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Understanding the Native Californian Diet: Identification of Condensed and Hydrolyzable Tannins in Tanoak Acorns (*Lithocarpus densiflorus*)

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The tanoak (*Lithocarpus densiflorus*) acorn was a staple food in the Native American diet and is still used in traditional dishes. Acorns from the genus *Quercus* have been shown to contain a large range of hydrolyzable tannins. However, neither hydrolyzable nor condensed tannins have been characterized in tanoak acorns. The aim of this study was to identify the full range of hydrolyzable and condensed tannins in extracts of tanoak acorns using liquid chromatography/electrospray ionization−mass spectrometry/mass spectrometry. Condensed tannins were identified as B type oligomers of (epi)-catechin (procyanidins) with a degree of polymerization up to six. Oligomers up to and including tetramers were identified by UV spectra and MS detection whereas pentamers and hexamers were detected only by MS. The total concentration of condensed tannins was 464 mg/100 g acorn pericarp. The concentration of propocyanidin monomers, dimers, trimers, and tetramers in acorn pericarp (mg/100 g acorn pericarp) were 95 ± 10.9, 148 ± 35.0, 90 ± 17.9, and 131 ± 1.9, respectively. No procyanidins were found in the acorn cotyledon tissue. A total of 22 hydrolyzable tannins were identified in methanolic extracts of acorn cotyledon tissue. Gallic acid derivatives predominated and included galloylated esters of glucose, hexahydrodiphenoyl esters of glucose, and methylated gallates. Galloylated esters of glucose were present as isomers of galloyl glucose, digalloyl glucose, and trigalloyl glucose. Mass spectral fragmentation patterns indicate the presence of one gallic acid-galloyl glucose isomer and two gallic acid-digalloyl-glucose isomers. No isomers of tetragalloyl glucose and pentagalloyl glucose were identified. Ellagic acid and ellagic acid pentoside were also identified.

KEYWORDS: Acorn; hydrolyzable tannin; condensed tannin; phenolic; antioxidant; procyanidins; *Lithocarpus densiflorus*

INTRODUCTION

The potential for foods to protect against chronic and degenerative diseases has prompted a reevaluation of the modern diet with an emphasis on increasing consumption of foods rich in micronutrients and bioactive phytochemicals. Many of these compounds are thought to have been higher in native diets. There is an ongoing effort to build a historical reservoir of information on foods, herbs, and medicinal plants important in native diets. The acorn from the tanoak tree (*Lithocarpus densiflorus*) was a dietary staple for Native Americans from the northern coastal regions of California. Traditional processing and cooking techniques have been preserved through the generations, and the acorn is still used today by Californian Native Americans for making acorn flour and soup. Acorns are also dietary constituents in other parts of the world, including Spain, Italy, Korea, China, and Japan, although the varieties consumed in these regions are from the genus *Quercus*, not *Lithocarpus*. Acorns are also an important wildlife food in areas where oaks occur; mice, squirrels, chipmunks, birds, and deer consume large amounts of acorns (*1*, *2*). Iberian ham, which is a highly prized meat, is derived from Iberian pigs, which feed on acorns. It is hypothesized that the high quality of the meat is due to the high level of antioxidants in the acorns, which help to prevent lipid oxidation (*3*). The U.S. Department of Agriculture National Nutrient Database indicates that acorns are a good source of vitamins and minerals as well as a calorically dense food (*4*). European acorns of the *Quercus* genus demonstrate high antioxidant activity because of high levels of hydrolyzable tannins (*3*).

The two major types of tannins in plants are hydrolyzable and condensed tannins, and these are classified by structure and susceptibility to acid hydrolysis. Hydrolyzable tannins are comprised of a polyol carbohydrate core (usually D-glucose) esterified to phenolic acids such as gallic or ellagic acid, forming gallotannins and ellagitannins, respectively (**Figure 1**). Mild acid hydrolysis of these tannins yields carbohydrates and phenolics.

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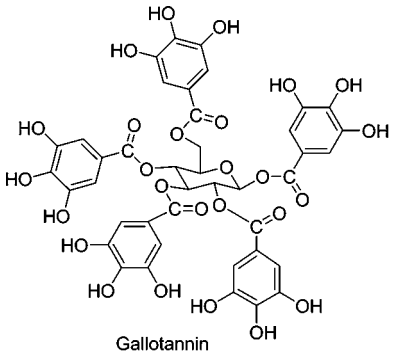

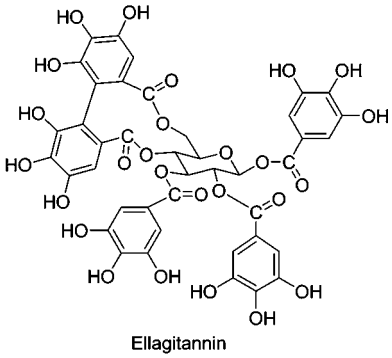

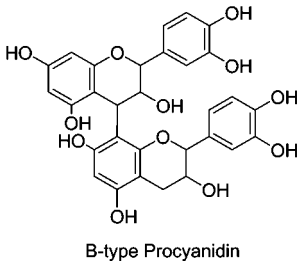

Figure 1. Structures of representative hydrolyzable tannins and condensed tannins.

Condensed tannins or proanthocyanidins are homogeneous or heterogeneous polymers of flavan-3-ol molecules (**Figure 1**), and acid hydrolysis yields flavan-3-ols. The monomers can be linked through C4→C8 or C4→C6 to form B type proanthocyanidins, or an additional C2→C7 linkage may be present to form doubly linked A type proanthocyanidins. Proanthocyanidins comprised solely of (epi)-catechin monomers are termed procyanidins.

Condensed and hydrolyzable tannins are associated with many foods with described health benefits (e.g., wine and cocoa) (*5*). Many hydrolyzable tannins are bioactive, possessing antimutagenic and anticarcinogenic properties (*6*, *7*). The type and quantity of hydrolyzable tannins present in a food ultimately affect its biological activity (*8*). Acorns from the *Quercus* genus contain approximately 300 μg of hydrolyzable tannins per gram (*3*). The hydrolyzable tannins in tanoak acorns have yet to be determined. The consumption of procyanidins is linked to reduced serum cholesterol, and in vitro assays indicate protection against low-density lipid oxidation and platelet aggregation (*9*−*11*). Procyanidins are found in many nut varieties, including walnuts, almonds, cashews, and pecans, all in which the B type form predominates (*5*). The composition of procyanidins in acorns has not been described. Their activity has been shown to be dependent on chain length, so accurate characterization of the type and distribution of procyanidins is imperative in determining the potential health benefits of tanoak acorns (*12*).

The following study describes the characterization of the full range of hydrolyzable and condensed tannins in tanoak acorns using liquid chromatography/electrospray ionization−mass spectrometry (LC/ESI-MS).

METHODS AND MATERIALS

Reagents. Acetic acid, methylene chloride, methanol, acetone, and ammonium acetate were obtained from Fisher Scientific (Houston, TX). Formic acid and procatechuic acid were purchased from Sigma (St. Louis, MO). Hexane was purchased from EMD Chemicals (Gibbstown, NJ). Reagent-grade water was generated by a Barnstead E-pure deionization system (Dubuque, IA). The cocoa procyanidin standard was a gift from M&M/Mars (Hackettstown, NJ).

Acorns. Mature acorns from eight *L. densiflorus* trees were harvested from Northwest Sonoma County, CA, on October 6, 2005, at which time mature acorns were dropping from tanoaks in the area. Trees were shaken, and the fallen acorns were collected on tarps. After collection, the acorns were refrigerated at 7 °C until they were delivered the next day to UC Davis, CA, where they were frozen at −20 °C. Acorns were pooled randomly to obtain a representative sample for analysis.

Extraction of Condensed Tannins. Four gram samples of acorn pericarp taken from 12 acorns were ground with a mortar and pestle and homogenized with a PowerGen 125 homogenizer (Fisher Scientific) for 1 min in 25 mL of extraction solvent comprised of acetone, water, and acetic acid (70:29.5.05) in triplicate as described previously (*13*). Procatechuic acid was added to the extract as a recovery standard, and recoveries were consistently ∼75%. The solution was then sonicated for 30 min with an FS30 Fisher sonicator, refrigerated for 30 min, and centrifuged at 1700*g* with a Beckman Accuspin FR (Fullerton, CA). A 20 mL aliquot of water was added to the supernatant before rotary evaporation under partial vacuum at 45 °C. The aqueous extract was loaded onto an Agilent AccuBond^{II} 6 mL ODS-C18 SPE column that had been conditioned with 5 column volumes of methanol and 5 column volumes of water. The column was rinsed with 10 volumes of water and dried for 2 min, and the procyanidins were eluted with 10 mL of extraction solvent. The eluent was filtered through a 0.45 μm PTFE membrane filter. A 50 μL sample of this solution was injected onto the high-performance liquid chromatography (HPLC). Acorn cotyledon tissue was prepared by the same method, except a 1 g sample was used after 3 g of cotyledon tissue was ground with a laboratory mill (IKA, Staufen, Germany), defatted three times with 15 mL of hexane, and dried under nitrogen.

Extraction of Hydrolyzable Tannins. A 2 g sample of acorn cotyledon tissue from five acorns was ground in a laboratory mill, defatted three times with 15 mL of hexane, and dried under nitrogen. Hydrolyzable tannins were extracted with 80% methanol, sonicated for 5 min, and centrifuged at 1700*g* for 10 min, and the supernatant was filtered through a 0.45 μm PTFE membrane filter. A 5 μL sample of this solution was injected onto the HPLC.

Condensed Tannin Snalysis by LC/ESI-MS. Chromatographic analyses were performed on a Shimadzu HPLC with an SIL-10A autoinjector, binary LC 10AD pumps, and a SPD-10A UV/vis detector. Separations were achieved using reversed phase HPLC with a 250 mm × 2.0 mm i.d., 5 μm Luna Silica column (Phenomenex, Torrance, CA) monitoring at 280 nm. The binary mobile phase consisted of (A) methylene chloride, methanol, water, and acetic acid (82:14:2:2 v/v) and (B) methanol, water, and acetic acid (96:2:2 v/v). Separations were achieved at a flow rate of 0.2 mL/min with the following series of linear gradients: 0−30 min, 0−18% B in A; 30−45 min, 18−31% B in A; and 45−50 min, 31−88% B in A. The HPLC system was interfaced to an ESI ZSPRAY Micromass Quattro LC (Beverly, MA) using the following settings: capillary voltage, −3.2 kV; cone voltage, −30 V; source temperature, 145 °C; desolvation gas temperature, 300 °C; nebulizer gas flow rate, 65 L/h; and desolvation gas flow rate, 450 L/h. A 10 mM concentration of ammonium acetate in methanol was introduced at 0.03 mL/min through a tee in the eluant stream as an ionization reagent. MS data were collected from 100 to 2000 *m*/*z* with a scan duration of 0.1 amu and processed using MassLynx software v 3.5. Calibration curves were made for the summed peak areas of all of the isomers for each oligomer in a composite cocoa standard. Identifica-

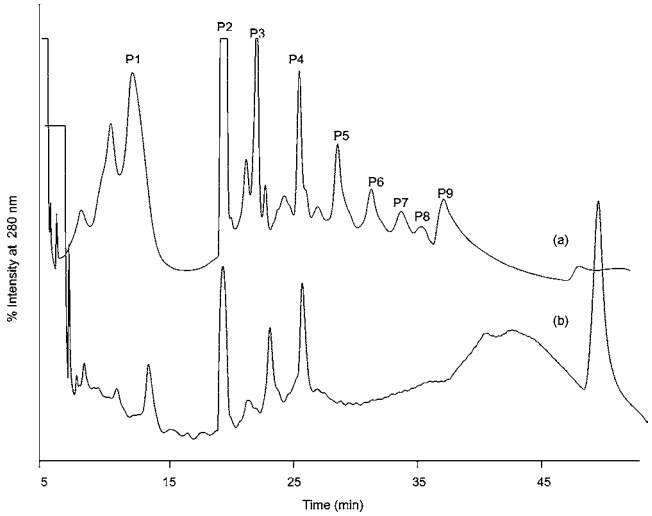

Figure 2. HPLC chromatograms of cocoa (**a**) and tanoak acorn (**b**) procyanidin extracts monitoring at 280 nm. Labels indicate the degree of procyanidin polymerization.

tion of the procyanidin oligomers in tanoak acorns was made by comparing LC retention times with those in the cocoa procyanidin standard and by filtering the total ion chromatogram (TIC) at *m*/*z* ratios corresponding to singly and multiply charged ions produced from each oligomer. Quantitation was achieved by comparing the summed peak areas for all isomers in the oligomeric group in the samples to the summed peak areas of the corresponding oligomers in the cocoa standard as described previously (*14*).

Hydrolyzable Tannin Analysis by LC/ESI-MS and LC−Diode Array Detection. Separations were performed on the same system as above using reversed phase HPLC with a 250 mm × 2.0 mm i.d., 5 μm Prodigy ODS column (Phenomenex) monitoring at 280 nm. The mobile phase consisted of (A) 1% formic acid in water and (B) 1% formic acid in methanol. Separations were achieved at a flow rate of 0.2 mL/min with the following series of linear gradients: 5−25% B in A, 0−6 min; 25−40% B in A, 6−30 min; and 40−60% B in A, 30−45 min. The ESI-MS conditions were as follows: capillary voltage, −3.25 kV; cone voltage, −25 V; source temperature, 140 °C; desolvation gas temperature, 300 °C; nebulizer gas flow rate, 65 L/h; and desolvation gas flow rate, 450 L/h. TICs were recorded over a range of 150−1000 *m*/*z* with a scanning duration of 0.1 amu. Peaks with *m*/*z* ratios corresponding to possible hydrolyzable tannins were fragmented by MS/MS to confirm peak identity from mass fragmentation patterns. Argon was used as the collision gas, and the collision energy was set to 20 V. UV/vis spectra were obtained on a Waters 2690 Alliance HPLC system using the same column as above with a 996 photodiode array detector scanning from 240 to 400 nm.

RESULTS AND DISCUSSION

LC/ESI-MS was used to identify both condensed and hydrolyzable tannins in tanoak acorns. Using this methodology, oligomers of homogeneous B type procyanidins were identified and quantified in acorn pericarp by comparison with retention times and peak areas from a B type procyanidin standard isolated from cocoa (**Figure 2**). Oligomers up to and including tetramers were identified by UV spectra and MS detection and by comparison of retention times with the oligomer standard. Two additional oligomers, pentamers and hexamers, not present in UV spectra were detected in mass spectra (**Figure 3**). Oligomer identity was confirmed by generating reconstructed ion chromatograms (RICs) from the TIC filtered for masses corresponding to singly $[M - H]^-$ and doubly $[M - 2H]^{2-}$ charged ions for each oligomer (**Figure 3**). B type procyanidins have been shown to generate doubly charged species and can be differentiated from singly charged species by isotope peak distribution (*15*). The concentration of each oligomer in acorn pericarps (as mg/100 g pericarp) was 95 ± 10.9 for the monomer, 148 ± 35.0 for the dimer, 90 ± 17.9 for the trimer, and 131 ± 1.9 for the tetramer.

The LC/ESI-MS methodology used allows for the separation and detection of A type procyanidins as well, which elute shortly before B type procyanidins. However, mass spectra demonstrated that A type oligmers are not present in acorn pericarp. Neither A type nor B type procyanidins were found in the acorn cotyledon tissue, the portion normally used for human consumption. This can be explained by the fact that proanthocyanidins accumulate in the seed coats of many plants (*16*), possibly to form a protective barrier against insects, microbial pathogens, and animals (*17*). An analysis of proanthocyanidins in peanuts also showed an abundance of several oligomers in the pericarp while no proanthocyanidins were detectable in the cotyledon (*13*). Procyanidins found acorn pericarp would play a larger dietary role in forage animal diets rather than in the human diet.

Using LC/ESI-MS, the full range of hydrolyzable tannins extracted from tanoak acorn cotyledon tissue was identified. Analysis was performed on acorn cotyledon tissue and not pericarp because a previous study showed only minor differences in the quantity of certain hyrolyzable tannins in the profiles of pericarp and cotyledon tissue (*3*). A total of 22 hydrolyzable tannins were identified in methanolic extracts of acorn cotyledon tissue (**Figure 4**). LC/MS spectra demonstrate that both gallotannins and ellagitannins are present with the gallotannins generally having shorter retention times (**Table 1**). Additionally, spectral data from a UV/vis photodiode array detector were used to classify each compound as gallic acid type (A) or ellagic acid type (B) based on the spectrum of gallic acid and ellagic acid standards from 240 to 400 nm (**Table 1**). The gallic acid derivatives identified in this study include galloylated esters of glucose, hexahydrodiphenoyl esters of glucose, and methylated gallates (**Table 1**). The galloylated esters of glucose were present as isomers of galloyl glucose (*1*, *2*), digalloyl glucose (*5*−*7*, *9*, *11*), gallic acid-galloyl-glucose (*13*), trigalloyl glucose (*8*, *14*), and gallic acid-digalloyl-glucose (*16*, *17*). Tetragalloyl glucose (*18*) and pentagalloyl glucose (*14*) were also identified, but no isomers were detected. Ellagic acid and its derivatives were also identified, including ellagic acid pentoside (*22*) and valoneic acid dilactone (*20*).

To further identify the hydrolyzable tannins identified in the RICs, MS/MS experiments were performed and retention times were compared to commercial standards when available (gallic acid and ellagic acid). The identity of each hydrolyzable tannin was confirmed by the presence of characteristic fragment ions. For example, galloylated esters of glucose lose multiple gallic acid moieties (*m*/*z* 169) during fragmentation. Galloyl glucose ($[M - H]^-$ at *m*/*z* 331) yielded fragment ions at *m*/*z* 169 after the loss of a gallic acid moiety, at *m*/*z* 125 after decarboxylation of the gallic acid moiety (M − H − 44), and at *m*/*z* 271 after cross-ring fragmentation of glucose (M − H − 60) (*18*). The existence and intensity of fragmentation ions that differed for each galloyl glucose peak, as well as for other identified hydrolyzable tannins, are due to different isomeric forms. Digalloyl glucose ($[M - H]^-$ at *m*/*z* 483) yielded fragment ions at *m*/*z* 313 after the loss of a gallic acid (M − H − 170), at *m*/*z* 331 after loss of a galloyl moiety (M − H − 152), and at *m*/*z* 465 after loss of water (M − H − 18) (**Figure 5A**) in addition to the fragment ions observed for galloyl glucose. One digalloyl glucose isomer (peak 13) had additional fragments at *m*/*z* 439 and 287 resulting from the decarboxylation (M − H − 44) of a gallic acid moiety still bonded to glucose (**Figure 5B**). These

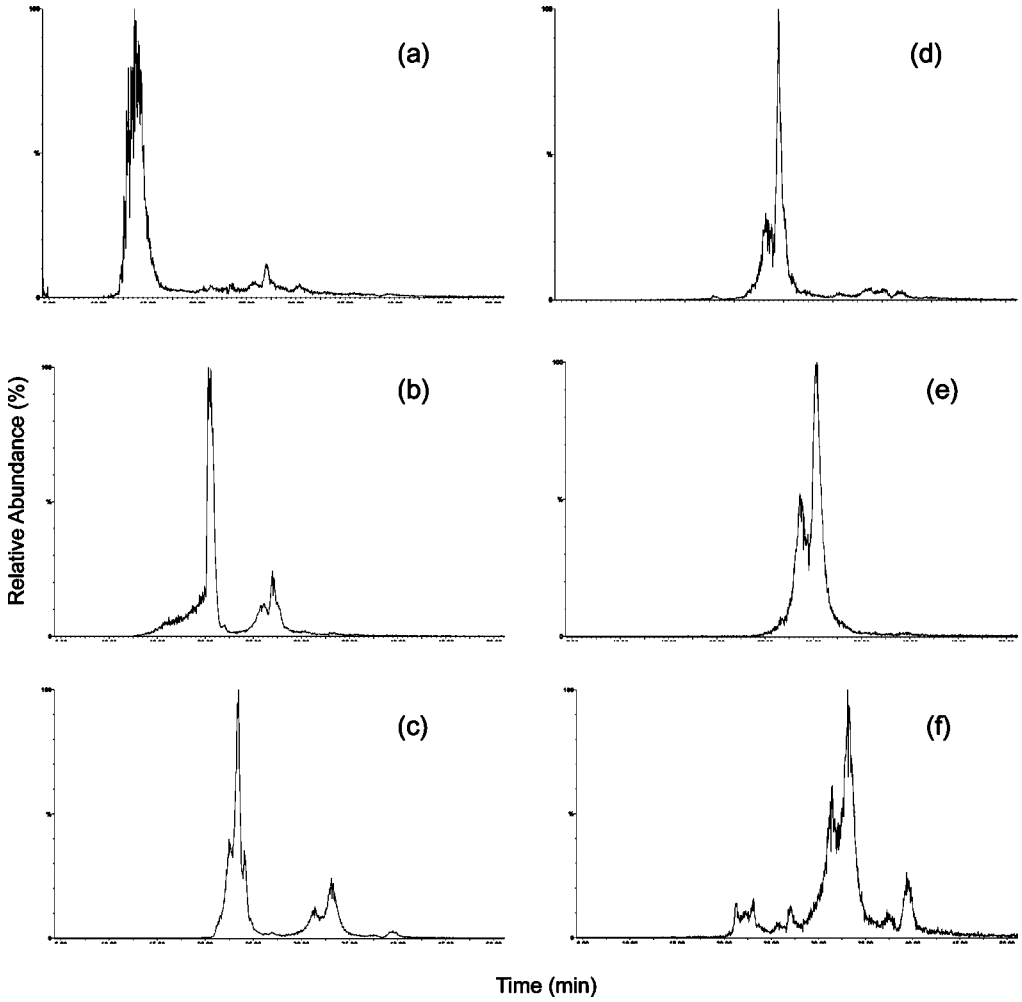

Figure 3. RICs of procyanidin oligomers in tan oak acorn extracts. RICs were generated by filtering the most abundant ions corresponding to (**a**) monomers $[M - H]^-$ 289, (**b**) dimers $[M - H]^-$ 577, (**c**) trimers $[M - H]^-$ 865, (**d**) tetramers $[M - 2H]^{2-}$ 576, (**e**) pentamers $[M - 2H]^{2-}$ 720, and (**f**) hexamers $[M - 2H]^{2-}$ 864.

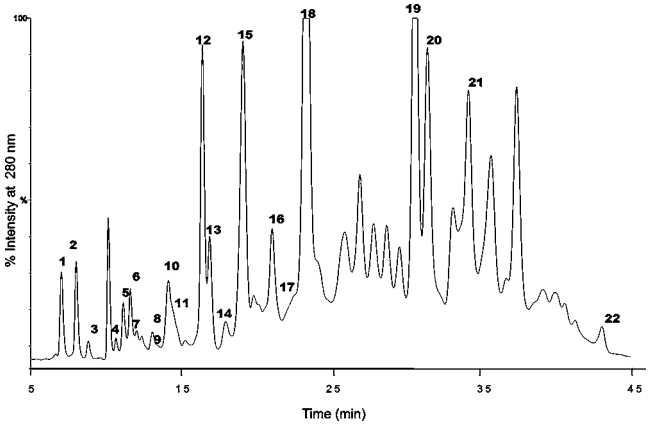

Figure 4. HPLC chromatogram of tanoak acorn hydrolyzable tannins monitoring 280 nm.

fragments indicate that this isomer, a gallic acid-galloyl-glucose rather than digalloyl glucose with one of the gallic acid moieties, bonded to glucose via an ether linkage through a hydroxyl group on the gallic acid rather than via an ester linkage through the carboxylic acid group (*19*). The loss of $-CO_2$ is possible for ortho- and para-substituted hydroxybenzoic acids; therefore, the gallic acid groups that can be decarboxylated are most likely linked at the C3 position. Trigalloyl glucose ($[M - H]^-$ at *m*/*z* 635) yielded fragments of *m*/*z* 483 and 313 after the sequential loss of two gallic acid moieties as well as *m*/*z* 465 from the loss of water. Two trigalloyl glucose isomers (peaks 16 and 17) demonstrate daughter ions at *m*/*z* 439 from the loss of a gallic acid moiety and decarboxylation of a gallic acid group. Again, these fragmentation patterns indicate that either one or more gallic acid groups are ether linked and not esterified to the glucose core and that the molecules are either gallic acid digalloyl glucose or digallic acid galloyl glucose. Tetragalloyl glucose and pentagalloyl glucose showed similar patterns of sequential gallic acid loss during fragmentation. Gallic acid itself ($[M - H]^-$ at *m*/*z* 169) produced a fragment of *m*/*z* 125 after the loss of a $-CO_2$ group.

Trigalloyl-hexahydrodiphenoyl-glucose ($[M - H]^-$ at *m*/*z* 937) and digalloyl-hexahydrodiphenoyl-glucose ($[M - H]^-$ at *m*/*z* 785) isomers produced fragments of *m*/*z* 785 and 633, respectively, after the loss of gallic acid (*m*/*z* 169) and fragments

Table 1. B Type Procyanidin Oligomers in Tanoak Acorns

	procyanidin oligomer concentration
monomer	95 ± 10.9 mg/100 g acorn skin
dimer	148 ± 35.0 mg/100 g acorn skin
trimer	90 ± 17.9 mg/100 g acorn skin
tetramer	131 ± 1.9 mg/100 g acorn skin
pentamer	ND[a]
hexamer	ND[a]

[a] ND, not detectable by UV/vis spectroscopy.

Table 2. Identification of Hydrolyzable Tannins in Tanoak Acorn Extracts[a]

peak	t_R (min)	compound	UV spectrum type	MS $[M-H]^-$ (*m*/*z*)	MS/MS $[M-H]^-$ (*m*/*z*)
1	6.5	galloyl glucose	A	331	271, 169, 125
2	7.4	galloyl glucose	A	331	169, 125
3	8.1	gallic acid	A	169	125
4	9.8	digalloyl glucose	A	483	331, 271, 169
5	10.2	digalloyl-hexahydrodiphenoyl-glucose	A	785	301
6	10.6	digalloyl glucose	A	483	331, 271, 169
7	11.0	digalloyl glucose	A	483	331, 271, 169
8	11.9	trigalloyl glucose	A	635	483, 465, 313, 169
9	12.3	digalloyl glucose	A	483	483, 331, 313, 271, 169
10	12.9	digalloyl-hexahydrodiphenoyl-glucose	A	785	301, 169
11	13.2	digalloyl glucose	A	483	331, 313, 271, 169, 125
12	15.0	methyl gallate	A	183	168, 124
13	15.4	gallic acid-galloyl-glucose	A	483	439, 331, 313, 287, 271, 169, 125
14	16.4	trigalloyl glucose	A	635	483, 465, 313, 271, 169
15	17.5	trigalloyl-hexahydrodiphenoyl-glucose	A	937	301
16	19.2	gallic acid-digalloyl-glucose	A	635	483, 465, 439, 331
17	20.4	gallic acid-digalloyl-glucose	A	635	483, 465, 439, 331
18	21.07	tetragalloyl glucose	A	787	635, 617, 465, 313
19	27.7	valoneic acid dilactone	B	469	301
20	28.6	pentagalloyl glucose	A	939	787, 769, 617
21	31.1	ellagic acid pentoside	B	433	301
22	39.1	ellagic acid	B	301	

[a] UV spectrum type indicates gallic acid type (A) or ellagic acid type (B).

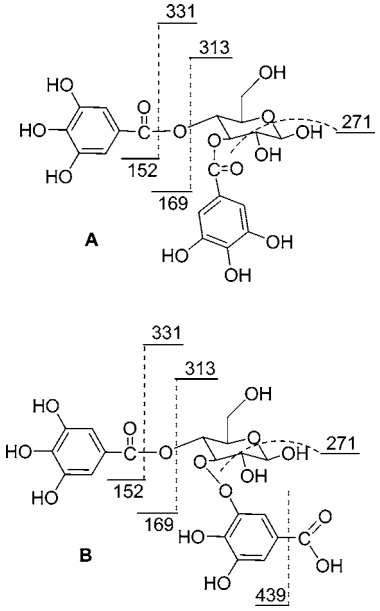

Figure 5. Schematic of digalloyl glucose (**A**) and gallic acid-galloyl-glucose (**B**) fragmentation by LC-ESI/MS/MS.

of 635 and 483, respectively, after the loss of the HHDP (*m*/*z* 302). The loss of a methyl group from methyl gallate ($[M-H]^-$ at *m*/*z* 183) produced a fragment of *m*/*z* 168, and decarboxylation of that fragment yielded another fragment of *m*/*z* 124. Valoneic acid dilactone ($[M-H]^-$ at *m*/*z* 469) was also identified by its ellagic acid fragment, *m*/*z* 301, and fragmentation allowed ellagic acid pentoside ($[M-H]^-$ at *m*/*z* 433) to be recognized by an ellagic acid fragment at *m*/*z* 301.

All of the hydrolyzable tannins identified in tanoak acorns in this study were common to acorns of the genus *Quercus*, with the exception of two methylated compounds (*3*). The major hydrolyzable tannins found in *Quercus* acorns by Cantos et al. (*3*) are isomers of trigalloyl glucose and isomers of trigalloyl-hexahydrodiphenoyl-glucose, gallic acid, and ellagic acid. Trigalloyl-hexahydrodiphenoyl-glucose was also a major peak in the tanoak extract. However, the other major peaks in tanoak acorns, methyl gallate, tetragalloyl glucose, valoneic acid dilactone, pentagalloyl glucose, and ellagic acid pentoside, were only minor peaks in the *Quercus* extract chromatogram. The differences in the range of hydrolyzable tannins found in tanoak acorns in this study and those found in *Quercus* acorns can be attributed to the fact that different plant varieties have been shown to produce different types and quantities of phenolic compounds (*20*).

Condensed tannins up through hexamers, as well as 22 different hydrolyzable tannins, were identified in tanoak acorns. The condensed tannins were procyanidins of the B type, and the hydrolyzable tannins were gallic acid and ellagic acid derivatives, including two methylated species. Condensed tannins were present at a concentration of 464 mg/100 g pericarp. No condensed tannins were detected in the acorn cotyledon tissue. Although most nuts, including pistachios, almonds, walnuts, peanuts, and cashews, have significant levels of condensed tannins in the nutmeat, those levels are lower than those found in acorn pericarp (*21*). Even though pericarps were removed and a leaching step was included in the acorn processing method, a significant amount of hydrolyzable tannins would still be consumed since acorns comprised upward of 50% of Native Californians' diets (*22*). These high-tannin diets may have had positive health benefits, as evidenced by both in vitro and in vivo data from tannin activity studies. These studies provide a first step toward understanding the composition of acorn polyphenolics in Native American diets, and they identify tanoak acorn pericarp as a good source for B type procyanidin oligmers.

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