HPLC-Mass Spectrometry Analysis of Isoflavones (44230)

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Abstract. **The current interest in the role of dietary isoflavonoids, particularly the soy isoflavone genistein, in lowering the risk of several chronic diseases, has led to the need for rapid, sensitive and precise assays for isoflavones and their metabolites in food matrices and in various physiological fluids and tissues. HPLC has the advantage over GC-based methods in that all the conjugated and unconjugated isoflavonoids and their metabolites can be separated and analyzed without the need for derivatization. An important advance in mass spectrometry has been the introduction of effective interfaces between the HPLC and the mass spectrometer, namely the electrospray ionization (ESI) and the heated nebulizer–atmospheric pressure chemical ionization (HN-APCI) interfaces. Because of the isoflavonoid concentrations in fluids such as bile or urine, preliminary extraction, so essential for GC-MS and many other analytical methods, is not necessary. This immediately overcomes the thorny issue of finding an effective solid-phase extraction procedure. Using reversed-phase HPLC-ESI-MS, it is possible to obtain a mass/intensity map of all isoflavonoid metabolites in a single 20 min analysis. Analysis of isoflavonoid conjugates in serum/plasma samples requires initial extraction, but the conjugates can be measured intact either by capillary reversed-phase HPLC-ESI-MS or on regular reversed-phase columns by HPLC-HN-APCI-MS. In both cases, specificity is obtained by causing the parent isoflavonoid molecular ions to undergo collision-induced dissociation to form specific daughter ions in a triple quadrupole MS instrument. When it is only necessary to measure the total isoflavonoids and their metabolites in blood, hydrolysis can be performed directly in serum/plasma samples and isoflavonoids recovered by ether or ethyl acetate solvent extraction. The isoflavone aglucones can be analyzed by HPLC-MS under isocratic solvent conditions, thereby drastically shortening analysis time and opening up prospects for automation. Therefore, HPLC-MS is a technique that is broadly applicable to the major issues in phytoestrogen research.**

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Due to the potential application in the prevention and treatment of several major chronic diseases (1), there is a very strong interest in the accurate and specific measurement of isoflavones in body fluids and tissues derived from animal experiments and clinical trials in humans, and in food samples (2). The range of concentrations in these various sample types varies from mg/g to submicrogram/g.

Traditionally, reversed-phase HPLC analysis combined with UV detection has been used to measure the isoflavones in soy products because of the high concentrations of isoflavones from these sources (3–7). However, foods with much lower amounts of isoflavones, or those with other phytoestrogens such as coumestrol or the lignans, present a greater technical challenge.

In most cases, measurement of isoflavonoids and their metabolites in blood or urine has been carried out by gas chromatography–mass spectrometry (GC-MS) (8–20). However, this technique, although sensitive and accurate, is quite laborious and expensive. It requires several solid-phase extraction and chromatography steps prior to deriva-

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tization of the compounds to be measured. Many steps are needed to separate the different conjugate forms of the isoflavonoids in the specimen since they all have to be hydrolyzed to the aglucones for final analysis. The multistep procedure leads to substantial losses during workup, which has necessitated the use of added, deuterated internal standards for each compound being examined (11, 12, 14). However, exchange reactions of some of the deuterated sites during the workup procedures occur leading to uncorrected errors in this procedure.

HLPC has the advantage over GC-MS in that it is not necessary to prepare volatile derivatives and that conjugated forms can be analyzed as easily as the aglucones. However, until recently, practically useful interfaces between HPLC instruments and the mass spectrometer did not exist. First, it was difficult to overcome the problem of delivering the mobile liquid phase into the mass spectrometer at flow rates up to 1 ml/min and yet maintain the high vacuum conditions required for analysis of the solute ions. The second problem was how to generate gas phase ions from solutes in the mobile liquid phase and then how to transfer them (but not the solvent molecules) into the mass spectrometer.

A thermospray ionization interface offered the first glimmer of hope that isoflavones could be analyzed by HPLC-MS (21, 22). Ions were formed by spraying the column eluate at high enough heat to evaporate the solvent. An electrolyte had to be present to obtain the ions. However, the sensitivity offered by this technique was no better than HPLC methods based on UV detection. Nonetheless, it offered a much greater specificity than HPLC-UV, particularly when tandem MS experiments were performed (22). This specificity is much needed when analyzing biological matrices.

The breakthrough in the application of HPLC-MS has come from the development of two interfaces, the electrospray ionization (ESI) interface and the heated nebulizer–atmospheric pressure chemical ionization (HN-APCI) interface (Fig. 1). In both cases, ionization occurs at atmospheric pressure.

In the case of ESI, the liquid phase from the HPLC column (at flow rates up to 100 μl/min) is forced out of a narrow capillary aimed close to but not at the orifice of the mass spectrometer. Under the influence of a strong electric field created by a 5-kV potential difference between the tip of the capillary and orifice of the mass spectrometer, the resulting spray of charged droplets disperses into a Taylor cone and begins to evaporate (Fig. 1A). The latter process can be accelerated by using a warm, dry nitrogen curtain gas travelling in the reverse direction to the droplets. As the droplets decrease in size, the electrostatic forces created by the charged solutes in the droplets cause either the formation of smaller and smaller droplets leading to solute transfer into the gas phase or direct ejection of the solute ions from the droplet surface. The ions are then attracted by the electrostatic gradient into the mass spectrometer. The solvent ions are not charged and, therefore, do not enter the

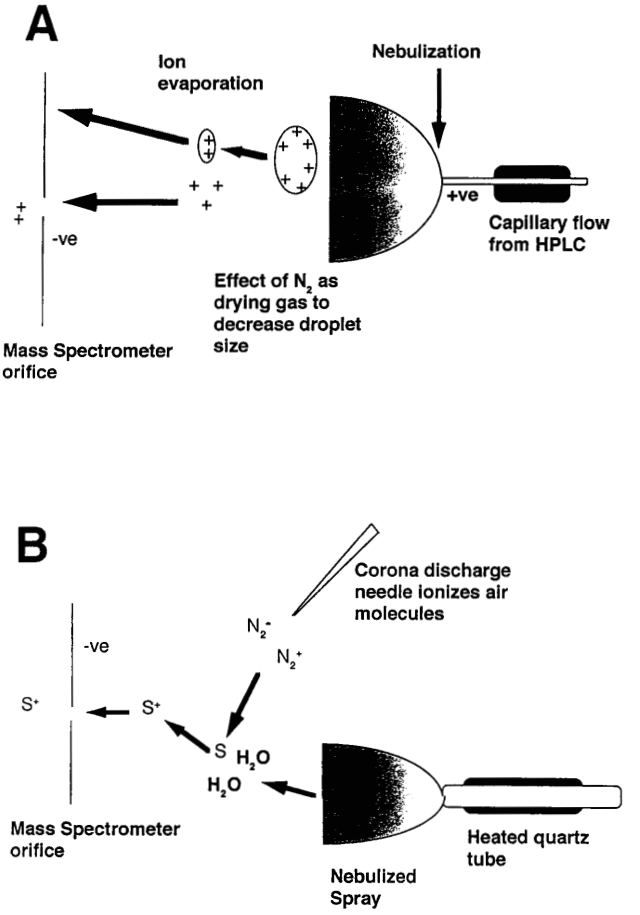

Figure 1. Interfaces used to combine HPLC with mass spectrometry. (A) electrospray ionization interface. (B) Heated nebulizer-atmospheric pressure chemical ionization mass spectrometry interface.

mass spectrometer. This method of ionization is sufficiently "soft" that even intact proteins can be transferred successfully as multiple-charged ions into the gas phase. The evaporation of the solvent causes a marked cooling effect on the droplets. This also permits thermally labile compounds to be ionized without decomposition.

In the HN-APCI interface, the mobile phase is passed down a quartz tube heated to 400°–500°C. The thermal capacity of the quartz tube is such that all the solvent has been evaporated once the sample (as a dispersed solute/solvent vapor mixture) leaves the tube. Ionization is accomplished by first ionizing the air within the interface by a corona discharge needle. The N_2^+ and N_2^- ions first ionize the solvent vapor molecules, which in turn ionize the solute molecules (hence the use of the term chemical ionization) (Fig. 1B). In this type of interface, sensitivity is determined by the total mass of solutes present, rather than their concentration, as in the case of ESI-MS. Since the solutes are heated to approximately 100°C during the evaporative nebulization, this interface is unsuitable for thermolabile compounds. However, as will be seen later, the partial decomposition of isoflavonoid glycosides during HN-APCI gives useful structural information.

In this manuscript, we describe the application of HPLC-MS to various types of isoflavonoids present in foods and physiological fluids.

Materials and Methods

Materials. Genistein and genistin were isolated from a concentrate prepared from soy molasses by Protein Technologies International (St. Louis, MO) as described previously (22). Daidzein was purchased from LC Labs (Woburn, MA) and biochanin A from Sigma Chemical Co. (St. Louis, MO). Equol, dihydrodaidzein, *O*-desmethylangolensin (ODMA), enterolactone, and enterodiol were each generous gifts from Dr. Kristina Wähälä, Department of Chemistry, University of Helsinki, Finland. The 4-sulfate and 7-sulfate esters of genistein and daidzein were prepared chemically (24). The 7-*O*-β-glucuronide of genistein was isolated from bile of a rat in which genistein was infused duodenally (24). 4-^{14}C-labeled genistein (14 mCi/mmol) was custom synthesized by Moravek Biochemicals (Brea, CA).

Methods. The isoflavones in soy foods were extracted with 80% aqueous methanol (10 volumes/g) as described (6).

Female Sprague-Dawley (Harland Sprague-Dawley, Indianapolis, IN) rats were placed on isoflavone-free AIN-76A diets at weaning. At 25 days of age, genistein was included in their diet (1 g/kg diet). They were fed the diet for a further 6 months and then placed in a metabolic cage where urine was collected on a daily basis. Samples were collected in beakers set in crushed dry ice. They were stored frozen at −80°C until analyzed.

Blood samples were collected from an antecubital vein of healthy volunteers who had consumed 20 g of an isolated soy protein beverage (Protein Technologies International, St. Louis, MO) twice a day for 2 weeks (24). Plasma samples were obtained by centrifuging the blood samples at 2500 × g for 10 min at room temperature. The glucuronides and sulfate esters of the isoflavonoids and their metabolites in the plasma were hydrolyzed to their aglucones by adding a mixed β-glucuronidase/sulfatase to the plasma. After incubation overnight at 37°C, the aglucones were recovered by extraction with diethyl ether (24).

HPLC-MS Procedures. All analyses were performed using a Hewlett Packard (Wilmington, DE) model 1050 HPLC and PE-Sciex (Concorde, Ontario, Canada) API III triple quadrupole mass spectrometer.

Analyses of isoflavonoids and other phytoestrogens in foods were carried out on a 10 cm × 4.6 mm i.d. 300 Å pore size, Aquapore C_8 reversed-phase HPLC column. The mobile phase was a linear gradient of 0%–50% acetonitrile in 10 m*M* ammonium acetate, pH 6.5, over 10 min at a flow rate of 1 ml/min. Solutes were introduced into the mass spectrometer *via* the HN-APCI interface operating in both the positive and negative modes.

Extracted samples of bile or urine were separated by reversed-phase HPLC on a 15 cm × 0.21 cm i.d. Brownlee Aquapore C_8 column (Varian, Walnut Creek, CA) using a linear 0%–50% gradient (5%/min) of acetonitrile in 10 m*M* ammonium acetate, pH 6.5 at a flow rate of 0.2 ml/min. The column eluate was split 1:1, and one stream passed into the IonSpray interface of the mass spectrometer operating in the negative ion mode, with an orifice potential of −60 V.

Plasma extracts were analyzed under isocratic conditions (30% acetonitrile in 10 m*M* ammonium acetate) at a flow rate of 1 ml/min. After chromatographic separation, the eluate stream was diluted with 13 μl/min of ammonium hydroxide provided by a Harvard infusion pump (South Natick, MA) and passed into the HN-APCI interface. The orifice potential of the mass spectrometer was set at −60 V.

In the full scan mode, ions entering the mass spectrometer were analyzed over a m/z range from 50–800. MS-MS daughter ion spectra were obtained by passing molecular ions selected by the first quadrupole into an argon gas collision cell and analyzing the fragment ions in a third quadrupole. Multiple reaction ion monitoring (MRM) was carried out in a similar manner to MS-MS by selection of specific ions not only in the first quadrupole, but also in the third quadrupole. Integration of peak areas was carried out using the program MacQuan, provided by the mass spec-

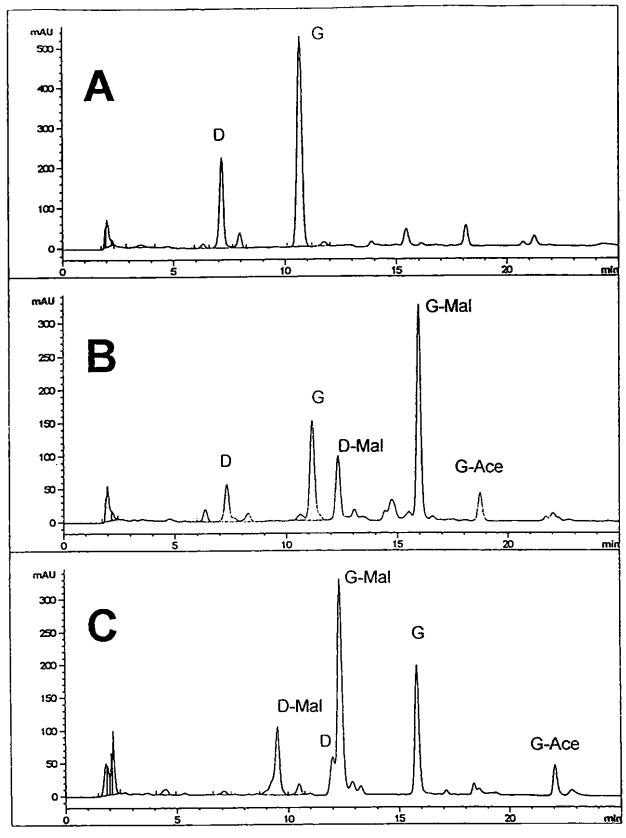

Figure 2. Reversed-phase HPLC analysis of isoflavone conjugates in isolated soy protein. (A) Extracted at 80°C for 4 hr. (B and C) Extracted at room temperature for 2 hr. The conjugates were separated using a 0%–50% linear gradient of acetonitrile in 0.1% TFA (A and B) or in 10 m*M* ammonium acetate, pH 6.5 (C). Peak identification: D = daidzin; G = genistin; D-Mal = 6′-O-malonyldaidzin; G-Mal = 6′-*O*-malonylgenistin; D-Ace = 6′-*O*-acetyldaidzin; G-Ace = 6′-*O*-acetylgenistin.

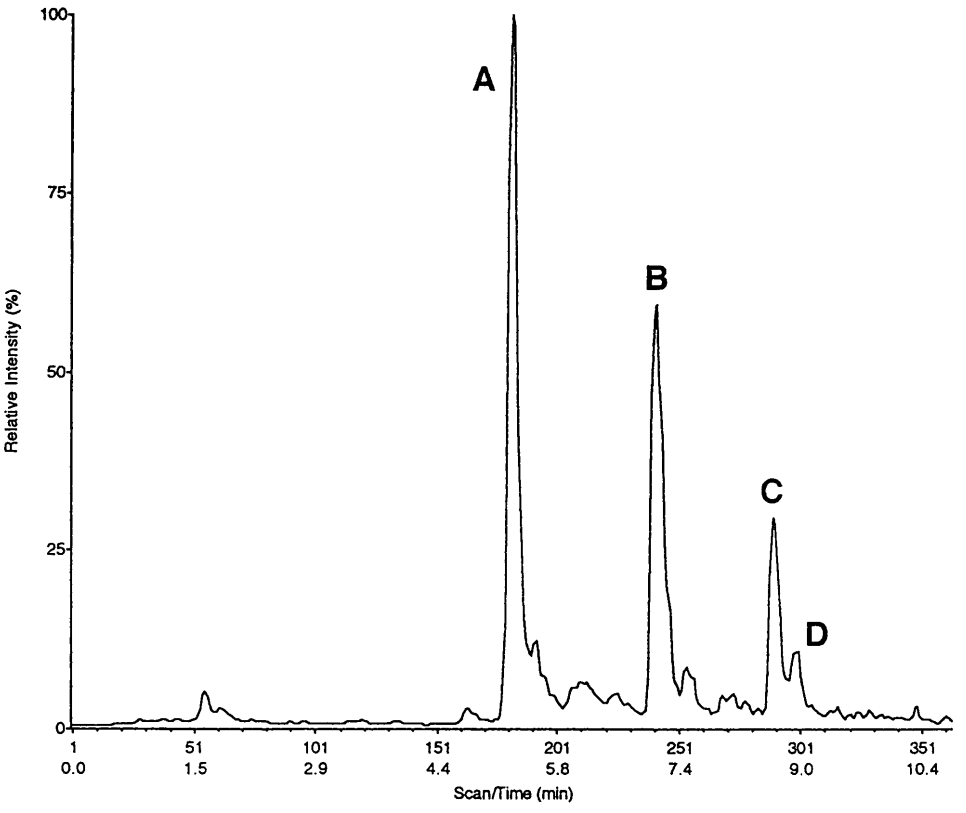

Figure 3. Selected ion chromatogram (m/z 271) for genistein conjugates in isolated soy protein by HPLC-HN-APCI-MS using a 0%–50% linear gradient of acetonitrile in 10 m*M* ammonium acetate, pH 6.5.

trometer manufacturer (Hewlett Packard). Areas were corrected by the peak area of the added internal standard biochanin A and compared to the areas of a set of known isoflavonoid standards in order to estimate the plasma isoflavonoid concentrations.

Results

Isoflavones in Soy Foods. Prior to 1994 investigators analyzed isoflavones in foods by first extracting the food with hot aqueous methanol or ethanol. Reversed-phase HPLC analysis suggested that in most foods, the β-glucosides predominated (Fig. 2A). However, when room temperature extraction conditions were used, a quite different chromatogram was obtained (Fig. 2B). Furthermore, when the pH of the mobile phase was raised to 6.5 by using ammonium acetate rather than TFA, several of the major peaks underwent notable retention time shifts, suggesting that they were organic acids (Fig. 2C).

Using the aglucone ion for genistein (*m/z* 271) to monitor for the different forms of genistein in an isolated soy protein preparation separated by HPLC, three distinct peaks were observed (Fig. 3). Mass spectra of each peak revealed that they had molecular weights of 518, 432, and 474, respectively (Fig. 4A, 4B, 4C). Peak I was the 6′-*O*-malonylglucoside of genistein (Fig. 4A). Peak II was the expected β-glucoside of genistein (Fig. 4B). Peak III was the 6′-*O*-acetylglucoside of genistein (Fig. 4C). In each case, the *m/z* 271 aglucone ion was the predominant ion in the mass spectrum, indicating that the bond between genistein and the glucoside was thermally labile or was susceptible to the energy provided by ion formation and ion acceleration. The mass spectrum of the 6′-*O*-malonylglucoside of genistein (Fig. 4A) contained a $[M+H-44]^+$ ion indicating that decarboxylation (loss of CO_2) occurred in the interface. In the negative ion mode, when the HPLC mobile phase contains formic or acetic acids or their ammonium salts, prominent $[M-H+46]^-$ or $[M-H+60]^-$ ions, respectively, are observed (Coward L, Kirk M, and Barnes S, unpublished observations).

Thus, it became clear that genistein in the soybean is the 6′-*O*-malonylglucoside, which is converted to the other conjugates and to genistein by the combined action of food processing and fermentation (Fig. 5). Regular soy milks and tofus that are prepared by first extracting soybeans with very hot water contain predominantly the β-glucosides, suggesting that the high aqueous heating caused the removal of the malonate ester group by hydrolysis. Recently introduced low fat (lite) or nonfat soy milk products have much lower amounts of isoflavones than the regular soy milk (25). "Lite" tofu also contains far less isoflavones than regular tofu.

Identification of a Biliary Genistein Metabolite in Rats Treated with ^{14}C-Labelled Genistein. 4-^{14}C-labeled genistein administered duodenally to rats fitted with an indwelling biliary cannula is rapidly taken up from the intestine with greater than 75% being excreted in bile in a 4-hr period (26). Only 2% of the dose was excreted in the urine in this period. HPLC analysis demonstrated that there was only one peak of radioactivity in bile, and it was sensitive to β-glucuronidase but not sulfatase (27). HPLC-ESI-

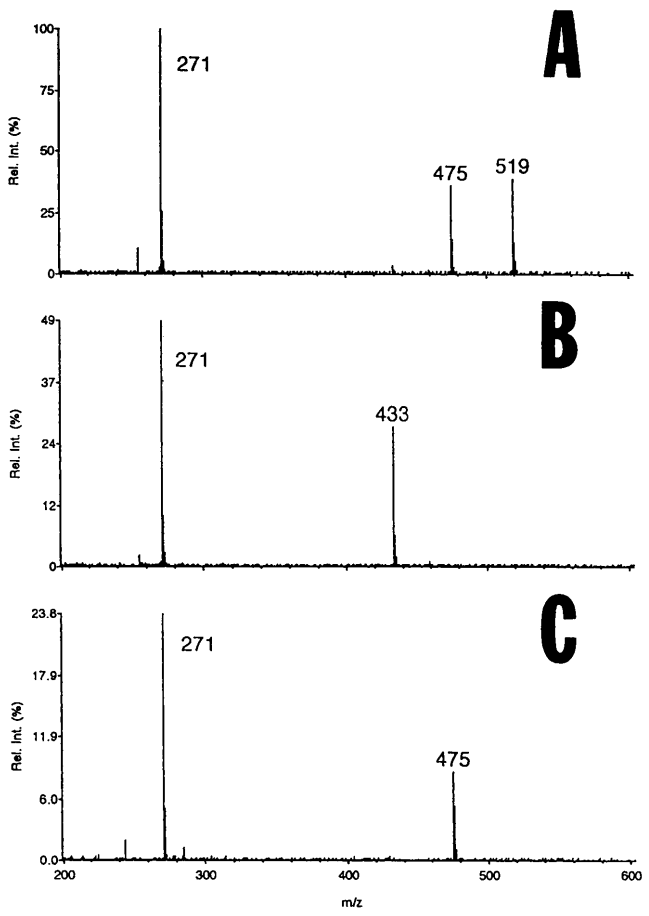

Figure 4. Positive ion mass spectra of the peaks (I, II & III) observed in Figure 3. (A) 6′-*O*- malonylglucosylgenistein. (B) genistin. (C) 6′-*O*-acetylglucosylgenistein.

MS showed that it had a molecular weight of 446. It was confirmed to be derived from genistein because of the marked +2 isotope peak (from the ^{14}C-label) (Fig. 6). Daughter ion spectra of the 445 $[M-H]^-$ molecular ion contained the expected 269 aglucone ion for genistein. Experiments using proton NMR showed that the metabolite was the 7-*O*-β-glucuronide of genistein (24).

Urinary Isoflavonoid Analyses. Isoflavones and their metabolites in urine (and plasma) are usually first extracted with a solid-phase cartridge, prior to further purification steps that precede GC-MS. Since most of the metabolites are conjugated with glucuronide or with sulfate, recoveries through this step have until recently (24) not been well documented. However, the solid phase cartridge is nothing more than a small HPLC column. So, instead of trying any purification, neat (in fact diluted) urine was analyzed by HPLC-ESI-MS. By inspecting the resulting contour plots of urines from rats on a control diet and the same diet plus genistein, the peaks coming from genistein metabolites can be readily identified.

Four major peaks were identified, genistein (*m/z* 269) and genistein β-glucuronide (*m/z* 445), and the glucuronide (*m/z* 297) and sulfate (*m/z* 201) of *p*-ethylphenol. These can be seen in the reconstructed selected ion chromatogram (Fig. 7). Genistein sulfate was not detected.

Measurement of the isoflavones and their metabolites in urine from those consuming a heavy soy diet can be carried out by HPLC-UV analysis (28), as well as by HPLC-MS. In the latter method, multiple reaction ion monitoring (MRM) was used, combining each parent molecular ion with a specific daughter ion produced by collision-induced dissociation. The data obtained from the two methods correlated quite well (correlation coefficients of 0.9672 for daidzein and 0.9206 for genistein, $n = 55$). However, the concentrations of daidzein detected by the HPLC-HN-APCI-MRM method were 40% lower tan those by HPLC-UV. This suggests that other nonphytoestrogen metabolites, not separated from the isoflavonoids and their metabolites, are present in urine and contributed to the absorbance at 262 nm, but were not detected by mass spectrometry (Fig. 8A). Similar data were obtained for genistein; however, the slope of the correlation between the two techniques for genistein was closer to 1.0 than for daidzein (Fig. 8B). At the low urinary isoflavone concentrations found in those consuming a regular American diet, HPLC-UV is inadequate, and a more sensitive and specific method such as HPLC-MS is necessary.

Isoflavonoids in Blood. In plasma or serum, the isoflavone concentrations are low (<1 μ*M*), and only limited amounts of blood are available. Typically, analyses have to be carried out starting with pmol amounts of analytes. For most investigators, the total concentrations of the isoflavones and their metabolites are sufficient. After trying several approaches (24), the Lundh method (29) developed in the late 1980s was chosen for this analysis. To check that hydrolysis occurred for each sample, phenolphthalein glucuronide (PPG), 4-methylumbelliferone sulfate (MUS), and biochanin A were added as internal standards. Biochanin A measured overall aglucone recovery and the PPG and MUS deconjugation. These assays were carried out on a regular 10 cm × 4.6 mm i.d. reversed-phase column using the HN-APCI interface (24).

Specificity of measurement of each compound was achieved by monitoring a combination of the molecular ion and a specific daughter ion for each isoflavonoid and their metabolites obtained by collision with an Ar-N_2 gas mixture. Using a high-pressure collision cell, much more extensive fragmentation and more importantly, sensitivity—in fact, about a 10-fold increase—was observed (Fig. 9).

Because of the high selectivity of the parent-daughter ion combination, the need for chromatographic separation of the analytes was drastically reduced. As a result, gradient elution became unnecessary. All the assays were carried out under isocratic conditions. This shortened the time for the assay of each sample from 25–30 min to 6 min and improved reproducibility.

The isoflavone and their metabolite concentrations were studied over a 2-week period in volunteers drinking two isolated soy protein beverages (42 mg genistein and 27

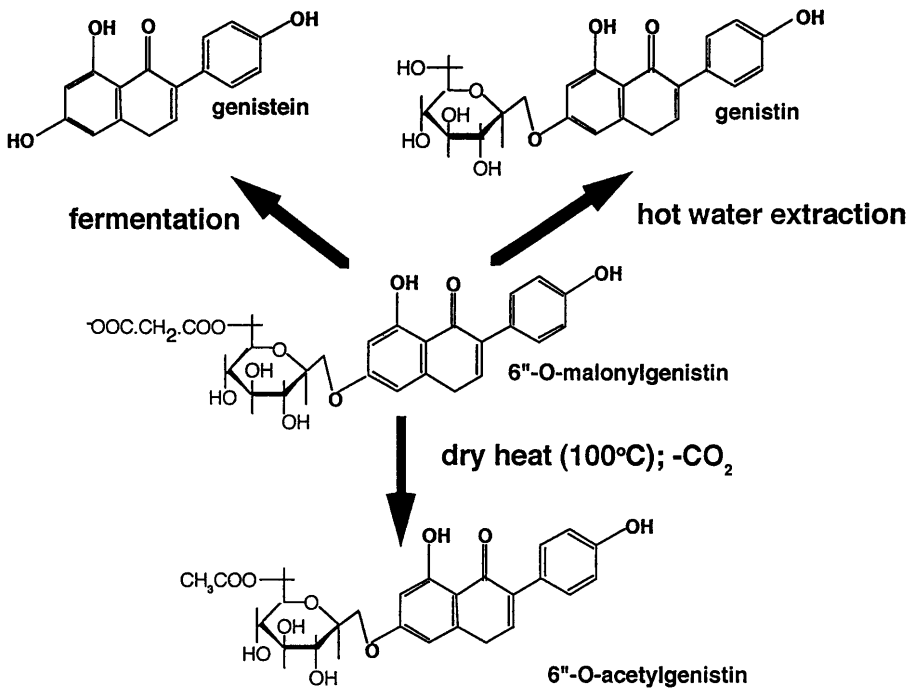

Figure 5. The chemical forms of genistein in soy foods.

mg daidzein) each day. The peak plasma concentrations of genistein (730 n*M*) and daidzein (620 n*M*) were reached on the first day and tended to decline thereafter, suggesting that an adaptive metabolism was induced. The ratio of genistein:daidzein in the blood was very similar to that in the soy beverage, also indicating that these isoflavones have similar bioavailabilities.

Discussion

HPLC-MS using ESI and HN-APCI interfaces is highly applicable to the analysis of isoflavones and their metabolites in foods and biological fluids. The interfaces permit the detection of diagnostic ions (usually molecular ions) without the need for derivatization, or in many cases without the need for multistep extraction procedures. The relative ease by which isoflavones can be measured using this technique will be invaluable to investigators in many areas of research on isoflavonoids.

ESI-MS is well suited to investigations of the metabolites of isoflavonoids in physiological fluids. Sulfate esters and glucuronides, which are thermally labile in the HN-APCI interface, form molecular ions in ESI. Genistein 7-*O*-β-glucuronide was identified in rat bile using this method (24, 27).

A particular advantage of the ESI interface is that it is sensitive to the concentration of the solutes. As in the case of UV-detection of tryptic peptides, the use of narrower bore columns rather than the standard 4.6 mm i.d. columns increases sensitivity. Because of this, peptides are analyzed by HPLC-ESI-MS using 75–300 μm i.d. reversed-phase columns (30). Similar increases in sensitivity for isoflavones are observed using a 300-μm i.d. reversed-phase column to analyze isoflavones (24). Such columns require well

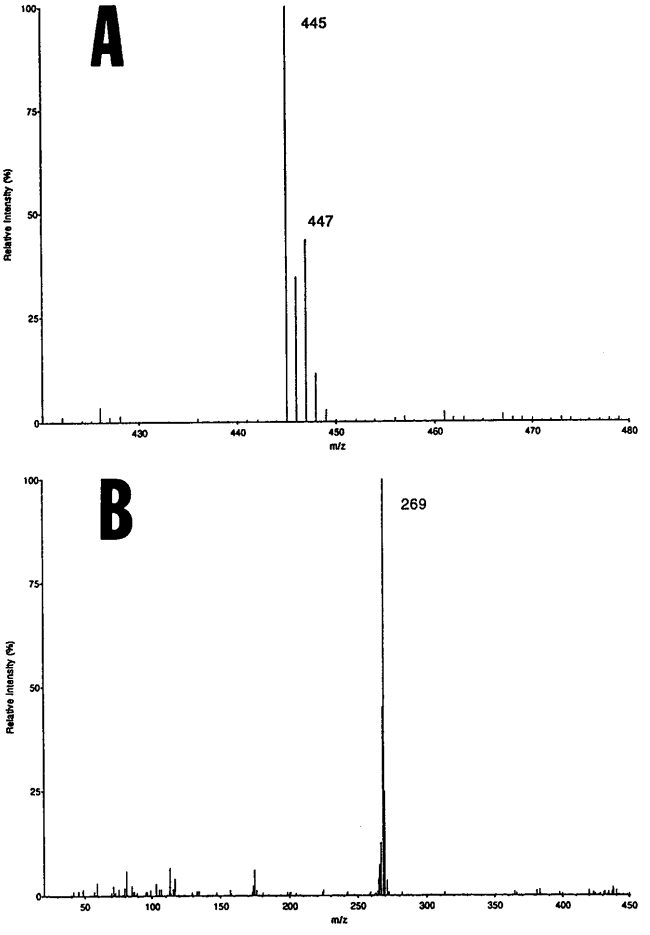

Figure 6. Negative ion mass spectra of biliary genistein metabolite by HPLC-ESI-MS. (A) Full scan mass spectrum of metabolites. (B) Daughter ion mass spectrum of the m/z 445 ion.

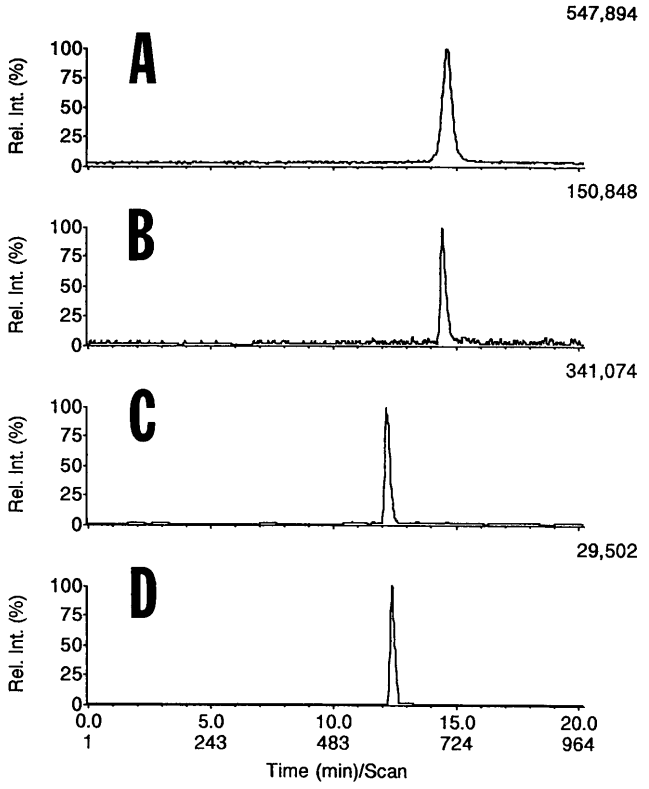

Figure 7. Reconstructed ion chromatograms for the principal genistein-derived ions in rat urine from HPLC-ESI-MS analysis. (A) m/z 201 (*p*-ethylphenol sulfate). (B) m/z 269 (genistein), (C) m/z 297 (*p*-ethylphenol glucuronide). (D) m/z 445 (genistein glucuronide).

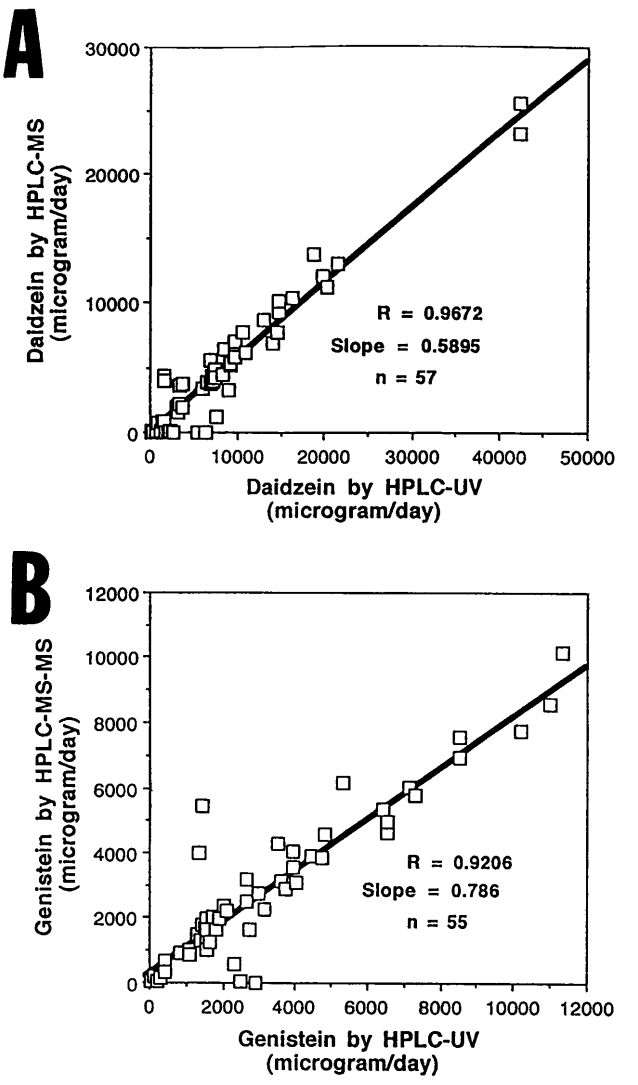

Figure 8. Correlation of analysis of urinary isoflavones by HPLC-HN-APCI-MRM and HPLC-UV. (A) daidzein, (B) genistein. Data are presented as μg excreted in the urine per day.

regulated flow rates in the range of 1–5 μl/min, which can be achieved by conventional HPLC pumps (operating at 50–250 μl/min) combined with a solvent stream splitter. The sample is introduced into the solvent stream after splitting has occurred.

Analysis of isoflavones has also been carried out using the HN-APCI interface (6, 24, 31). This interface is well suited to simply applying previous HPLC methods in which isoflavones are detected by their UV absorbance. It can accommodate flow rates up to 1.0 ml/min without splitting. However, unlike ESI, it is a mass analyzer, lowering the flow rate confers no advantage. Although glycosidic conjugates of isoflavones found in soy foods are stable enough to be detected as their molecular ions in the HN-APCI interface (6), the physiologic conjugates (β-glucuronides and sulfates) decompose, yielding the aglucone ions. Identification of the these types of conjugates using the HN-APCI interface can only be based on chromatographic mobility.

MS-MS experiments have provided useful structural information of the isoflavone conjugates and allow the distinction between the isoflavonoids and other nonisoflavonoid compounds in biological matrices that have the same unit mass. A form of MS-MS, multiple reaction ion monitoring, has enabled isoflavones and their metabolites to be measured accurately in blood (24) and urine (32) with high reproducibility, and more importantly, rapidly. Even with manual injections, at least 50 samples/day can be analyzed. Given the speed of this analysis, the data obtained in the present study demonstrate that HPLC-MRM-MS is the preferred technique over HPLC-UV methods because the latter are not suited to the study of low isoflavonoid excretion rates.

The recent development of capillary electrophoresis (CE) for the separation of isoflavonoids (33) offers the potential of tremendous further improvements in their analysis. The low flow rates associated with capillary electrophoresis (less than 1 μl/min) are suited to the use of the ESI interface. Already, Aramendia *et al.* (34, 35) have developed a CE-ESI-MS method using selected ion monitoring in which a linear standard curve was obtained for injections in the range 0.2–20 fmol. Further improvements in sensitivity and specificity using this technique can be expected by using selected parent ion/daughter ion combinations rather than molecular ions to measure individual isoflavonoids. The very high sensitivities possible may overcome one of

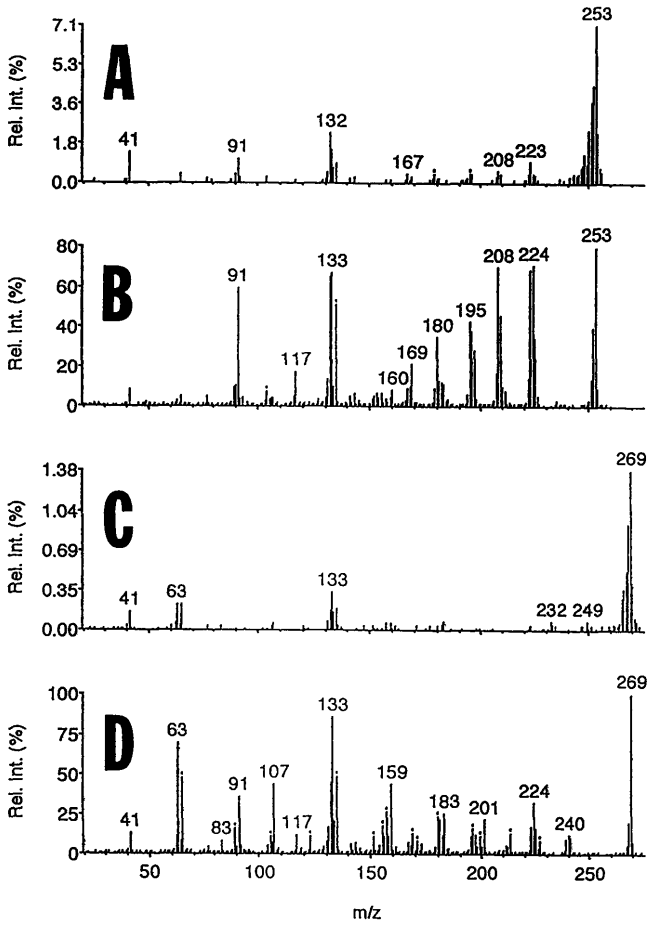

Figure 9. Daughter ion CID mass spectra of isoflavones. (A and B) Daidzein. (C and D) Genistein. A and C, data obtained using low pressure collision cell, B and D, data obtained using high-pressure collision cell.

the principal limitations of CE-ESI-MS, the very small amounts of sample that can be loaded into the capillary.

In summary, MS is a vital tool for investigators in isoflavone and phytoestrogen research. The combination of liquid chromatography techniques (HPLC and capillary electrophoresis) with MS greatly extends the range of analysis hitherto possible with GC -MS methods.

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