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Variation in the Flavonol Glycoside Composition of Almond Seedcoats As Determined by MALDI-TOF Mass Spectrometry

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Seedcoats of 16 almond varieties were screened for flavonol glycosides by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Flavonol glycosides were extracted by a simple methanolic extraction followed by a quick cleanup procedure with a Sep-Pak C_{18} cartridge. Each of the 16 seedcoat samples exhibited a unique composition. Four flavonol glycosides, isorhamnetin rutinoside, isorhamnetin glucoside, kaempferol rutinoside, and kaempferol glucoside, were detected and quantified with use of rutin as an internal standard. Individual peak ratios were very consistent across triplicate analyses of all samples; the average standard deviation was 9%. In all almond varieties, isorhamnetin rutinoside was the most abundant flavonol glycoside, and the total content ranged from 75 to 250 μg/g.

KEYWORDS: MALDI-TOF; mass spectrometry; almond seedcoats; screening; flavonol glycosides

INTRODUCTION

Almonds are the seeds of varieties of *Prunus amygdalus*, a member of the Rosaceae family, which also includes apples, pears, peaches, and plums. The flesh of the seed is encased in a brown leathery coating, called the seedcoat, which protects the almond from oxidation and microbial contamination. Many food applications of almonds in bakery and confectionary items, cereals, snack formulations, and marzipan, require the flesh of the almond alone without the seedcoat. During the process of blanching, moist heat is used to loosen the seedcoat from the almond flesh, followed by gentle agitation to complete the separation.

Almond seedcoats are usually discarded after they are removed by blanching. Yet, recent investigations into the phytochemical composition of almonds have shown that the seedcoats may contain many potentially beneficial compounds, opening up new possibilities for the value of almond seedcoats. Studies by Brieskorn and Betz (*1*) revealed that almond seedcoats are a rich source of sterols. Some types of sterols have been shown to reduce serum cholesterol levels (*2*). Takeoka et al. (*3*) described three triterpenoids, betulinic acid, oleanoic acid, and ursolic acid, which have reported antiinflammatory (*4*), anti-HIV (*5*), and anti-cancer activities (*6*).

Oxidative stress has been linked to the aging process, and to the development of arteriosclerosis and cancer. One potential way to combat oxidative stress is to consume a variety of antioxidants in the diet. The primary source of naturally occurring dietary antioxidants is plant foods. Commonly found in edible plants are phenolic compounds which are thought to exhibit antioxidant activity through scavenging free radicals (*7*). Knowledge about the absorption, bioavailability, and metabolism of dietary phenolics is also important to fully evaluate their potential health benefits.

Sang et al. (*8*, *9*) have isolated and characterized several important antioxidant phenolic compounds from almond seedcoats. Flavonoids are a subclass of phenolic compounds, which can be further classified into several structural subcategories, including proanthocyanidins and flavonol glycosides. Proanthocyanidins of varying degrees of polymerization have been identified in the almond seedcoat, almond flesh, and almond fruit (*10*−*14*) and are likely responsible for the reddish-brown hue of the seedcoat.

Our previous work has identified four flavonol glycosides in almond seedcoats: isorhamnetin rutinoside, isorhamnetin glucoside, kaempferol rutinoside, and kaempferol glucoside (**Figure 1**), using a technique known as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (*15*). We demonstrated the potential of this instrument to rapidly analyze flavonol glycosides in a complex extract. Also, we were able to reliably quantify these four analytes by using an internal standard and correcting for in-source fragmentation. These findings prompted us to investigate the differences in flavonol glycoside composition across many varieties of almond seedcoat, using MALDI-TOF MS as a screening tool. In this paper we describe the variation in the seedcoat flavonol glycoside composition among 16 varieties of almonds using MALDI-TOF MS.

MATERIALS AND METHODS

Materials and Reagents. Seedcoats from 16 varieties of almonds (*Prunus amygdalus*) were supplied by the Almond Board of California (Modesto, CA). The varieties were Aldrich, Butte, Carmel, Fritz, Le Grand, Mission, Monterey, Nonpareil, Padre, Peerless, Price, Ruby, Sauret, Sonora, Thompson, and Wood Colony. Isorhamnetin-3-rutinoside was obtained from Extrasynthese S.A. (Genay Cedex, France),

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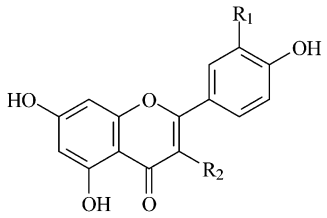

Flavonol	R_1	R_2
kaempferol	H	OH
quercetin	OH	OH
isorhamnetin	OCH3	OH
kaempferol-3-glucoside	H	*O*-glucose
quercetin-3-glucoside	OH	*O*-glucose
isorhamnetin-3-glucoside	OCH3	*O*-glucose
kaempferol-3-rutinoside	H	*O*-rutinose
quercetin-3-rutinoside (rutin)	OH	*O*-rutinose
isorhamnetin-3-rutinoside	OCH3	*O*-rutinose

Figure 1. Structure of flavonol glycosides.

and rutin (quercetin-3-rutinoside) was purchased from Sigma Chemical Co. (St. Louis, MO). The matrix 2′,4′,6′-trihydroxyacetophenone monohydrate was obtained from Aldrich Chemical Co. (Milwaukee, WI). All water was deionized by a Milli-Q water purification system (Millipore Corp., Bedford, MA).

Extraction of Flavonol Glycosides. Extraction conditions were a modification of our earlier procedure (*15*). Each variety of almond seedcoat was ground to a fine powder in a blender and sieved through a 20-mesh screen. An internal standard, rutin (500 μL of a 1 mg/mL solution in 90% methanol), was added to 5.0-g samples of each almond variety. The seedcoats were extracted twice by stirring each time with 50 mL of 70% methanol for 30 min at room temperature, and the extracts were combined. Methanol was removed under reduced pressure, and the volume of the aqueous residue was adjusted to 25 mL with water. A Sep-Pak C_{18} cartridge was preconditioned with 5 mL of methanol followed by 5 mL of water. Each extract was filtered through a 0.22 μm membrane and 5 mL was loaded onto a cartridge. The cartridges were washed with 30 mL of water and eluted with 2 mL of 0.01 M NaCl in 70% methanol. The reddish-brown extract was refrigerated until analysis.

MALDI-TOF MS. MALDI-TOF MS analyses were performed by using a Proflex III instrument with a linear flight tube (Bruker Analytical Systems Inc., Billerica, MA). A solution (20 mg/mL) of 2′,4′,6′-trihydroxyacetophenone monohydrate in acetone (0.7 μL) was applied to the stainless steel probe and allowed to air-dry, followed by application of 0.7 μL of analyte solution. Analytes were ionized by using a 3 ns nitrogen laser pulse (337 nm) and accelerated under 20 kV, using delayed extraction before entering the time-of-flight mass spectrometer. Laser strength was adjusted to provide optimal signal-to-noise ratios and samples were analyzed in the positive ion mode.

Quantification of Flavonol Glycosides. Three spectra of 80 laser shots each were obtained for each almond sample, and the results were averaged. Peak heights of the proton and sodium adduct ions for all flavonol species were determined relative to rutin and reported as μg of flavonol per g of seedcoat. Linearity ($R^2 = 0.99$), response ratios (0.5127 for isorhamnetin; 0.8481 for kaempferol), and quantification calculations were determined as described in Frison-Norrie and Sporns (*15*).

RESULTS AND DISCUSSION

Minor modifications were made to the sample preparation method reported in Frison-Norrie and Sporns (*15*) for the purpose of improving the extraction efficiency and the consistency in conditions across the analysis of many samples. The particle size of the ground almond seedcoats was limited to that which could pass through a 20-mesh screen. Two 30-min extractions of 50 mL of 70% methanol were used instead of one 30-min extraction of 100 mL of 70% methanol. The internal standard, rutin, was added prior to extraction instead of prior to the column cleanup step to account for losses during workup.

The ionic environment during MALDI-TOF MS ionization has the potential to alter the analyte response ratios, depending on the types of ions that are preferentially formed. To test the possibility that response ratios are dependent on the types of cations present, the relative responses of two standards (rutin and isorhamnetin-3-rutinoside) were studied under three conditions: in the presence of no added cations, in the presence of excess sodium (0.01 M), and in the presence of excess potassium (0.01 M) (**Figure 2**). Excess sodium completely suppressed the formation of potassium adducts and created $[M + 2Na - H]^+$ ions. Excess potassium had the same effect, substituting potassium for sodium. In both cases, the proton adducts were unaffected. Proton, sodium, and potassium adducts of the rutinosides all appeared in the spectrum with no added cations. Only the rutinosides showed a preference for alkali metal adducts. In this particular case, the fragment ions (glucosides and aglycones) appeared only as protonated species, although glucosides are also known to form sodium adducts (*15*).

Table 1 shows the response ratios for isorhamnetin-3-rutinoside and its fragment ions as compared to the total rutin response. The relative responses given in the table represent the averages of three different MALDI-TOF MS analyses for each set of conditions. Sodium and potassium seem to be equally preferred for ionization, although sodium produces slightly lower variability on an individual peak basis. In the absence of added cations, the rutinoside response is de-emphasized, which consequently distorts the relative abundance of the fragment ions. Conversely, because the rutinosides have an affinity for alkali metal adducts, their response is accentuated in the presence of either sodium or potassium, again affecting the relative abundance of the fragment ions. At the same time, however, addition of sodium or potassium offers an advantage by significantly decreasing the spectrum-to-spectrum variability in the responses of the parent and fragment ions of the analyte (isorhamnetin-3-rutinoside) with respect to the response of the internal standard (rutin). Alternatively, it is possible that the presence of sodium or potassium suppresses fragmentation.

The ratio of the total isorhamnetin-3-rutinoside response (H^+ and Na^+ adduct ions of rutinoside plus fragment ions) to the total rutin response remains similar across all three conditions, implying that only the relative responses of individual peaks are affected by differences in the cation environment. The total rutin response remains constant despite changes in the relative ratios of individual ion peaks. Thus, to approximate the true flavonol composition in the almond seedcoat extracts by the mass spectrum, a correction factor is necessary to account for changes in the apparent abundance of fragment ions. As discussed in our previous work (*15*), the fragmentation pattern of rutin can be used to predict the fragmentation pattern of the flavonol analytes, which accounts for this variability in response.

Figure 3 shows the variation in total flavonol glycoside content and the abundance of isorhamnetin rutinoside, isorhamnetin glucoside, kaempferol rutinoside, and kaempferol glucoside in the seedcoats of 16 varieties of almonds. Only these four flavonol glycosides were detected in all of the samples tested, and isorhamnetin rutinoside was consistently the major species. The results for each almond variety represent the average of three MALDI-TOF MS analyses. The relative ratios of the individual flavonol glycoside peaks were very consistent; the average standard deviation among triplicate analyses for all

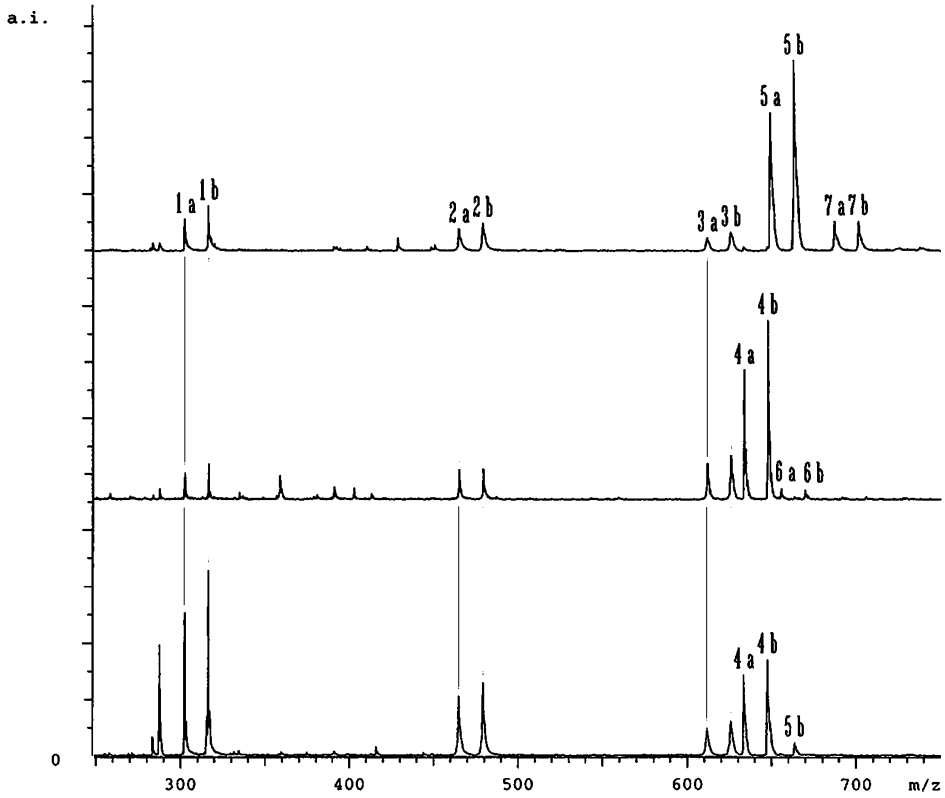

Figure 2. MALDI-TOF MS positive ion spectra of a mixture of rutin and isorhamnetin-3-rutinoside standards in a 1:1 concentration ratio (each at 0.5 mg/mL in 90% methanol). Bottom: Solution prepared with no added cations. Middle: Solution prepared with NaCl at a concentration of 0.01 M. Top: Solution prepared with KCl at a concentration of 0.01 M. (1a) [quercetin + H]$^+$, (1b) [isorhamnetin + H]$^+$, (2a) [quercetin-3-glucoside + H]$^+$, (2b) [isorhamnetin-3-glucoside + H]$^+$, (3a) [quercetin-3-rutinoside + H]$^+$, (3b) [isorhamnetin-3-rutinoside + H]$^+$, (4a) [quercetin-3-rutinoside + Na]$^+$, (4b) [isorhamnetin-3-rutinoside + Na]$^+$, (5a) [quercetin-3-rutinoside + K]$^+$, (5b) [isorhamnetin-3-rutinoside + K]$^+$, (6a) [quercetin-3-rutinoside + 2Na − H]$^+$, (6b) [isorhamnetin-3-rutinoside + 2Na − H]$^+$, (7a) [quercetin-3-rutinoside + 2K − H]$^+$, (7b) [isorhamnetin-3-rutinoside + 2K − H]$^+$.

Table 1. Comparison of Response Ratios of Isorhamnetin-3-rutinoside and Rutin Standards under Different Ionic Conditions

response ratios[a]	no cations added	Na^+ added	K^+ added
isorhamnetin[b]/rutin total[c]	0.47 ± 0.13	0.15 ± 0.01	0.16 ± 0.03
isorhamnetin-3-glucoside[b]/rutin total[c]	0.24 ± 0.05	0.16 ± 0.02	0.09 ± 0.03
isorhamnetin-3-rutinoside/rutin total[c]	0.55 ± 0.12	1.00 ± 0.08	1.05 ± 0.10
isorhamnetin-3-rutinoside total[d]/rutin total[c]	1.27 ± 0.04	1.31 ± 0.11	1.29 ± 0.07

[a] Values are the averages of three spectra. [b] Isorhamnetin and isorhamnetin-3-glucoside are fragment ions of isorhamnetin-3-rutinoside. [c] Refers to the sum of the peak heights of all of the ions associated with rutin and its fragment ions. [d] Refers to the sum of the peak heights of all of the ions associated with isorhamnetin-3-rutinoside and its fragment ions.

samples was 9%. Quantification of flavonol glycosides was achieved according to the following equation:

$$[\text{analyte}] = [\text{rutin}] \times (\text{peak height analyte/peak height rutin}) \times \text{dilution factor} \times \text{response factor} \times \text{fragmentation correction factor} \quad (1)$$

For a detailed discussion of the quantification method, which is quite complex due to differential responses and fragmentation patterns, refer to Frison-Norrie and Sporns (*15*). Within the varieties studied, the total flavonol glycoside content ranged from 75 μg/g (per gram of almond seedcoat) to nearly 250 μg/g. Further study is needed to determine to what extent the variation in composition reflects inherent varietal differences as opposed to differences in growing conditions, handling, processing, and storage.

In a recent study by Sang et al. (*8*), nine antioxidative phenolic compounds were isolated from almond seedcoats: four flavonol glycosides, one flavanone glycoside, and three benzoic acid derivatives. In keeping with our results, they confirmed the presence of isorhamnetin-3-rutinoside, isorhamnetin-3-glucoside, and kaempferol-3-rutinoside. However, their work did not report kaempferol-3-glucoside, which we had previously detected by MALDI-TOF MS and also identified by its high-performance liquid chromatography (HPLC) retention time (*15*). Instead, they reported isorhamnetin-3-galactoside. We could not have differentiated this compound from isorhamnetin-3-glucoside by MALDI-TOF MS since these two compounds have identical molecular weights. Furthermore, the resolution we had previously achieved using HPLC probably would not have been sufficient to resolve the two isomers. Sang et al. (*8*) performed an extensive chromatographic separation where isolation of the galactoside from the glucoside could have been achievable. Therefore, it is possible that both of these isomers exist in almond seedcoats, but that our methodology cannot differentiate them.

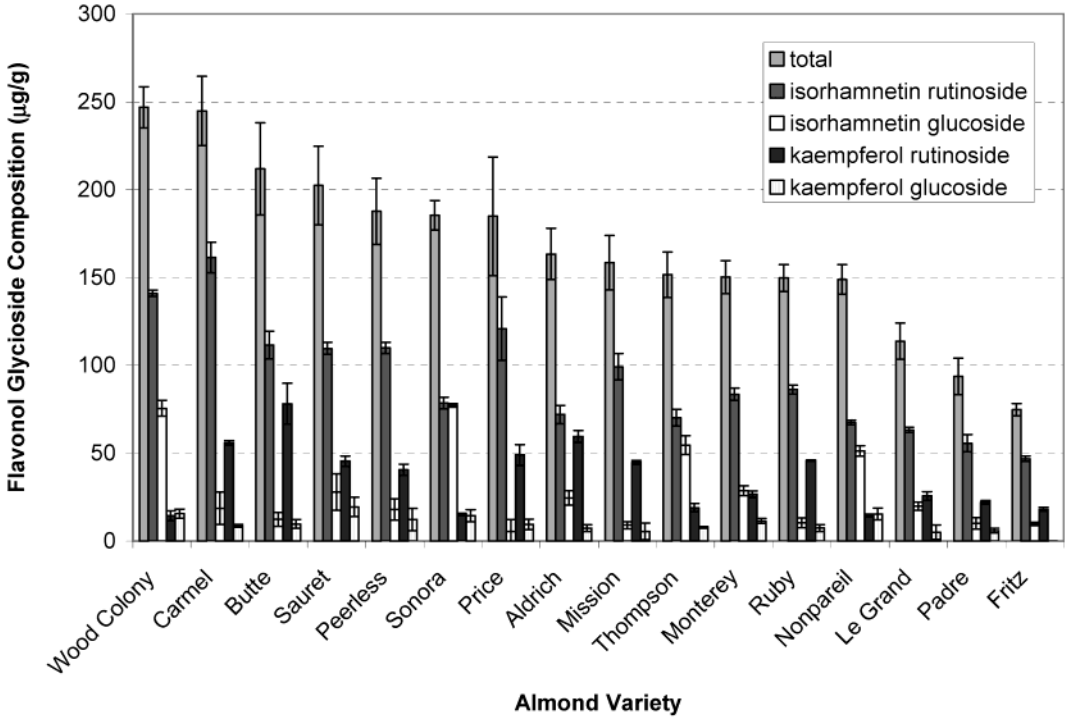

Figure 3. Total flavonol glycoside content and individual flavonol glycoside composition of 16 almond varieties. Results for each variety are averages based on three MALDI-TOF MS spectra.

The flavanone glycoside reported by Sang et al. (*8*), naringenin-7-glucoside (prunin), is chemically similar to the flavonol glycosides, but has a unique molecular weight. Also, according to their yield data, prunin is present in amounts comparable to the flavonol glycosides. Despite these features, it was not detected by our MALDI-TOF MS protocol.

The simple extraction with 70% methanol and crude purification on a Sep-Pak C_{18} cartridge is not selective for flavonol glycosides. The reddish-brown color of the extract is a testament to the presence of other compounds, as flavonol glycosides themselves are yellow or nearly colorless. When considering the number of phytochemicals that have been identified in almond seedcoats (terpenoids, sterols, proanthocyanidins, benzoic acid derivatives, prunin, and flavonol glycosides), it is obvious that many compounds present in the extract are not detectable by our MALDI-TOF MS method. Differences in ionizability may play a role. Depending on their chemical nature and the sample preparation conditions, some families of compounds ionize preferentially, suppressing the ion formation of other groups of compounds. Only those which ionize preferentially will be detected. This feature partially explains the relative tolerance to impurities demonstrated by MALDI-TOF MS. It also confers the advantage of selectivity as long as the analytes of interest are easily ionized. However, this selectivity places certain limitations on the MALDI-TOF MS technique as a general screening tool, since not all compounds in the extract mixture will necessarily be detected.

There is the potential for MALDI-TOF MS to be used in screening the composition of other types of plant foods, if one is aware of the variation in ionization and fragmentation. In this study, the simple extraction procedure coupled with the speed and reliability of the MALDI-TOF MS analysis provide a powerful combination for comparing and characterizing the flavonol glycoside composition in seedcoats of different almond varieties.

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