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# Hop (*Humulus lupulus* L.) Proanthocyanidins Characterized by Mass Spectrometry, Acid Catalysis, and Gel Permeation Chromatography

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Proanthocyanidins extracted from hops (*Humulus lupulus* L. cv. Willamette) were subjected to Sephadex LH-20 column chromatography using a step gradient of methanol, water, and acetone. The resulting fractions were analyzed by acid catalysis, electrospray ionization and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), and gel permeation chromatography (GPC). The proanthocyanidins contained catechin and epicatechin as monomers and as terminal and extension units. Epigallocatechin was found as extension units. The mean degree of polymerization (mDP) of the crude proanthocyanidins was 7.8, but heptamers were the largest oligomers visible in mass spectra of the whole. In the last-eluted fraction (mDP = 22.2), polymers as large as 20-mers were detected by MALDI-TOF-MS, demonstrating the effectiveness of prior separation in improving MS detection. GPC data correlated well with acid catalysis results, confirming the presence of large polymers that were not detected by MS.

**KEYWORDS: Proanthocyanidin; hops; *Humulus lupulus*; acid catalysis; gel permeation chromatography (GPC); electrospray ionization (ESI); matrix-assisted laser desorption/ionization (MALDI); time-of-flight (TOF); mass spectrometry (MS)**

## INTRODUCTION

Proanthocyanidins are polymers of the polyphenolic flavan-3-ols and are widely distributed in the plant kingdom. They account for the astringent qualities of many commonly consumed fruits and their beverage products, and they are of current interest to researchers for their antioxidant and other potentially health-promoting qualities (*1*). Hops, the female inflorescences of *Humulus lupulus* L., are used in the brewing of beer to add aroma and flavor, especially bitterness. Beer contains a rich variety of polyphenols from hops and malt. The proanthocyanidins are of additional concern to brewers because of their ability to complex with proteins to form hazes (*2*). Hops are known to contain catechin and epicatechin, as well as dimeric and trimeric proanthocyanidins (*3*), and tetramers and pentamers have been detected by mass spectrometry (*4*).

Proanthocyanidins can differ in polymer length (degree of polymerization or DP), subunit composition, and type of linkage between subunits (*5*). As polymer size increases, the possible number of isomers increases as well, making chromatographic resolution into pure compounds increasingly difficult. As proanthocyanidins usually occur in complex mixtures in nature, this problem complicates research into both their chemical and biological properties.

In the study of the chemical properties of the proanthocyanidins, methods such as acid catalysis in the presence of nucleophiles (*6*, *7*) or $^{1}H$ and $^{13}C$ NMR (*8*, *9*) yield information about subunit composition and DP, but only that of the average composition of the bulk mixture being analyzed. Despite its shortcomings (*10*), high-performance liquid chromatographic analysis (HPLC) of the products of acid catalysis with nucleophiles is the more accessible of the two types of bulk analysis. Fractionation of a complex proanthocyanidin mixture is a useful step to provide supplementary information to characterize mixtures by acid catalysis (*10*−*12*). Although the individual fractions are still mixtures, a better picture of size distribution is revealed than could be gained from analysis of the whole.

The size distribution of a mixture of intact proanthocyanidin molecules can be elucidated by gel permeation chromatography (GPC) (*13*) and by mass spectrometry (MS). However, mass spectra are usually dominated by the lower molecular weight species, even though chemical methods indicate that large proanthocyanidins are present in appreciable quantities. This phenomenon is seen in the analysis of proanthocyanidins from several sources by different mass spectral methods (*14*−*17*). Thus, MS may not reflect the actual abundance of proanthocyanidins of different DP, but only the relative ease of ionization

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of the molecules. Perhaps prior size separation would benefit mass spectral analysis, as well.

The occurrence of proanthocyanidins in complex mixtures also makes it difficult to firmly establish the effect of molecular size on the biologically important properties of proanthocyanidins. Most studies of the biological properties of purified proanthocyanidins are based on the readily resolved and easily identified monomeric polyphenols or smaller oligomers (*18*, *19*). The oft-cited paper by Haslam (*20*) examined mostly the properties of gallotannins; of the proanthocyanidins, the tannins generally found in foods and beverages, only one dimer and one trimer were studied. Because of the analytical difficulties, only a few workers have gone beyond these simple compounds: for example, a tetramer in a study of color stability of model solutions of oenin (*21*) and a pentamer for a study of the effect of molecular size on the formation of beer haze (*22*). Lea and Arnold (*23*) fractionated proanthocyanidins to compare the role of dimers through pentamers and unresolved larger polymers in the perception of bitterness and astringency in ciders. Other workers (*24*, *25*) have prepared fractions of proanthocyanidin extracts for their experiments, allowing the properties of still complex mixtures of small and larger polymers to be separately measured. Obviously, improvement in the separation of the proanthocyanidins is needed to better examine the relationship of size to their chemical and biological properties.

A variety of methods have been proposed to fractionate proanthocyanidins. Labarbe (*26*) and Saucier (*27*) and their coworkers proposed separation methods based on the relative solubility of proanthocyanidins of different molecular sizes in different solvents and solvent mixtures. It should be noted that the second group raised concerns that the first method may not be reproducible because a key material may not be readily available. A few investigators (*28*, *29*) have used countercurrent distribution. Several chromatographic methods for separating proanthocyanidins, using size exclusion, normal phase, or reverse phase, have been proposed and used during the past three decades (*26*, *30*). Sephadex LH-20 has been one of the more widely used materials for proanthocyanidin chromatography since 1974 (*31*), both for size fractionation using alcohol−water gradients and, more frequently, for sample cleanup, to remove sugars, glycosides, and monomeric polyphenols from larger proanthocyanidins. Sephadex LH-20 is readily available worldwide and is likely already in use in most laboratories involved in proanthocyanidin research. Therefore, separation methods based on it are more likely to be reproducible by and useful to other researchers.

Generally, 60−70% acetone in water is used as a final wash following 100% methanol, typically eluting all of the proanthocyanidins larger than tetramers in a single fraction (*24*). We are not aware of anyone using a step gradient of 10, 20, and 30% acetone in an effort to improve fractionation with Sephadex LH-20. In this paper, we report on the use of such a gradient to preparatively separate the proanthocyanidins of hops (*H. lupulus* L.). Eight distinct fractions resulted, five with mean DP (mDP) larger than tetramers. The subsequent analysis of these fractions clearly demonstrates that prior separation can improve the mass spectral detection of the proanthocyanidins. Previously, study of the health-related (*1*) or haze-forming (*2*) properties of hop proanthocyanidins has been largely restricted to dimers and trimers. The improved separation and the knowledge that hops contain much larger polymers in larger quantities than previously reported (*3*, *4*) should enable a significant extension of the study of the proanthocyanidins of this basic raw material of the brewing industry.

**Table 1.** Solvent Mixes for Refined Separation

| | vol (mL) | acetone (vol %) | methanol (vol %) | water (vol %) |
|---|---|---|---|---|
| A | 800 | 0 | 60 | 40 |
| B | 800 | 0 | 75 | 25 |
| C | 800 | 0 | 90 | 10 |
| D | 600 | 10 | 80 | 10 |
| E | 600 | 20 | 65 | 15 |
| F | 600 | 30 | 40 | 30 |
| G | 800 | 60 | 0 | 40 |

## MATERIALS AND METHODS

**Materials.** Acetone, acetonitrile, dichloromethane, *N*,*N*-dimethylformamide, and methanol were of HPLC grade and purchased from Fisher Scientific (Santa Clara, CA). Also purchased from Fisher Scientific were glacial acetic acid, lithium chloride, and sodium acetate. Phloroglucinol and 3-indoleacrylic acid (IAA) were purchased from Sigma (St. Louis, MO). Sephadex LH-20 was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Water was purified to HPLC grade on a Millipore Milli-Q apparatus (Bedford, MA). All solvent−water mixtures used in column chromatography contained 0.1% (v/v) formic acid (Fluka brand, Sigma-Aldrich, Milwaukee, WI) and were degassed by helium sparging prior to use.

Willamette hop cones (*Humulus lupulus* L. cv. Willamette) from the U.S. Department of Agriculture−Oregon State University Experimental Hop Yard, Corvallis, OR, were harvested at maturity in September 2000, kiln-dried, baled, and stored at −15 °C until needed.

**Extraction and Initial Purification.** Whole hops (50 g, 13% moisture) were extracted three times with 1 L of dichloromethane (DCM) (1 h with occasional stirring) to remove pigments, lipids, etc., and then air-dried in a fume hood overnight. The dry hops were ground in a Wiley mill to pass 20 mesh and then extracted three times with 800 mL of 7:3 v/v acetone/water (2 h with continuous stirring). At the end of each of the first two extractions, stirring was stopped for 15 min and the supernatant poured off and retained; fresh 70% acetone was added to the settled solids, and stirring was resumed. The final extraction was filtered through Whatman no. 1 paper on a Büchner funnel by vacuum; the solids were washed with an additional 200 mL of 70% acetone. The combined extracts were rotary evaporated under vacuum at 35 °C to remove the acetone.

The resulting extract (∼1 L) was washed first with 800 mL of hexane, followed by 400 mL of DCM, to remove more pigments and nonpolar material, and then rotary evaporated to remove the residual organic solvents. The washed extract was loaded onto a 4 cm diameter × 30 cm long Sephadex LH-20 column equilibrated with 1:3 v/v methanol (MeOH)/water. The column was then washed with 1:3 v/v MeOH/water (400 mL), 1:1 v/v MeOH/water (1040 mL), and 4:1 v/v MeOH/water (400 mL) to elute glycosides and other materials and then catechin/epicatechin monomers and dimers. Fractions were monitored with two-dimensional (2D) TLC on cellulose plates developed first with 3:1:1 v/v/v *tert*-butyl alcohol/water/acetic acid, dried, then developed in the second dimension with 6% v/v aqueous acetic acid, then visualized with vanillin−HCl reagent. Finally, 7:3 v/v acetone/water (500 mL) was used to elute the proanthocyanidin polymers. Fractions containing all of the proanthocyanidins collected after the bulk of monomers and some dimers had been eluted were pooled and rotary evaporated to remove organic solvents, then lyophilized to dryness to yield 1.940 g of crude proanthocyanidins.

**Refined Separation of Proanthocyanidins.** The crude proanthocyanidins were dissolved in 50.0 mL of MeOH. An aliquot of 5.0 mL was withdrawn for the "total" sample. The remainder was diluted with an equal volume of 0.5% aqueous formic acid to yield 90 mL of 1:1 v/v MeOH/water solution and then applied to a 4 cm diameter × 45 cm long Sephadex LH-20 column equilibrated with 1:1 v/v MeOH/water. The column was developed with the sequence of solvent mixtures shown in **Table 1**. Fractions (∼15 mL) were collected and monitored

**Table 2.** Results of Acid Catalysis of Hop Proanthocyanidin Pooled Fractions

| sample | sample wt[a] (g) | mDP by acid catalysis | mol % epicatechin in terminal units | overall *cis*/*trans* molar ratio[b] | tri-OH/di-OH molar ratio[c] | mass yield of acid catalysis[d] (%) |
|---|---|---|---|---|---|---|
| total | 1.940 | 7.8 | 12.5 | 3.08 | 0.28 | 60.6 |
| 1 | 0.042 | 1.8 | 19.7 | 0.80 | 0.06 | 12.0 |
| 2 | 0.152 | 2.3 | 18.6 | 0.60 | 0.05 | 41.0 |
| 3 | 0.193 | 3.8 | 12.9 | 1.39 | 0.13 | 45.6 |
| 4 | 0.186 | 5.4 | 12.2 | 2.04 | 0.16 | 61.0 |
| 5 | 0.226 | 7.6 | 12.0 | 2.89 | 0.21 | 58.9 |
| 6 | 0.229 | 10.2 | 12.0 | 3.69 | 0.25 | 61.6 |
| 7 | 0.242 | 13.4 | 12.1 | 4.50 | 0.30 | 61.7 |
| 8 | 0.451 | 22.2 | 11.7 | 6.46 | 0.44 | 64.7 |

[a] Recovered from column = 1.721 g or 88.7%. [b] 2,3-*cis* to 2,3-*trans* units = (epicatechin + epigallocatechin)/(catechin). [c] Ratio = (epigallocatechin)/(catechin + epicatechin). [d] % = 100 × (calculated mass of all subunits)/(measured mass of sample analyzed).

by 2D TLC and by ESI-MS and then pooled into eight parts (see Results and Discussion). The pooled fractions were rotary evaporated to remove organic solvents, lyophilized to dryness, dissolved in minimal MeOH, and stored at −15 °C.

**Acid Catalysis of Proanthocyanidin Fractions.** Aliquots of the "total" and eight pooled fractions were analyzed for subunit composition and mDP by acid catalysis in the presence of excess phloroglucinol (*7*) except for a modified HPLC method to determine the products. This reversed-phase method consisted of two Chromolith RP-18e (100 × 4.6 mm) columns connected in series and protected by a guard column (Purospher STAR RP-18e, 4 × 4 mm, 5 μm), all purchased from EM Science (Gibbstown, NJ). The procedure utilized a binary gradient of 1% v/v aqueous acetic acid (mobile phase A) and acetonitrile containing 1% v/v acetic acid (mobile phase B). Eluting peaks were monitored at 280 nm. Elution conditions were as follows: 3.0 mL/min; 3% B for 4 min, a linear gradient from 3 to 18% B in 10 min, and 80% B for 2 min. The column was washed with 3% B for 2 min before the next injection. This HPLC method was used over the previously published one (*7*) because of its reduced run time (from 70 to 18 min) in addition to an improvement in analyte resolution.

**Gel Permeation Chromatography (GPC).** The GPC method used to analyze the proanthocyanidins consisted of two PLgel (300 × 7.5 mm, 5μm, 500 × 100 Å) columns connected in series and protected by a guard column containing the same material (50 × 7.5 mm, 5 μm), all purchased from Polymer Laboratories (Amherst, MA). Sample injection amount was typically 40 μg. The isocratic method utilized a mobile phase consisting of *N*,*N*-dimethylformamide containing 1% v/v glacial acetic acid, 5% v/v water, and 0.15 M lithium chloride. The flow rate was maintained at 1 mL/min with a column temperature of 60 °C, and elution was monitored at 280 nm.

**Electrospray ionization mass spectrometry (ESI-MS)** was performed on a PE Sciex AP III Plus triple-quadrupole in the single MS positive mode. Samples diluted to 1 mg/mL were loop-injected into 2:1 v/v MeOH/0.5% aqueous formic acid flowing at 5−10 μL/min to the electrospray source. Ionization voltage was 5000 V, and the orifice was set at 60 V.

**Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)** was performed on a custom-built instrument with delayed extraction source in the linear positive mode. Samples diluted to 1−2 mg/mL were mixed with 3-indoleacrylic acid in saturated solution as the matrix, with acetonitrile the most commonly used solvent. A YAG laser was used to irradiate the samples, and the acceleration voltage was 23 kV. Each recorded spectrum was the sum of 30 consecutive pulses.

## RESULTS AND DISCUSSION

As part of an ongoing program of research on the polyphenols of hops at Oregon State University, proanthocyanidins were occasionally studied by mass spectrometry as new methods and instruments became available. As recently as 1997, pentamers had been the largest proanthocyanidins observed in electrospray ionization (ESI) mass spectra of crude mixtures in our laboratory (*4*). This was extended to heptamers when analysis was performed by a Fourier transform−ion cyclotron resonance (FT-ICR) instrument. MALDI-TOF MS allowed the detection of octamers at first. Later, the use of 3-indoleacrylic acid, reported by Ohnishi-Kameyama and co-workers (*15*) to be the best MALDI matrix for proanthocyanidins, resulted in the detection of polymers as large as tridecamers. In all spectra, however, the lowest molecular weight species produced the most abundant ions. Compared to their relative proportions as measured by UV absorbance, catechin and epicatechin produced ions in greater abundance than did dimers in MS of mixtures of hop proanthocyanidins. The possibility that ion peak intensity might not reflect the actual proportion of different size polymers and that even larger proanthocyanidins existed in hops that might not be detectable in the presence of more readily ionized small molecules led to our effort to separate the proanthocyanidins by size and then analyze the resulting fractions.

The extraction of 50.2 g (43.8 g of dry matter) of Willamette hop cones and the subsequent cleanup separation on Sephadex LH-20 yielded 1.940 g of crude oligomeric and polymeric proanthocyanidins, which were subjected to additional fractionation. The collected fractions were monitored by 2D-TLC and by ESI-MS, and fractions containing largely the residual monomers and dimers were pooled as samples 1 and 2, respectively. Separation of the different size classes was not complete, and divisions between pools were therefore somewhat arbitrary. Sample 3 contained fractions including mostly trimers, whereas the fourth sample contained the fractions dominated by peaks of *m*/*z* 1155 and 1171, or tetramers, eluted by the 10% acetone mixture.

The final pool of fractions (sample 8) contained the obvious brown solvent front of the 60% acetone and all subsequent fractions collected. ESI-MS monitoring of fractions between the fourth pool and this front showed a steady increase in the average mass of proanthocyanidin oligomers and in the proportion of doubly charged ions, but no obvious points of division. No colored fronts or bands were observed either, so the remaining fractions were arbitrarily combined into three pools of equal volume (samples 5−7).

The "total" sample and the eight pooled samples were analyzed by acid catalysis in the presence of excess phloroglucinol to determine their subunit composition and mDP (**Table 2**). The proanthocyanidins of Willamette hops are a complex mixture of monomers and polymers with a large range of molecular weights. Catechin and epicatechin occur as monomers and as both terminal and extension units in the polymeric proanthocyanidins. Overall, catechin is the main terminal unit, and epicatechin is the major extension unit found. The prodelphinidin epigallocatechin comprises 25% of the extension units. No evidence of galloylation was observed.

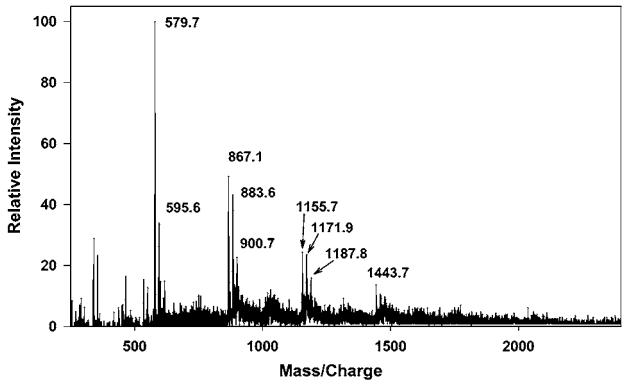


**Figure 1.** ESI mass spectrum of total hop proanthocyanidins before separation. See **Table 4** for identification of labeled peaks.

In the chromatograms of the acid catalysis cleavage products, a minor peak (~5 mol %) was observed to elute shortly before epigallocatechin-(4$\beta$→2)-phloroglucinol and to have an identical UV spectrum. This indicates that this peak may also derive from a prodelphinidin occurring as an extension unit and is likely a gallocatechin−phloroglucinol compound. However, rigorous identification including MS and NMR is required before it can be claimed that hop proanthocyanidins contain gallocatechin. It was not included in the results in **Tables 2** and **5**.

The increase in the proportion of prodelphinidins in the later fractions (**Table 2**) is probably an artifact of the separation mechanism and not a real correlation with mDP. As fractions were collected and monitored by ESI-MS, the relative intensities of peaks containing one or more prodelphinidin residues were seen to increase relative to the all-procyanidin oligomers of a given size; that is, proanthocyanidins of *m*/*z* 595 eluted later than 579 and *m*/*z* 883 later than 867. The number of hydroxyls in proanthocyanidins can increase both by a greater DP and by a greater proportion of the trihydroxy prodelphinidin units. These observations are consistent with hydrogen bonding between the hydroxyls of the proanthocyanidin molecules and the Sephadex stationary phase being a major mechanism for retention of the analyte molecules on the column.

The mass yield from the acid catalysis was fairly consistent for the later samples, ~60%. On the other hand, yields were lower in the early fractions. These contained much non-proanthocyanidin material, judging by both the 2D TLC plates and the ESI mass spectra. For example, sample 3 was dark green in color, despite the earlier washes with hexane and DCM. Also, the division between samples 3 and 4 was based not just on the first appearance of tetramers but also on the disappearance of *m*/*z* 341 and 355, ions recognizable as desmethylxanthohumol and xanthohumol, the major prenylflavonoids of hops (*32*). These two compounds ionize strongly, and detectable traces remained even after the DCM wash and the first Sephadex column.

Of the 1.721 g of proanthocyanidins recovered in the final separation, 26% of the mass was in sample 8, which had an mDP of 22 by acid catalysis. Five of the samples, containing 78% of the mass recovered, had mDP values of ≥5. In contrast, proanthocyanidins of lower mDP appear far more abundantly in mass spectra of the "total" sample. The most intense peak observed is the lowest MW detectable by each of the instruments used: an all-procyanidin dimer in the ESI spectrum (**Figure 1**) and an all-procyanidin trimer in the MALDI-TOF spectrum (**Figure 2**). Detection of lighter ions was not attempted on our MALDI-TOF instrument because of noise and matrix interference problems. Both mass spectra show that the hop proanthocyanidins are a mixture of different sizes of polymers, as large as pentamers in the ESI spectrum. In the MALDI-TOF spectrum, the peak at *m*/*z* 2345.2 corresponds to an octamer with one prodelphinidin subunit (expected *m*/*z* 2347.1), demonstrating the ability of MALDI-TOF MS to detect larger proanthocyanidins than ESI can. Because the mDP of the "total" sample was 7.8, the mass spectra clearly did not reveal the full range of polymers in the mixture.

**Table 3.** Expected and Observed $[M + Na]^+$ Ions in MALDI-TOF Mass Spectra

| DP[a] | PD[b] | expected *m*/*z*[c] | observed *m*/*z*[d] |
|---|---|---|---|
| 3 | 0 | 889.8 | 888.4, 888.6 |
| | 1 | 905.8 | 904.2 |
| | 2 | 921.8 | 919.8, 920.1 |
| 4 | 0 | 1178.0 | 1176.9, 1170.0, 1178.5 |
| | 1 | 1194.0 | 1192.4, 1192.9, 1193.8 |
| | 2 | 1210.0 | 1208.2, 1208.7, 1210.9 |
| 5 | 0 | 1466.3 | 1464.4, 1465.8, 1466.7 |
| | 1 | 1482.3 | 1480.3, 1481.2, 1481.5 |
| | 2 | 1498.3 | 1496.6, 1497.4, 1498.5 |
| | 3 | 1514.3 | 1512.8, 1513.5 |
| 6 | 0 | 1754.6 | 1752.6, 1752.8, 1755.2 |
| | 1 | 1770.6 | 1768.4, 1769.9, 1770.2 |
| | 2 | 1786.6 | 1785.2, 1785.4, 1786.3 |
| | 3 | 1802.6 | 1801.1, 1801.2, 1803.1 |
| 7 | 0 | 2042.8 | 2040.5, 2042.4, 2042.8 |
| | 1 | 2058.8 | 2056.9, 2058.1, 2059.1 |
| | 2 | 2074.8 | 2072.2, 2075.1 |
| | 3 | 2090.8 | 2090.2 |
| 8 | 0 | 2331.1 | 2328.2, 2331.4 |
| | 1 | 2347.1 | 2344.9, 2345.2, 2347.6 |
| | 2 | 2363.1 | 2362.6 |
| | 3 | 2379.1 | 2378.6 |
| | 4 | 2395.1 | 2395.5 |
| 9 | 0 | 2619.3 | 2618.7 |
| | 1 | 2635.3 | 2634.7 |
| | 2 | 2651.3 | 2651.7 |
| 10 | 1 | 2923.6 | 2922.2 |

[a] DP = degree of polymerization. [b] PD = number of prodelphinidins. [c] Average mass calculation based on natural isotope abundance. [d] Observed *m*/*z* from labeled peaks in **Figures 2** and **3**.

Both mass spectra confirm the presence of the (epi)-gallocatechin identified in the HPLC analysis of the products of acid catalysis. Following the all-procyanidin peak of each DP are ion peaks of 16, 32, or 48 greater *m*/*z*. As the atomic weight of oxygen is 16 Da, these can be presumed to be the ions of proanthocyanidins containing one, two, or three prodelphinidin units, respectively. Assignments of identity of the labeled peaks in all displayed spectra can be found in **Table 3** for MALDI-TOF spectra and in **Table 4** for ESI spectra.

The pooled fractions were analyzed separately by MS to learn more about the molecular weight distribution of the hop proanthocyanidins and to determine if the separation improved the detection of larger polymers. MALDI-TOF MS yielded spectra (**Figure 3**) with ion peaks corresponding to the calculated mass/charge ratios of singly charged sodium adducts of proanthocyanidins, as shown in **Table 3**. Use of the simulated average mass based on natural isotope abundance as the "expected" *m*/*z* was appropriate for the resolution of the instrument. Because 1.1% of carbon occurs as $^{13}C$, this distinction is important for the larger polymers. An all-procyanidin tetramer containing only $^{12}C$ will have an MW of 578.14 Da; tetramers containing the natural distribution of isotopes will have an average mass of 578.53 Da. For a comparable decamer, the MW values are 2882.65 and 2884.58, respectively.

Interpretation of the ESI spectra (**Figure 4**; **Table 4**) was more complex. Lower MW proanthocyanidins produced ions

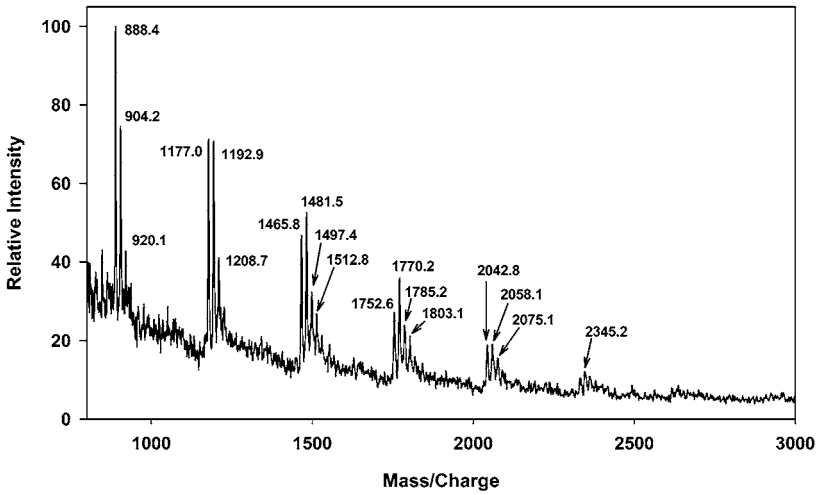


**Figure 2.** MALDI-TOF mass spectrum of total hop proanthocyanidins before separation. See **Table 3** for identification of labeled peaks.

**Table 4.** Expected and Observed $[M + H]^+$ and $[M + 2H]^{2+}$ Ions in ESI Mass Spectra

| | | calcd *m/z*[c] | | |
|---|---|---|---|---|
| DP[a] | PD[b] | $[M + H]^+$ | $[M + 2H]^{2+}$ | observed *m/z*[d] |
| 2 | 0 | 579.5 | | 579.7 |
| | 1 | 595.5 | | 595.6 |
| 3 | 0 | 867.8 | | 867.1, 867.8 |
| | 1 | 883.8 | | 883.4,[e] 883.6, 883.7 |
| | 2 | 899.8 | | 899.9, 900.7 |
| 4 | 0 | 1156.1 | | 1155.3,[e] 1155.7, 1155.8 |
| | 1 | 1172.1 | | 1171.2,[e] 1171.7, 1171.9 |
| | 2 | 1188.1 | | 1187.6,[e] 1187.8, 1188.6 |
| 5 | 0 | 1444.3 | | 1443.5, 1443.6,[e] 1443.7 |
| | 1 | 1460.3 | | 1459.8,[e] 1460.7 |
| | 2 | 1476.3 | | 1475.3,[e] 1476.7 |
| 6 | 1 | 1748.6 | | 1748.6,[e] 1749.2 |
| | 3 | | 891.2 | 890.8 |
| | 5 | | 906.8 | 907.1 |
| 7 | 1 | | 1018.9 | 1019.0, 1019.9 |
| | 2 | 2052.8 | 1026.9 | 2053.1, 1027.1 |
| 8 | 0 | | 1155.0 | 1155.3[e] |
| | 1 | | 1163.0 | 1163.3 |
| | 2 | | 1171.0 | 1171.2[e] |
| | 3 | | 1179.0 | 1179.2 |
| | 4 | | 1187.0 | 1187.6[e] |
| 9 | 0 | | 1299.2 | 1299.6, 1299.9 |
| | 1 | | 1307.2 | 1307.3, 1308.1 |
| | 2 | | 1315.2 | 1315.4 |
| | 3 | | 1323.2 | 1323.5 |
| 10 | 0 | | 1443.3 | 1443.6[e] |
| | 1 | | 1451.3 | 1451.5 |
| | 2 | | 1459.3 | 1459.8[e] |
| | 4 | | 1475.3 | 1475.3 |
| | 5 | | 1483.3 | 1483.4 |
| | 6 | | 1491.3 | 1492.7 |
| 12 | 6 | | 1779.6 | 1780.4 |
| 13 | 7 | | 1931.7 | 1931.9 |
| 14 | 5 | | 2059.8 | 2061.2 |
| 15 | 7 | | 2219.9 | 2216.9 |

[a] DP = degree of polymerization. [b] PD = number of prodelphinidins. [c] Average mass calculation based on natural isotope abundance. [d] Observed *m/z* from labeled peaks in **Figures 1**, **4**, and **5**. [e] Observed ion may be singly or doubly charged and is compared with both expected values.

consistent with singly charged protonated ions, whereas larger molecules were more likely to be detected as doubly charged, doubly protonated ions (*33*). The charge state can be seen in three ways. First, in the ESI spectrum of sample 4 (**Figure 4A**), groups of peaks are separated by intervals of *m/z* 288, corresponding to the incremental mass of (epi)catechin extension units. In the spectrum of sample 6 (**Figure 4B**), the groups are separated by half, or *m/z* 144, because some ions are doubly charged. Most easily recognized are ions from odd DP proanthocyanidins, such as heptamers and nonamers, because their doubly charged ion peaks do not overlap other peaks, as do singly charged tetramers and some doubly charged octamers, for example. Second, the interval between peaks within individual DP groups is *m/z* 16 in **Figure 4A**, but *m/z* 8 in **Figure 4B**. This is especially clear in the expanded inset spectrum in each figure. In both cases, this is the result of additional O atoms from the substitution of prodelphinidin subunits, in the first case in a singly charged ion, in the second, a doubly charged one. Third, in higher resolution scans, the interval between isotope peaks can be determined. Singly charged ions differing only in the numbers of $^{13}C$ atoms will be found *m/z* 1 apart, whereas those in doubly charged ions are separated by *m/z* 0.5. Scans (not shown) of sample 4 in the region of *m/z* 1443 contained well-resolved peaks *m/z* 1 apart in a pattern consistent with the expected isotope peaks of a singly charged pentamer. Scans of sample 6 revealed a more complex pattern. Resolution was not sufficient to clearly show peaks separated by *m/z* 0.5, nor was the mass accuracy good enough to prove a shift from an all-$^{12}C$ base peak, as expected with a singly charged pentamer, to a base peak resulting from the presence of a single $^{13}C$ in a doubly charged decamer. Most likely, the peak at *m/z* 1443.6 contains both singly and doubly charged ions. Therefore, the identity assignments shown in **Table 4** rely on the intervals between polymers of different DPs and different numbers of prodelphinidin units per molecule. No clear evidence could be seen in the ESI spectra for triply or higher charged proanthocyanidin ions.

**Table 5** summarizes the range and identity of the most abundant polymer size in the "total" sample and the eight pooled samples as determined by acid catalysis and by ESI and MALDI-TOF MS. Two main points are apparent. First, as the mDP increases, the molecular size estimate by mass spectrometry, as measured both by overall range and by most abundant detected ion, falls farther and farther short of the acid catalysis result. Second, larger proanthocyanidins are detectable in the later eluted fractions, which were not apparent in the "total" spectra. For example, in the ESI spectrum of sample 4, nonamers are clearly present at *m/z* 1299.9 and above. The improvement in the detection of larger proanthocyanidins following fractionation is even more obvious in the spectra of sample 6 (MALDI, **Figure 3B**; ESI, **Figure 4B**).

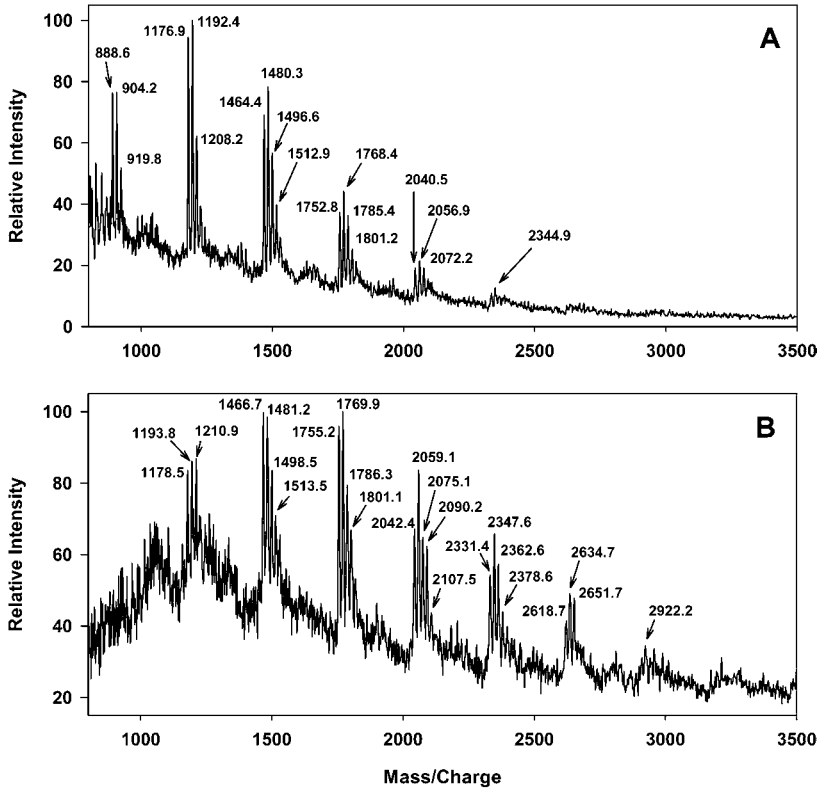


**Figure 3.** MALDI-TOF mass spectra: A, sample 4; B, sample 6. See **Table 3**.

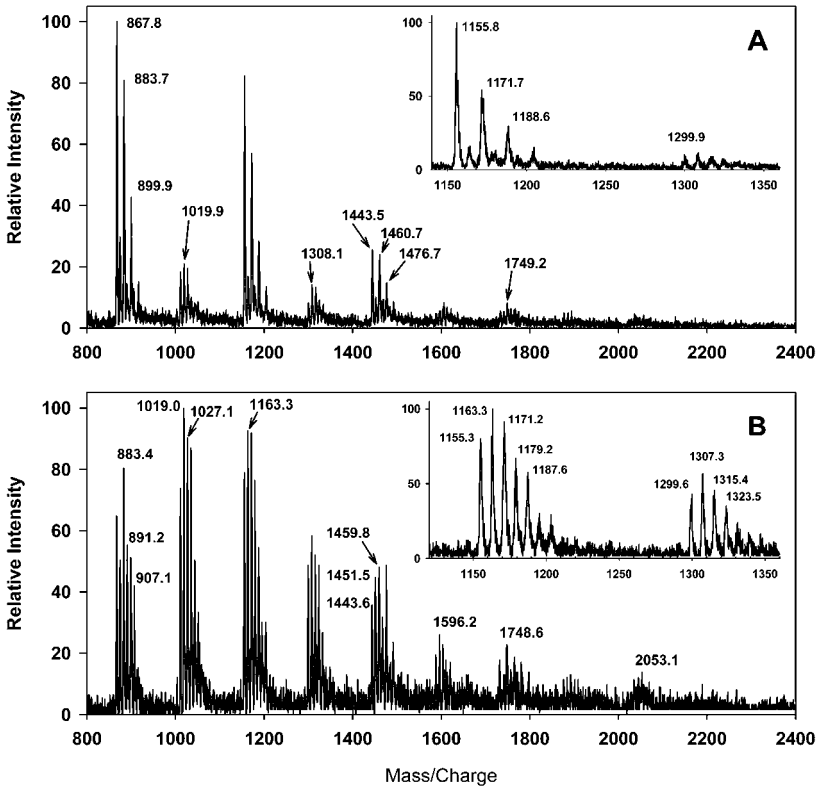


**Figure 4.** ESI mass spectra: A, sample 4; B, sample 6. See **Table 4** and text for explanation.

Unfortunately, the improvement is limited, as can be seen in the spectra of sample 8 (**Figure 5**). Not only are the MS polymer size estimates farthest from the mDP by acid catalysis, but the spectra are not easy to interpret. The ESI spectrum is quite noisy, although the more intense peaks are spaced ~144 Da apart, indicating that doubly charged ions are dominant. Assignments for some of the major peaks are given in **Table 4**. The MALDI spectrum is simpler in appearance, with peaks grouped at intervals corresponding to DP differences. The less intense peaks near the front of each of these groups correspond to the calculated masses of proanthocyanidins of a given DP with zero to four prodelphinidins, but most of the individual peaks cannot be unambiguously assigned, especially in the higher mass range. The prominent peaks at *m*/*z* 3042, 3632, 4513, and 5986 are

**Table 5.** Comparison of Mass Range Estimates by Acid Catalysis and Mass Spectrometry

| sample | mDP by acid catalysis | polymer range in ESI | most intense polymer in ESI | polymer range in MALDI | most intense polymer in MALDI |
|---|---|---|---|---|---|
| 1 | 1.8 | 1 | 1 | *a* | *a* |
| 2 | 2.3 | 1–5 | 2 | *a* | *a* |
| 3 | 3.8 | 2–5 | 3 | 3–7 | 3 |
| 4 | 5.4 | 3–9 | 3 | 3–8 | 4 |
| 5 | 7.6 | 3–11 | 4–7 | 4–10 | 5 |
| 6 | 10.2 | 3–13 | 7 | 4–10 | 6 |
| 7 | 13.4 | 3–13 | 8 | 4–13 | 8 |
| 8 | 22.2 | 3–14 | 10 | 4–20 | 10 |
| total | 7.8 | 1–5 | 2[b] | 3–8 | 3[b] |

[a] Not measured. [b] See text for explanation.

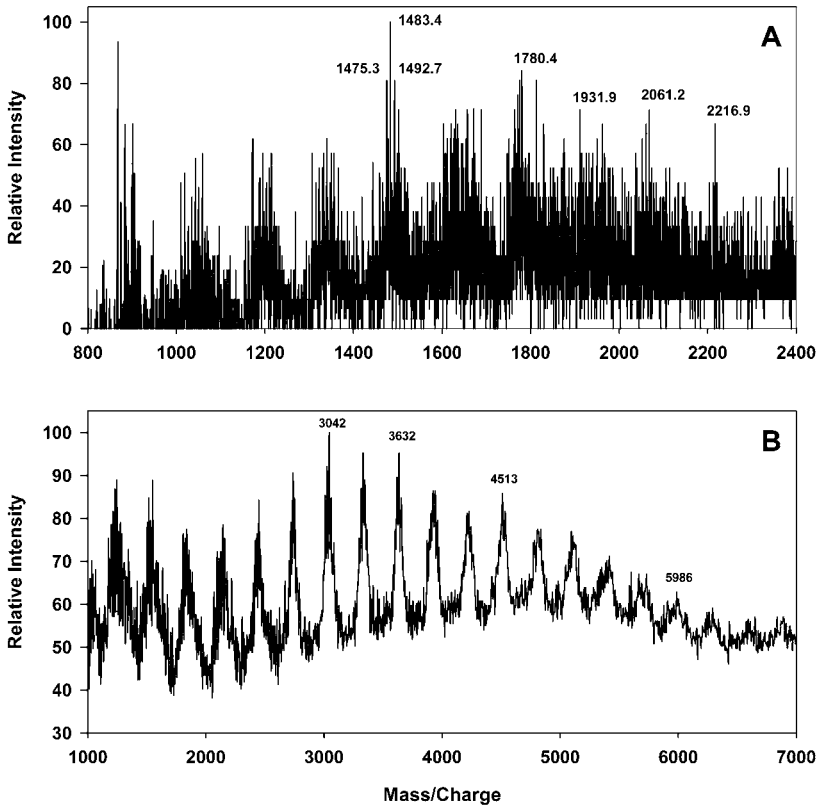


**Figure 5.** Mass spectra of sample 8: A, ESI; B, MALDI-TOF. See text for explanation.

apparently a 10-mer, a 12-mer, a 15-mer, and a 20-mer, respectively, all possibly with a high proportion of prodelphinidin subunits, but they are all halfway between calculated expected masses, outside the normal mass accuracy of our MALDI instrument. For example, a sodiated decamer with eight prodelphinidins has a calculated average mass of 3035.6 Da; with nine prodelphinidins, the expected mass is 3051.6. The formation of ion clusters may explain the difference: a non-covalent tetrameric aggregate of two dimers and two trimers, with eight prodelphinidins and a single sodium among them, would have a combined mass of 3041.6 Da. The laser energy had been increased with this sample in an effort to produce detectable ions, and it is possible that larger polymers fragmented in the source, which then combined into non-covalent clusters. Another possibility is the presence of a second sodium atom, without additional charge (*34*); in a decamer with seven prodelphinidins, the expected mass is 3042.6. High-resolution or MS-MS experiments would be needed to test these possibilities. The peaks at *m*/*z* 3042, 3632, and 4513 were also seen in the spectra of sample 7, another difficult sample on MALDI, but not in the lower mDP samples. Dimeric ion clusters were confirmed in the FT-ICR spectra of some samples. Although prior separation enabled better detection of larger oligomers than was possible in the total mixture, mass spectrometry still does not provide a full picture of the mass distribution of the proanthocyanidins.

Researchers have observed that MALDI-TOF spectra of synthetic polymers that have polydispersed molecular weight distributions often show a discrimination against the high MW components in the mixture that is not seen in the spectra of polymers with a narrow MW distribution (*35*). This phenomenon has been attributed to a number of factors, including sample preparation (*36*−*38*) and detection limitations (*39*, *40*). All samples in the present study were prepared and spotted on the MALDI probe in the same manner. Although the technique could be optimized, sample preparation alone does not account for the failure to see the larger proanthocyanidin molecules in the "total" sample. Delayed detection did not improve the peak intensities of heavier proanthocyanidins in recorded spectra, so

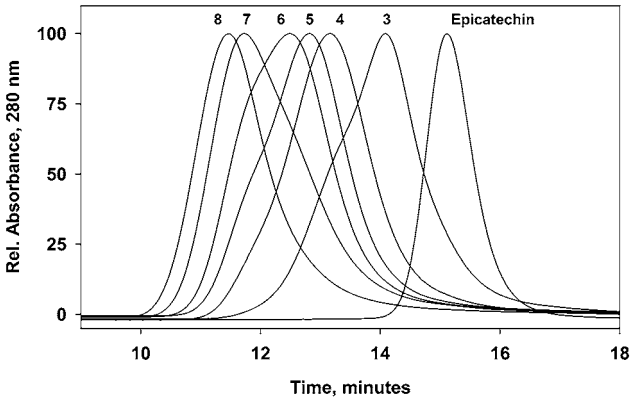


**Figure 6.** Gel permeation chromatograms of samples 3–8 and epicatechin.

detector saturation by low MW species was not an explanation, either.

Mass discrimination has also been observed in the ESI mass spectra of polydisperse mixtures of lipids (*41*) and synthetic polymers (*42*). In ESI, the phenomenon is thought to be caused by partitioning of analyte molecules between the surface and inner volume of droplets, with differential ionization and transfer to the gas phase as a result (*43*). Whether the properties of small proanthocyanidin oligomers and larger polymers are sufficiently different to cause such partitioning is not known.

Mass spectrometry measures ion intensity on a number, not weight, basis. Using the mDP from acid catalysis, the trimer sample (3) contains ~6 times as many moles as sample 8 on a per weight basis. On the basis of the proportion of those samples in the total, however, the proanthocyanidin molecules of sample 3 are ~2.5 times the number of those represented by sample 8. In contrast, the ion intensity of the base peak in the ESI spectrum of sample 3 was 30-fold greater than in sample 6 and in MALDI-TOF, 13-fold. Also, in spectra of the total sample, the large proanthocyanidins were not visible above the baseline. Thus, it seems that discrimination against the mass spectral detection, for whatever reason, of larger proanthocyanidins did occur.

Some of the hop proanthocyanidin samples, including the "total," were analyzed by ESI coupled to a FT-ICR mass spectrometer. Data from this high-resolution instrument confirmed the identities assigned to ions in the spectra shown above. Byrd and McEwen (*44*) discussed the importance of signal-to-noise problems for detection by MALDI-TOF MS of high molecular weight polymers in a polydisperse mixture, and that is the case with the hop proanthocyanidin spectra, as well. The improved signal-to-noise ratio of the FT-ICR allowed the detection of heavier proanthocyanidins in each of the samples analyzed. In general, the range of detected proanthocyanidins was extended by one or two additional DP; the most abundant oligomer was the same or one DP larger than recorded in **Table 5**. In the spectrum of the "total" sample, tetramers were the most intense peaks, and polymers as large as dodecamers were present, a clear improvement over the spectrum in **Figure 1**.

Polymer scientists have used size exclusion and gel permeation chromatography to independently characterize the MW distribution of polymer mixtures examined in the studies of mass discrimination cited above. On the other hand, some workers have applied prior separation by size exclusion (*44*) and gel permeation (*46*, *47*) chromatography to produce fractions of lesser molecular weight dispersity to avoid the problem of mass discrimination in the mass spectrometry of polydisperse mixtures. MS analysis of these fractions yielded more accurate spectra, and the spectra provided mass calibration for the size fractionation of the chromatography.

The hop proanthocyanidin fractions were analyzed by high-performance GPC to provide another source of molecular weight distribution information. The resulting average sample retention times (determined by measuring the retention time of each sample at 50% elution) show that the higher numbered samples elute earlier from the GPC column (**Figure 6**). Thus, proanthocyanidins eluting later from Sephadex LH-20 possess greater hydrodynamic volumes. Excluding samples 1 and 2, which were not analyzed, GPC shows that average molecular weight increases with the order of elution from Sephadex, consistent with the phloroglucinol analysis. In fact, a comparison of the average molecular weight of the samples [determined using the compositional and mDP information from acid catalysis (**Table 2**)] with their respective GPC retention times indicates that these analytical results have a strong relationship ($R^2 = 0.996$, **Figure 7**). The sample chromatograms indicate that each contains a different molecular size range, although the ranges do overlap. The peak maxima, nearly equivalent to the 50% retention times, were well separated. This again indicates that mass spectrometry is providing an incomplete understanding of the proanthocyanidin samples, as the mass spectra show a distribution skewed toward the lower *m*/*z* ions within each sample.

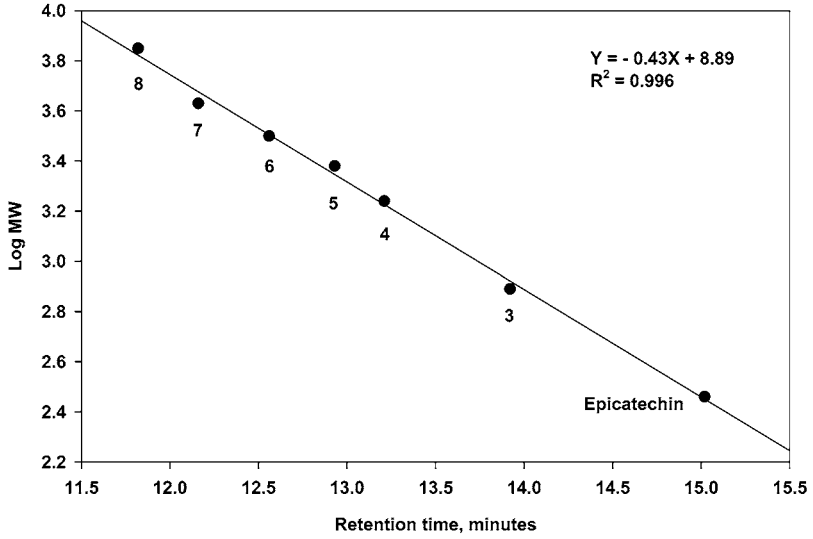


**Figure 7.** Relationship of molecular weight calculated from acid catalysis data to retention time from GPC.

In summary, we have characterized the proanthocyanidins of Willamette hops with complementary methods. Acid catalysis in the presence of excess phloroglucinol yielded the subunit composition of the proanthocyanidin samples, as well as their average molecular weights. Mass spectrometry confirmed that hop proanthocyanidins contain both procyanidin and prodelphinidin residues and gave more information on the MW distribution, but was unable to detect the full range of masses present. GPC, although unable to give any information on the subunit composition or direct measurements of MW, did reveal a more complete picture of the distribution of molecular weights in the samples. All methods confirm that hops contain proanthocyanidins of much higher DP than previously reported.

Prior size separation of the hop proanthocyanidins by the use of the acetone, methanol, and water step gradient on Sephadex LH-20 allowed more information to be gained from each of the analytical methods. In particular, mass spectral detection of larger molecules was significantly improved, but separation alone was insufficient to allow a complete analysis of MW distribution by MS. Further progress will depend on better resolution and mass accuracy, as well as optimization of ionization. Like other fractionation methods published, this one did not produce complete separation to pure compounds. To judge by reported fraction mDP values (*10*, *26*, *27*), however, the present method worked at least as well to produce fractions with distinctly different properties. The method uses readily available and relatively inexpensive components, and further refinement is possible through manipulation of the proportions of the three solvents.

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