LC-Electrospray Ionization and LC-FABMS Study of Flavonoid Glycosides Extracted from Peanut Meal

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ABSTRACT: HPLC, HPLC-electrospray ionization (LC-ESI), and LC-FABMS were used to characterize flavonoid glycosides in the methanol extract from peanut meal. Five isoflavones, daidzin, glycitin, genistin, daidzein, and genistein, were separated by HPLC and characterized by comparison with known standards using ESI-MS. The flavonoid methylquercetin (rhamnetin) was present in the methanol extract from peanuts and identified by ESI-MS. Four other flavonoids, two quercetin diglycosides, one quercetin monoglucoside, and isorhamnetin glucoside, were found to be present in the methanol extract based on their reversed-phase elution pattern, mass ions, and fragment ions using flow-FABMS.

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KEY WORDS: ESI, FAB, flavonoids, HPLC, isoflavones, mass spectrometry.

Flavonoid-type compounds are widespread in nature and are found frequently in fruits, vegetables, grains, and legumes. There are over 4,000 known flavonoids, which include 12 subclasses (1). All plant constituents with a structure based on the aromatic heterocyclic 3-phenylbenzopyrene fall within this classification (2). Isoflavones and flavonoids are isomeric to each other and are generally found in the glycosylated form. Flavonoids are found in nearly every plant, whereas isoflavones are usually treated separately from other subclasses because they are found in significant concentrations chiefly in soybeans and soy-related foods (3,4).

Both isoflavone and flavonoid compounds are antioxidants and are believed to have similar health effects (5). They are hydrogen-donating compounds and act as radical scavengers suppressing superoxide and other free radical reactions. From a health standpoint these compounds have shown activity against allergies, inflammation, cancer cells, carcinogens, arthritis, and hypertension (6–10). Ascorbic acid is regenerated by flavonoids, which in turn regenerates vitamin E (11). Therefore, a daily diet that includes foods and food products containing flavonoids is important to health and nutrition.

HPLC was first used in 1976 for the separation of flavonoid-type compounds (12). Since then, numerous papers have been published on flavonoid separation using different solvent systems, but most separations have been accomplished on C_8 and C_{18} reversed-phase columns. Soybeans (and soy products) have been the most widely studied legume for isoflavone content primarily because of the levels present. Isoflavones and their derivatives were isolated and identified from several soybean products in studying extraction conditions and analysis by HPLC and electrospray ionization (ESI)-MS (13). Other legumes contain isoflavones, but the levels are much lower (14). In peanuts a C-glycoside was found in the leaves of peanuts and tentatively identified as vitexin (15). Earlier research on the extract of Spanish peanuts also suggested the presence of a flavone, dihydroquercetin; and 5,7-dimethoxy isoflavone has been isolated and identified as a component of peanut cotyledons (16,17). In a preliminary classification scheme using UV spectrometry and varieties of white testa peanuts, the aglycones of quercetin, rhamnetin, and isorhamnetin and two other aglycones were tentatively identified as isoflavones (2). In a study on the biotic and abiotic elicitation of phytoalexins in peanut leaves, daidzein, formononetin, medicarpin, and other flavonoid-type compounds were identified by their absorption at 280 nm (18). In this paper we describe methodology for the extraction, separation, and identification of isoflavones and flavonoid compounds using hyphenated techniques of HPLC, ESI, and FABMS.

MATERIALS AND METHODS

Materials. Solvents used for the extractions of flavonoid compounds and separations on HPLC columns were HPLC grade and purchased from Fisher Scientific Co. (Fairlawn, NJ). Isoflavone standards used for comparisons of retention times on reversed-phase columns and ESI standard spectra were daidzin, glycitin, genistin, daidzein, and genistein obtained from Indofine Chemical Co., Inc. (Somerville, NJ). Samples were either peanut hearts or whole peanut cotyledons.

Sample preparation and extractions. Peanuts were ground in a coffee grinder as finely as possible without producing a paste. The neutral lipid and polar fractions from peanuts were extracted using temperature and pressure (19). Approximately 10 g of peanuts was ground and placed in a high-pressure vessel. The acylglycerol fraction (~98% of the lipid fraction) was removed by three static extractions using hexane as the solvent. After removal of the neutral lipid fraction, the sample was re-extracted with methanol using three static extractions at 120°C and at 3000 psi. The methanol extract was stored in an Erlenmeyer flask and placed in a refrigerator

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overnight or until the sample could be further processed. This polar extract was filtered through filter paper and the methanol removed by flash evaporation. The residue was redissolved in a small amount of water. This aqueous solution was placed on a C_{18} solid-phase extraction (SPE) cartridge (10 g, 60 cc Varian Mega Bond Elut C_{18}) that had previously been conditioned with water. The SPE column was then washed with water and eluted with 70% aqueous methanol collected in an Erlenmeyer flask. A final wash of the SPE column was done with 100% methanol. Each fraction was collected separately and stored in a refrigerator for further analysis. The aqueous methanol fraction was reduced in volume, centrifuged, and filtered through a 0.45 µm syringe filter.

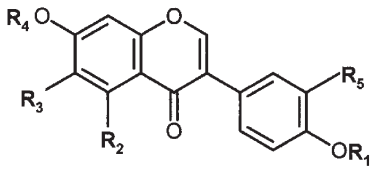
HPLC separation. The filtered 70% aqueous methanol extract from the SPE purification step was analyzed on a C_{18} reversed-phase column (250 × 4.6 mm; Supelco, Bellefonte, PA) using a Varian 5000 HPLC unit equipped with a UV-vis detector at 262 nm. The components in the extract were separated using a linear gradient consisting of 1 to 50% acetonitrile (solvent A) and water (solvent B) in a background of 1 mM ammonium acetate using a flow rate of 1 mL/min. Components in the mixture were detected at 262 nm. A heart-cut was made from this column as determined by previously running a standard isoflavone mixture. The collected fraction was reduced in volume, and the isoflavones were separated on a C_8 reversed-phase column (250 × 4.6 mm; Zorbax SB, Fisher Scientific Co.) using a BioRad model 52001T HPLC unit. Eluting components were monitored using a BioRad model 1706 UV-vis detector at 262 nm. A linear gradient of 1 to 50% acetonitrile (solvent A) and water (solvent B) in a background of 1 mM ammonium acetate at a flow rate of 1 mL/min was used to separate the sample, and the components were detected at 262 nm. The linear gradient covered a period of 50 min.

HPLC/ESI-MS. Isoflavones in the mixture were separated on a C_8 reversed-phase column (150 × 2.1 mm; Zorbax, Fisher Scientific Co.) with a linear gradient from 5 to 50% acetonitrile and water in a background of 1 mM ammonium acetate at a flow rate of 150 µL/min using an Applied Biosystems 140 dual syringe pump attached to the inlet of a Hewlett-Packard Micromass Quattro II Electrospray Triple Quadropole mass spectrometer equipped with a data collection system. The eluted isoflavones were detected at 262 nm prior to the effluent being split and a portion of the stream directed into the ESI interface. Mass accuracies of greater than 0.01% are obtainable on molecules up to 150 daltons. Both positive and negative ions can be determined.

HPLC/FABMS. A portion of the crude methanol extract was analyzed by flow-FABMS using an Applied Biosystems 140 dual syringe pump attached to the inlet of a Jeol JMS-HX double-focusing FAB mass spectrometer with a pneumatic splitter. Resolution was 1000, scan mass range 0–1300, scan speed 5 s, cycle time 3 s, ion source temperature 33–37°C, and the mode was either negative or positive depending on the information to be obtained. The components in the crude methanol fraction were separated on a C_8 reversed-phase column (150 × 2.1 mm; MacMod Analytical, Inc., Chadds Ford, PA). A solvent system consisting of MeOH/hexane/1 mM ammonium acetate/glycerol (75:2:21:2) was used to separate the component mixture. An isocratic separation was used and the run was terminated at 25 min.

RESULTS AND DISCUSSION

HPLC of isoflavones. Flavonoid-type compounds are widespread throughout the plant kingdom and occur chiefly as their glycoside conjugates (20,21). The general structure of the isoflavone molecule is shown in Figure 1 along with the position of the various functional groups for each isoflavone and the M.W. Each of the isoflavones is numbered in this figure, and these numbers are consistent for the isoflavones throughout the manuscript. Figure 2 shows the separation of



Name of isoflavone	R_1	R_2	R_3	R_4	R_5	MW
1. DAIDZIN	H	H	H	Glc	H	416
2. GLYCITIN	H	H	OMe	Glc	H	446
3. GENISTIN	H	OH	H	Glc	H	432
4. DAIDZEIN	H	H	H	H	H	254
5. GENISTEIN	H	OH	H	H	H	270

FIG. 1. Isoflavone skeleton, positional specificity, and M.W.

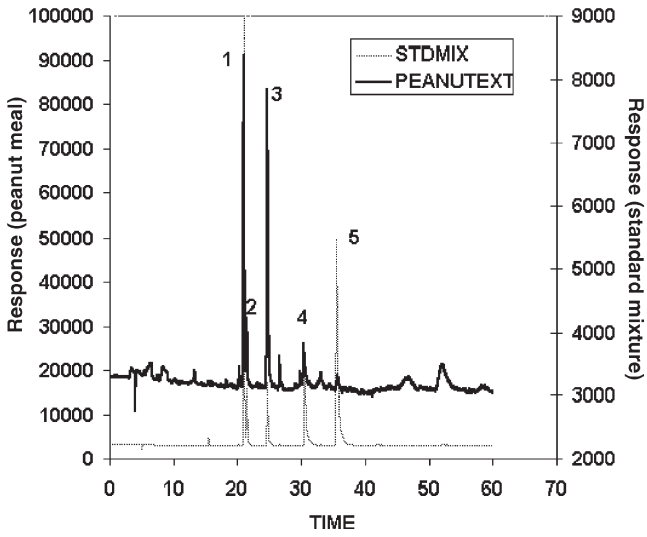


FIG. 2. HPLC chromatogram overlay of the MeOH extract from peanut meal and a standard mixture of the isoflavones daidzin (1), glycitin (2), genistin (3), daidzein (4), and genistein (5).

isoflavones extracted from peanut meal on a C_8 reversed-phase column using acetonitrile and water as the eluting solvents in a background of 1 mM ammonium acetate and detection at 262 nm. The upper chromatographic trace represents the isoflavones in the polar fraction from peanuts, and the lower chromatographic trace represents a standard mixture of isoflavones consisting of (1) daidzin, (2) glycitin, (3) genistin, (4) daidzein, and (5) genistein. These compounds are found in relatively high concentrations in soybeans but in much lower concentrations in other legumes (14). The upper chromatogram represents a heart-cut from a separation of the MeOH extract on a C_{18} reversed-phase column. This was necessary owing to the numerous compounds in the extract and resulted in a more suitable and less complex sample for further analysis using ESI-MS. In peanuts, the isoflavones occur primarily as glycoside conjugates as found in soybeans and other plant material. The aglycons daidzein and genistein are present but in much lower levels. The aglycon of glycitin was not found in this study.

LC-ESI-MS analysis of flavonoid compounds. A hyphenated technique of LC-ESI-MS has been used to characterize five isoflavones and one flavonoid compound extracted from peanut meal. Figure 3C shows the separation of the methanol extract on a C_8 microbore column and detection at 262 nm prior to splitting the effluent into the ESI mass spectrometer. When using HPLC with MS it is necessary to use very low flow rates; therefore this requires microbore columns. This method was optimized on an HPLC unit prior to being connected to the mass spectrometer using a linear gradient of 5–50% acetonitrile with water in a background of 1 mM ammonium acetate at a flow rate of 150 µL/min on a 150 × 2.1 mm C_8 reversed-phase column. The order of elution of the isoflavones shown in a UV analog chromatogram (Fig. 3C) is the same as shown in Figure 2, namely, (1) daidzin, (2) glycitin, (3) genistin, (4) daidzein, and (5) genistein. Retention times (t_R) are printed on the UV analog chromatogram. Selected ion traces are shown in trace A representing the $[M + H]^+$ ion of glycitin (t_R 24.57, *m/z* 447) and trace B representing the $[M + H]^+$ ion of daidzin (t_R 24.05, *m/z* 417) (Fig. 3). The ESI-MS was run in the positive mode. There is an offset of 0.35-min t_R between the UV trace and the ESI-MS analog trace.

The mass spectrum of daidzin is shown in Figure 4. The ion at *m/z* 417 represents the $[M + H]^+$ ion and the ion at *m/z* 255 represents the $[M - Glu]^+$, which is the aglycon of daidzin. Verification of spectra was made by chromatography and MS of a standard mixture of isoflavones. The potassium adduct ion was not present in the spectra. The ESI spectrum shown in Figure 5 is a mixed spectrum of daidzin and glycitin. The ion at *m/z* 447 represents the $[M + H]^+$ of glycitin, and the ion at *m/z* 285 represents the $[M - Glu]^+$ ion, which is the aglycon of glycitin. Ions at *m/z* 417 and 255 are the ions related to daidzin. The $[M + H]^+$ ions of both of these isoflavones are labeled with their component names on the spectra (Fig. 5). These two isoflavones elute very close to each other, and therefore a mixed spectrum is to be expected. Loss of the sugar moiety is important in determining the aglycon fragment. Ions below *m/z* 255 are apparently low M.W. phenolic compounds in the extract. Similar ions were also produced during the ESI-MS of standard isoflavones. The ESI spectrum shown in Figure 6 is a mixed spectrum showing the presence of the isoflavone genistin and of a flavonoid compound. The mass ion at *m/z* 433 represents the $[M + H]^+$ ion of genistin, and the ion at *m/z* at 271 $[M - Glu]^+$ represents the aglycon of genistin. The ion at *m/z* 317 is the $[M + H]^+$ ion of the flavonoid methylquercetin (rhamnetin). The $[M + H]^+$ ions of these compounds are labeled on the spectra. This

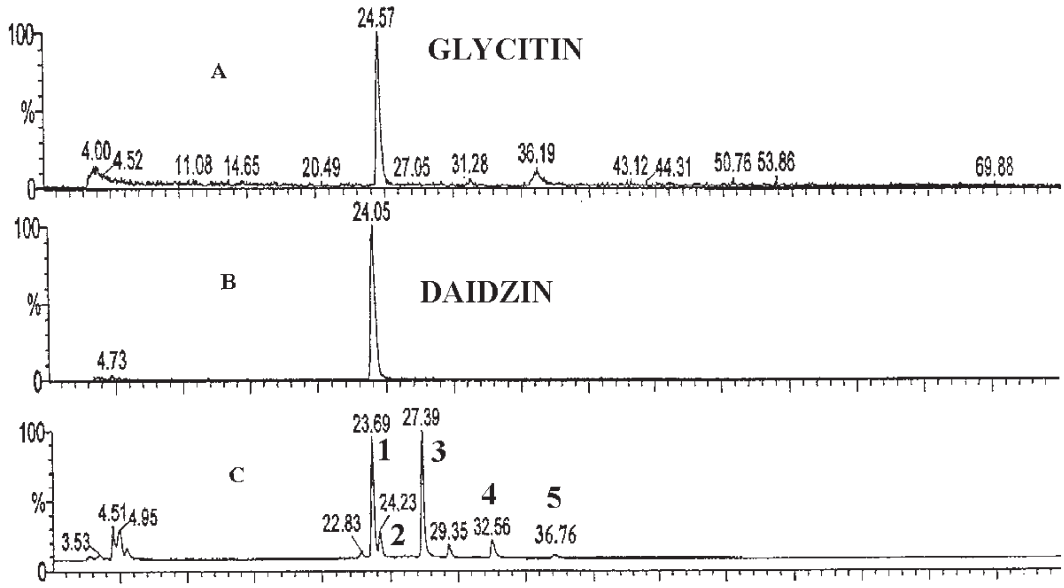


FIG. 3. Electrospray ionization UV analog and selected ion trace chromatograms: (A) glycitin ion trace, (B) daidzin ion trace, (C) UV analog chromatogram for the peanut extract (1, t_R 23.69–daidzin; 2, t_R 24.23–glycitin; 3, t_R 27.39–genistin; 4, t_R 32.56–daidzein; 5, t_R 36.76–genistein).

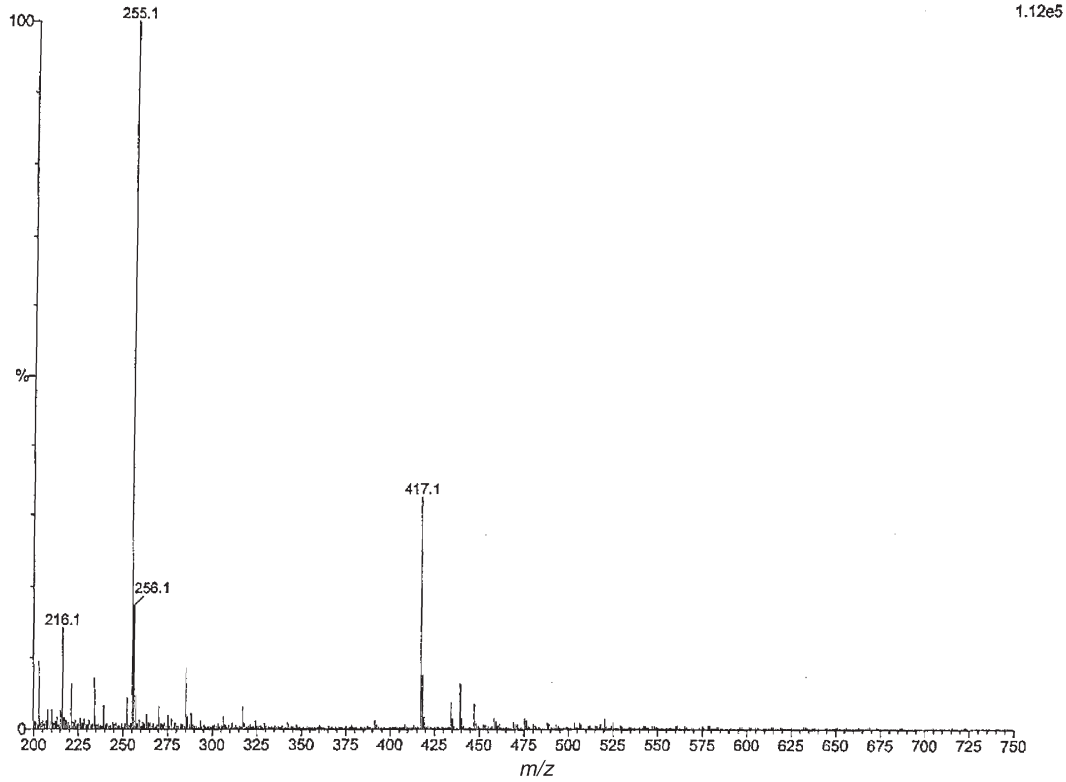


FIG. 4. Electrospray ionization mass spectrum of daidzin.

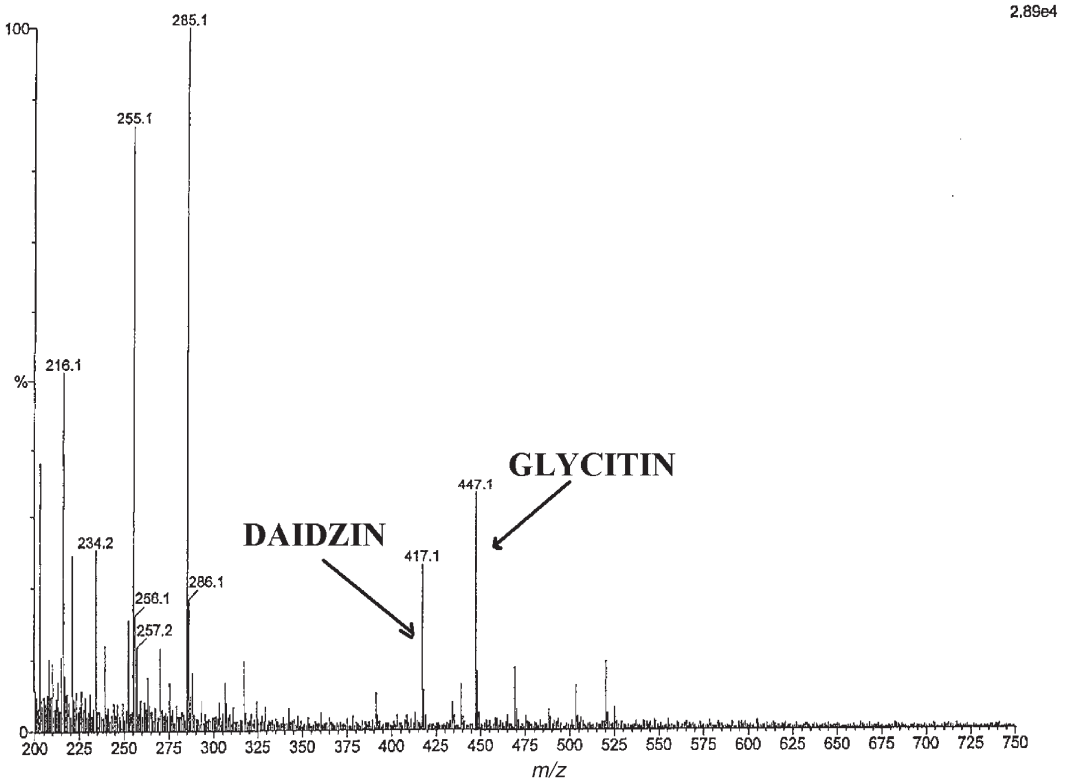


FIG. 5. Electrospray ionization mixed mass spectra of daidzin and glycitin.

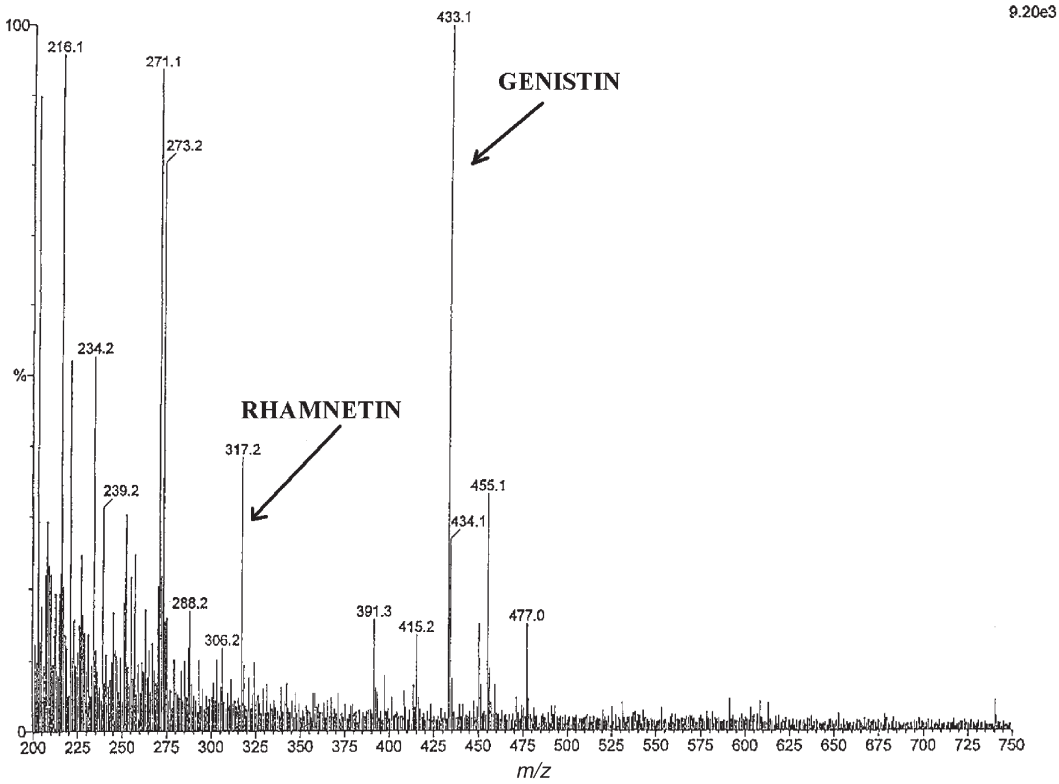


FIG. 6. Electrospray ionization mixed mass spectra of rhamnetin and genistin.

flavonoid compound also was found in the ESI spectra of the standard genistin. HPLC-ESI analysis of the methanolic extract of peanuts confirms the presence of methylquercetin as proposed previously by researchers using HPLC and UV spectrometry in classifying white testa peanuts (2). Mass ions for daidzein (*m/z* 255 $[M + H]^+$) and genistein (*m/z* 271 $[M + H]^+$) were also in their respective spectra (data not shown).

LC-FABMS of methanolic extract from peanut meal. A similar column was used in this analysis (150 × 2.1 mm C_8 reversed-phase column); however, a more polar solvent, MeOH/hexane/1 mM ammonium acetate/glycerol (75:2:21:2), was used in place of the acetonitrile and water in the analysis of isoflavones. The system was operated isocratically at a faster flow rate (300 µL/min) without UV detection. With this elution profile, saponins (previously characterized in peanuts) eluted first, and flavonoid diglycosides, flavonoid monoglycosides, and isoflavones were also found (22). Flavonoid compounds are isomeric to the isoflavones. The mass chromatogram ion traces for ions at *m/z* 595 and 478 are shown in Figure 7. A general flavonoid skeleton structure is shown in Figure 8 along with peak number, peak assignment, t_R, and M.W. The upper trace of Figure 7 (mass chromatogram *m/z* 595) indicates the presence of two isomers with identical M.W. The FAB mass spectra of the isomer at t_R 5.80 (peak no. 2) is shown in Figure 9. The mass ion at *m/z* 595 is $[M - H]^-$ because the FAB instrument was operated in the negative mode. A loss of 162 amu (*m/z* 433) indicates a hexose sugar moiety [M – H – hexose] has been cleaved, and the ion at *m/z* 301 [M – H – hexose – pentose] indicates it is a flavonoid diglycoside. The loss of two sugar moieties would indicate the aglycon to be quercetin (M.W. = 302). MS cannot differentiate between isomers nor can this technique determine the point of attachment of the sugar moiety on the molecule. A standard was not available at the time of analysis. The mass ion, fragmentation pattern, and point of elution on the C_8 column under the described conditions indicate that this compound is a quercetin diglycoside (Fig. 8). The isomer at t_R 5.25 (Fig. 8, peak no. 1) probably is another quercetin diglycoside with different glycosides attached. A fragment at *m/z* 315 in the spectra (not shown) indicates that the aglycon is methylquercetin. Therefore, the ion at *m/z* 315 [M – deoxy hexose – pentose] indicates the compound to be a quercetin diglycoside. These data are consistent with published data where researchers found quercetin aglycons in peanuts (2).

The ion trace at *m/z* 478 (Fig. 7) shows two peaks with the same mass. One has a t_R of 7.50 min (peak no. 3) and the other one of 16.00 min (Fig. 7, peak no. 4). These two isomers are widely separated, and this alone indicates a quercetin monoglycoside and an isorhamnetin monoglycoside. The addition of a methoxy functional group on ring B of the flavonoid structure increases the t_R of this compound vs. the methoxy group on ring A when using reversed-phase columns. The spectra for the *m/z* ion 478 at t_R 16.0 show a large mass peak at *m/z* 478 (Fig. 10). This is the mass ion for isorhamnetin glucoside.

In this study we have developed methodology for the separation, detection, and characterization of isoflavones and some flavonoid compounds in peanuts. Both of these subclasses

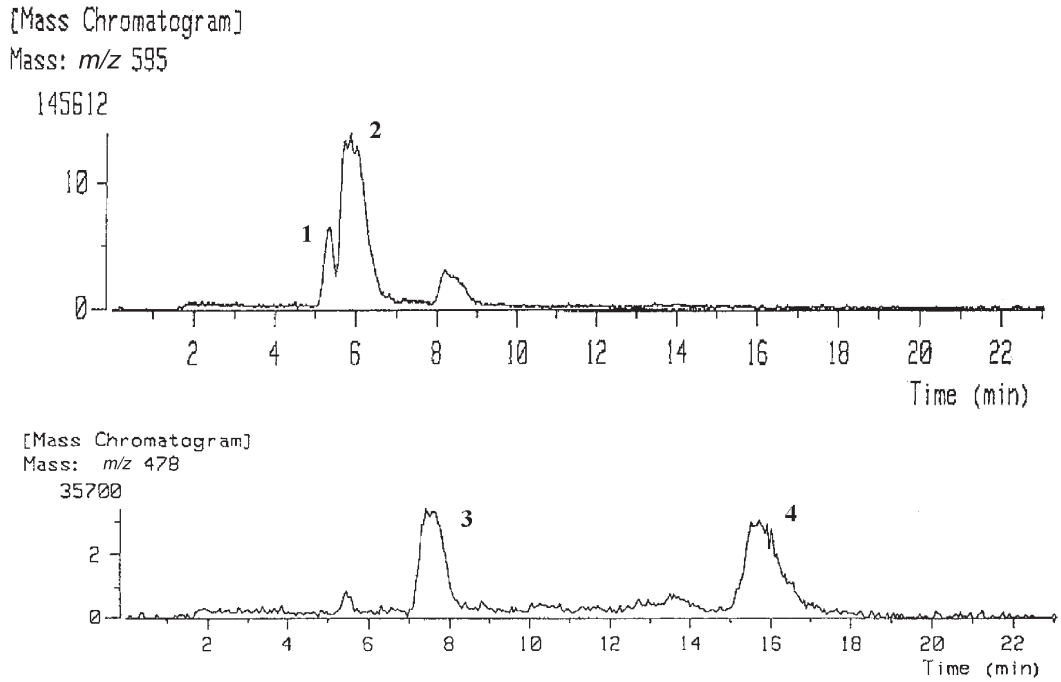
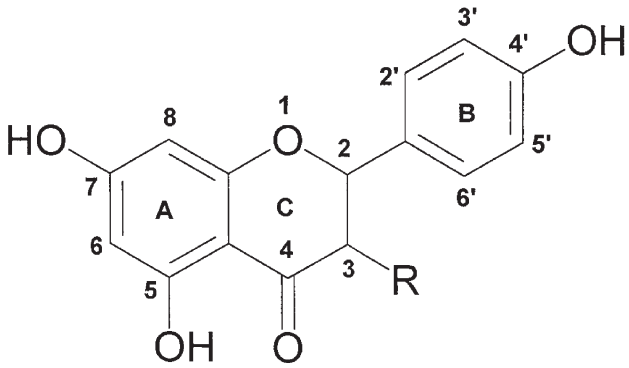


FIG. 7. Flow FAB mass chromatograms of flavonoid di- and monoglycosides in the MeOH extract from peanut meal: upper trace, diglycosides; lower trace, monoglycosides.

have potential health benefits. Quercetin and the conjugated glycosides are among the most biologically active and common flavonoids found in foods (23). These flavonoids have recently been shown to reduce carcinogenic activity in cooked-food mutagens, reduce enzyme activity associated with tumor cells, and enhance the activity of anticancer agents (24). Therefore, diets that include peanuts and other foods containing these compounds may have health benefits.



Peak Assigment for the Methanol Extract of Peanut Meal

	Compound	t_R	[M−H] (m/z)	Fragment ion (m/z)	MW
1.	Quercetin diglycoside	5.25	595	316	596
2.	Quercetin diglycoside	5.80	595	433, 301	596
3.	Quercetin glucoside	7.50	478[a]	316	478
4.	Isorhamnetin glucoside	16.00	478	316	478

[a] Mass ion

FIG. 8. Flavonol skeleton and peak assignment for di- and monoglycosides.

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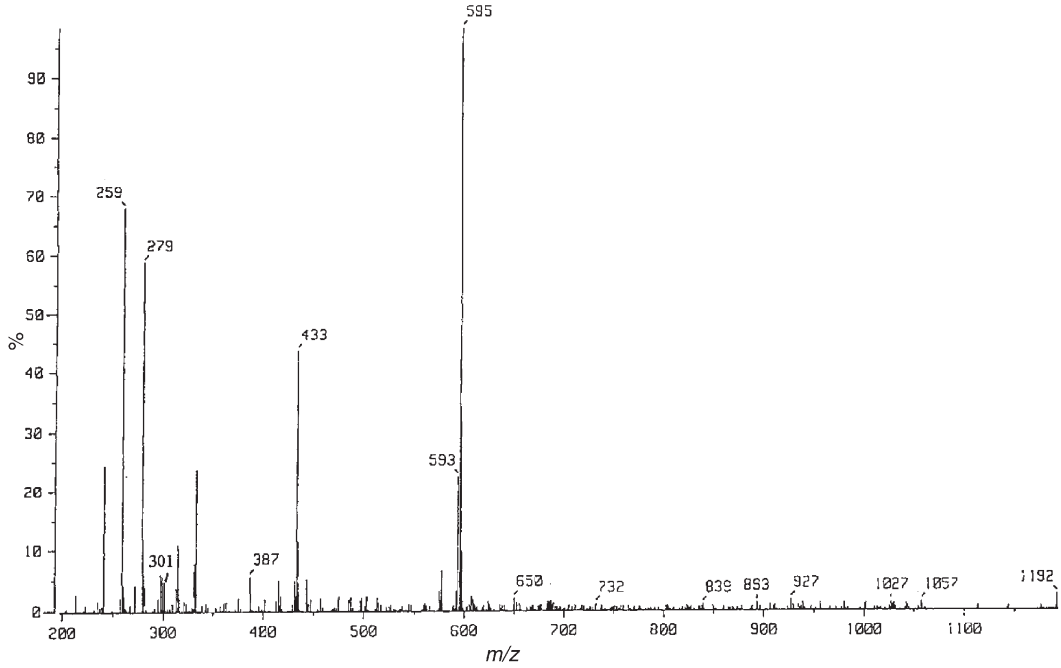

FIG. 9. FAB mass spectrum of a flavonoid diglycoside at t_R 5.8 (peak no. 2).

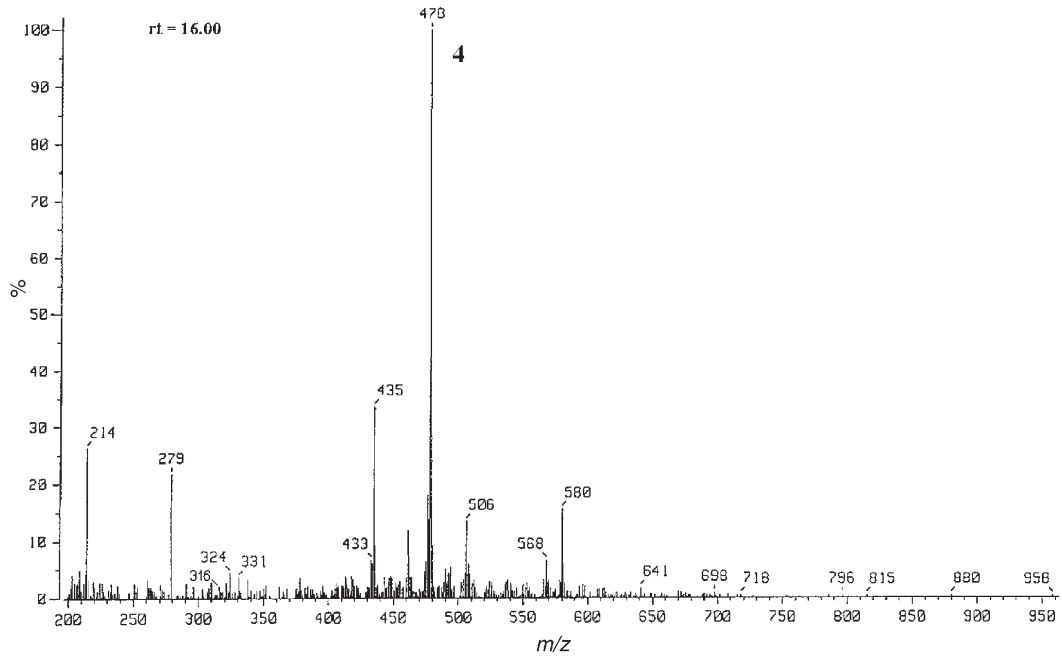

FIG. 10. FAB mass spectrum of flavonoid monoglycoside at t_R 16.00 (peak no. 4).

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