Application of LC/ESI/MS and LC/EI/MS to the Characterization of Tannins and Flavonoids from the Acorns of *Quercus macrocapra*

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INTRODUCTION

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. The development of a LC/ESI/MS method coupled with diode array detection was undertaken in an attempt to provide a more efficient method of separating and direct method of identifying the constituents. The chromatographic separation method was subsequently utilized in LC/EI/MS. In LC/EI/MS effluent from an HPLC is introduced into an Electron Ionization source giving typical EI spectra that can be searched on a common mass spectral library. Each method yields different fragmentation patterns, but in combination provide additional information of a complementary nature for structural elucidation.

SAMPLE PREPARATION FOR MASS SPECTROSCOPY

Cotyledons and embryo of *Quercus macrocarpa* (Burr Oak) were treated with the following method. The cotyledons and embryo were removed from the seed coat and pulverized with a mortar and pestle. Two grams of the dry material was added to eight 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 – PARTICLE BEAM LCMS INTERFACE

For particle beam LCMS, the system included the following components. The liquid chromatograph used was an Agilent Model 1100 modular system with quaternary pump, vacuum degasser, 100 vial autosampler and variable wavelength detector. The HPLC column used was a Zorbax SB-C18 (Agilent pn 830990-902), narrow bore 2.1 x 150 mm 3.5 micron. The Genesis II particle beam interface (CSS Analytical Co. Inc.) was attached to an Agilent 5973 MSD so that samples can be analyzed by LC/MS with electron impact and chemical ionization. The Genesis II is an improved particle beam interface, which delivers a higher amount of analyte to the ion source, when compared to previous commercial interfaces. The mass spectrometer used was an unmodified Agilent 5973 Mass Selective Detector (Agilent Technologies, Inc., Palo Alto California) with turbo molecular pump. The Agilent 5973 is a benchtop quadrupole mass spectrometer with mass range of 1.6 to 800 mass units, 10,000 volt HED, and is available with EI or EI/CI capabilities.

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 µL/min, injection volume was 0.5µL, and column temperature was 25 °C. The ESI parameters were as follows: nebulizer, 15 psi; dry gas (N_2), 5.00 L/min; dry temperature, 325 °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]

MASS SPECTRAL ANALYSIS

The data collected from the Particle Beam EI Ionization of the chromatographic separation was analyzed with AMDIS (Automated Mass Spectral Deconvolution and Identification System), version 2.1, DTRA/NIST, 2002. Since no library was available for searching for the ESI, spectral identifications were made either by comparing the parent ion molecular weight with those obtained from literature reports or deduction based upon previous results.

FIGURE 1. HPLC TRACE MEASURED AT 254nm

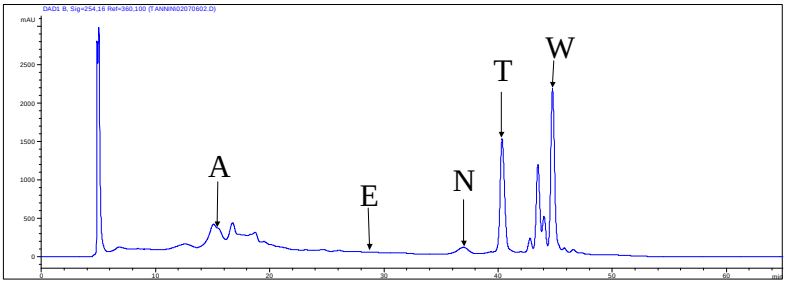
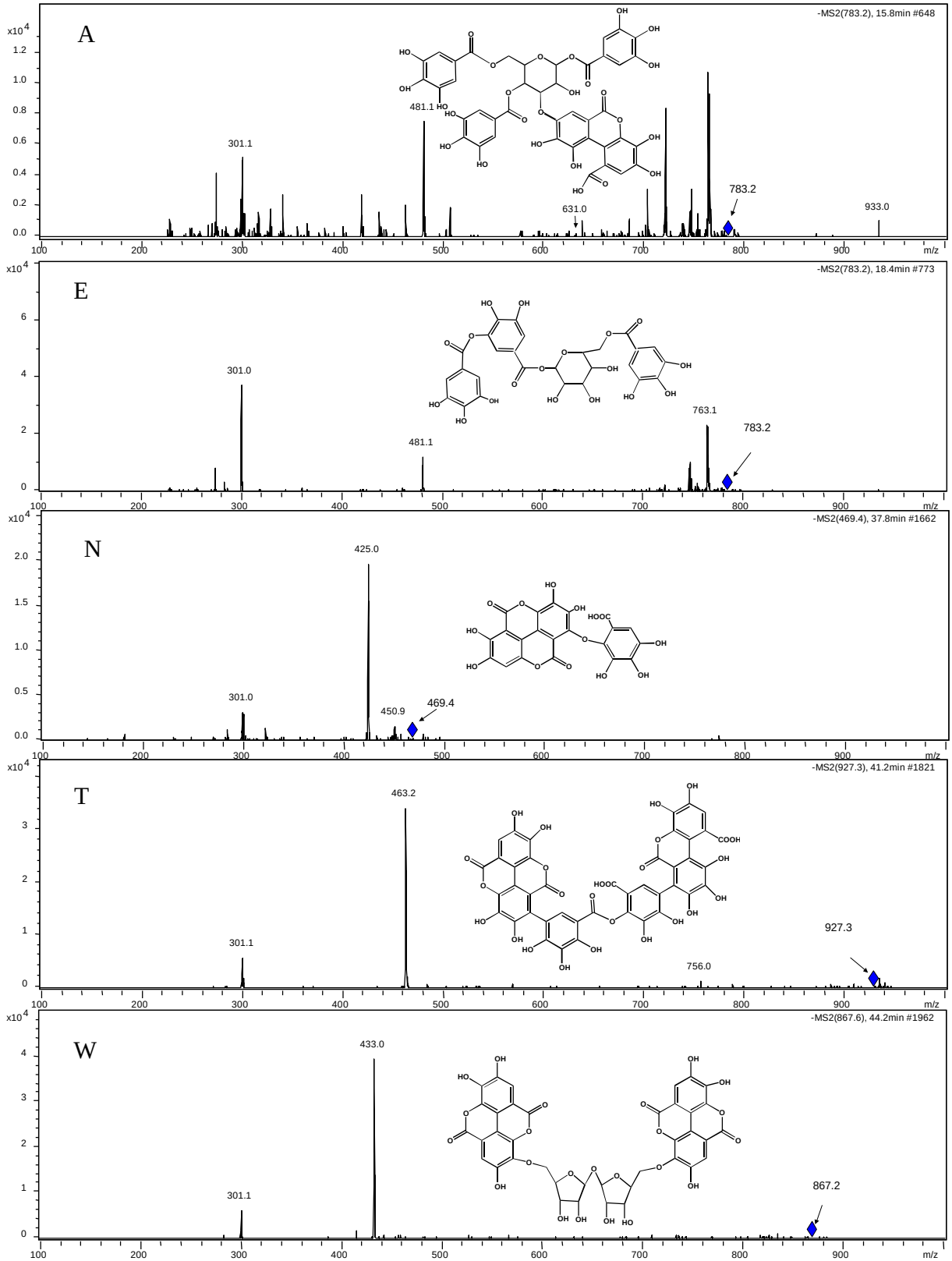


FIGURE 2. MS/MS SPECTRAL DATA



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 approximately 45 constituents from the extract obtained from the dried and crushed cotyledon of *Quercus macrocarpa* (Burr Oak) under the conditions previously described. When this separation was coupled with the ESI and EI mass spectrometers we were able to obtain mass spectral data in the negative mode quite successfully for ESI, but despite attempts at reducing the keV in the EI we were unable to obtain parent ion data for the constituents. The collected data for the EI LCMS was submitted for deconvolution and extracted ion analysis using the AMDIS program. The tannins proved too labile under the analysis conditions and yielded fragments corresponding to gallic acid and sugar residues. We were able to identify 24 constituents in the ESI analysis as tannins by comparison to previously published accounts in the literature and also by deduction. Several other possible tannins were also detected, but are still under investigation at this time to ascertain their identities.

To this point we have identified several tannins possessing the same parent ion mass, but differing daughter ion formation and fragmentation. These compounds migrate significantly differently in the LC indicating some type of difference in the attachment of the identified residues to the core structures. While we were able to match several of these with previously identified tannins from other sources of oak acorns, we did identify several tannins not previously reported in the literature and have proposed structures for those molecules.[2]

Tannins A & T represent structures possessing open lactones in their ellagic acid moieties that have not been previously mentioned in the literature. These structures could possibly be artifacts of the isolation or analysis procedure and further analysis is needed to confirm their presence.

TABLE 1. IDENTIFIED COMPOUNDS

	RT (min)	Parent (m/z)	Parent Compound	MSMS Ion	MSMS Ion	MSMS Ion	MSMS Ion
A	15.8	933.4	Trigalloyl-ellagoyl-Glucose	783.2	631	481.1	301.1
B	16.9	933.2	Trigalloyl-HHDP-Glucose	631.1	569.1	468.2	301.2
C	17.3	933.1	Pentagalloyl-glucose	631.1	569.2	425.1	301.2
D	17.4	783.4	Tetragalloyl-Glucose	764.1	746	481	301.1
E	18.4	783.2	Tetragalloyl-Glucose	763.1	481.1	301	N/A
F	19.2	633.4	Trigalloyl-Glucose	613.1	481.1	301	
G	19.4	783.6	Tetragalloyl-Glucose	764.4	651.1	481.1	301
H	19.9	633.1	Trigalloyl-glucose	613.1	481.1	301.1	
I	20	783	Tetragalloyl-glucose	764.0	746	481.1	301.1
J	21.3	613.5	Dehydrated tergallic-C-glucoside	595.5	523.6	493.2	301.1
K	22.8	613.7	Dehydrated tergallic-C-glucoside	493.1			
L	24.5	633.2	Trigalloyl-glucose	614.1	467.7	301.1	

	RT (min)	Parent (m/z)	Parent Compound	MSMS Ion	MSMS Ion	MSMS Ion	MSMS Ion	MSMS Ion
M	28.4	631.4	Tergallic-O-glucoside	626.7	528.1	451.1	301.1	
N	37.8	469.4	Valoneic Acid Dilactone	425	301			
O	38	469.0	Valoneic Acid Dilactone	425.1	300.9			
P	38.4	469.2	Valoneic Acid Dilactone	424.9	300.9			
Q	40.8	595.5	Identity Unknown	463.3	301			
R	41	927.4	Valoneic Acid Dimer	463.1	301			
S	41.2	933.3	Trigalloyl-HHDP-glucose	756.6	463.2	301.1		
T	41.2	927.3	Valoneic Acid Dimer	463.2	301.1			
U	41.6	468.9	Valoneic Acid Dilactone	301.1				
V	42.1	561.5	Identity Unknown	543.1	479.2	409.2	271	169
W	44.2	867.6	Ellagic Acid Pentoside dimer	433	301			
X	46.5	850.0	Identity Unknown	821.1	804.4	677.7	451.5	301.1

CONCLUSIONS

The identification and uniqueness of the tannins found in *Quercus macrocapra* (Burr Oak) acorns provides our research group and others with additional insight into the complexity of this class of biomolecules. The ecological role of tannins in nature is still poorly understood and we hope to identify, isolate, and through the utilization of bioassays make an attempt to characterize the possible role of specific tannins in plant-predator interactions. 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 macrocapra* (Burr 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. While identification of the tannins is possible by ESI MS/MS with regards to components present, the wide variety of isomeric structures possible because of the numerous connection points on the tannins makes absolute confirmation difficult.

FUTURE WORK

We plan to develop an ESI MS/MS search library for the chemical constituents of the Burr Oak acorn and the additional acorns under investigation in our laboratories to help expedite the identification of the tannins as our research expands. Isolation of the tannins will be necessary to investigate their biological activities and to assign absolute structural identities by NMR.

REFERENCES

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