, using an acquisition time of 4.1 s and a 90° pulse width of 7.3 μs. Carbon-13 NMR spectra were obtained on the Varian Unity Nova 750 spectrometer. Spectra were obtained with a 10 mm broadband probe at 20 °C, at an operating frequency of 188.55 MHz over a sweep width of 45 kHz, using a 90° pulse width of 9.5 μs, an acquisition time of 0.75 s, and a recycle delay of 3.0 s. Samples for ^{1}H NMR were prepared using deuterated dimethyl sulfoxide (DMSO-d_6) as a solvent; samples for ^{13}C NMR were prepared using wet acetone as a solvent. All samples were stored at −5 °C prior to use.

Antiproliferation Assay. *Cell Culture*. Hepa 1c1c7 murine liver cancer cells (ATCC CRL 2026) were maintained in α-minimal essential medium supplemented with 10% fetal bovine serum. LNCaP human prostate cancer cells (ATCC CRL 1740) were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium with 0.25% glucose, 0.238% HEPES, 0.011% sodium pyruvate, 0.15% $NaHCO_3$, and 10% fetal bovine serum (FBS). Both were kept in a humidified incubator at 37 °C with 5% CO_2 and were subcultured when 75 mm flasks were 90% confluent. In the past, these cell lines have been successfully used to test the effects of phytochemicals on in vitro cancer cell growth (*27, 28*).

Treatment of Cells with Blueberry Fractions or Flavonoid Standards. Each blueberry fraction was dissolved in DMSO to make a 4 mg/mL stock solution. A quercetin standard was dissolved in DMSO to make a 50 mM stock solution. Hepa 1c1c7 cells were plated at a minimum density of 5000 cells/well in a 96-well plate and incubated for 24 h. After 24 h, old medium was removed and replaced with fresh medium containing 20 μg/mL of blueberry fraction, 2.38 mM quercetin standard, or 0.5% DMSO control, maintaining 0.5% DMSO for each treatment or control. Plates were incubated for 48 h, after which bioassays were performed. LNCaP cells were handled in a similar manner except that cell density was adjusted to 20000 cells/well in a 96-well plate and the treatment incubation period was 48 h.

A CellTiter 96 assay kit (Promega Corp., Madison, WI) was used to determine the relative number of viable cells remaining after incubation with treatments. For the assay, old medium was removed from the 96-well plates and replaced with 100 μL of fresh medium per well. Next, 20 μL of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium plus phenazine methosulfate (MTS/PMS) reagent was added to each well followed by a 3 h incubation period at 37 °C and 5% CO_2. Plates were read using an ELx800 microplate reader (Bio-Tek, Winooski, VT) at 490 nm. A blank row corrected for background noise and data was expressed as a percent of the DMSO control. Each treatment was tested on three different plates with eight wells on each plate for a total of 24 wells. Active fractions were tested for dose−response at concentrations of 40, 30, 20, 12, 15, 10, and 5 μg/mL.

Cytotoxicity Assay. To be sure the blueberry treatments were not causing cell necrosis in the antiproliferation assay, a CytoTox 96 assay (Promega Corp.) was performed. This assay measures lactate dehydrogenase (LDH) produced by the cells. LDH is released during cell lysis resulting from necrosis. Bovine heart LDH and lysed cells were used as positive controls to verify performance of system components and provide an upper limit for data comparison. To lyse cells for a positive control, 10 μL of lysis solution (9% v/v Triton X-100) was added to eight control wells followed by incubation for 45 min in a humidified incubator at 37 °C and 5% CO_2. To start the assay, 50 μL aliquots of media from treatment and control wells were transferred into a fresh 96-well plate. For the LDH positive control, 50 μL of LDH positive control solution and 50 μL of fresh medium were added to a row of wells (eight wells total). A blank row of fresh medium unexposed to cells was used to correct for culture medium background noise resulting from residual LDH occurring in serum-containing media. Reconstituted substrate mix (50 μL) was added to each well, and the plate was incubated in the dark at room temperature for 30 min. Stop solution (1 M acetic acid, 50 μL) was added to each well, and the plate was read at 490 nm on an ELISA plate reader. To calculate cytotoxicity, data were expressed as a percentage of control. Average absorbance values from the culture medium control were subtracted from experimental and maximum LDH release values. Percent cytotoxicity is expressed as

$$\% \text{ cytotoxicity} = \text{exptl LDH release/max LDH release}$$

Each fraction with significant activity in the MTS/PMS antiproliferation assay was tested in the cytotoxicity assay using one plate with eight well replicates.

Bacterial Antiadhesion Assay. The antiadhesion bioactivity of the blueberry fractions was tested by measuring the ability of the fractions to suppress agglutination of human red blood cells (HRBC) (A_1, Rh+) (*29*) following incubation with P-fimbriated *E. coli*. Uropathogenic *E. coli* strains were isolated from the urine of human patients diagnosed with urinary tract infections. Strains specific for P fimbrial adhesins were subcultured in tryptose broth at 37 °C for 16 h, transferred to colonization factor agar (CFA) plates (*29*), and grown overnight at 37 °C to enhance production of P fimbriae. Strains were harvested by centrifugation, washed once, and suspended in phosphate-buffered saline solution (PBS) at pH 7.0 at a concentration of 5×10^8 bacteria/mL. The dried blueberry fractions were dissolved in PBS at a starting concentration of 1.2 mg/mL, and the pH was checked for neutrality (pH 7) to ensure that there were no acids in the fractions that could interfere with the bacteria in the bioassay. Serial 2-fold dilutions were prepared from each blueberry fraction. Each dilution (30 mL) was incubated with 10 mL of bacterial suspension on a 24-well polystyrene plate for 10 min at room temperature on a rotary shaker. A 3% v/v suspension of HRBCs in PBS was prepared, and 10 mL of the diluted blood was added to test suspensions. Suspensions were incubated for 20 min on a rotary shaker at room temperature and evaluated microscopically for the ability to prevent agglutination. The final dilution concentration was recorded at which agglutination suppression by the blueberry fractions occurred. Wells containing bacteria plus PBS, HRBC plus PBS, bacteria plus test fraction, and HRBC plus test fraction served as negative controls for agglutination, and wells containing bacteria plus HRBC served as a positive control for agglutination.

Statistical Analysis. Statistical analysis was conducted using Sigma Plot (SPSS Inc., Chicago, IL, version 8.02). Differences among treatments were calculated using *t* tests. Significance of relationships was calculated using regression analysis to obtain *R* values.

RESULTS AND DISCUSSION

Bioassays. *Antiproliferation Assay*. Fraction 5 from the Toyopearl extraction isolated with 50% aqueous acetone significantly reduced proliferation of Hepa 1c1c7 and LNCaP cells, with dose-dependent 73 and 89% growth inhibitions at 20 μg/mL and IC_{50} values of 15.5 and 13.1 μg/mL, respectively. Subfraction 2 from F5 (F5.2) was slightly less active than the F5 parent fraction, inhibiting growth by 64% at 20 μL/mL (**Figure 1**), whereas subfraction 1 (F5.1) was difficult to dissolve and had no apparent antiproliferative activity.

Cytotoxicity. Although none of the blueberry fractions appeared to be cytotoxic when viewed under a microscope, a quantitative assay was necessary for certainty. Due to the expense of the assay, only fractions active in the antiproliferation assay were tested. F5 and F5.2 had low but statistically significant cytotoxicity in both cell lines, similar to a 2.38 mM treatment with quercetin (**Figure 2**).

Bacterial Antiadhesion Assay. Fractions 3, 4, 5, and 5.2 had in vitro activity thresholds below 1.0 mg/mL, whereas fraction 2 had an activity threshold of 4.0 mg/mL. Fractions 4 and 5.2 demonstrated the lowest activity thresholds at 0.25 mg/mL. Lower activity thresholds represent a higher level of antiadhesion activity because it takes less of the fraction to elicit an antiadhesive response in the bacteria. As an example, Foo et al. (*20*) found that ethyl acetate extracts of Sephadex LH20-purified proanthocyanidins from American cranberry exhibited potent antiadhesion activity at a concentration of 75 μg/mL, a much lower concentration than found in wild blueberry extracts.

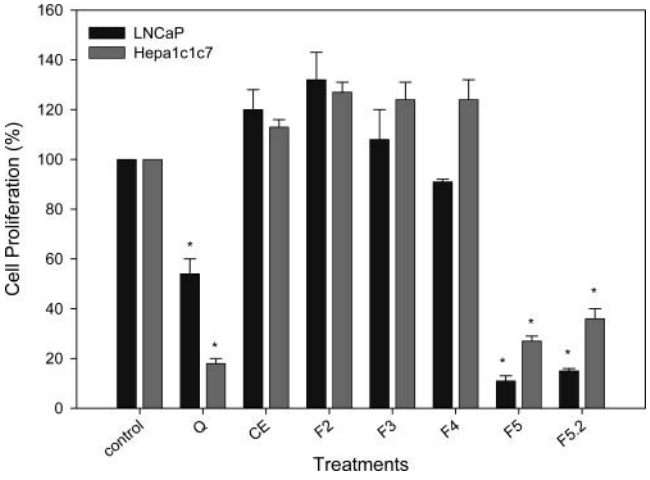

Figure 1. Proliferation of human prostate (LNCaP) and murine liver (Hepa 1c1c7) cancer cell lines treated with blueberry extracts. $Q = 2.38$ mM quercetin; CE = crude extract. The final concentration of all blueberry fractions was 20 μg/mL. Asterisks indicate treatments that were significantly different from control ($p \leq 0.05$).

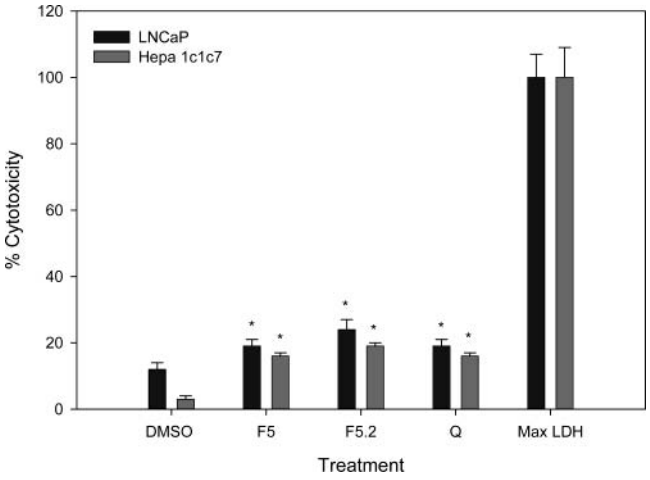

Figure 2. Percent cytotoxicity of blueberry fractions on LNCaP and Hepa 1c1c7 cell lines. Max LDH = maximum LDH release control; $Q = 2.38$ mM quercetin. The final concentration of F5 and F5.2 was 20 μg/mL. The final concentration of DMSO was 0.5%.

Effective Subfractionation. With bioassay data as a guide, F5 was subfractionated using various methods in an attempt to isolate and identify the active components. HPLC generated a chromatogram with several broad peaks all absorbing at 280 nm, the same absorption spectra as catechin and epicatechin. Unfortunately, all bioactivity was lost when samples were separated on HPLC using a C18 column. Open column chromatography proved to be a very successful means of separating some of the components in F5 without a loss of bioactivity. Sephadex LH20 was chosen as the support of choice because it acts by adsorption and release of proanthocyanidins, separating them without irreversible binding and subsequent bioactivity loss. F5.1 from Sephadex LH20 dried to a black powder and was highly insoluble, even in DMSO. F5.2 had a light purple color and was highly soluble. Both were tested in the antiproliferation assay; F5.2 had slightly less activity than the F5 parent fraction. F5.1 had no antiproliferation activity (**Figure 1**).

Compound Identification. TLC was performed on all of the fractions in an effort to determine general composition of fractions. Fraction 2 contained anthocyanins and many simple flavonoids. The presence of proanthocyanidins in F3, F4, F5, and F5.2 was established by viewing TLC plates with vanillin−HCl, a specific reagent for flavan-3-ols (*25*). We did not see exceptional resolution of any fraction, suggesting that the compounds may be copigmented in some cases. This chemical reaction can occur during oxidizing conditions and results in polymeric hybrids between tannins and anthocyanins (*30*) and may also explain the purple color of F5 and F5.2, even after chromatographic procedures that should have removed the pigments.

The vanillin proanthocyanidin quantification assay showed that F4 had the highest concentration of proanthocyanidins (7.25 mg/mL) and F2 the lowest (<1 mg/mL). When these data were compared to those of the bioassay (**Figures 1** and **2**), there was a significant linear relationship between proanthocyanidin content and antiadhesion activity ($R = 0.95$) and a less significant relationship between proanthocyanidin content and antiproliferation activity in the LNCaP cell line ($R = 0.68$) and the Hepa 1c1c7 cell line ($R = 0.59$). Fraction 4 had a greater concentration of proanthocyanidins than F5, yet in both cell lines, F5 had greater antiproliferation power than F4 in the antiproliferation assay. It is possible that F5 contained proanthocyanidins capable of reducing cancer cell growth that were absent or less concentrated in F4.

Because F4 and F5 were exceptionally bioactive, they were analyzed by NMR spectroscopy and mass spectrometry to determine the types of proanthocyanidins present. TLC and the vanillin assay showed that both F4 and F5 contained proanthocyanidins but did not yield information about purity, structure, or degree of polymerization. F5 was subfractionated into F5.1 and F5.2 to obtain fractions that retained the activity of the parent fraction F5 but were more suitable for NMR. Even though the yield of F4 was too small to be fractionated in a similar manner, it was possible to obtain NMR and MS data from the original fraction.

The proanthocyanidin content of F4 and F5.2 was confirmed by both ^{1}H and ^{13}C NMR spectroscopy techniques, used in conjunction with negative ion ESI and MALDI mass spectrometry. For both samples, ^{1}H NMR spectra revealed the presence of a series of oligomeric proanthocyanidins, with signals matching published spectra (*31*). The spectra were composed of a series of broad signals (*31*, *32*). Varying the temperature at which the spectra were acquired had no effect on observed line widths, indicating that the broadening does not arise from either inter- or intramolecular exchange or rotational processes. The observed line widths are due to the presence of many similar proanthocyanidin structures, resulting in a wide range of overlapping signals with similar chemical shifts. Signals from 6.4 to 7.0 ppm correspond to protons at the 2′-, 5′-, and 6′-positions. Those from 4.8 to 5.2, from 4.2 to 4.8, and from 3.6 to 4.1 ppm correspond to protons at the 2-, 3-, and 4-carbon positions, respectively. Aromatic hydroxyl group signals were apparent from 8.5 to 9.0 ppm. The 6-position proton signals are found at 5.8 ppm with very minimal 8-proton signals at 6.0−6.2 ppm. This, along with the location of the 4-position proton signal, shows the proanthocyanidins are linked with linear 4→8 bonds. If the molecules were composed of 4→6 bonds, the protons at the 8-position would be present and the 4-position proton signals would be shifted downfield. There were no other major signals present in F5.2, ruling out the presence of other types of flavonoid molecules, coumarate, cinnamate, or proanthocyanidins, based on other units. However, in F4, in addition to the proanthocyanidin signals, there were also peaks at 7.1−7.3 ppm and at 1.0−1.5 ppm. These peaks match the signals of

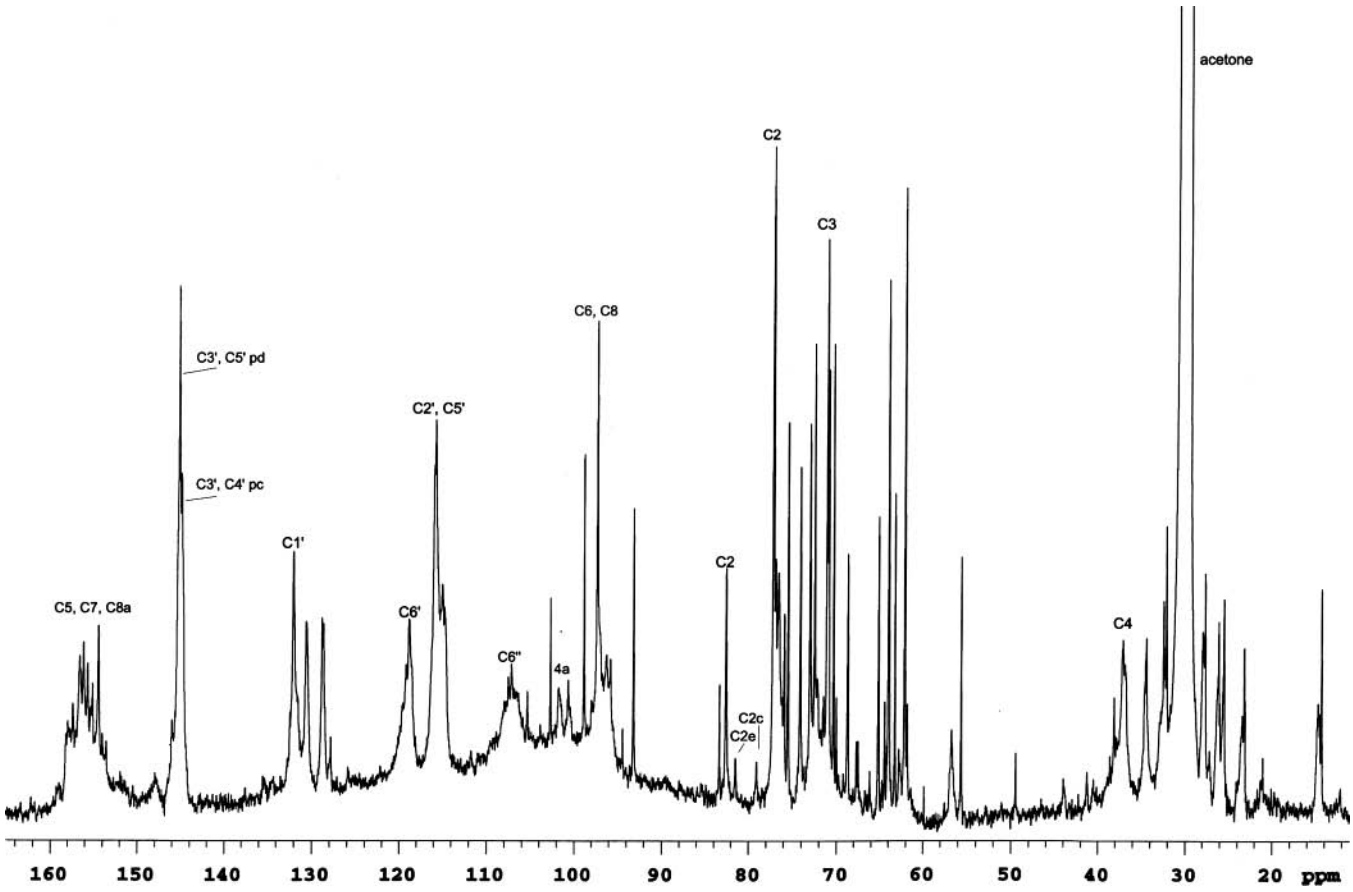

Figure 3. ^{13}C NMR spectra of wild blueberry fruit fraction 4.

rhamnose, lipid impurities, and perhaps a flavonoid glycoside, but it is difficult to make a positive identification using just ^{1}H NMR. To make a more positive identification of the structural differences of F4 and F5.2, additional analytical methods were employed.

Carbon-13 NMR spectra (**Figures 3** and **4**) confirmed the ^{1}H NMR observations that F4 and F5.2 contained primarily proanthocyanidin oligomers (**Figure 5**). Peaks at 115−116 and 119−121 ppm correspond to the chemical shifts of C2′, C5′, and C6′, respectively, of the catechol B-ring (*20*). Other chemical shifts found in the flavan-3-ol skeleton include C3′, C4′, C5′, and C1′ of the B-ring at 145 and 132 ppm, respectively. The major peak at 145 corresponds to C3′ and C5′ of prodelphinidin, whereas the minor peak corresponds to the C3′ and C4′ from procyanidin structures (*33*). The presence of both peaks indicates that both procyanidin and prodelphinidin types of proanthocyanidins are present; the only difference is an additional hydroxyl group at the C5′-position in prodelphinidin. Prodelphinidins have been reported in *Vaccinium* berries (*30*), and delphinidin is a common component of *Vaccinium* anthocyanins (*34*). Signals from C5, C7, and C8 of the A-ring were seen at 155−159 ppm and C-ring carbons from 65 to 80 ppm. These C-ring carbons are particularly sensitive to stereochemical changes (*33*). C3 peaks from cis and trans isomers occur at 73 ppm, but those for C2 appear at 76 ppm for the cis and at 84 ppm for the trans form. As the area between 65 and 80 ppm contains several signals, especially in F4, there may be molecules of various stereochemistries present.

There were several differences between the ^{13}C NMR spectra of F4 and F5.2. The spectrum of F4 indicated the presence of several non-proanthocyanidin impurities that are not seen in the spectrum of F5.2. These included signals at 129−131, 193, 62−65, and 15 ppm. The signals resemble the chemical shift of flavonoid glycosides (*35*). These compounds were not evident on TLC plates or HPLC chromatograms; they occur in small quantities relative to the proanthocyanidins. One other difference between F4 and F5.2 was that F4 had weak carbon signals at 151−152 and 104.5 ppm, whereas F5.2 did not have any signal present in this region. These chemical shifts are in the region of C5 and C7 of a doubly linked phloroglucinol A-ring (*20*). Because the signals were small in F4 and absent in F5, A-type-linked proanthocyanidins probably make up a small percentage of the total proanthocyanidin fraction from blueberries. In addition, the data from the proton spectra indicate primarily single 4→8 bonds. This is in contrast to the American cranberry (*Vaccinium macrocarpon*), which has been shown to contain a much larger portion of A-type proanthocyanidins (*20*). In the relation of structures to bioactivity, it should be noted that in the antiadhesion assay, isolated cranberry proanthocyanidins were more active than fractions F4 or F5.2 from blueberry.

Another structural difference that has been shown to effect bioactivity is the degree of polymerization (DPn) of the proanthocyanidin polymers. DPn can significantly affect the ability of a proanthocyanidin to associate with proteins and polysaccharides (*30*, *36*, *37*). The antioxidant, antifungal, antienzymic, antisecretory, and antitumor properties of proanthocyanidins may also correlate with their DPn (*30*). Negative ion ESI (**Figures 6** and **7**) and MALDI (**Figure 8**) mass spectra were used to identify and calculate the DPn of oligomeric proanthocyanidins in F4 and F5.2. The ESI mass spectra from F4 demonstrated the presence of a series of proanthocyanidins ranging from singly charged monomers (DP1, catechin or epicatechin) to doubly charged octamers (DP8). In F5.2, the series of proanthocyanidins ranged from doubly charged tet-

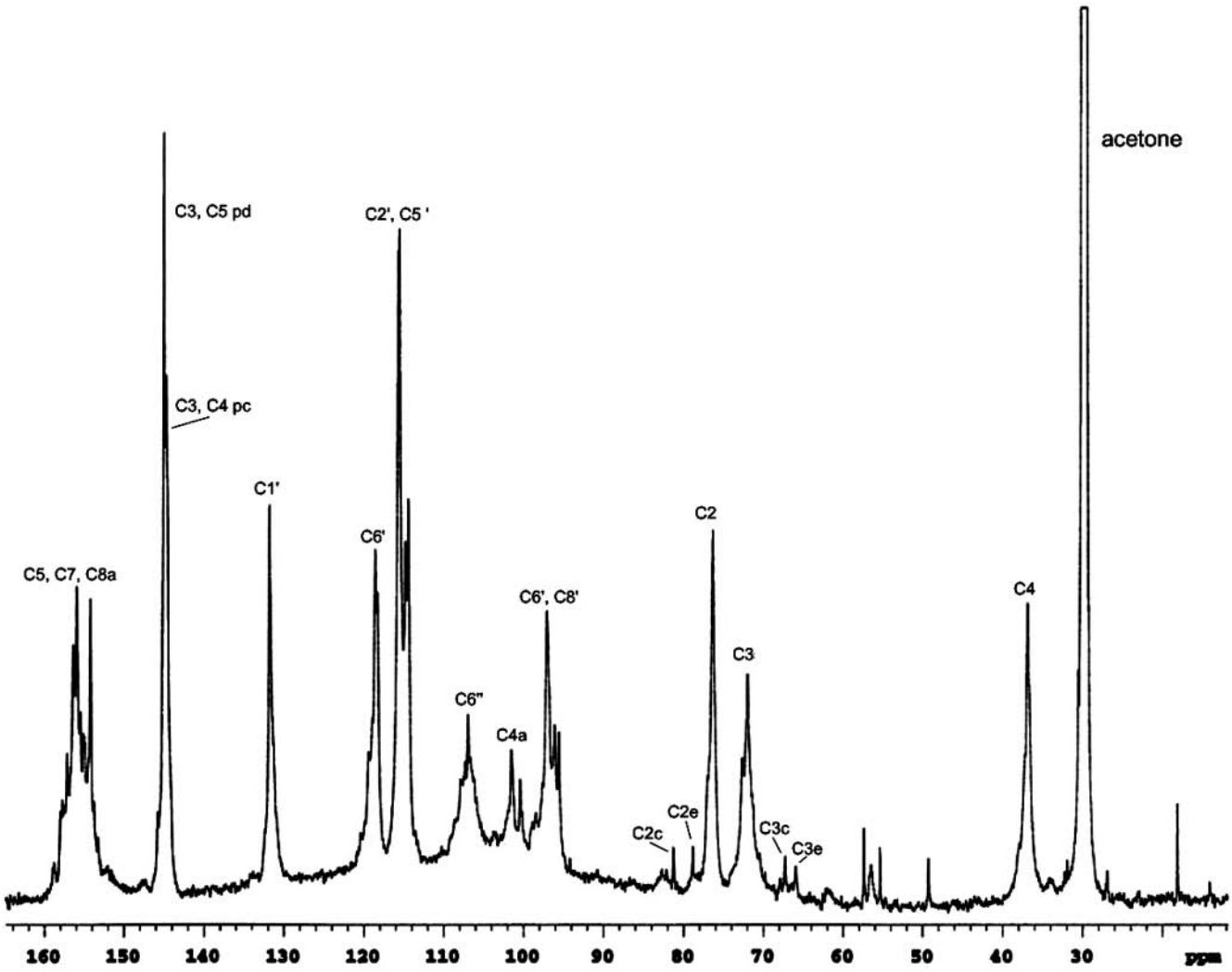

Figure 4. ^{13}C NMR spectra of wild blueberry fruit fraction 5.2.

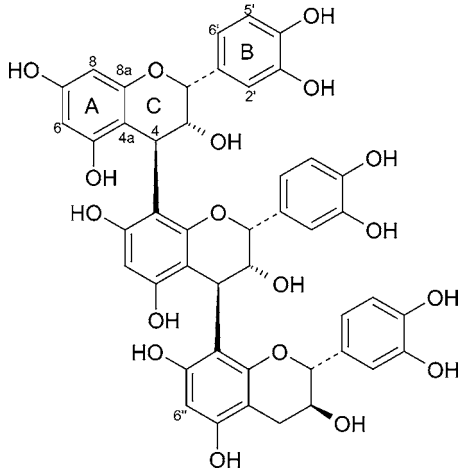

Figure 5. Structure of a typical proanthocyanidin molecule (DPn = 3) with single 4→8 bonds.

ramers (DP4) through doubly charged octamers (DP8); there were no measurable monomers or dimers present. A small peak at *m*/*z* 432 indicated a minimal amount of doubly charged trimers present. In both samples, there were signals corresponding to doubly charged ions of the proanthocyanidin tetramer (DP4, *m*/*z* 576) pentamer (DP5, *m*/*z* 720), hexamer (DP6, *m*/*z* 865), heptamer (DP7, *m*/*z* 1009), and octamer (DP8, *m*/*z* 1153). A singly charged pentamer peak was visible in F5.2 at *m*/*z* 1441. There may also be a singly charged tetramer obscured at *m*/*z* 1153 by the doubly charged octamer peak. As Foo and Lu (*38*) observed, doubly charged species are expected for larger polymers, most likely from the increased charge separation, which minimizes the electrostatic repulsive forces. When the ESI mass spectrum scale from F5.2 was expanded, the doubly charged tetramer peak (*m*/*z* 576.6) had a primary isotope signal at half integral spacing (*m*/*z* 577.1), confirming the peak is from a doubly charged ion (*39*). There were several differences in the ESI mass spectra for F4 and F5.2. F4, in addition to the presence of smaller molecular weight proanthocyanidins (epicatechin/catechin monomers through trimers), contained a few peaks that did not correspond to proanthocyanidin signals and were not found to be artifacts of the run. Peaks at *m*/*z* 301, 463, and 513 corresponded to the molecular weights of quercetin, quercetin glucoside, and perhaps quercetin xyloside malonate. These peaks were not seen in F5.2 ESI mass spectra and are in agreement with ^{13}C and ^{1}H NMR spectra of F4 that suggested the presence of flavonoid glycosides.

To confirm the results of the ESI mass spectra, MALDI mass spectra were recorded in the positive ion mode. MALDI mass spectra showed a series of repeating proanthocyanidin polymers (**Figure 8**) ranging from the sodium adducts of trimer (DPn3) to nonamer (DPn9). Additionally, there may be doubly charged polymers up to 17 units occurring at locations similar to the

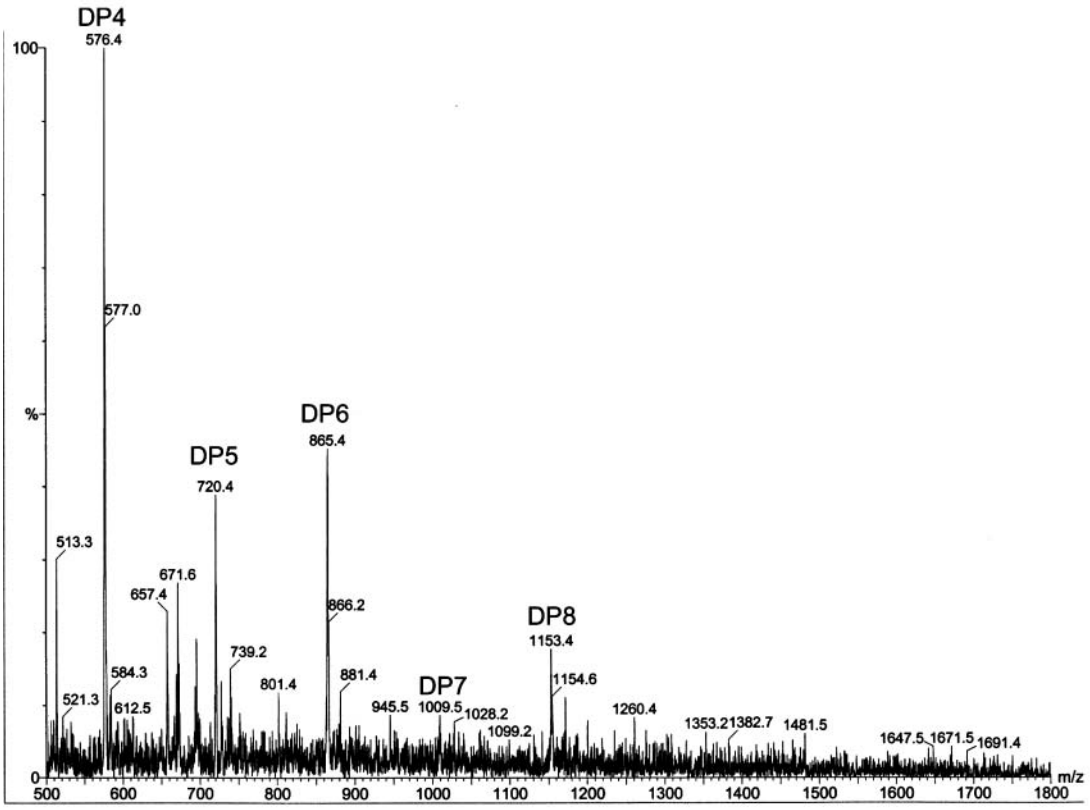

Figure 6. Negative ion ESI mass spectrum of wild blueberry fruit fraction 4.

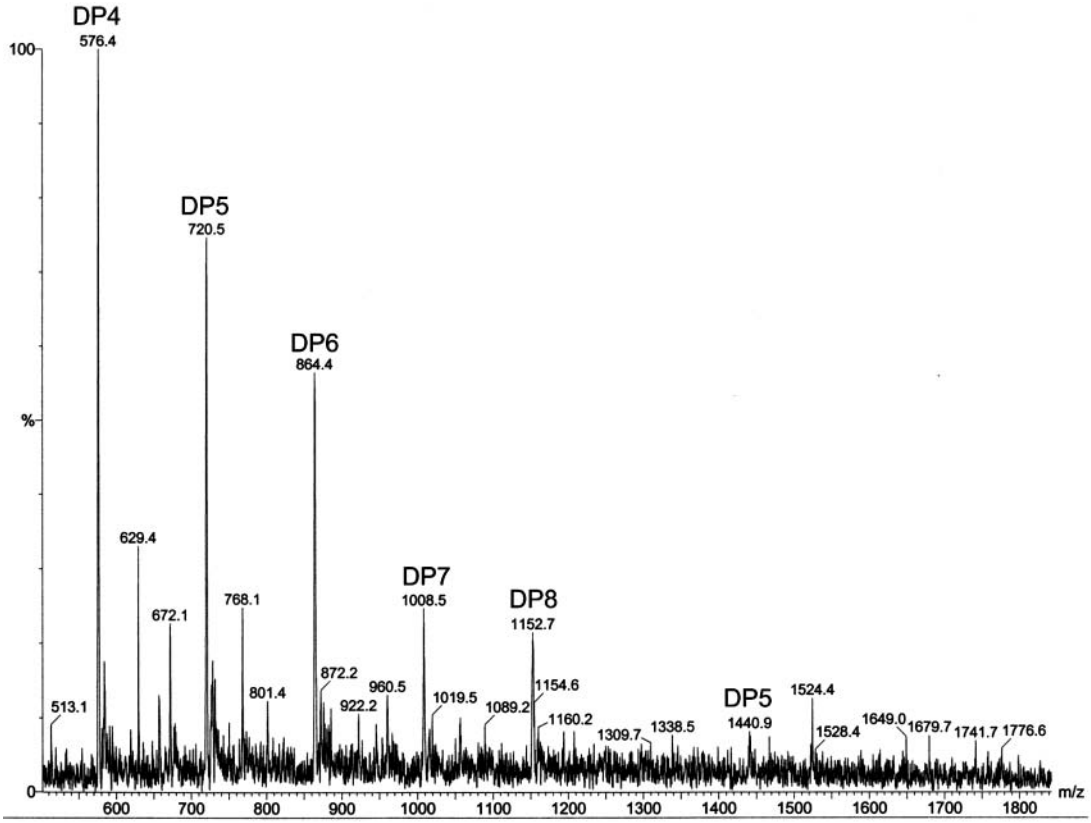

Figure 7. Negative ion ESI mass spectrum of wild blueberry fruit fraction 5.2.

singly charged molecules in the spectra. As the molecular weight of the oligomers increases, the signal intensity decreases. This is not surprising, as both MALDI and ESI mass spectroscopy discriminate in favor of the lower molecular weight ions, reducing the apparent abundance of the larger oligomers (*20*). Overall, ESI and MALDI mass spectra corroborate that both F4 and F5 contain a series of proanthocyanidin polymers. In F5.2, lower molecular weight proanthocyanidins (monomer

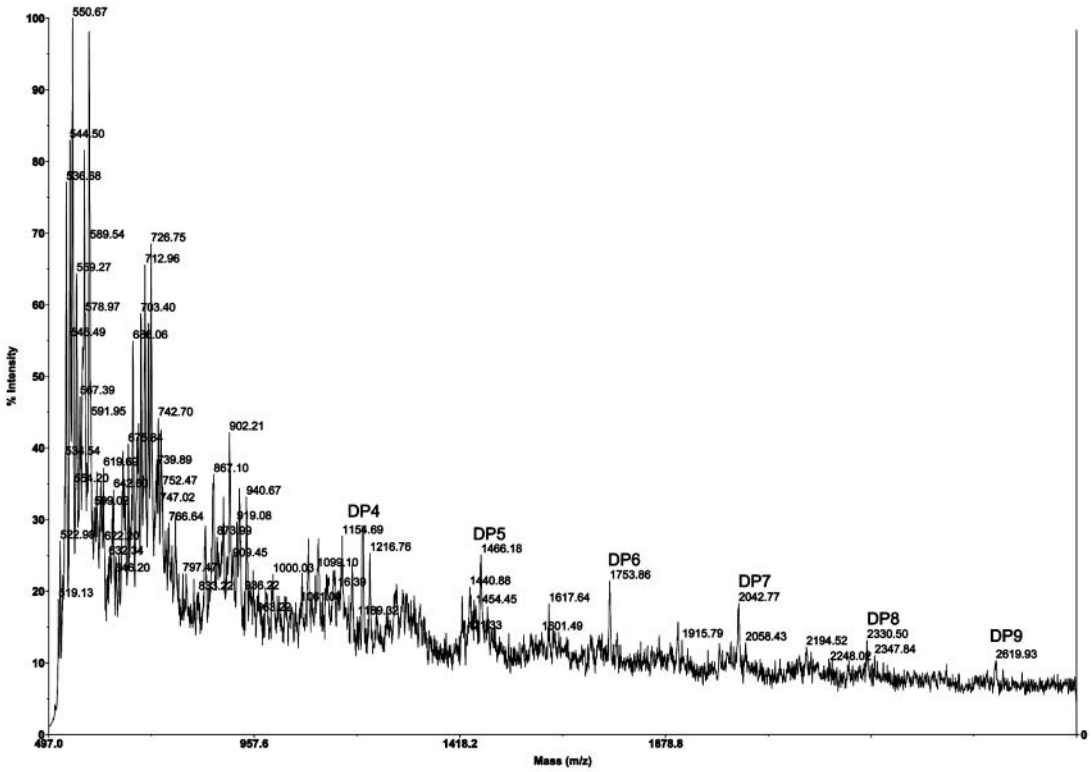

Figure 8. MALDI mass spectrum of F5.2 showing a series of proanthocyanidin oligomers.

through trimer) make up an insignificant percentage of the total proanthocyanidin content, whereas F4 has a greater abundance of lower molecular weight proanthocyanidins in addition to some signals that may correspond to flavonoid glycosides.

^{1}H and ^{13}C NMR spectroscopy techniques were used to estimate the degree of polymerization. In the absence of doubly linked A-type bonds, the average molecular weight can be determined directly from the spectra by comparing the areas of the C3 resonances of the terminal and extender flavan-3-ol units, at 67−68 and 72−73 ppm, respectively (*40*). The terminal unit is defined as the lower-most flavan-3-ol unit, which contains C6″ in **Figure 5**. Comparing the intensity of the signal at 67.2 ppm to that at 72.6 ppm in the ^{13}C NMR spectra of F4 gives an intensity ratio of 1:2.5, which represents a DPn of 3.5. For F5.2, the ^{13}C NMR spectra showed an intensity ratio of 1:4, indicating the DPn equals 5. ^{1}H NMR was also used to estimate DPn. Guyot et al. (*41*) showed that by integrating the A-ring proton signals between 5.8 and 6.5 ppm in ^{1}H NMR spectra and comparing them to the intensity of the H4 signals of the terminal units between 2.4 and 3.0 ppm it is possible to derive the DPn. Using this procedure gives DPn values of 3.0 for F4 and 6.3 for 5.2.

The NMR DPn estimations for F4 and F5.2 gave overall average DPn values of 3.25 for F4 and 5.65 for F5.2. Because F4 had a larger ratio of lower molecular weight to higher molecular weight proanthocyanidins than F5.2, this may explain differences found in the antiproliferation bioassay. Degree of polymerization can significantly affect the interactions between proanthocyanidins and other molecules, with large molecules often having greater biological activity (*30*, *36*, *39*). Using current analytical techniques, it has not been possible to isolate individual high molecular weight proanthocyanidins, determine the precise structures, predict molecular shape, and then relate individual structures to biological activity on a molecular level.

In conclusion, NMR and mass spectra show that F4 and F5.2 from wild blueberries were composed of a series of high molecular weight (largely tetramer to octamer) proanthocyanidin molecules linked with single 4→8 bonds. Although the blueberry fractions tested are similar in composition to that of the American cranberry (*V. macrocarpon* Ait.), A-type double linkages make up only a small percentage of proanthocyanidins present. A-type linkages may play an important role in the ability of proanthocyanidins to interact with *E. coli* responsible for urinary tract infections (*15*), which could explain why cranberry proanthocyanidins had higher antiadhesion activity than blueberry proanthocyanidins. In addition, the degree of polymerization of F5.2 was slightly higher than that reported for similar fractions of American cranberry fruit (DPn = 4.7) (*15*), being estimated at 5.0 from the ^{13}C NMR spectra and 6.3 from the ^{1}H NMR spectra. Because F4 and F5.2 were composed primarily of oligomeric proanthocyanidins, it is likely that one or a mixture of several high molecular weight proanthocyanidins is responsible for the antiproliferation and antiadhesion activity measured and reported, yet these compounds remain difficult to separate and purify. Chemical methods must therefore be developed to separate individual proanthocyanidin molecules for identification while retaining them unchanged for simultaneous bioactivity testing.

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