

Identification of Tannins and Determination of Concentration in *Quercus palustris* Acorns

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ABSTRACT

Hydrolyzable and condensed tannins are representatives of a large group of polyphenolic compounds found in plants. There is great speculation as to the importance and function of these compounds in plant-predator relationships.¹ The identification of and characterization of these molecules from plant materials could greatly increase the likelihood of elucidating a role and mechanism of action. Cotyledons and embryo of the *Quercus palustris* acorn were pulverized with a mortar and pestle after removing the seed coat. Tannins were extracted from this material and subjected to ESI/LC/MS. The tannins were identified based upon the fragmentation patterns and comparison with prior work. The concentration of the individual tannins in the developing *Quercus palustris* acorn was followed throughout the growing season to observe the effect of maturation.

SAMPLE PREPARATION FOR MASS SPECTROSCOPY

Cotyledons and embryo of *Quercus palustris* (Pin Oak) were treated with the following method. The cotyledons and embryo were removed from the seed coat and pulverized with a mortar and pestle. One gram of the dry material was added to ten mL of a solution of MeOH/water (80:20 v/v) containing 0.8 mM NaF to prevent sample oxidation. The solution was shaken on a Glas-Col bench top shaker for one hour and allowed to settle. The supernatant was removed and filtered with a 0.2 μm hydrophilic nylon membrane filter. The filtered extract was analyzed using LC/ESI/MS AND LC/EI/MS.

INSTRUMENTATION – HPLC/DAD/ESI-MS/MS Analyses

LC/ESI/MS/MS experiments were performed on an Agilent MSD XCT ion trap mass spectrometer (Palo Alto, CA) equipped with an electrospray ionization (ESI) interface, 1100 HPLC, a DAD detector, and Chemstation software. The column used was a 150 x .5 mm i.d., Zorbax XDB- C18 3.5 μm (Agilent, Palo Alto, CA). Flow rate was 5.00 $\mu\text{L}/\text{min}$, injection volume was 0.5 μL , and column temperature was 25 $^{\circ}\text{C}$. The ESI parameters were as follows: nebulizer, 15 psi; dry gas (N_2), 5.00 L/min; dry temperature, 325 $^{\circ}\text{C}$; trap drive, 78.0; skim 1, -40 V; lens 1, 5.00 V; octopole RF amplitude, 200.0 Vpp; capillary exit, -200 V. The ion trap mass spectrometer was operated in negative ion mode scanning from m/z 50 to m/z 2200 at a scan resolution of 13000 amu/s. Trap ICC was 70000 units and maximal accumulation time was 200000 μs . MS-MS was operated at a fragmentation amplitude of 1.0 V, and threshold ABS was 20,000 units.

LIQUID CHROMATOGRAPHIC SEPARATION

The constituents were separated using a water (A) and methanol (B) gradient (each containing 0.1% formic acid). Initial conditions were 3% methanol increasing to 25% methanol at 6 minutes increasing to 35% at 25 minutes increasing to 90 % at 35 minutes holding at 90% to 40 minutes and returning to starting conditions at 45 minutes. The detection wavelength was 254nm. This separation method was utilized on both the ESI and PB instruments.²

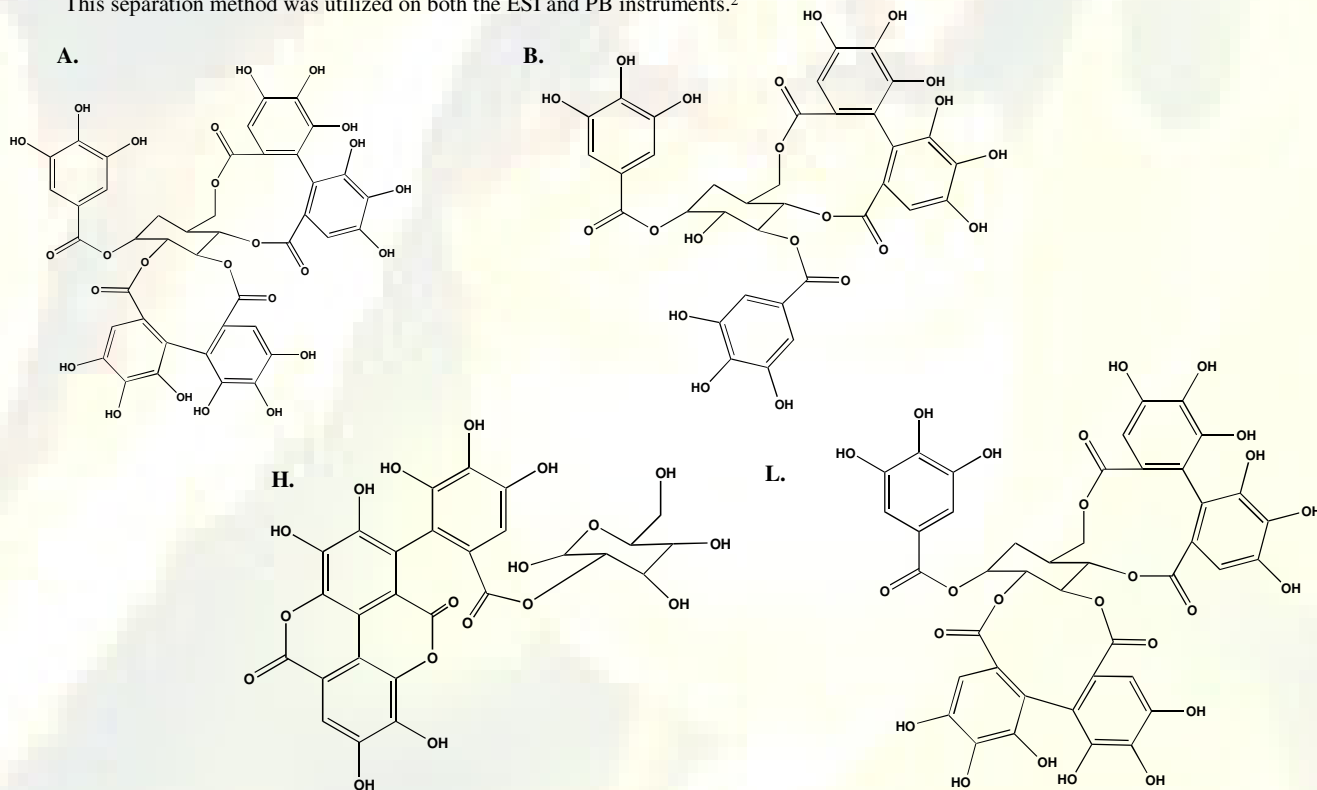


FIGURE 1. HPLC TRACE MEASURED AT 254nm

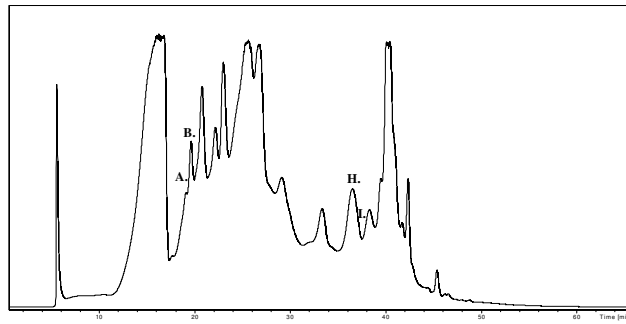
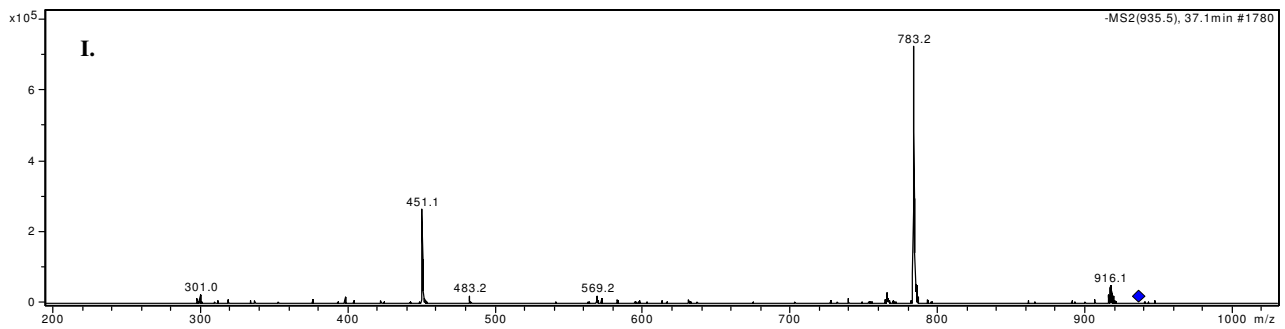
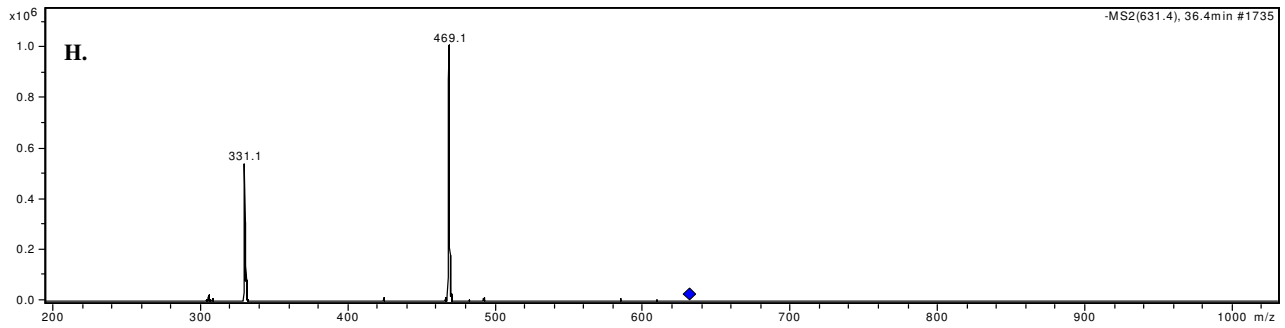
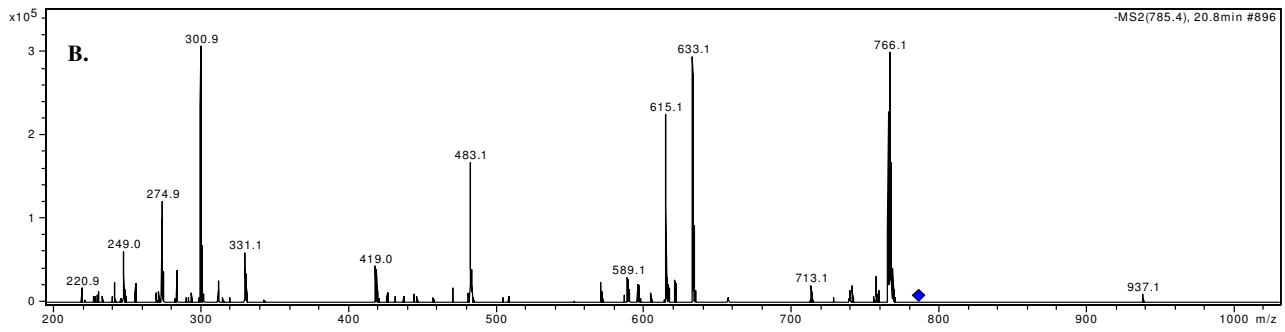
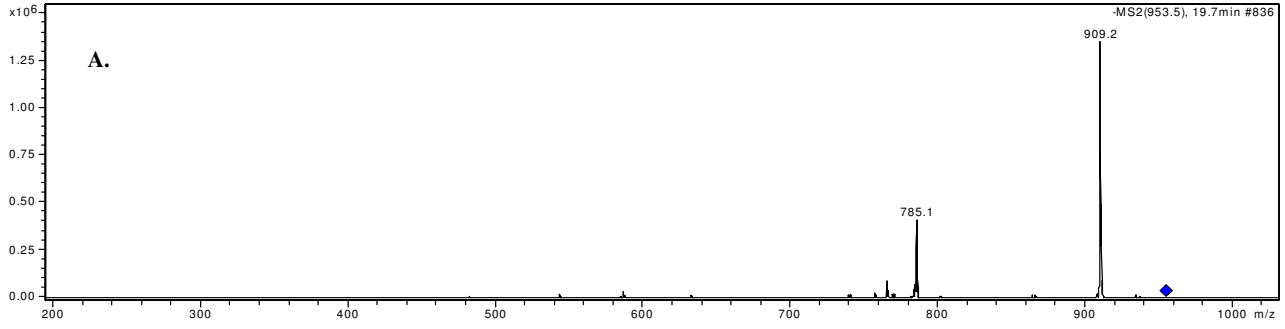


FIGURE 2. MS/MS SPECTRAL DATA



RESULTS

We were able to successfully implement a chromatographic method using a reverse-phase column with UV detection at 254 nm that could resolve the constituents of the acorn nut extract. The LC/ESI/MS was able to separate several constituents from the extract obtained from the dried and crushed cotyledon of *Quercus palustris* (Pin Oak) under the conditions previously described. Several individual tannins were deduced by comparing the fragmentation patterns of the *Quercus palustris* acorns with previously published results². Many of the tannins were the same as those found in the previous literature; however, there were also a number that were identified and for which we have proposed structures (Structures A, B, and I). See Table 1 for a list of several tannins identified. Several other possible tannins were also detected, but are still under investigation at this time to ascertain their identities.

Eleven of the tallest peaks were identified from the mass spectral data. The relative concentrations of these peaks were then followed throughout one month of the growing season. The first sample date was 9/14, the second date was 10/03, and the third was 10/14. The relative concentrations have been recorded in Table 2. The relative tannin concentrations show marked increase in tannin concentrations over the last two weeks of the sample period. The concentrations remain relatively unchanged in most cases between the first two weeks of the sampling period.

TABLE 1. IDENTIFIED COMPOUNDS

	RT (min)	Parent (m/z)	Parent Compound	MSMS Ion	MSMS Ion	MSMS Ion	MSMS Ion
A	19.7	953.5	Galloyl-di-HHDP glucose (with ester bond to HHDP hydrolyzed)	909.1	785.1		
B	20.8	785.4	Digalloyl-HHDP-glucose	766.1	633.1	483.1	300.9
C	22.4	935.2	Galloyl-di-HHDP glucose	916.2	783.5	451.3	301.2
D	23.0	860.1	Identity Unknown	785.2	677.1	483.4	300.8
E	25.8	953.6	Galloyl di-HHDP glucose (w/ ester bond to HHDP hydrolyzed)	909.2	785.3		
F	29.2	785.2	Digalloyl-HHDP-glucose	766.2	633.3	483.2	301.2
G	31.6	937.1	Trigalloyl-HHDP-glucose	916.5	783.2	633.1	300.9
H	36.4	631.4	Tergallic-O-glucoside	469.1	331.1		
I	37.1	935.5	Galloyl-di-HHDPglucose	916.1	783.2	451.1	301.0
J	38.2	935.2	Galloyl-di-HHDP glucose	916.3	782.8	485.1	301.2
K	41.1	935.7	Galloyl-di-HHDP glucose	915.8	783.1	485.3	300.8

TABLE 2. RELATIVE TANNIN CONCENTRATIONS

	RT (min)	Parent (m/z)	Parent Compound	Relative Concentration on 10/14	Relative Concentration on 10/3	Relative Concentration on 9/14
A	19.7	953.5	Galloyl-di-HHDP glucose (with ester bond to HHDP hydrolyzed)	5.99	1.08	1
B	20.8	785.4	Digalloyl-HHDP-glucose	3.71	1.39	1
C	22.4	935.2	Galloyl-di-HHDP glucose	1	1.25	1.13
D	23.0	860.1	Identity Unknown	1.27	1	1.09
E	25.8	953.6	Galloyl di-HHDP glucose (w/ ester bond to HHDP hydrolyzed)	5.16	1.26	1
F	29.2	785.2	Digalloyl-HHDP-glucose	2.81	1	1.08
G	31.6	937.1	Trigalloyl-HHDP-glucose	1.57	1	1.62
H	36.4	631.4	Tergallic-O-glucoside	4.51	1	1.77
I	37.1	935.5	Galloyl-di-HHDPglucose	3.11	1.14	1
H	38.2	935.2	Galloyl-di-HHDP glucose	3.70	1.08	1
K	41.1	935.7	Galloyl-di-HHDP glucose	2.11	1	1.17

CONCLUSIONS

The identification and uniqueness of the tannins found in *Quercus palustris* (Pin Oak) acorns provides our research group and others with additional insight into the complexity of this class of biomolecules. Several tannins were identified from comparison of the fragmentation patterns to published data. A number of other tannins were also identified which were unique to the *Quercus palustris* (Pin Oak). We have been able to deduce possible structures for some of these tannins from their mass spectral data.

Eleven of the largest peaks were selected from the mass spec and their concentrations were studied over a one month growing period. Acorns were sampled at the middle of September, and the beginning and middle of October. The relative concentrations of most of the tannins studied showed a large increase in concentration over the last two weeks of the sampling period. These concentrations, however had rather insignificant changes between the first two weeks of the sampling period.

In summary, an evaluation of our experiments showed the following:

1. The utilization of ESI in negative mode works very well for the characterization of the tannins from a variety of acorn producing trees.
2. We were able to identify several tannins in the acorns of *Quercus palustris* (Pin Oak) that have been characterized in at least one other variety of acorn.
3. Additionally we have proposed unique structures for several previously unreported tannins.
4. A large increase in relative tannin concentrations was observed over the last two weeks of the sampling period.

FUTURE WORK

We hope to continue studying the changes of tannin concentrations through the growing season. We also hope to collect mass spectral data on several other species of oak acorns. We also hope to determine if there may be some type of connection between the tannin concentrations and any function they may have in any plant-predator relationships.

REFERENCES

1. Tannins: Does Structure Determine Function? An Ecological Perspective. William V. Zucker. The American Naturalist, **1983**, volume 121, issue 3, 335-365.
2. Phenolic Compounds and Fatty Acids From Acorns (*Quercus Spp.*), The Main Dietary Constituent of Free-ranged Iberian Pig. Emma Cantos, Juan Carlos Espin, Clemente Lopez-Bote, Lorenzo De La Hoz, Juan A. Ordonez, and Francisco A. Tomas-Barberan. J. Agric. Food Chem. **2003**, 51, 6248-6255.