



Identification of Ingested Tannins and Their Localization in the Tissues of

Curculio spp. by LC-ESI-MSn

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ABSTRACT

Typical acorn weevil larva (*Curculio spp.*) develop through five instars or growth stages while living inside acorns utilizing the cotyledon as a source of nourishment. While these weevil larvae are able to consume the acorn meat, they however also ingest large amounts of tannins which have been implicated in predator-plant relationships as a defense mechanism. In addition, weevil larvae tend to feed on the basal region of the acorn more often than that of the apical region presumably due to the lower concentrations of tannins present there. The identification of these molecules and where they are localized in various tissues of the weevil larva could greatly increase the likelihood of elucidating a role and mechanism of action. The samples of weevil larvae were obtained from acorns derived from two white oak trees (*Quercus macrocarpa*-Bur Oak) located in Swope Park, Kansas City, Missouri. The larvae were dissected and individual tissue types were separated and prepared. Four tissue types were examined for the presence of tannins and were subjected to liquid chromatography electrospray ionization mass spectroscopy (LC-ESI-MSn); these included the skin, head, fat, and gut. The tannins found in the various tissues of the weevil larvae come from two classes: ellagitannins and gallotannins. While this is the case, tannins from the ellagitannin class appeared much more frequently which agrees with previous research on white oaks.

INTRODUCTION

Tannins are defined as “naturally occurring water soluble polyphenols of varying molecular weight (Spencer et al. 1988) and “are known to form insoluble molecular aggregates with a plethora of chemicals, including proteins (unique feature of tannins), polysaccharides, lipids, alkaloids, and polyvalent metal ions” (Barbehenn, 1998). Tannins are secondary metabolites of plants and are also the second most abundant group of plant phenolics. They are distributed into the two following major groups based on their structures and properties: hydrolysable and nonhydrolysable or condensed tannins. Hydrolysable tannins break down into two classes-ellagitannins and gallotannins. Hydrolysable tannins have a wide prevalence in a variety of plants and are found in the bark, wood, leaves, fruit, roots and seed. They are readily hydrolyzed by acids, bases, or certain enzymes.

TABLE 1. TANNINS CATEGORIZED by M/Z AND LOCATION IN ACORN AND WEEVIL LARVAE TISSUES

Sample	Hydrolysable Tannin															
	433	447	463	469	481	483	561	595	613	625	631	633	783	850	927.3	933
Bur Oak Acorn	42.2	42.9	34.8, 35.4, 36.7, 37.1, 37.7	-	7.4, 8.0	-	-	-	-	-	22.7	17.9, 21.0, 21.9	12.9, 13.4, 16.1, 17.6, 18.5	-	-	11.9, 15.6
Larvae Fat	-	-	-	-	-	-	-	37.4	-	25.8, 27.0, 27.9, 29.1	-	-	-	-	-	-
Larvae Gut	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Larvae Skin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Larvae Head	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Larvae Composite	-	-	-	-	-	-	-	37.4, 37.9, 38.4	-	25.6, 26.8, 28.9	-	-	-	-	-	-
Preserving Solution	-	-	-	-	-	-	-	37.4	-	26.8, 27.3, 28.2	-	-	-	-	-	-

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.

CONCLUSIONS

Based on previous research on white oaks, our data supports the prevalence of ellagitannins over gallotannins in the Bur Oak. Additionally, several tannins previously found in *Quercus macrocarpa* were not found in our analysis and these are included in Table 1. This is presumably due to the seasonal variation in tannin content of the oak structures. Analyses of the various tissues of *Curculio spp.* identified two ellagitannins proposed as Ellagic Acid-Glucoside Pentoside (Compound J) and Ellagic Acid-Diglucoside (novel proposed structure) (Compound H). The ellagitannins were found only in the fat tissues of the larvae. Ellagic Acid-Glucoside Pentoside and Ellagic Acid-Diglucoside were not found in the Bur Oak acorn, however, Ellagic Acid-Pentoside and Ellagic Acid-Glucoside were present. Thus, it is proposed that the glucose moiety is added to Ellagic Acid-Pentoside and Ellagic Acid-Glucoside by glucosyltransferases after its absorption through the midgut. Therefore, some form of the tannin must have been able to cross the peritrophic membrane and the midgut epithelia. The peritrophic membrane provides four mechanisms to potentially protect herbivorous insects: adsorption, ultrafiltration, polyanion exclusion, and as antioxidants (Berbehenn, 2001). Gut alkalinity, digestive B-glucosidases, and gut surfactants may also confer immunity to polyphenols. Interactions between tannins and proteins are strongly influenced by pH, ionic strength, detergents, and the concentrations of certain specific ions. Examination of the composite weevil larvae found no other hydrolysable tannins present. The concentration of Ellagic Acid-Diglucoside was approximately six-fold higher than Ellagic Acid-Glucoside Pentoside in the composite sample. It is not known why these ellagitannins are only being sequestered in the fat tissue or what their function is. However, it has been known that hydrolysable tannins defend primarily against chewing phytophagous insects or animals (Lewis and Yamamoto, 1989) and that usually these tannins are responsible for gut lesions in non-adaptive insects (Schultz, 1989). On the other hand, evidence indicates some insects can utilize phenolic compounds to stabilize proteins in the cuticle. The same utilization of tannins could be present in this case as well. It is not surprising that Ellagic acid was also not found individually in the larval tissues seeing as how it is an antinutritional factor because of its ability to bind essential metals (Harborne, 1989). It is possible that these two ellagitannins would be involved in protein or other macromolecular binding but it is still unclear at this point. Lastly, weevil larvae preserved in methanol/water (80:20 v/v) containing 0.8mM NaF were dissected and analyzed and results found the two tannins had diffused into the other tissues. The tannins were present in the preserving solution used as well. Thus, this preserving solution will not allow for viable samples to be used for future tissue-specific analyses.

SAMPLE PREPARATION FOR MASS SPECTROSCOPY

The tissues samples derived from five fresh self-evacuated fifth instar *Curculio spp.* larvae from Bur Oak acorns collected in the Fall of 2008 were treated with the following method. The skin, head, fat, and gut of each weevil larva were separated using various dissection tools. Two drops of a solution of methanol/water (80:20 v/v) containing 0.8mM NaF were added to each tissue to prevent sample oxidation. The samples were then pulverized with a stirring rod and then sonicated for a period of 30 minutes in a 5200 Branson Sonicator bath. The extract was removed and filtered with a 0.45 µm low protein binding hydrophilic LCR (PTFE) membrane filter. The filtered extract was then analyzed using LC-ESI-MSn. The composite sample (ten whole weevils larvae) was treated with the same method with the exception that a ratio of 1g of live samples was added to 1mL of the solution indicated and a crude filtration was done with a cotton plugged pasteur pipet prior to the LCR membrane filtration. The cotyledons and embryos of *Quercus macrocarpa* were treated with the following method. The cotyledons and embryos were removed from the seed coat, allowed to dry at 37°C and then pulverized with a mortar and pestle. A ratio of 100mg of the dry material was added to 1mL of a solution of methanol/water (80:20 v/v) containing 0.8mM NaF to prevent sample oxidation. The solution was shaken on a Glas-Col bench top shaker for two weeks and allowed to settle. The supernatant was removed and a crude gravity filtration was performed using a Whatman Filter paper. The filtrate was then filtered with a 0.45 µm low protein binding hydrophilic LCR (PTFE) membrane filter. The filtered extract was analyzed using LC/ESI/MSn.

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

LC/ESI/MS/MSn 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 0.5 mm i.d., Zorbax XDB – C18 3.5 µm (Agilent, Palo Alto, CA). Flow rate was 5.00 µL/min, injection volume was 0.5 µL, and column temperature was 25°C. The ESI parameters were as follows: nebulizer, 15 psi; dry gas (N₂), 5.00 L/min; dry temperature, 325°C; trap drive, 78.0; skim 1, -40V; lens 1, 5.00V; octopole RF amplitude, 200.0 Vpp; capillary exit, -200V. 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 2000000 µs. MS-MS was operated at a fragmentation amplitude of 1.0V, and threshold ABS was 20000 units.

TABLE 2. PROPOSED IDENTITIES of TANNINS by RETENTION TIME

ID	RT	Structure	LC/MS (M - H) m/z	MS/MS m/z	Presence
A	7.4, 8.0	Hexahydroxydiphenoyl-glucose	481	421, 301	Bur Oak Acorn
B	11.9, 15.6	Trigalloyl-HHDP-glucose	933	915, 631, 613, 569, 301	Bur Oak Acorn
C	12.9, 13.4, 16.1, 17.6, 18.5	Tetragalloyl-glucose	783	764, 481, 301	Bur Oak Acorn
D	17.9, 21.0, 21.9	Trigalloyl-glucose	633	613, 481, 301	Bur Oak Acorn
E	21.3	Dehydrated tergallic-C-glucoside	613	595.5, 523.6, 493.2, 301.1	Not Present
F	22.7	Tergallic C-glucoside	631	613, 493, 469, 301	Bur Oak Acorn
G	24.0-24.6	Digalloyl-glucose	483	331, 301, 271, 169	Not Present
H	25.8, 27.0, 27.9, 29.1	Ellagic Acid-diglucoside	625	463, 301	Weevil Larvae Fat, Weevil Larvae Composite
I	34.8, 35.4, 36.7, 37.1, 37.7	Ellagic acid-glucoside	463	415, 301	Bur Oak Acorn
J	37.4, 37.9, 38.4	Ellagic acid-glucoside pentoside	595	463, 301	Weevil Larva Fat & Weevil Larvae Composite
K	37.8	Valoneic acid dilactone	469	425, 301	Not Present
L	39.3, 40.1	Identity Unknown	561	543, 479, 409, 271, 169	Not Present
M	41.2	Valoneic Acid Dimer	927.3	463.2, 301.1	Not Present
N	42.2	Ellagic Acid pentoside	433	301	Bur Oak Acorn
O	42.9	Dihydroxybenzoic acid-ellagic acid	447	301	Bur Oak Acorn
P	46.5	Identity Unknown	850	821.1, 804.4, 677.7, 451.1, 301.1	Not Present

TABLE 3. PERCENTAGE OF TANNINS IN BUR OAK ACORN AND WEEVILS

	Bur Oak Acorn		
	Parent	Count	% Total
J MW 595 Ellagic Acid-Glucoside Pentoside	433	12758743	9.7
	447	3513946	2.7
	463	15041134	11.4
	481	1515364	1.2
	631	2625596	2.0
	633	6361463	4.8
	783	15636299	11.9
	933	74248954	56.4
	Total	131701499	100.0
	Weevil Larvae Composite		
H MW 625 Ellagic Acid-Diglucoside	Parent	Count	% Total
	595	119955	13.8
	625	750839	86.2
Total	870794	100	