

# Characterization of Hop and Beer Tannins by Electrospray and MALDI-TOF Mass Spectrometry

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## OVERVIEW

### Purpose

- Improve MS detection of large proanthocyanidins (tannins) by use of prior chromatographic fractionation
- Elucidate the size distribution of tannins in hops and beer

### Methods

- Chromatography on Sephadex LH-20 columns using a new solvent elution scheme to separate crude tannin mixtures into different size classes

### Results

- MS (ESI-Q and MALDI-TOF) of fractions reveals much larger tannins than can be detected by MS of crude mixtures, but the largest still remain undetected even though they are among the most abundant tannins in hops

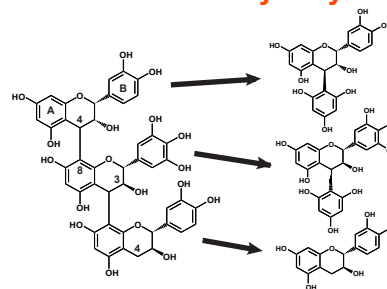
## Introduction

□ Proanthocyanidins, or condensed tannins, are polymers of the polyphenolic flavan-3-ols, most commonly catechin and epicatechin. They are widely distributed in the plant kingdom, and they contribute to the astringent qualities of such common beverages as tea, red wine, and beer. They are currently of great interest because of their properties as anti-oxidants and radical scavengers (for example, Stevens *et al.*, 2002, and Takahata *et al.*, 2001). Tannins in hops (*Humulus lupulus* L.) are of concern to brewers because they combine with proteins to form beer haze. Size, or polymer chain length, may influence many of these properties, but this has been difficult to study because tannins occur in complex mixtures. Only the smallest components of these mixtures are readily resolved chromatographically or detected by mass spectrometry.

□ Sephadex LH-20 has been used widely for tannin chromatography (Karchesy *et al.*, 1989), most commonly for removing such contaminants as carbohydrates and glycosides, but also for size separation through the use of alcohol-water gradient elution. Typically, 60 - 70% acetone (aq.) is used as a final wash to complete the elution of the tannins, but to our knowledge no one has reported the use of acetone as a component of a solvent gradient. Could this be used to better resolve the larger tannins?

□ Mass spectra of tannin mixtures from a variety of sources are always dominated by the lowest MW components, with peak intensity diminishing as polymer length increases, in a pattern resembling exponential decay. Other evidence, acid hydrolysis for example, indicates that heavy tannins are often more abundant in those same mixtures. Would better separation of the heavy tannins from the more readily ionized light tannins result in improved detection by MS?

## Structures and Hydrolysis



□ The common flavan-3-ols found in tannins are distinguished by the orientation of the OH at C-3 (catechin vs. epicatechin) and by the number of OH's on the B ring (catechin vs. gallo catechin). They are most often linked by a C-4 to C-8 bond, with the orientation at C-4 opposite that of the OH bond at C-3. Thus, the tannin on the left above is **epicatechin-(4β→8)-gallo catechin-(4α→8)-catechin**.

□ The subunit composition of a tannin can be revealed by acid hydrolysis in the presence of excess phloroglucinol (Kennedy and Jones, 2001). Terminal units (with only H at C-4) are released as free flavans. Extension units (bonded to another flavan through C-4) are released as carbocations, which then bond to a nucleophile such as phloroglucinol. The identity of all products can be identified and quantified by UV-HPLC. The molar ratio of the total of subunits to the terminal units is the **mean degree of polymerization (mDP)** or average length of the proanthocyanidin chain.

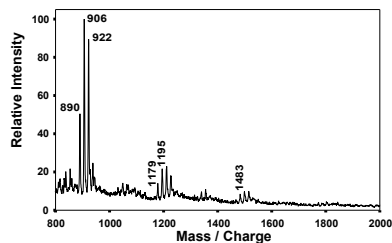
## Extraction and Initial Purification

- Whole Willamette hops (50 g, frozen 2000 crop, 13% moisture) were extracted 3X with 1 L methylene chloride (DCM) (1 hour with occasional stirring) to remove pigments, lipids, etc., then air-dried in hood overnight.
- Dry hops were ground in Wiley mill to pass 20 mesh, then extracted 3X with 800 ml of 70% aq. acetone (2 hours with continuous stirring). All extracts pooled and rotary evaporated under vacuum to remove acetone.
- Extract (~1 L) was partitioned first with 800 ml hexane, then 400 ml DCM, to remove more pigments and nonpolar material, then rotary evaporated to remove solvents.
- Washed extract was loaded onto 4 cm diam. x 30 cm long Sephadex LH-20 column equilibrated with 25% aq. methanol (MeOH). Column then washed with 25% MeOH (400 ml), 50% MeOH (1040 ml), and 80% MeOH (400 ml) to elute glycosides and other materials and then catechin/epicatechin monomers and dimers.
- Elution was monitored with 2D TLC on cellulose plates developed first with 3:1:1 *tert*-butanol:water:acetic acid, dried, then developed in the second dimension with 6% acetic acid (aq.), then visualized with vanillin-HCl reagent.
- Finally, 70% acetone (500 ml) was used to elute tannin polymers. Fractions containing all trimer and larger proanthocyanidins were pooled and rotary evaporated to remove organic solvents, then lyophilized to dryness to yield 1.940 g of crude tannin.

## Refined Separation of Tannins

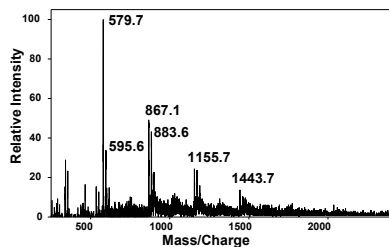
- Crude proanthocyanidins were dissolved in 50.0 ml of MeOH. 5.0 ml were withdrawn for "Total" sample.
- Remainder was diluted with 0.5% aq. formic acid to yield 90 ml of 50% MeOH solution, then applied to 4 cm diam. x 45 cm long Sephadex LH-20 column equilibrated with 50% MeOH.
- Column was developed with:
  - 60% MeOH (800 ml) (all contain 0.2% formic acid)
  - 75% MeOH (800 ml)
  - 90% MeOH (800 ml)
  - 10% acetone, 80% MeOH, 10% water (600 ml)
  - 20% acetone, 65% MeOH, 15% water (600 ml)
  - 30% acetone, 40% MeOH, 30% water (600 ml)
  - 60% acetone, 40% water (800 ml)
- Fractions were monitored by 2D TLC and by ESI-MS, then pooled as follows:
  - #1: catechin/epicatechin rich, eluted by 75% MeOH
  - #2: dimer rich, eluted by 75% - 90% MeOH
  - #3: trimer rich, eluted by 90% MeOH
  - #8: large proanthocyanidins eluted by 60% acetone (obvious brown band)
  - #4, 5, 6, 7: monitoring by TLC and MS shows a continual increase in the size of proanthocyanidins, but no obvious cutoffs or fronts. Therefore, fractions collected between #3 and the front of #8 were divided into four equal parts and pooled.
- All pooled fractions were rotary evaporated, lyophilized to dryness, dissolved in MeOH, and stored in the freezer.

## Tannins of a Typical Beer



- A major brand U. S. pilsener beer (40 fl. oz. or 1.2 L) was degassed by sparging with helium and by ultrasonication. The beer was then passed through a Sephadex LH-20 column to adsorb the tannins. The column was washed and the oligomeric tannins (16 mg) eluted by the procedure used for crude hop extracts.
- MALDI-TOF shows trimers as the most intense peaks, with tetra- and pentamers clearly present. Within each size class, tannins containing one or more (epi)gallo catechin units are more abundant than all-(epi)catechin tannins, possibly because of greater solubility. The peaks shown are assumed to be sodium adducted. Acid hydrolysis with phloroglucinol results in an mDP of 5.3; that is, an average tannin in this beer is, in fact, a pentamer.

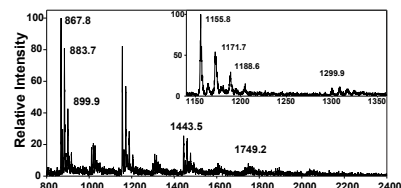
## Electrospray MS of Hop Tannins



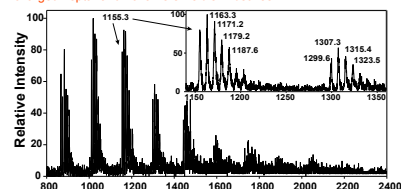
ESI Mass Spectrum of "Total" Hop Tannins

Electrospray mass spectrometry was performed on a Sciex AP III Plus, in the single MS positive mode. Samples diluted to 1 mg/ml were loop-injected into 2:1 MeOH:0.5% aq. formic acid flowing at 5 - 10  $\mu$ l/min to the electrospray source. Ionization voltage was 5000 V and the orifice set at 60 V.

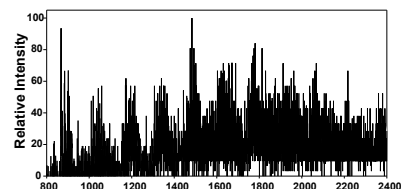
Dimers composed of all (epi)catechin produce the most intense peak. The four possible combinations, the so-called procyanidins B1 - B4, can not be distinguished by MS. Next is the all (epi)catechin trimer. The expected  $m/z$  of the protonated ion equals  $290 \times (n-1)288 + 1$ , yielding the 579, 1155, 1443 sequence above. Within each oligomer size class, peaks at 16, 32, etc., units heavier indicate the presence of one or more (epi)gallocatechin units. The pentamers are the largest tannins clearly visible in the spectrum. Only singly charged ions are apparent.



Fraction 4: singly charged trimers through hexamers, with doubly charged hepta- and nonamers visible in between



Fraction 6: note peaks 8, not 16, apart in each group: doubly charged ions most intense in hepta- to decamer range



Fraction 8: Too complex to interpret clearly. Most peaks of higher  $m/z$  than in other fractions, but base peak intensity lowest of all fractions

## Summary

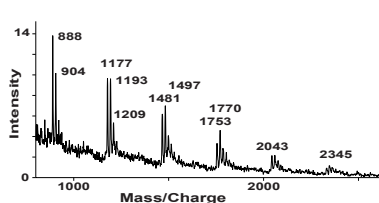
Fractionation of proanthocyanidins by size greatly improves the detectability of larger molecules by MS, with MALDI more effective at ionizing the larger tannins than ESI. However, the largest of the tannins still remain undetectable by MS.

### References

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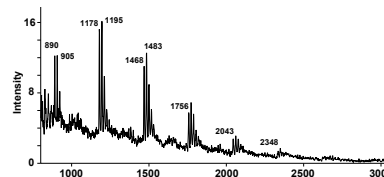
## MALDI-TOF MS of Hop Tannins



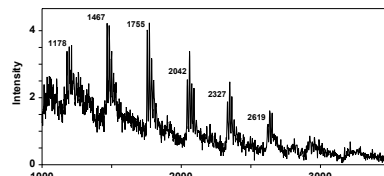
MALDI-TOF Spectrum of "Total" Hop Tannins

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 as the matrix (Ohnishi-Kameyama *et al.*, 1997), with acetonitrile as the most common 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 "shots."

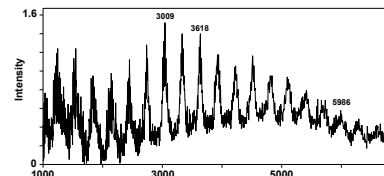
Monomers and dimers have masses below the recorded range, thus trimers are the most intense ions in the spectrum of a total mixture. Polymer sizes up to octamers are clearly seen, and each size class contains the peaks 16, 32, etc.,  $m/z$  units apart indicating chains containing one or more (epi)gallocatechins. In spite of great care during the purification of the tannins, all peaks are apparently sodium adducted, rather than the protonated ions seen in ESI.



Fraction 4: Tetramers are the most intense peaks, octamers the largest polymers clearly present



Fraction 6: Penta- and hexamers are the most intense peaks and nonamers are easily seen



Fraction 8: Tannins up to 20-mers are visible, decamers most intense. Note how much simpler the spectrum is to interpret than ESI

## Method Comparison

Fraction	Weight (mg)	Mean Degree of Polymerization	Polymer Range in ESI	Most Intense Polymer in ESI	Polymer Range in MALDI	Most Intense Polymer in MALDI
1	44	1.8	1	1	*	*
2	134	2.4	1 - 5	2	*	*
3	196	3.7	2 - 5	3	3 - 7	3
4	194	5.4	3 - 9	3	3 - 8	4
5	205	7.4	3 - 11	4 - 7	4 - 10	5
6	208	9.2	3 - 13	7	4 - 10	6
7	246	12.7	3 - 13	8	4 - 13	8
8	470	22.0	3 - 14	10	4 - 20	10
Total	1940	7.4	1 - 5	2	3 - 8	3

All but the earliest Sephadex fractions contain tannin polymers larger than can be detected by mass spectrometry of crude mixtures. Within each fraction, the most intense peaks are of the smaller polymers, just as in whole mixtures. Thus, relative peak intensity appears to be more a reflection of ease of ionization than of concentration of a particular tannin in the mixture. With acid hydrolysis as a benchmark, MS is still unable to detect large tannins or yield useful quantitation.

