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Identification and Quantification of Flavonol Glycosides in Almond Seedcoats Using MALDI-TOF MS

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Interest in the molecular composition of almonds is growing, due to their popularity in a wide variety of food formulations. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a powerful new technique that can be used to rapidly identify and quantify possible bioactive compounds in these popular tree nuts. Four flavonol glycosides were identified in almond seedcoats for the first time: isorhamnetin rutinoside, isorhamnetin glucoside, kaempferol rutinoside, and kaempferol glucoside. A MALDI-TOF MS methodology was developed using rutin (quercetin-3-rutinoside) as an internal standard to quantitatively determine each of the four flavonol glycosides. Results of MALDI-TOF MS analysis were verified by high performance liquid chromatography.

KEYWORDS: Almond seedcoats; flavonol glycosides; MALDI-TOF MS; quantfication; isorhamnetin; kaempferol

INTRODUCTION

The search for dietary components that confer health benefits beyond those traditionally ascribed to the macro- and micronutrients is an emerging area of nutritional research. Particular attention has been directed toward identifying certain phytochemicals that may protect against the development of chronic diseases. Several epidemiological studies have linked nut consumption with reduced risk of coronary heart disease (*1*−*3*). Furthermore, nuts have been shown to favorably alter serum lipid levels whereby the magnitude of the effect is substantially greater than would be predicted by the fatty acid profile alone (*4*−*6*). This evidence suggests that the numerous bioactive constituents in nuts may contribute considerably to the overall health benefits. For this reason, the characterization and quantification of these compounds is an important first step toward determining their specific actions and efficacies.

Almonds are an attractive subject of study due to their widespread consumption and increasing popularity. Although published information on almond flavonoid composition is scarce, the presence of certain polyphenols has been confirmed. Several studies report procyanidins of varying degrees of polymerization occurring in the almond seedcoat, almond flesh, and almond fruit (*7*−*11*). Other flavonoids are known to occur naturally in all nuts (*12*), but currently, no characterization in almonds has been reported. As antioxidants, flavonoids help to quench free radicals and regenerate other antioxidants. Several in vitro studies have demonstrated the ability of flavonoids to inhibit the oxidation of low-density lipoprotein (LDL) (*13*), modulate platelet activation (*14*), and inhibit cancer cell proliferation (*15*).

The importance of flavonol glycosides, a subclass of flavonoids, has led to the development of a number of methods for identification and quantification. Traditionally, flavonol glycosides have been characterized using paper chromatography, thin-layer chromatography, and UV spectroscopy (*16*). Recently, the application of high-performance liquid chromatography (HPLC) for their separation and quantification has become predominant (*17*, *18*). The coupling of HPLC to mass spectrometry methods such as electrospray has been adopted to provide molecular weight information and to generate characteristic fragment ions for structural elucidation. (*19*, *20*). However, the most powerful techniques for molecular structure determination of flavonol glycosides remain ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy (*21*, *22*).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a new analytical technique first introduced in 1987 (*23*). Originally developed for the analysis of large biomolecules, MALDI-TOF MS has been successfully applied to a number of analytical problems in the food area, although its food applications are still limited (*24*). The MALDI-TOF MS technique offers several advantages over other methodologies including ease of sample preparation, rapid generation of spectra, tolerance of impurities, and minimal fragmentation allowing direct access to molecular weight. However, isomers with the same molecular weight cannot be differentiated. Qualitative and quantitative analysis of anthocyanins, which are structurally similar to flavonols, has been reported by Wang and Sporns (*25*). The first application of MALDI-TOF MS to study food flavonol glycosides appeared in 2000 (*26*), but quantitative analysis was not addressed. The objectives of the current research were to develop and validate a MALDI-TOF MS methodology for qualitative and quantitative analysis of flavonol glycosides in almond seedcoats.

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MATERIALS AND METHODS

Materials and Reagents. A composite sample of almond seedcoats (*Prunus dulcis*), which had been removed from the almond meat by blanching, was supplied by the Almond Board of California (Modesto, CA). Rutin (quercetin-3-rutinoside) was purchased from Sigma Chemical Co. (St. Louis, MO). Isorhamnetin-3-rutinoside, isorhamnetin-3-glucoside, kaempferol-3-rutinoside, and kaempferol-3-glucoside were obtained from Extrasynthese S. A. (Genay Cedex, France). The matrix 2′,4′,6′-trihydroxyacetophenone monohydrate (THAP) was from Aldrich Chemical Co. (Milwaukee, WI). All water was double-deionized (Milli-Q water purification system, Millipore Corp., Bedford, MA).

Extraction and Isolation of Flavonol Glycosides. A 5.0 g sample of almond seedcoats, ground to a coarse powder, was stirred with 100 mL of 70% methanol for 30 min and then filtered by gravity through Whatman No. 4 filter paper. Methanol was removed from the filtrate using a rotary evaporator with a bath temperature of 35 °C. The filtrate volume was adjusted to 25 mL with water. The methanol-free filtrate (5 mL) was loaded onto a Sep-Pak C_{18} cartridge (Waters Corp., Milford, MA), which had been preconditioned with 5 mL of methanol and 5 mL of water. The cartridge was washed three times with 10 mL of water and eluted with 2 mL of 70% methanol for samples analyzed by HPLC. Alternatively, flavonol glycosides were eluted with 2 mL of 0.01 M NaCl in 70% methanol for MALDI-TOF MS analysis. The orange-brown extracts were refrigerated until use.

MALDI-TOF MS. MALDI-TOF MS analysis was performed using a Proflex III instrument with a linear flight tube (Bruker Analytical Systems Inc., Billerica, MA). A saturated solution of THAP in acetone (1 μL) was applied to the stainless steel probe and allowed to air-dry, followed by application of 1 μL of analyte solution. Analytes were ionized 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 ratio, and samples were analyzed in the positive ion mode.

Determination of Rutin Linearity and Response Factors by MALDI-TOF MS. Stock solutions of rutin, isorhamnetin-3-rutinoside, and kaempferol-3-rutinoside were prepared by dissolving each in 70% methanol at 1.00 mg/mL. A series of dilutions were prepared to give concentration ratios of rutin to either isorhamnetin-3-rutinoside or kaempferol-3-rutinoside as follows: 0.25, 0.5, 1.0, 1.5, and 2.0. Each of these solutions was analyzed by MALDI-TOF MS, and the peak height ratios (using the sum of all proton and sodium adduct peaks for both fragment ions and parent ions) were compared to the concentration ratios to obtain the response factors.

Quantification Using MALDI-TOF MS. Quantification of flavonol glycosides was achieved using rutin as an internal standard. A 40 μL aliquot of rutin stock solution (1.00 mg/mL in 70% methanol) and 360 μL of 0.01 M NaCl in 70% methanol were added to 600 μL of almond seedcoat eluent to give a final rutin concentration of 40 μg/mL. This solution was spotted on five separate positions on the MALDI probe. A single spectrum was then generated for four of these positions by randomly collecting 120 laser shots. The probe was then washed and spotted a second time at five positions, and four more spectra were generated for a total of eight spectra. Peak heights for proton and sodium adducts were determined for all flavonol species from each spectrum.

HPLC Analysis. The HPLC system was composed of a Varian Vista 5500 pump (Varian Canada Inc., Mississauga, Ontario, Canada), a Varian 9090 autosampler, and a SpectroMonitor III UV detector (LDC/Milton Roy, Riviera Beach, FL). The system was fitted with a 75 mm × 4.5 mm i.d. preinjection C18 saturator column containing silica-based packing (12 μm) and a 50 mm × 4.6 mm i.d. guard column containing Supelco LC-18 reversed-phase packing, 20−40 μm (Supelco, Bellefonte, PA). Flavonol glycosides were separated on a Supelcosil SPLC-DB-18 250 mm × 10 mm i.d. (5 μm) preparative reversed-phase column (Supelco). The solvent system consisted of HPLC grade water (solvent A) and acetonitrile (solvent B). Flow rate was maintained at 5 mL/min, with a linear gradient of solvent A and the following proportions (v/v) of solvent B: 0 min, 15% B; 0−40 min, 15−18% B; 40−44 min, 18−31% B; 44−46 min, 31% B; 46−48 min, 31−15% B. Total run time was 55 min, and detection was at 354 nm. Injection volume of standard and sample solutions was 90 μL. A Shimadzu CLASS-VP chromatography data system (Shimadzu Scientific Instruments Inc., Columbia, MD) was used to monitor and to integrate the eluted peaks.

Quantification Using HPLC. A standard curve was prepared using four concentrations of a standard mixture. The relative proportions of flavonol glycosides in the standard mixtures are similar to those found naturally in the almond seedcoat extract. A stock solution was prepared with isorhamnetin-3-rutinoside, isorhamnetin-3-glucoside, kaempferol-3-rutinoside, kaempferol-3-glucoside, and rutin (internal standard) at concentrations of 120, 40, 20, 12, and 120 μg/mL, respectively, in 90% methanol. The stock solution was then diluted by a factor of 2, 4, and 8. Three replicate injections of almond seedcoat extract containing rutin as an internal standard were performed.

RESULTS AND DISCUSSION

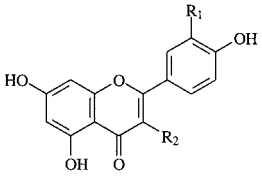
MALDI-TOF MS analysis is commonly used for molecular identification purposes, as molecular weights can be determined rapidly and accurately. More challenging is the application of this tool for quantification purposes, as several obstacles must be overcome. Difficulties in achieving reproducible homogeneous crystal beds can lead to inherent variability among identical preparations. Multiple analytes in solution can compete for ionization energy or exhibit variations in ionization potential. Multiple ion adducts may appear for single compounds, with different compounds displaying different preferences for particular ion forms. Finally, fragmentation, while diagnostic for identification purposes, can complicate the issue of quantification if fragment ions and analyte ions are identical.

Controlling Variability. One factor that can affect the degree of variability among replicates is the uniformity of the matrix−analyte crystal bed. 2′4′6′-Trihydroxyacetophenone monohydrate was selected as a matrix because it has proven successful in our lab in the past for analyzing flavonol glycosides (*26*), and it demonstrated good spot-to-spot repeatability and tolerance of impurities. When dissolved in acetone, this matrix evaporates quickly to produce a lawn of small homogeneous crystals (*27*). However, the nature of the solvent system, which is subsequently spotted on top of the matrix, can affect the homogeneity of the final crystal formation. Solutions prepared in 90% methanol completely redissolved the matrix and caused very irregular crystals. Conversely, solutions prepared in 70% methanol only redissolved a small proportion of the matrix. The remaining undissolved matrix acted as seed crystals for the rapid recrystallization of analyte and matrix in a more regular fashion. Multiple analyses of standards in 90% methanol showed much higher standard deviations in relative responses than did standards dissolved in 70% methanol (data not shown). For this reason, all MALDI-TOF MS analyses were carried out using solutions prepared in 70% methanol.

As a further measure to reduce variability, 10 positions on the probe were spotted and one spectrum was generated at each position. Of these 10 spectra, the eight best ones were chosen for further analysis. Spectrum selection criteria included high response, good resolution, good signal-to-noise ratio, and consistency in relative response with respect to the other spectra.

Response Factors of Flavonol Glycosides in MALDI-TOF MS. Despite large variations in absolute response, relative responses remained relatively constant across many trials. On this basis, the relative response of analytes to an internal standard can be used for quantification purposes. Rutin was chosen as an internal standard for almond seedcoat analysis because of its absence in the sample and its structural similarity to the analytes (**Table 1**). However, because of inherent differences in crystallization and ionizability, chemically similar compounds may exhibit different responses at the same concentration. Thus,

Table 1. Structure of Flavonol Glycosides



flavonol	R_1	R_2
kaempferol	H	OH
quercetin	OH	OH
isorhamnetin	OCH_3	OH
kaempferol-3-glucoside	H	*O*-glucose
quercetin-3-glucoside	OH	*O*-glucose
isorhamnetin-3-glucoside	OCH_3	*O*-glucose
kaempferol-3-rutinoside	H	*O*-rutinose
quercetin-3-rutinoside (rutin)	OH	*O*-rutinose
isorhamnetin-3-rutinoside	OCH_3	*O*-rutinose

Table 2. Identification of Flavonol Glycoside Peaks in Mass Spectrum of Almond Seedcoat Extract

flavonol glycoside	exact mass	MALDI-MS mass	obsvd adduct ions
kaempferol	287.26	286.57	$[M + H]^+$
isorhamnetin	317.29	316.69	$[M + H]^+$
kaempferol glucoside	449.42	449.07	$[M + H]^+$
	471.40	471.09	$[M + Na]^+$
isorhamnetin glucoside	479.45	479.17	$[M + H]^+$
	501.43	501.16	$[M + Na]^+$
kaempferol rutinoside	595.58	595.34	$[M + H]^+$
	617.56	617.41	$[M + Na]^+$
isorhamnetin rutinoside	625.61	625.46	$[M + H]^+$
	647.59	647.46	$[M + Na]^+$
	669.58	669.48	$[M + 2Na - H]^+$

it is important to determine the analyte response factors prior to quantification.

The response of rutin was linear with standards of isorhamnetin-3-rutinoside and kaempferol-3-rutinoside ($R^2 = 0.99$ for both), and response factors were determined to be 0.5127 and 0.8481, respectively. In other words, at the same concentration, the response of rutin will be roughly 51% as intense as isorhamnetin-3-rutinoside and 85% as intense as kaempferol-3-rutinoside. The only structural differences among these molecules are in the aglycone (**Table 1**). Because the rutinoside analogues share an identical aglycone with their glucoside analogues, as well as fragment to produce them, these response factors were assumed to be valid for the respective glucosides as well.

Ionization Patterns of Flavonol Glycosides. Quantification using MALDI-TOF MS can be further complicated by multiple ionization. Peak heights of all flavonol glycosides in the sample (including internal standard peaks) were calculated using the software. On the basis of molecular weight, four flavonols were identified in the almond seedcoat extract as isorhamnetin rutinoside, isorhamnetin glucoside, kaempferol rutinoside, and kaempferol glucoside (**Table 2**). Excess sodium was added to the almond extracts in order to suppress the formation of potassium adduct ions. Under these conditions, all three types of almond seedcoat flavonols (isorhamnetin, kaempferol, and quercetin derivatives) exhibited similar ionization patterns. **Figure 1** shows that $[M + H]^+$ adducts were produced for aglycone peaks, $[M + H]^+$ and $[M + Na]^+$ adducts for glucoside peaks, and $[M + H]^+$, $[M + Na]^+$, and $[M + 2Na - H]^+$ adducts for rutinoside peaks, for a total of six peaks per type of

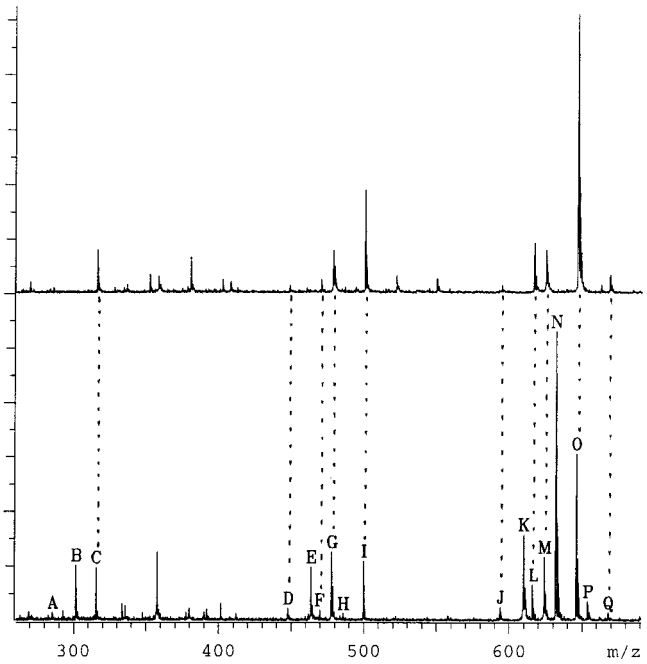


Figure 1. MALDI-TOF MS positive ion spectra of almond seedcoat extract with added sodium. Top, almond seedcoat extract; bottom, almond seedcoat extract with rutin added as internal standard. Spectra are the sum of 120 laser pulses. Internal standard peaks are indicated in bold. (A) $[kaempferol + H]^+$, (**B**) $[quercetin + H]^+$, (C) $[isorhamnetin + H]^+$, (D) $[kaempferol\ glucoside + H]^+$, (**E**) $[quercetin\text{-}3\text{-}glucoside + H]^+$, (F) $[kaempferol\ glucoside + Na]^+$, (G) $[isorhamnetin\ glucoside + H]^+$, (**H**) $[quercetin\text{-}3\text{-}glucoside + Na]^+$, (I) $[isorhamnetin\ glucoside + Na]^+$, (J) $[kaempferol\ rutinoside + H]^+$, (**K**) $[quercetin\text{-}3\text{-}rutinoside + H]^+$, (L) $[kaempferol\ rutinoside + Na]^+$, (M) $[isorhamnetin\ rutinoside + H]^+$, (**N**) $[quercetin\text{-}3\text{-}rutinoside + Na]^+$, (O) $[isorhamnetin\text{-}3\text{-}rutinoside + Na]^+$, (**P**) $[quercetin\text{-}3\text{-}rutinoside + 2Na - H]^+$, and (Q) $[isorhamnetin\ rutinoside + 2Na - H]^+$. Unlabeled peaks are matrix fragments.

flavonol. Kaempferol rutinoside, a minor compound, did not express the double sodium minus proton adduct. It appears that as the carbohydrate component increases, preference for the sodium ion form also increases. The total analyte response was taken to be the total of all associated adduct peaks. Therefore, peak heights of the two glucoside adduct peaks and the three rutinoside adduct peaks were summed to give the total response for the respective compounds.

Fragmentation of Flavonol Glycosides. Although diagnostic for identification purposes, fragmentation can cause considerable difficulty for quantification. As shown in **Figure 2**, in-source fragmentation of rutinoside standards to yield both glucoside and aglycone ions can be responsible for a substantial proportion of the response in MALDI-TOF MS. The characteristic peaks at $[M\text{-}146]^+$, indicated the loss of the labile rhamnose residue, resulting in the glucoside. Subsequent loss of another 162 mass units, equivalent to a glucose residue, resulted in the aglycone peak. Although aglycone peaks were observed in the almond mass spectrum, it is assumed that these arose entirely from fragmentation of the parent rutinoside and glucoside ions, since HPLC data did not indicate aglycones in the sample. However, chromatography data did confirm the presence of glucosides in the sample. Therefore, the observed glucoside peak response in the mass spectra was due to a combination of glucoside present originally in the sample and that resulting from fragmentation of the rutinoside. In contrast, all of the aglycone response was due to fragmentation. As illustrated in **Figure 3**, one can construct a picture of the original concentration of

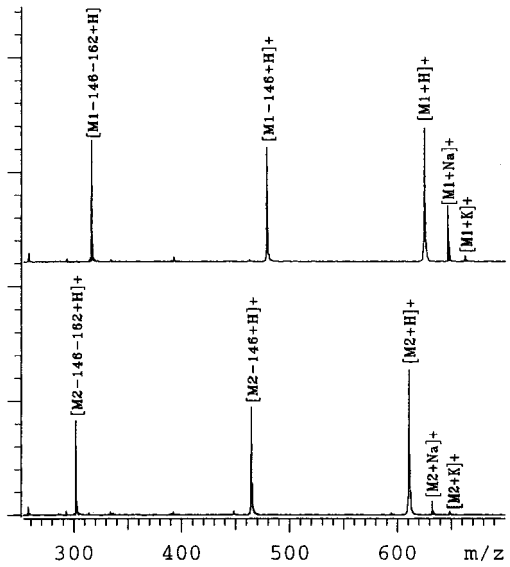


Figure 2. MALDI-TOF MS natural cation spectra of flavonol glycoside standards. Top, M1 = isorhamnetin-3-rutinoside (1.5×10^{-3} M in 70% methanol); bottom, M2 = rutin (1.5×10^{-3} M in 70% methanol).

flavonol glycosides in the almond sample by determining the proportion of fragment ion response attributable to each parent ion.

A comparison of fragmentation ratios (peak height ratio of fragment ions to parent ions) between rutin and isorhamnetin-3-rutinoside showed considerable variation in the absolute value as indicated by large standard deviations (**Table 3**). However, within any single MALDI-TOF MS run, the relative fragmentation ratios of these two flavonol rutinosides were similar. Previous work by Wang and Sporns (*26*) also reported similar fragmentation among all flavonol glycosides using MALDI-TOF MS. For this reason, it was concluded that the fragmentation pattern of the rutin internal standard was predictive of the fragmentation patterns of rutinosides present in the sample. Using the internal standard fragmentation pattern as a model, the proportion of glucoside and aglycone response due to rutinoside fragmentation was calculated for each replicate. The remaining proportions of glucoside and aglycone responses were therefore representative of the glucoside concentration. The proportion of parent glucoside and rutinoside ions that appear in the mass spectrum as fragment ions were accounted for by a fragmentation correction factor.

The analyte concentration (in μg/g, fresh weight basis) was calculated according to eq 1 (where "peak height rutin" is the sum of all proton and sodium adduct peak heights associated with rutin and its fragment ions). See also the Supporting Information.

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

Comparison of MALDI-TOF MS and HPLC Results. HPLC is currently the most common method for analyzing flavonol glycosides, both qualitatively and quantitatively. For this reason, an HPLC protocol was chosen to compare with the MALDI-TOF MS results, to evaluate the effectiveness of this mass spectrometric method, and to validate the assumptions made. Further evidence for the identity of the four compounds of interest in almond seedcoat was provided by peak retention times similar to those of pure standards. **Table 4** shows the results of flavonol glycoside analysis in almond seedcoats using both MALDI-TOF MS and HPLC methods. The HPLC calculations were based on a linear relationship and response factors when compared with rutin of 0.4460, 0.3172, 0.8344, and 0.5491 for isorhamnetin-3-rutinoside, isorhamnetin-3-glucoside, kaempfer-

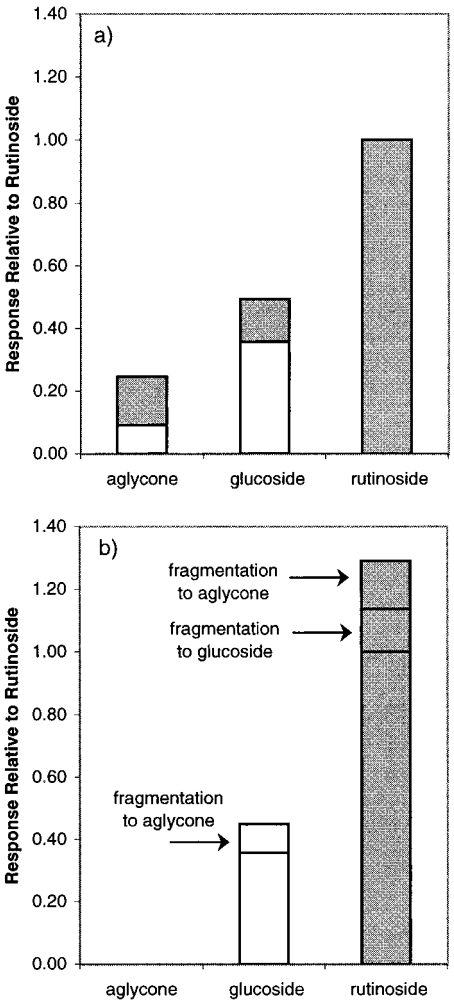


Figure 3. Schematic representation of the method used to correct for fragmentation of the rutinoside and glucoside analytes in MALDI-TOF MS analysis. ■ represents proportion of response from rutinoside, and □ represents proportion of response from glucoside. (a) Relative responses observed in a typical spectrum for aglycone, glucoside, and rutinoside ions. (b) Relative responses of these ions after correcting for fragmentation.

Table 3. Ratios of Fragment Ions to Parent Ions for Mixtures of Rutin and Isorhamnetin-3-rutinoside Standards[a]

	isorhamnetin-3-rutinoside	rutin	isorhamnetin-3-rutinoside/rutin
[M-146][b]	0.52 (0.12)	0.54 (0.12)	0.96 (0.07)
[M-146-162][c]	0.86 (0.36)	0.83 (0.35)	1.04 (0.08)
total (fragment ions[d]/ parent ions[e])	1.40 (0.52)	1.37 (0.51)	1.02 (0.07)

[a] All data are means of 21 replicates, and numbers in parentheses represent the standard deviations. Flavonol glycoside standards were prepared by mixing appropriate amounts (v/v) from 1.00 mg/mL stock solutions in 70% methanol to give the following concentration ratios of rutin to isorhamnetin-3-rutinoside: 0.25, 0.5, 1.0, 1.5, 2.0, 4.0, and 5.0. Three replicate spectra were obtained at each concentration. [b] Proton and sodium adduct ions from loss of rhamnose residue. [c] Proton adduct ions from loss of rutinose residue. [d] Total of proton and sodium adduct ions from loss of either one or both carbohydrate residues. [e] Total of unfragmented proton and sodium adduct molecular ions.

Table 4. Comparison of MALDI-TOF MS and HPLC Determination of Flavonol Glycoside Concentration in Almond Seedcoat (μg/g)[a]

	MALDI-TOF MS[b]	HPLC[c]
isorhamnetin rutinoside	51 (5)	53 (3)
isorhamnetin glucoside	18 (3)	13 (1)
kaempferol rutinoside	18 (3)	22 (1)
kaempferol glucoside	6 (1)	7 (1)
total	93 (12)	95 (6)

[a] Concentration determined on a fresh weight basis. [b] Numbers in parentheses represent standard deviations where $n = 8$. [c] Numbers in parentheses represent standard deviations where $n = 3$.

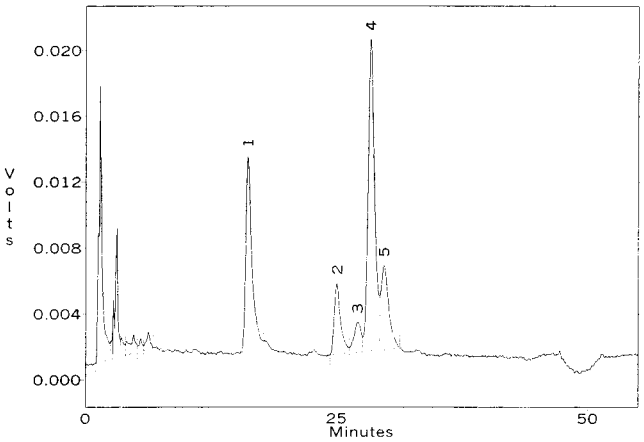


Figure 4. HPLC chromatogram of almond seedcoat extract with added rutin. (1) rutin, (2) kaempferol rutinoside, (3) kaempferol glucoside, (4) isorhamnetin rutinoside, and (5) isorhamnetin glucoside.

ol-3-rutinoside, and kaempferol-3-glucoside, respectively, at 354 nm. The different response factors for compounds with the same aglycone likely arose because of variation in the solvent. Thus, for HPLC quantification purposes, the need for standards is evident, in that response factors will differ slightly for each variation in solvent conditions.

The results for individual flavonol glycosides and the total show good correlation between the two analytical methods. The HPLC method may have slightly overestimated the isorhamnetin rutinoside concentration and slightly underestimated the isorhamnetin glucoside concentration, as these two peaks were not completely resolved (**Figure 4**). No modifiers such as TFA were added, due to the possibility of hydrolyzing the rhamnose residue. Of the two methods, MALDI-TOF MS exhibited a higher variability. However, MALDI-TOF MS is much faster than HPLC, requiring about 20 min to determine peak heights for eight probe positions, whereas one HPLC analysis lasted nearly an hour. Another advantage of MALDI-TOF MS is the ability to identify peaks based on mass, rather than on comparison with retention times of standards, which may be unavailable, as for HPLC.

Carbohydrate moieties can bind to various positions on the parent flavonoid, but the MALDI-TOF MS technique is unable to distinguish among these isoforms. Characteristic fragmentation provides some structural information, but to achieve more detailed information would require further analysis using other techniques such as NMR. However, it is postulated that the carbohydrates are attached at the 3-position, as this form is the most common (*28*), and because chromatography data showed similar retention times between flavonol-3-glycoside standards and the unknown compounds in the sample.

Because there was a high correlation between the results of the mass spectrometric and chromatographic quantitative analyses, the assumptions made for MALDI-TOF MS quantification are valid in this system. Therefore, MALDI-TOF MS is an excellent complement to conventional analytical methods. As such, there is potential for MALDI-TOF MS to be used as a rapid and definitive screening tool for a wide variety of almond samples for the purposes of exploring varietal differences and determining authenticity.

Supporting Information Available: Explanation of calculation for fragmentation correction factors. This material is available free of charge via the Internet at http://pubs.acs.org.

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