JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Characterization of Ellagitannins, Gallotannins, and Bound Proanthocyanidins from California Almond (*Prunus dulcis*) Varieties

Liyang Xie, Anna V. Roto, and Bradley W. Bolling*

Department of Nutritional Sciences, University of Connecticut, 3624 Horsebarn Road Extension, Unit 4017, Storrs, Connecticut 06269-4017, United States

Supporting Information

ABSTRACT: Extractable and bound proanthocyanidins and hydrolyzable tannins were characterized in Nonpareil, Carmel, and Butte almond varieties from California, with $n = 3$ samples/variety. Bound proanthocyanidins were recovered from extracted defatted almond residue by hydrolysis with 4 N sodium hydroxide and represented 3−21% of the total proanthocyanidin content among varieties. The bound proanthocyanidins were recovered primarily as monomers and dimers. In contrast, acid hydrolysis of extracted almond residue did not yield bound proanthocyanidins. Hydrolyzable tannins were characterized in aqueous acetone extracts of defatted almond using two-dimensional TLC and further quantitated by HPLC following acid hydrolysis. Almond hydrolyzable tannin content was 54.7 ± 2.3 mg ellagic acid and 27.4 ± 7.3 mg gallic acid per 100 g almond among varieties. The tannin contents of Nonpareil, Carmel, and Butte almond varieties were not significantly different. Thus, bound proanthocyanidins and hydrolyzable tannins significantly contribute to almond polyphenol content.

KEYWORDS: *almonds, Prunus dulcis, polyphenols, tannin, proanthocyanidins, ellagitannin, gallotannin*

INTRODUCTION

Almonds (*Prunus dulcis*) are a rich dietary source of polyphenols.[1] The most abundant class of polyphenols in almonds is proanthocyanidins, followed by flavonoids and phenolic acids.[2] Almond flavonoids include flavonols, flavanones, and flavanols, in their monomeric, oligomeric, and polymeric forms; these latter also called proanthocyanidins or condensed tannins.[3−5] Almond proanthocyanidins are composed of flavan-3-ols with various degrees of polymerization.[4−7] However, although proanthocyanidins have been characterized in almond, data about their distribution among varieties is lacking.[4] Less is known about the content of hydrolyzable tannins in almonds. Tannins and other polyphenols may contribute to the health-promoting potential of almonds. Almond flavonoids and proanthocyanidins are bioavailable and extensively metabolized in vivo by phase II enzymes or intestinal microbiota.[8] These polyphenols and their metabolites may contribute to the antioxidant and anti-inflammatory effects of almonds observed in animal studies.[9,10]

A growing body of research suggests that the polyphenol content of foods could be underestimated because solvent extraction does not completely liberate polyphenols covalently bound to cell walls.[11,12] Previously, noncovalently bound phenolic acids and flavonoids (e.g., free or extractable) from almond skin extracts were quantitated by HPLC-MS.[13−15] However, Mandalari et al.[16] reported a portion of almond phenolic acids, mainly *p*-hydroxybenzoic acid, vanillic acid, and ferulic acid, was covalently bound and released by acid hydrolysis. Flavonoids, phenolic acids, and proanthocyanidins can also be released by acid or alkaline hydrolysis from other plant materials.[11,17] Thus, more work is needed to determine the relative proportion of free and bound polyphenols in almonds. The objectives of this study were to further characterize almond tannins by quantitating extractable and bound proanthocyanidins and screening for hydrolyzable tannins. We utilized Nonpareil, Carmel, and Butte almonds, as these are the top three cultivated California almond varieties.

MATERIALS AND METHODS

Safety. Precautions should be taken during methanolysis due to the exothermic nature of sulfuric acid dilution and neutralization.

Reagents and Materials. Methanol, dichloromethane, and acetic acid were of HPLC grade from Fisher Scientific (Fair Lawn, NJ, USA). Water was of ultrapure grade. All other chemicals and reagents were acquired from Sigma-Aldrich (St. Louis, MO, USA). Samples of whole, unpasteurized Butte, Carmel, and Nonpareil almonds grown in California from harvest year 2011 were provided by the Almond Board of California. Each variety had three unique samples representing different orchards, for a total of $n = 9$ samples. Almonds were stored at −20 °C until analysis.

Extraction of Proanthocyanidins. Frozen almonds were finely ground using an IKA A11 basic grinder (Wilmington, NC, USA). Almond powder (4 g) was homogenized in 40 mL of hexane using an IKA Ultra-Turrax T18 homogenizer for 2 min at a power setting of 4. The homogenate was centrifuged and the supernatant removed. The residue was similarly homogenized twice, and the resulting supernatants were combined and dried under a nitrogen gas stream. The mass of the extracted, dried lipids was recorded to determine almond lipid content. The defatted almond residue was then extracted with acidified aqueous acetone (70:29.5:0.5 mixture of acetone, distilled water, and acetic acid), according to methods described by Prior et al.[7] with modifications. Acidified aqueous acetone (40 mL) was added to the defatted nut residue, vortexed for 30−40 s, and sonicated in an ultrasonic cleaner for 10 min at 37 °C. The sample was then agitated on a tube rocker for 50 min at 23 °C and centrifuged, and the supernatant was filtered through a

Received: August 24, 2012
Revised: November 20, 2012
Accepted: November 20, 2012
Published: November 20, 2012

0.2 μm nylon filter. The pelleted almond residue was dried under a nitrogen gas stream and stored at −20 °C until hydrolysis. The filtered supernatant was dried under a nitrogen gas stream to remove acetone, frozen at −80 °C, lyophilized to a powder, and stored at −80 °C.

Acid and Alkaline Hydrolyses. Residual defatted or defatted, extracted almond powder was subjected to acid or alkaline hydrolysis. Acid hydrolysis was based on previously described methods.[18,19] Almond residue was resuspended in 20 mL of 1.2 N hydrochloric acid in methanol/water (80:20, v/v) and 0.5 g/L *tert*-butylhydroquinone and heated at 75 °C in a dry bath for 3 h. Incubates were then cooled over ice brought to pH 5.5 with 3.7 M ammonium acetate. The hydrolysis solution was centrifuged, and the supernatant was dried of residual methanol under nitrogen gas at 40 °C. The resulting aqueous solution was stored at −80 °C until further analysis.

Alkaline hydrolysis was performed according to a previously described method.[17] Extracted residue from defatted almonds was resuspended in 20 mL of 4 N aqueous sodium hydroxide. Resuspended residue was incubated in a water bath at 60 °C for 15 min with 30 s of vortexing every 3 min. The hydrolyzed sample was cooled over ice and neutralized with 4 N hydrochloric acid. The neutralized sample was centrifuged, and the supernatant was frozen at −80 °C, lyophilized to a powder, and stored at −80 °C until further testing.

Isolation of Tannins. Tannins were isolated by stepwise elution from Sephadex LH-20 columns, as described previously.[7] Neutralized aqueous hydrolysis solutions or dried almond extracts were reconstituted in 2−4 mL of 30% methanol in water and were loaded on a 1 cm diameter column containing ∼4 g of Sephadex LH-20. Sugars and salts were eluted with 50 mL of methanol/water (30:70 v/v) and discarded. Proanthocyanidins were eluted with 100 mL of acetone/water (70:30 v/v). The proanthocyanidin fraction was dried of residual acetone by a nitrogen gas stream at 37 °C. The resulting aqueous residue was frozen at −80 °C and lyophilized to a powder prior to HPLC analysis.

HPLC Analysis of Proanthocyanidins. Proanthocyanidins were quantitated according to a modified method[7] using a Dionex Ultimate 3000 HPLC equipped with a refrigerated autosampler, column oven, diode array detector, and fluorescence detector (Sunnyvale, CA, USA). Proanthocyanidin isolates were reconstituted in 100 μL of methanol/dichloromethane (1:1), and 20 μL was injected on a 260 mm × 4.60 mm i.d., 5 μm, Hypersil silica column (ThermoFisher, Bellefonte, PA, USA). Samples were resolved by a 1 mL/min gradient of 100% dichloromethane (A) and 100% methanol (B) with a constant 4% of acetic acid/water (50:50, v/v) (C). The gradient increased linearly from 14% B at 0 min to 28.4% B at 30 min, to 39.6% B at 45 min, to 86% B at 55 min, held until 60 min, and then decreased to 14% B until 75 min. Proanthocyanidins were quantitated by fluorescence, with excitation at 276 nm and emission at 316 nm, using (+)-catechin, (−)-epicatechin, and proanthocyanidin B2 as standards (Figure 1). Proanthocyanidins were tentatively identified by degree of polymerization by comparison to a previous study that utilized LC-MS/MS analysis of almond proanthocyanidins,[20] and HPLC−fluorescence data were integrated using a constant baseline.[7] A check standard of cocoa proanthocyanidins purified in-house was used to control for intraday variation.

Thin-Layer Chromatography (TLC) of Hydrolyzable Tannins. Almond hydrolyzable tannins were characterized by two-dimensional TLC.[21] Duplicate 20 × 20 cm, 100 μm, cellulose plates (Selecto Scientific Inc., Suwanee, GA, USA) were spotted with 50 μL of the almond tannin fraction reconstituted in 100% methanol. Plates were developed in glass chambers saturated with 100−150 mL of a 14:1:5 (v/v/v) mixture of butan-2-ol/acetic acid/water until the solvent front was at least 18 cm. Plates were then removed from the chambers and dried at ambient temperature. Plates were then rotated 90° and placed into a glass chamber saturated with 100−150 mL of a 2:98 (v/v) mixture of acetic acid/water. The plates were dried at ambient temperature. Plates were sprayed with a saturated potassium iodate solution to detect gallotannins or 4% sodium nitrite (w/v) in 50% aqueous acetic acid (v/v) to detect ellagitannins.

Gallotannin and Ellagitannin Hydrolysis. Defatted almond was hydrolyzed according to a previously described method.[22] Defatted almond powder (20 mg) was reconstituted in 2 mL of methanol in 50 mL glass screw-top test tubes. Sulfuric acid (18% in water, 100 μL) was slowly added to samples in two aliquots, and the solution was manually swirled between additions. Samples were then incubated in a dry bath at 85 °C for 20 h, cooled over ice, and neutralized by carefully adding 4 aliquots of 50 μL of ethanolamine to each sample. Caffeic acid or *trans*-stilbene (5 μg/mL) was used as internal standard for analysis of gallic acid or ellagic acid, respectively (Figure 1). Solutions were brought to 2 mL with water and centrifuged. Supernatants were passed through a 0.2 μm nylon syringe filter if cloudy or used directly for HPLC analysis.

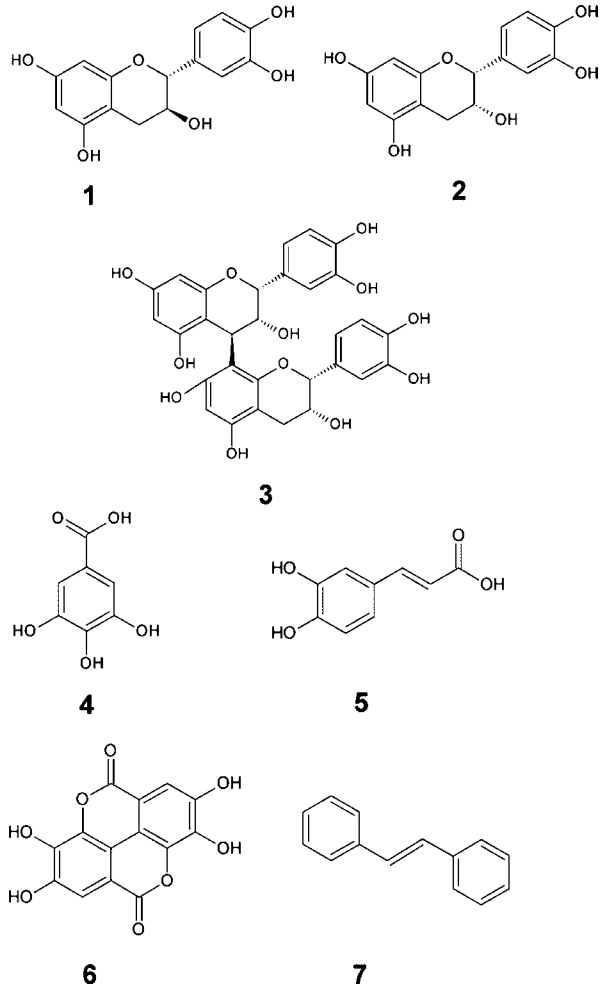

Figure 1. Structures of standard compounds utilized for HPLC analysis of almond polyphenols: (**1**) (+)-catechin; (**2**) (−)-epicatechin; (**3**) proanthocyanidin B2; (**4**) gallic acid; (**5**) caffeic acid; (**6**) ellagic acid; (**7**) *trans*-stilbene.

HPLC Analysis of Gallic Acid and Ellagic Acid. HPLC was performed using the previously described Dionex Ultimate 3000 system. Diluted, neutralized hydrolysis solutions or standards (20 μL) were injected onto a 50 mm × 2.10 mm i.d., 2.2 μm, Dionex Acclaim 120 Å RSLC PolarAdvantage column (ThermoScientific, Sunnyvale, CA, USA). Gradients of 1% aqueous acetic acid (A) and methanol (B) were used to resolve samples at 0.2 mL/min. For gallic acid, 10% B was held to 3 min, followed by a linear gradient to 40% B at 7 min, returning to 10% B at 12 min, and held for 5 min. For ellagic acid, 10% A was held for 3 min, followed by a linear gradient to 100% B at 10 min, returning to 10% B at 12 min, and held for 8 min. Gallic acid and ellagic acid were used as external standards for quantitation. Gallic acid and ellagic acid were identified in hydrolysis solution by comparison of UV spectra analysis and retention time to authentic standards. Methyl gallate eluted at 4.4 min but was not detected below 0.12 ng on-column in hydrolysis solutions. Gallic acid and caffeic acid were quantitated at 280 nm, and ellagic acid and *trans*-stilbene were quantitated at 320 and 250 nm, respectively. Inter- and intra-assay RSDs for gallic acid analysis were 14.6 and 7.4%, respectively. Inter- and intra-assay RSDs for ellagic acid

analysis were 1.1 and 3.0%, respectively. Gallic acid, caffeic acid, ellagic acid, and *trans*-stilbene eluted at 1.9, 8.7, 12.4, and 15.2 min, respectively.

Statistics and Data Analysis. Data are the mean ± standard deviation of at least duplicate samples. Statistical significance was determined by two- or one-way ANOVA as indicated, followed by Tukey's multiple-comparison test using GraphPad Prism 5.01 software (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

Tannins are the most abundant class of polyphenols in almonds.[2] Little is known about the abundance of bound proanthocyanindins and hydrolyzable tannins in almonds or their distribution between varieties. Therefore, we characterized and quantitated extractable and bound proanthocyanidins and hydrolyzable tannins in Nonpareil, Carmel, and Butte almond varieties.

Extractable Proanthocyanidins. The extractable proanthocyanidins from almond consisted of (+)-catechin and (−)-epicatechin and three dimer peaks, including proanthocyanidin B2, as well as trimers and greater oligomers (Figure 2A).

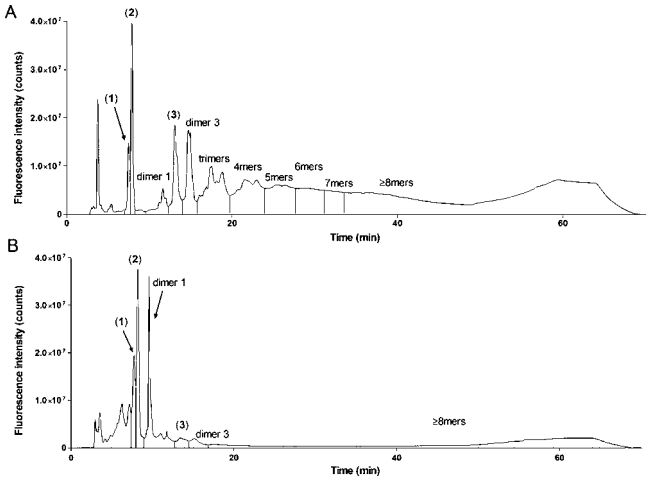

Figure 2. Representative HPLC fluorescence chromatograms of (A) a solvent-extractable almond proanthocyanidin fraction and (B) an alkaline-hydrolyzed almond residue proanthocyanidin fraction.

Normal-phase HPLC resolution of proanthocyanidins in California almond varieties was similar to that of Spanish almonds.[20] Almond proanthocyanidins have (epi)afzelechin, (epi)catechin, and (epi)gallocatechin as constituent units, with both A-type and B-type linkages and an average degree of polymerization of 12.7.[5,20,23] Almond proanthocyanidins with polymerization of 6 or more have only B-type linkages.[20]

Among the three almond varieties, Nonpareil had ∼2-fold more extractable proanthocyanidin B2 equivalents (PE) (107.3 mg/100 g almond) than Butte (25.4 mg/100 g almond) and Carmel (28.1 mg/100 g almond) because of increased oligomer content (Table 1). Nonpareil had 2-fold the monomers of Butte and 3-fold the monomers of Carmel. Nonpareil had 3-fold the dimers of Butte and Carmel and 4-fold the trimers of Butte and Carmel. Nonpareil also had ∼3 fold the oligomers (4−7-mers) and 8-mers and greater polymers of Butte and Carmel almonds. The extractable proanthocyanidin profiles, for example, as percent of the sum of proanthocyanidins, varied among the three almond genotypes (Figure 3A). 8-mers and greater polymers were the most abundant proanthocyanidins among the three almond genotypes, ranging from 27.2 to 33.3% of extractable proanthocyanidins, with the greatest polymer proportion in Carmel. Nonpareil had the greatest relative proportion of proanthocyanidin B2, trimers, and 4-mers. Butte had the highest proportions of (+)-catechin (0.9%), (−)-epicatechin (6.8%), dimer 1 (2.4%), and dimer 3 (15.9%). Carmel had the highest proportions of 5-mers, 6-mers, and 7-mers, which were 10.8, 7.5, and 7.4%, respectively.

Proanthocyanidins and hydrolyzable tannins contribute a significant portion of the polyphenol content of almonds and other tree nuts. Almonds have more extractable proanthocyanidins than walnuts, but less than hazelnuts, pecans, and pistachios.[4] Gu et al.[4] reported 184.1 mg extractable proanthocyanidins/100 g almond in a commercial almond sample using similar extraction conditions. Although the distribution of proanthocyanidin polymers was similar to this study, the content was higher than the 25−107 mg PE/100 g obtained in the present study. The use of proanthocyanidin B2 equivalents may contribute to this difference. Also, preharvest factors such as climate and environment and postharvest factors such as storage and processing may contribute to differences among almond samples.[2] However, our values appear greater than those in a study by Garrido et al.,[3] adjusted by the relative proportion of almond skins to 100 g almond. The present work is limited by a lack of suitable analytical standards for HPLC analysis. Further effort is required to purify proanthocyanidins of similar composition, and linkages and with various polymerizations to validate methods for quantitation of almond proanthocyanidins.

The differences in extractable proanthocyanidins among almond varieties are greater than other almond polyphenols. In the present study, Nonpareil had more extractable proanthocyanidins than Butte and Carmel, whereas there were no differences in flavonoids and phenolic acids between these varieties.[14] Nonpareil almonds had significantly greater (−)-epicatechin content than the same study, with 3.4 mg/100 g almonds.[14] It is unclear if this difference is due to yearly variation in polyphenol content or the different extraction conditions between studies.

Bound Proanthocyanidins. The yield of proanthocyanidins after alkaline hydrolysis of almond was maximized at 4 N sodium hydroxide. Proanthocyanidins recovered from alkaline hydrolysis of extracted almond residue were quantitated by HPLC (Figure 2B). Only proanthocyanidin monomers, dimers, and oligomers with polymerization of ≥8 were recovered after hydrolysis (Table 1). Alkaline hydrolysis of extracted almond residue yielded an additional 3.4, 6.8, and 6.4 mg PE/100 g almond in Nonpareil, Butte, and Carmel almonds, respectively (Table 1). These yields added 22−66% toward monomer content and 45−81% to dimer 1 content. Nonpareil almonds had the lowest recovery of bound proanthocyanidins, despite having the highest yield of extractable proanthocyanidins among almond varieties.

The release of monomers and dimers upon alkaline hydrolysis of almond is consistent with a previous study in cranberry pomace, alkaline hydrolysis of which liberated 4-fold the monomer to hexamer proanthocyanidins than conventional extraction.[17] Alkaline hydrolysis of cranberry polymeric proanthocyanidins also yielded primarily monomers and dimers.[17]

In the present study, alkaline-hydrolyzed proanthocyanidins were 3.1−26.8% of the extractable proanthocyanidins (Table 1). This proportion was less than that of apple, peach, and nectarine, of which bound proanthocyanidins were 6−20-fold of the extractable proanthocyanidins measured by HPLC.[11] Other bound polyphenols were 2−6-fold of the extractable polyphenols

Table 1. Contents (Milligrams per 100 g Almond) of Extracted and Alkaline-Hydrolyzed Proanthocyanidins (PACs) of California Almond Cultivars[a,b]

	Nonpareil			Butte			Carmel		
PAC	extracted	hydrolyzed	total	extracted	hydrolyzed	total	extracted	hydrolyzed	total
(**1**) (+)-catechin	0.3 ± 0.1	0.1 ± 0.1	0.4 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.1
(**2**) (−)-epicatechin	3.4 ± 0.5	1.2 ± 0.9	4.6 ± 1.2	1.2 ± 1.3	2.6 ± 1.1	3.9 ± 1.1	0.8 ± 0.3	1.8 ± 1.0	2.6 ± 1.2
PAC dimer 1	2.0 ± 1.6	1.4 ± 0.9	3.4 ± 1.4	0.4 ± 0.4	1.8 ± 0.5	2.2 ± 0.1	0.4 ± 0.1	1.9 ± 1.2	2.3 ± 1.3
(**3**) PAC B2	8.3 ± 2.9	0.03 ± 0.03	8.3 ± 2.9	1.5 ± 1.8	0.8 ± 1.3	2.3 ± 1.5	1.2 ± 0.5	0.1 ± 0.0	1.2 ± 0.5
PAC dimer 3	8.4 ± 2.2	0.02 ± 0.01	8.4 ± 2.2	2.5 ± 2.0	0.2 ± 0.3	2.7 ± 1.8	2.4 ± 0.2	0.1 ± 0.1	2.6 ± 0.2
trimers	14.0 ± 3.3		14.0 ± 3.3	3.0 ± 3.8		3.0 ± 3.8	2.7 ± 1.5		2.7 ± 1.5
4-mers	15.1 ± 3.5		15.1 ± 3.5	3.2 ± 4.0		3.2 ± 4.0	3.3 ± 2.1		3.3 ± 2.1
5-mers	10.9 ± 4.1		10.9 ± 4.1	2.2 ± 2.9		2.2 ± 2.9	3.1 ± 1.9		3.1 ± 1.9
6-mers	7.2 ± 2.3		7.2 ± 2.3	1.6 ± 2.1		1.6 ± 2.1	2.2 ± 1.6		2.2 ± 1.6
7-mers	6.3 ± 2.1		6.3 ± 2.1	1.7 ± 2.1		1.7 ± 2.1	2.2 ± 1.6		2.2 ± 1.6
≥8-mers	31.3 ± 8.9	0.6 ± 0.1	31.9 ± 8.8	7.9 ± 10.5	1.2 ± 1.0	9.1 ± 10.1	9.7 ± 6.7	2.3 ± 0.3	12.0 ± 7.0
sum	*107.3*	*3.4*	*110.6*	*25.4*	*6.8*	*32.2*	*28.1*	*6.4*	*34.5*

[a]Data are the mean ± standard deviation, n = 3/cultivar. Proanthocyanidins with polymerization of two and greater are expressed as proanthocyanidin B2 equivalents. [b]Statistical significance levels among extractable PACs by two-way ANOVA were $P < 0.0001$ for PACs and $P < 0.0001$ for genotype and $P = 0.9977$ for their interaction. Statistical significance levels among base-hydrolyzable PACs by two-way ANOVA were $P < 0.0001$ for PACs and $P = 0.0036$ for genotype and $P = 0.7349$ for their interaction. Statistical significances among total PACs by two-way ANOVA were $P < 0.0001$ for PACs and $P < 0.0001$ for genotype and $P = 0.4417$ for their interaction.

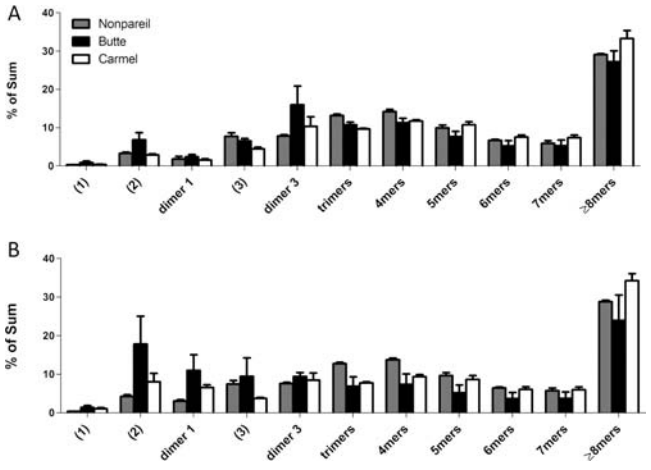

Figure 3. (A) Relative extractable proanthocyanidin profile of California almond genotypes. Data are the mean ± SEM of Nonpareil, Butte, and Carmel almonds (n = 3/genotype). By two-way ANOVA, $P = 0.2921$ for genotype and $P < 0.0001$ for proanthocyanidin type, with $P = 0.0087$ for their interaction. (B) Relative total proanthocyanidin profile of California almond genotypes. Data are the mean ± SEM of Nonpareil, Butte, and Carmel almonds (n = 3/genotype). By two-way ANOVA, $P = 0.5959$ for genotype and $P < 0.0001$ for proanthocyanidin type, with $P = 0.0019$ for their interaction.

in apple, peach, and nectarine measured by LC-MS.[11] The bound polyphenol content of tree nuts may be less than those of other fruits and vegetables. In walnuts and heartnuts, bound total phenolic contents were 20−77% of the extractable total phenolic contents measured by Folin−Ciocalteu assay.[24] In a study of 29 foods, bound phenolic compounds accounted for nearly the same or more antioxidant capacity than free phenolic compounds measured.[25] Thus, the proportion of bound polyphenols may depend on plant varieties, genotypes, and polyphenol composition. Further work is needed to define the contribution of bound polyphenols to almond bioactivity.

In contrast to alkaline hydrolysis, acid hydrolysis of extracted almond residue did not yield additional proanthocyanidins by HPLC fluorescence analysis (<0.02 mg/100 g almond). Strongly acidic conditions, such as in simulated gastric juice, degrade purified catechins and proanthocyanidins B2 and B5.[26] While the methanolic acid hydrolysis conditions were insufficient to release additional bound proanthocyanidins in almond, previous studies have used this method to liberate catechins from foods.[18,19]

Total Proanthocyanidins. The sum of extractable and bound proanthocyanidins reflects the total proanthocyanidin content of almonds (Table 1). Nonpareil almonds had the most total proanthocyanidins with 110.6 mg PE/100 g, representing 3.4- and 3.2-fold of Butte and Carmel varieties, respectively. Butte almonds had the greatest relative proportion of monomers and dimers, contributing ∼50% to the total proanthocyanidin content. In contrast, trimers and greater oligomers contributed >70% of the proanthocyanidin content of Nonpareil and Carmel almonds.

The proanthocyanidin profile of Butte and Carmel almonds, as percent of sum, changed when extractable and bound proanthocyanidins were considered (Figure 3B). This was due to the high content of monomers, dimers, and trimers in Butte and Carmel almonds following alkaline hydrolysis of their extracted residue. For oligomers (>3) and polymers, the total proanthocyanidin profile reflected the extractable proanthocyanidin profile.

Hydrolyzable Tannins. Extractable almond tannin fractions were resolved by two-dimensional TLC to screen for the presence of gallotannins and ellagitannins. In each almond variety, at least five or six unique spots reacted with potassium iodate, indicating potential gallotannin content. Nonpareil and Butte almonds each had four orange-brown spots after a sodium nitrate spray, indicating the presence of ellagitannins.[21] Carmel almonds had a unique ellagitannin profile, with three orange-brown and three pink spots after the sodium nitrate spray. Low retention factors in the second TLC dimension tentatively indicated the presence of large molecular weight ellagitannin and gallotannins.[21]

Hydrolyzable tannins vary according to their polymeric level of gallic acid and ellagic acid.[27] A number of ellagitannins have been identified in walnut, including pedunculagin, glansrins, casuar-

ictin, and others.[28,29] Thus, almonds may also contain a diverse ellagitannin profile. Precaution should be taken in the selection of almond tannin TLC bands for further analysis, because potassium iodate also reacts with catechins. Further work is needed to isolate and identify these compounds from almonds.

Gallic acid and ellagic acid were present in hydrolysis solutions of defatted almonds (Figure 4). Nonpareil, Butte, and Carmel

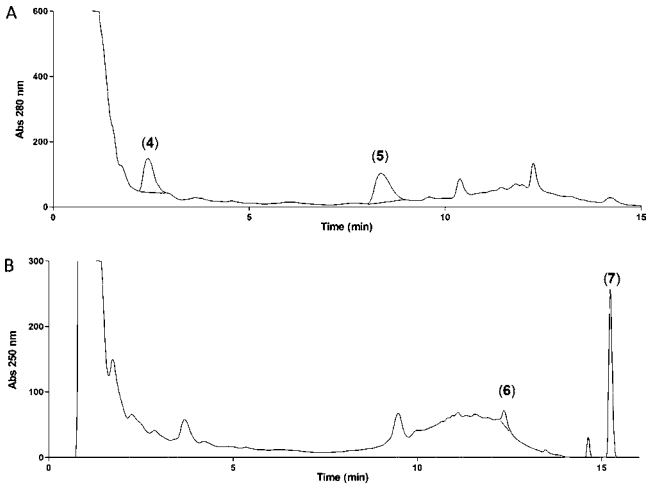

Figure 4. Representative HPLC chromatograms of (A) (**4**) gallic acid and (B) (**6**) ellagic acid following hydrolysis of defatted almond powder.

almonds contained 19.6−34.1 mg gallic acid/100 g and 43.3−57.4 mg ellagic acid/100 g with no significant differences between varieties (n = 3/variety) (Table 2). In comparison to

Table 2. Hydrolyzable Tannin Content of California Almond Cultivars Determined by Hydrolysis and HPLC Analysis[a,b]

hydrolyzable tannin	value	almond cultivar		
		Nonpareil	Butte	Carmel
mg gallic acid (**4**)/100 g	mean	34.1 ± 2.4	28.5 ± 10.5	19.6 ± 5.0
	range	32.6−36.8	22.4−40.6	14.1−23.8
mg ellagic acid (**6**)/100 g	mean	57.4 ± 6.4	53.3 ± 1.5	53.4 ± 5.4
	range	50.5−63.2	51.6−54.3	48.7−59.3

[a]Data are the mean ± standard deviation, n = 3/cultivar. [b]Statistical significance levels among almond cultivars by one-way ANOVA were P = 0.1016 for gallotannins and P = 0.5490 for ellagitannins.

previous studies, almond ellagitannins are 3−10-fold less than walnut, which have 150−546 mg of ellagic acid/100 g.[24,29] A previous study reported almond contained little to no gallotannins and ellagitannins following hydrolysis.[30] However, that study employed a more moderate hydrolysis condition than the present study. Thus, almond hydrolyzable tannin content may have been previously underestimated. Ellagic acid consumption in the United States is estimated to be 3.4−15.1 mg/1000 kcal energy in adult females, varying by whether they meet fruit and vegetable intake recommendations.[31] Therefore, consuming a 43 g (1.5 oz) serving of almond could more than double the estimated ellagic acid intake.

Although originally recognized as antinutrients, a number of putative health effects are attributed to tannins. Gallic acid is antioxidant and apoptotic to SH-SY5Y and U 937 cancer cells in vitro.[32,33] Likewise, ellagic acid has in vitro antioxidant potential, anti-inflammatory activity, estrogenic and/or antiestrogenic roles, and antimicrobial and prebiotic effects.[34] However, ellagic acid is bioavailable only after it is metabolized to urolithins, which may be a marker for nut intake.[35] Almond proanthocyanidins are bioavailable and tend to be metabolized to (epi)catechin conjugated metabolites by phase II enzymes at 2−6 h after consumption in healthy adults.[36] There is a need to characterize the bioaccessibility of bound phenolics in almonds and other polyphenol-rich foods. Thus, future studies characterizing the effects of almond polyphenol consumption should consider hydrolyzable tannins and bound proanthocyanidins.

In conclusion, almonds are a source of diverse polyphenols, including phenolic acids, flavonoids, proanthocyanidins, ellagitannins, gallotannins, and likely other uncharacterized constituents. Alkaline hydrolysis yielded additional polyphenols from extracted almond residue, increasing the total proanthocyanidin content 3−21% in California almonds. Almonds were also found to contain 27.4 mg gallic acid and 54.7 mg ellagic acid/100 g following hydrolysis. Database values and future analytical research efforts should consider the presence of "bound" polyphenols, ellagitannins, and gallotannins in California almonds.

■ ASSOCIATED CONTENT

Supporting Information

Additional table and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 1 (860) 486-2180. Fax: 1 (860) 486-3674. E-mail: bradley.bolling@uconn.edu.

Funding

Financial support was provided by the Almond Board of California, Modesto, CA, USA. Additional funding was provided by the University of Connecticut College of Agriculture and Natural Rescources.

Notes

A portion of this work was presented April 24, 2012, at the Experimental Biology meeting: Bolling, B.; Roto, A.; Kimball, K.; Xie, L. Extractable and nonextractable proanthocyanidins of Nonpareil, Carmel, and Butte California almonds. *FASEB J.* **2012**, *26* (1), 1025−1026.

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful for the technical assistance of Kerilyn Kimball, Elizabeth Wallett, and Rod Taheri. Almond materials were provided by the Almond Board of California, Modesto, CA, USA.

■ REFERENCES

(1) Pérez-Jiménez, J.; Neveu, V.; Vos, F.; Scalbert, A. Identification of the 100 richest dietary sources of polyphenols: an application of the Phenol-Explorer database. *Eur. J. Clin. Nutr.* **2010**, *64*, S112−S120.

(2) Bolling, B. W.; Chen, C. Y. O.; McKay, D. L.; Blumberg, J. B. Tree nut phytochemicals: composition, antioxidant capacity, bioactivity, impact factors. A systematic review of almonds, Brazils, cashews, hazelnuts, macadamias, pecans, pine nuts, pistachios and walnuts. *Nutr. Res. Rev.* **2011**, *24*, 244−275.

(3) Garrido, I.; Monagas, M.; Gómez-Cordovés, C.; Bartolomé, B. Polyphenols and antioxidant properties of almond skins: influence of industrial processing. *J. Food Sci.* **2008**, *73*, C106−C115.

(4) Gu, L.; Kelm, M. A.; Hammerstone, J. F.; Beecher, G.; Holden, J.; Haytowitz, D.; Gebhardt, S.; Prior, R. L. Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *J. Nutr.* **2004**, *134*, 613−617.
(5) Gu, L.; Kelm, M. A.; Hammerstone, J. F.; Beecher, G.; Holden, J.; Haytowitz, D.; Prior, R. L. Screening of foods containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic degradation. *J. Agric. Food Chem.* **2003**, *51*, 7513−7521.
(6) Monagas, M.; Garrido, I.; Lebrón-Aguilar, R.; Carmen Gómez-Cordovés, M.; Rybarczyk, A.; Amarowicz, R.; Bartolomé, B. Comparative flavan-3-ol profile and antioxidant capacity of roasted peanut, hazelnut, and almond skins. *J. Agric. Food Chem.* **2009**, *57*, 10590−10599.
(7) Prior, R. L.; Gu, L. Occurrence and biological significance of proanthocyanidins in the American diet. *Phytochemistry* **2005**, *66*, 2264−2280.
(8) Urpi-Sarda, M.; Garrido, I.; Monagas, M.; Gómez-Cordovés, C.; Medina-Remón, A.; Andres-Lacueva, C.; Bartolomé, B. Profile of plasma and urine metabolites after the intake of almond [*Prunus dulcis* (Mill.) D.A. Webb] polyphenols in humans. *J. Agric. Food Chem.* **2009**, *57*, 10134−10142.
(9) Chen, C. Y.; Milbury, P. E.; Lapsley, K.; Blumberg, J. B. Flavonoids from almond skins are bioavailable and act synergistically with vitamins C and E to enhance hamster and human LDL resistance to oxidation. *J. Nutr.* **2005**, *135*, 1366−1373.
(10) Mandalari, G.; Bisignano, C.; Genovese, T.; Mazzon, E.; Wickham, M. S.; Paterniti, I.; Cuzzocrea, S. Natural almond skin reduced oxidative stress and inflammation in an experimental model of inflammatory bowel disease. *Int. Immunopharmacol.* **2011**, *11*, 915−924.
(11) Arranz, S.; Saura-Calixto, F.; Shaha, S.; Kroon, P. A. High contents of nonextractable polyphenols in fruits suggest that polyphenol contents of plant foods have been underestimated. *J. Agric. Food Chem.* **2009**, *57*, 7298−7303.
(12) Arranz, S.; Silván, J. M.; Saura-Calixto, F. Nonextractable polyphenols, usually ignored, are the major part of dietary polyphenols: a study on the Spanish diet. *Mol. Nutr. Food Res.* **2010**, *54*, 1646−1658.
(13) Bolling, B. W.; Blumberg, J. B.; Chen, C. Y. O. The influence of roasting, pasteurisation, and storage on the polyphenol content and antioxidant capacity of California almond skins. *Food Chem.* **2010**, *123*, 1040−1047.
(14) Bolling, B. W.; Dolnikowski, G.; Blumberg, J. B.; Chen, C. Y. O. Polyphenol content and antioxidant activity of California almonds depend on cultivar and harvest year. *Food Chem.* **2010**, *122*, 819−825.
(15) Bolling, B. W.; Dolnikowski, G.; Blumberg, J. B.; Chen, C. Y. O. Quantification of almond skin polyphenols by liquid chromatography-mass spectrometry. *J. Food Sci.* **2009**, *74*, C326−C332.
(16) Mandalari, G.; Tomaino, A.; Arcoraci, T.; Martorana, M.; Turco, V. L.; Cacciola, F.; Rich, G. T.; Bisignano, C.; Saija, A.; Dugo, P.; Cross, K. L.; Parker, M. L.; Waldron, K. W.; Wickham, M. S. J. Characterization of polyphenols, lipids and dietary fibre from almond skins (*Amygdalus communis* L.). *J. Food Compos. Anal.* **2010**, *23*, 166−174.
(17) White, B. L.; Howard, L. R.; Prior, R. L. Release of bound procyanidins from cranberry pomace by alkaline hydrolysis. *J. Agric. Food Chem.* **2010**, *58*, 7572−7579.
(18) Harnly, J. M.; Doherty, R. F.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Bhagwat, S.; Gebhardt, S. Flavonoid content of U.S. fruits, vegetables, and nuts. *J. Agric. Food Chem.* **2006**, *54*, 9966−9977.
(19) Merken, H. M.; Beecher, G. R. Liquid chromatographic method for the separation and quantification of prominent flavonoid aglycones. *J. Chromatogr., A* **2000**, *897*, 177−184.
(20) Prodanov, M.; Garrido, I.; Vacas, V.; Lebronaguilar, R.; Duenas, M.; Gomezcordoves, C.; Bartolome, B. Ultrafiltration as alternative purification procedure for the characterization of low and high molecular-mass phenolics from almond skins. *Anal. Chim. Acta* **2008**, *609*, 241−251.
(21) Mueller-Harvey, I. Analysis of hydrolysable tannins. *Anim. Feed Sci. Technol.* **2001**, *91*, 3−20.
(22) Lei, Z.; Jervis, J.; Helm, R. F. Use of methanolysis for the determination of total ellagic and gallic acid contents of wood and food products. *J. Agric. Food Chem.* **2001**, *49*, 1165−1168.
(23) Monagas, M.; Garrido, I.; Lebrón-Aguilar, R.; Bartolome, B.; Gómez-Cordovés, C. Almond (*Prunus dulcis* (Mill.) D.A. Webb) skins as a potential source of bioactive polyphenols. *J. Agric. Food Chem.* **2007**, *55*, 8498−8507.
(24) Li, L.; Tsao, R.; Yang, R.; Liu, C.; Zhu, H.; Young, J. C. Polyphenolic profiles and antioxidant activities of heartnut (*Juglans ailanthifolia* var. *cordiformis*) and Persian walnut (*Juglans regia* L.). *J. Agric. Food Chem.* **2006**, *54*, 8033−8040.
(25) Pellegrini, N.; Serafini, M.; Salvatore, S.; Del Rio, D.; Bianchi, M.; Brighenti, F. Total antioxidant capacity of spices, dried fruits, nuts, pulses, cereals and sweets consumed in Italy assessed by three different in vitro assays. *Mol. Nutr. Food Res.* **2006**, *50*, 1030−1038.
(26) Zhu, Q. Y.; Holt, R. R.; Lazarus, S. A.; Ensunsa, J. L.; Hammerstone, J. F.; Schmitz, H. H.; Keen, C. L. Stability of the flavan-3-ols epicatechin and catechin and related dimeric procyanidins derived from cocoa. *J. Agric. Food Chem.* **2002**, *50*, 1700−1705.
(27) Chung, K.-T.; Wong, T. Y.; Wei, C.-I.; Huang, Y.-W.; Lin, Y. Tannins and human health: a review. *Crit. Rev. Food Sci. Nutr.* **1998**, *38*, 421−464.
(28) Fukuda, T.; Ito, H.; Yoshida, T. Antioxidative polyphenols from walnuts (*Juglans regia* L.). *Phytochemistry* **2003**, *63*, 795−801.
(29) Cerdá, B.; Tomás-Barberán, F. A.; Espín, J. C. Metabolism of antioxidant and chemopreventive ellagitannins from strawberries, raspberries, walnuts, and oak-aged wine in humans: identification of biomarkers and individual variability. *J. Agric. Food Chem.* **2005**, *53*, 227−235.
(30) Molyneux, R. J.; Mahoney, N.; Kim, J. H.; Campbell, B. C. Bioassay-directed isolation and identification of antiaflatoxigenic constituents of walnuts. In *Bioactive Natural Products: Detection, Isolation and Structural Determination*; Colegate, S. M., Molyneux, R. J., Eds.; CRC Press: Boca Raton, FL, 2008; pp 421−437.
(31) Murphy, M. M.; Barraj, L. M.; Herman, D.; Bi, X.; Cheatham, R.; Randolph, R. K. Phytonutrient intake by adults in the United States in relation to fruit and vegetable consumption. *J. Acad. Nutr. Diet.* **2012**, *112*, 222−229.
(32) Saeki, K.; Yuo, A.; Isemura, M.; Abe, I.; Seki, T.; Noguchi, H. Apoptosis-inducing activity of lipid derivatives of gallic acid. *Biol. Pharm. Bull.* **2000**, *23*, 1391−1394.
(33) Lu, Z.; Nie, G.; Belton, P. S.; Tang, H.; Zhao, B. Structure-activity relationship analysis of antioxidant ability and neuroprotective effect of gallic acid derivatives. *Neurochem. Int.* **2006**, *48*, 263−274.
(34) Landete, J. M. Ellagitannins, ellagic acid and their derived metabolites: a review about source, metabolism, functions and health. *Food Res. Int.* **2011**, *44*, 1150−1160.
(35) Tulipani, S.; Urpi-Sarda, M.; García-Villalba, R.; Rabassa, M.; López-Uriarte, P.; Bulló, M.; Jáuregui, O.; Tomás-Barberán, F.; Salas-Salvadó, J.; Espín, J. C.; Andrés-Lacueva, C. Urolithins are the main urinary microbial-derived phenolic metabolites discriminating a moderate consumption of nuts in free-living subjects with diagnosed metabolic syndrome. *J. Agric. Food Chem.* **2012**, *60*, 8930−8940.
(36) Garrido, I.; Urpi-Sarda, M.; Monagas, M.; Gómez-Cordovés, C.; Martín-Álvarez, P. J.; Llorach, R.; Bartolomé, B.; Andrés-Lacueva, C. Targeted analysis of conjugated and microbial-derived phenolic metabolites in human urine after consumption of an almond skin phenolic extract. *J. Nutr.* **2010**, *140*, 1799−1807.