SPECIAL GUEST EDITOR SECTION

# An Accurate and Reproducible Method for the Quantitative Analysis of Isoflavones and Their Metabolites in Rat Plasma Using Liquid Chromatography/Mass Spectrometry Combined with Photodiode Array Detection

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**To study the safety and potential health benefits of soy isoflavones, a rapid and simple method based on liquid chromatography combined with mass spectrometry (LC/MS) and photodiode array detector (PDA) was developed for the determination of isoflavones in rat plasma. The analytes included daidzein, genistein, glycitein, equol, 4-ethyl phenol, and biochanin A over a concentration range of 1.0–4320.0 nM using 75 μL of rat plasma. Rat plasma samples were hydrolyzed by adding an enzyme mixture from *Helix pomatia* containing glucuronidase and sulfatase to convert the isoflavone β-glycosides daidzin, genistin, and glycitin to their active aglycone forms. A liquid–liquid extraction method using ethyl acetate as the extraction solvent was used to extract aglycones and the internal standards (phenolphthalein β-D glucuronide, 4-methylumbelliferyl sulfate, and apigenin) from digested plasma samples. The extract was evaporated to dryness under a nitrogen stream, reconstituted with 0.1% formic acid in water–acetonitrile (85 + 15), and injected into a Zorbax SB-CN reversed-phase column (4.6 × 75 mm, 3.5 μm particle size). The Micromass ZQ detector was operated in the positive ion selected-ion monitoring mode. The flow rate for LC was 1.0 mL/ min, with a split where 25% of the effluent was introduced into the electrospray ionization probe of the MS instrument and 75% into the PDA. The chromatographic run time was 16.0 min, with delay of 10 min/injection. The interday precision and accuracy of the standard samples were <2.6% relative standard deviation and <10% relative error, respectively. Recovery of the reported isoflavones with this method varied from 86 to 100%.**

The current AOAC INTERNATIONAL *Official Methods of Analysis* (1) does not provide a method for the analysis of isoflavones and their metabolites in biological fluids. The only existing AOAC Method, **2001.10** (1), is for the determination of isoflavones in soy and selected foods containing soy by using extraction, saponification, and liquid chromatography (LC). Because of sample matrix differences, this method may not be applicable for biological fluids.

Development of a suitable analytical method for determining isoflavones and their metabolites in biological matrixes has been difficult due to the large number of phytoestrogens that exist and the range of chemical forms in which they can occur within various biological matrixes (2).

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In 1985, gas chromatography/mass spectrometry (GC/MS) was the principle method for the quantitative analysis of isoflavones and other polyphenols in biological fluids (3). These methods are relatively long, requiring purification, fractionation, hydrolysis, and derivatization, and they analyze only specific isoflavones of interest (4–8). During the last decade, there have been advancements in the field of MS with the development of new interfaces and ionization and detection techniques. High-performance LC/MS has become the method of choice for the analysis of isoflavones and their metabolites in food, rat plasma, and urine (9–11). Most previous studies have determined daidzein and/or genistein in plasma samples using a chromatographic separation of 30 min or longer (10, 12). Compared with GC/MS, isoflavones can be analyzed using HPLC/MS without the need for derivatization and extraction from the biological matrixes, so the LC/MS procedure is much simpler. Furthermore, the sensitivity of LC/MS (1–500 fmol) is higher compared with GC/MS (50 fmol; 3).

To our knowledge, the method described in the present study is the first method for the determination of soy isoflavone aglycones derived from dietary sources present in rat blood samples, as well as their metabolites. The method involves a single run of 16.0 min using LC/MS combined with a photodiode array detector (PDA). This state-of-the art LC/MS method will be a key determinant in generating reproducible and reliable data that will be used subsequently in the evaluation and interpretation of pharmacokinetic and bioavailability studies involving soy products, and it could be useful for the determination of isoflavones derived from other plant sources and fed to other animals.

## Experimental

### Reagents

(**a**) *Hydrolytic enzyme*.—A mixture of sulfatase and glucuronidase from *Helix pomatia* type H-5 (S3009), containing 400–600 units/mg of glucuronidase activity and 15–40 units/mg of sulfatase activity (Sigma-Aldrich Co., St. Louis, MO).

(**b**) *HPLC grade acetonitrile (ACN) and ethyl acetate*.—EMD Chemicals Inc. (Gibbstown, NJ)

(**c**) *Formic acid*.—Sigma-Aldrich Co.

(**d**) *Dimethyl sulfoxide (DMSO)*.—99.9% HPLC grade (Sigma-Aldrich Co.).

(**e**) *Water*.—Deionized, NANO-pure [Diamond ultra-violet (UV) ultrapure water purification system; Barnstead International, Essex, UK].

(**f**) *ACN–water*.—50 + 50 (v/v).

(**g**) *Mobile phase A*.—0.1% formic acid in water.

(**h**) *Mobile phase B*.—0.1% formic acid in ACN.

(**i**) *0.1% Formic acid in water–ACN*.—85 + 15 (v/v).

(**j**) *Sodium citrate buffer (25 mM, pH 5.0)*.—Prepared by weighing an appropriate amount of sodium citrate (Sigma-Aldrich Co.) in water and adjusting the pH of the solution to 5.0 using acetic acid.

### Standards

(**a**) *Daidzin, genistin, glycitin, daidzein, genistein, glycitein, and equol (metabolite of daidzein)*.—LC Labs (Woburn, MA).

(**b**) *Biochanin A, 4-ethyl phenol (metabolite of genistein) and 3 internal standards: phenolphthalein* β-*D glucuronide, 4-methylumbelliferyl sulfate, and apigenin*.—Sigma-Aldrich Co.

(**c**) *Stock standard solution of each standard (genistein, daidzein, glycitein, genistin, daidzin, glycitin, biochanin A, equol, 4 ethylphenol) and 3 internal standards (phenolphthalein* β-*D glucuronide, 4-methylumbelliferyl sulfate, and apigenin)*.—Prepared by accurately weighing out each standard into 10 mL DMSO. The concentration of the stock was calculated to be 8 mM. Stability of the stock solutions was checked over a period of 1 year (Table 1). This solution was stable for 1 month at room temperature.

(**d**) *Working standards 1 (8* μ*M)*.—Prepared by appropriately diluting the stock solution with ACN–water (50 + 50, v/v).

(**e**) *Working standards 2 (1.0–4320.0 nM)*.—Prepared from the working standards 1 by adding appropriate volumes of analyte and internal standards into 0.1% formic acid in water and ACN mixture (85 + 15, v/v). Working standards 1 and 2 were prepared daily. Each working standard 2 was initially infused into the mass spectrometer in the positive selected-ion monitoring (SIM) mode (span = 0.05 Da and interchannel delay time = 0.1 s) in order to determine the right cone voltage and maximum peak intensity

**Table 1. Stability test of the stock standards in DMSO at room temperature**

| | % Degradation (months) | | | | |
|---|---|---|---|---|---|
| Compound | 1 | 3 | 4 | 7 | 11 |
| Daidzein | 0.0 | 0.0 | 0.0 | 4.2 | 4.2 |
| Glycitein | 0.0 | 0.0 | 0.0 | 4.2 | 3.4 |
| Equol | 0.0 | 5.0 | 8.2 | 18.3 | 29.6 |
| Genistein | 0.0 | 3.8 | 3.8 | 4.2 | 4.6 |
| Biochanin A | ND[a] | ND | 14.9 | 18.9 | 20.7 |

[a] ND = Not determined.

**Table 2 Mass spectrometer operating conditions**

| MS | Waters ZQ 2000 |
|---|---|
| Ion mode | ESI+ |
| Capillary cone voltage, kV | 3.2 |
| Extractor, V | 3.0 |
| Source temperature, °C | 120 |
| Desolvation temperature, °C | 350 |
| Cone gas flow, L/h | 50 |
| Desolvation gas flow, L/h | 600 |

for each $[M + H]^+$ ion. Mass spectrometer operating conditions are reported in Table 2.

### *Enzyme*

Enzyme sulfatase type H-5 from *H. pomatia*, containing 29 units/mg solid sulfatase activity, was purchased from Sigma-Aldrich Co. A solution containing 23 units of sulfatase activity was prepared according to (10) by dissolving an appropriate amount of enzyme in 1.0 mL sodium citrate buffer.

### *Apparatus*

(**a**) *Analytical evaporator (N-EVAP)*.—Complete with heated water bath and nitrogen tank attached; capable of holding 12 vessels/run (Organomation Associates Inc., Berlin, MA).

(**b**) *Balance*.—Analytical, weighing to 0.00001 g (Mettler Toledo, Columbia, MD).

(**c**) *HPLC*.—The LC separations were performed by using a Waters (Milford, MA) Alliance 2695 liquid chromatograph and a Zorbax SB-CN reversed-phase column (4.6 × 75 mm, 3.5 μm particle size; Agilent Technologies, Wilmington, DE) with gradient elution of (0.1% formic acid and 85 + 15% water–acetonitrile) using the gradient steps shown in Table 3. The flow rate was 1.0 mL/min, with a split (using a Tee union splitter) where 25% of the effluent was introduced into the electrospray ionization (ESI) probe of the MS instrument and 75% into the PDA. Solvents were degassed by the online degasser of the Alliance 2695 system.

(**d**) *PDA*.—UV detection was monitored at 276.4 nm for the detection of 4-ethyl phenol in the same run using a Waters 996 PDA.

(**e**) *MS system*.—A Waters Micromass ZQ single quadrupole mass spectrometer was operated in the positive ion SIM mode (span = 0.05 Da, and interchannel delay time = 0.1 s). For glycoside and aglycone analysis, 6 time functions were utilized: first time function (0.0–8.0 min), second time function (8.0–10/8), and third time function (10.8–12.5) monitored the $[M+H]^+$ ions for 4-methylumbelliferone (*m/z* 177.1, dwell time = 0.3 s) at cone voltage 35V, daidzin (*m/z* 417.3, dwell time = 0.3 s) and genistin (*m/z* 433.8, dwell time = 0.3 s) and glycitin (*m/z* 447.4, dwell time = 0.3 s) at cone voltage 25 V. In the second time function (8.0–10.80 min), daidzein (*m/z* 255.2, dwell time = 0.4 s) and glycitein (*m/z* 285.2 , dwell time = 0.6 s) were monitored at a cone voltage of 45V. In the third time function (10.80–12.50 min), the $[M+H]^+$ ions for equol (*m/z* 243.1, dwell time = 0.7 s) and genistein (*m/z* 271.2, dwell time = 0.3 s) were monitored at cone voltages of 22V and 45V, respectively. In the fourth time function (12.5–14.0 min), the $[M+H]^+$ ions for phenolphthalein (*m/z* 319.2, dwell time = 1.0 s) were monitored at a cone voltage of 30V. In the fifth time function (14.0–16.0 min), biochanin A *(m/z* 285.2, dwell time = 0.5 s) was monitored at a cone voltage of 45 V and apigenin (*m/z* 271.2, dwell time = 0.5 s) at a cone voltage of 50V. Mass spectrometer operating conditions are reported in Table 2. The entire system from sample injection to data acquisition was computer-controlled with Empower software (Waters).

### *Standard Curve*

Three linear calibration curves (low, medium, and high) with a minimum of 5 concentrations/curve (total of 17 levels) were prepared daily by diluting the stock solutions with 0.1% formic acid and water–acetonitrile (85 + 15). A constant concentration of internal standard (4-methylumbelliferone, 800.0 nM) was chosen for all 3 calibration curves to achieve the following range of the standard concentrations: low (1.0–48.0 nM), medium (48.0–720.0 nM), and high (720.0–4320.0 nM) for all the standards except equol and 4-ethyl phenol. Equol ranges were 30.0–1920.0 nM and 1920.0–11526.0 nM for the medium and high calibration curve, respectively (no low calibration curve was set for equol). 4-Ethyl phenol standard concentrations ranges were 1200.0–3600.0 nM and 3600.0–21620.0 nM for medium and high calibration curve, respectively (no low calibration curve was set for 4-ethyl phenol).

These standard mixtures (all 17 levels) were injected before or after analysis of the unknowns. Also, 2 levels of

**Table 3. HPLC pump gradient[a] elution of the isoflavones for each run**

| Step | Time, min | Flow, mL/min | %A, 0.1% FA[b] in $H_2O$ | %B, 0.1% FA[b] in ACN |
|---|---|---|---|---|
| Initial | 0 | 1 | 85 | 15 |
| 2 | 7.5 | 1 | 82 | 18 |
| 3 | 8.5 | 1 | 70 | 30 |
| 4 | 12.0 | 1 | 70 | 30 |
| 5 | 13.0 | 1 | 55 | 45 |
| 6 | 16.0 | 1 | 55 | 45 |
| 7 | 17.0 | 1 | 5 | 95 |
| 8 | 18.0 | 1 | 5 | 95 |
| 9 | 19.0 | 1 | 85 | 15 |

[a] All gradients are linear.
[b] FA = Formic acid.

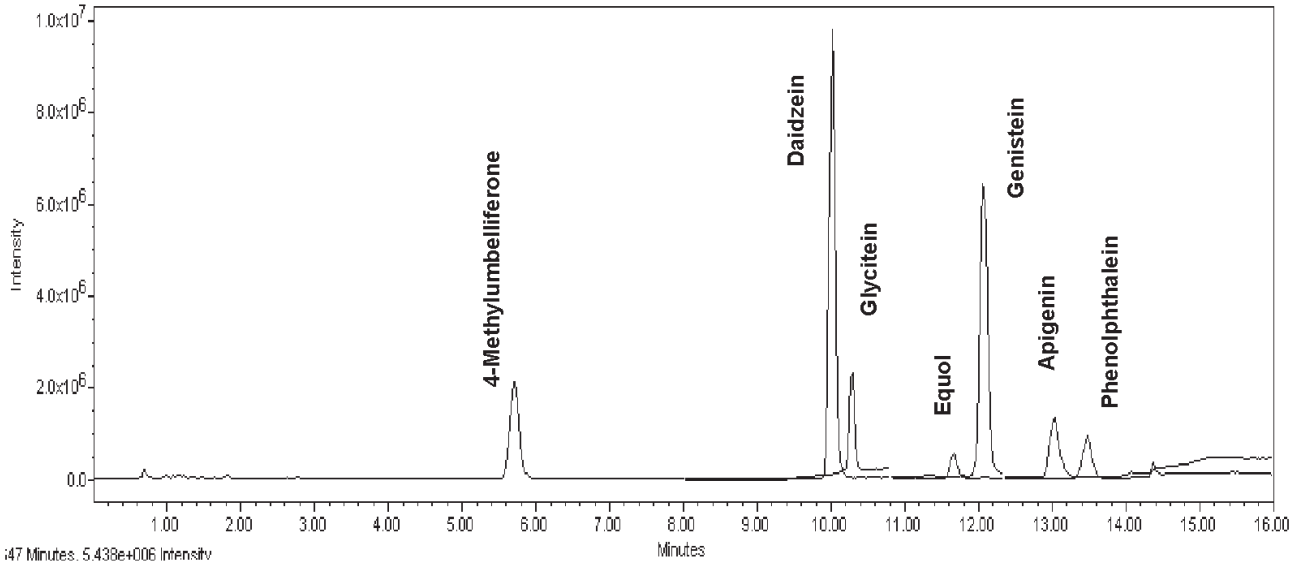


**Figure 1. A representative HPLC/MS chromatogram of isoflavone in rat plasma sample after enzyme hydrolysis.**

standards (low and high) were injected between every 2 unknown samples to ensure system stability during the whole run time. Only calibration curves from all analytes ($r^2 > 0.998$) in the expected calibration range were used (data not shown). The calibration of the instrument was repeated when the calibration curves showed an $r^2$ lower than 0.998.

For each calibration curve, peak area ratios of the analyte to the internal standard were plotted against the known analyte concentration. The resulting slope and y-intercept values for each analyte were used to determine analyte values in unknown specimens using the best-fit line equation

$$(y = mx + b)$$

All of the analytes were detected against 4-methylumbelliferone internal standard. Analytes below the limit of detection (LOD) were reported as nondetectable. Samples exceeding the highest standard of the high standard curve were diluted and reanalyzed in the linear range of the assay.

### *Rat Plasma Sample Collection*

Blood samples (0.5 mL) from isoflavone-fed and control rats dosed with 1000 mg/kg of soy protein isolate plus NOVASOY™ (Archer Daniels Midland Company, Decatur, IL) and casein (90% purity, ICN Biomedicals, Costa Mesa, CA), respectively, were collected in microtainer tubes with lithium heparin and plasma separator (Becton Dickinson and Company, Franklin Lakes, NJ). After blood collection, capped tubes were inverted several times. Tubes were centrifuged at 4°C, 8000 rpm in an IEC Centra MP4R refrigerated centrifuge, Cat. No. 2438 (Needham Heights, MA) for 3 min and about 250 μL of the plasma (supernatant) was transferred to 0.5 mL tubes and frozen at –80°C until the day of the analysis.

### *Extraction and Cleanup*

On the day of the analysis, plasma samples were thawed at room temperature and mixed on a Vortex mixer, and 75 μL aliquots were added to equal volumes of ACN and 10 μL of the internal standard solution (12.8 μM) in 1.5 mL Eppendorf tubes. Tubes were mixed on a Vortex mixer, sonicated for 10 min, and centrifuged at 14 000 rpm for 6 min at 4°C to precipitate proteins. An aliquot of the supernatant (100 μL) was combined with 1 mL sodium citrate buffer and incubated at 37°C for 45 min.

The time course of isoflavone hydrolysis by the mixture of enzymes was determined at 37°C over the intervals of 0, 15, 30, 45, 60, 120, 180, and 240 min, and 24 h. Maximum hydrolysis was monitored by the disappearance of the conjugated forms of the internal standards (phenolphthalein β-D-glucuronide and 4-methylumbelliferyl sulfate) and the appearance of the deconjugated forms (phenolphthalein and 4-methylumbelliferone). Failure to detect unconjugated phenolphthalein and 4-methylumbelliferone in the expected amounts can identify incomplete enzyme hydrolysis with this method (data not shown).

After 45 min of incubation at 37°C, isoflavone aglycones were extracted into ethyl acetate solvent (3 × 2 mL). Addition of ethyl acetate to the hydrolyzate causes enzyme precipitation. After 15 min of sonication, followed by 5 min of centrifugation at 3000 relative centrifugal force (RCF) at 8°C, the supernatant was removed and evaporated to dryness under a nitrogen stream using the N-EVAP at 37°C. Following the 3 extractions, the residue was reconstituted in 100 μL of HPLC mobile phase [0.1% formic acid in water–acetonitrile in (85 + 15)]. Ten μL of each prepared sample was injected into a Zorbax SB-CN reversed-phase column. The isoflavone aglycone content of plasma was determined by omitting enzymatic hydrolysis.

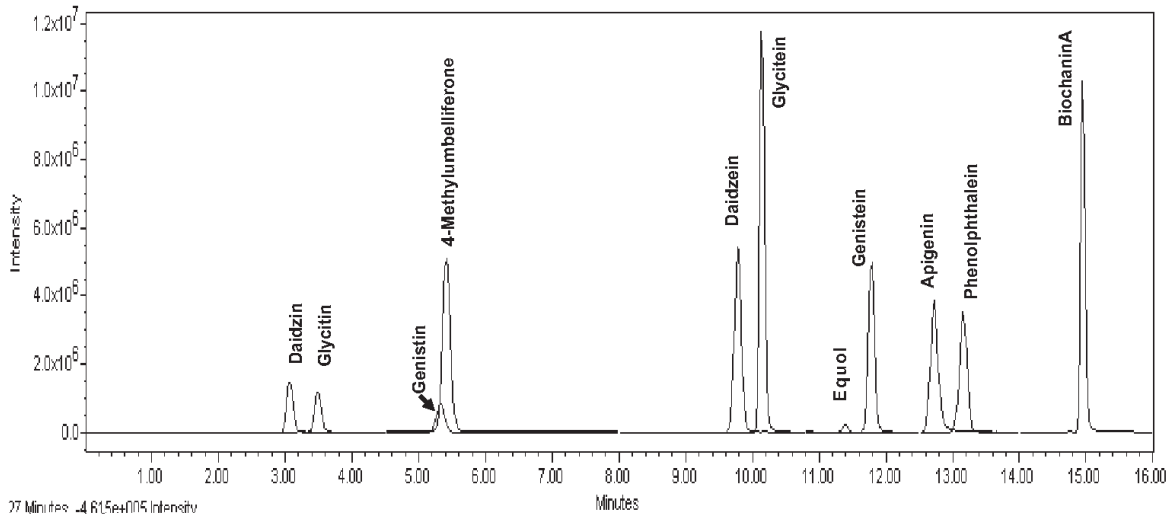


**Figure 2. A representative HPLC/MS chromatogram of isoflavone standards.**

**Table 4. Accuracy evaluation using isoflavone standard solutions fortified into control rat plasma**

| Isoflavone | Expected concn, nM | Mean of observed concn[a], nM | Relative error, % | Accuracy, % added |
|---|---|---|---|---|
| Daidzein | 2.0 | 2.0 | 0.6 | 100 |
| | 12.0 | 13.0 | 9.2 | 108 |
| | 24.0 | 23.0 | 1.4 | 96 |
| | 240.0 | 239.0 | 0.2 | 100 |
| | 480.0 | 464.0 | 3.1 | 97 |
| | 960.0 | 920.7 | 4.1 | 96 |
| Genistein | 2.0 | 2.0 | 1.7 | 100 |
| | 12.0 | 11.0 | 0.5 | 92 |
| | 24.0 | 22.0 | 4.2 | 92 |
| | 239.0 | 220.0 | 8.3 | 92 |
| | 479.0 | 434.0 | 9.5 | 91 |
| | 959.0 | 876.0 | 8.7 | 91 |
| Glycitein | 2.0 | 2.0 | 5.4 | 100 |
| | 12.0 | 12.0 | 2.3 | 100 |
| | 24.0 | 25.0 | 5.9 | 104 |
| | 240.0 | 249.0 | 3.8 | 104 |
| | 480.0 | 483.0 | 0.7 | 101 |
| | 960.0 | 971.0 | 1.2 | 101 |
| Equol | 30.0 | 29.0 | 4.7 | 97 |
| | 128.0 | 127.0 | 1.1 | 102 |
| | 640.0 | 631.0 | 1.5 | 105 |
| | 1921.0 | 1873.0 | 2.5 | 106 |
| | 3842.0 | 3871.0 | 0.7 | 101 |
| | 6403.0 | 6358.0 | 0.7 | 99 |

[a] The mean isoflavone concentrations ($n$ = 3) are shown with the respective relative error (%), and the accuracy compares all measurements with the concentrations added.

### *Calculations*

The concentrations (μM) of all isoflavones and their metabolites in the sample extract were determined from linear regression analysis of the appropriate standard curve. In case of using external calibration curves, the concentration of each isoflavone in test samples was calculated by using the following equation:

$$\text{Isoflavone } (\mu M) = C_s \times (V_m / V_i) \times (V_e / V_a) \times (1 / V_p)$$

where $C_s$ = concentration of isoflavone calculated from the standard curve (μM), $V_m$ = mobile phase volume added (100 μL), $V_i$ = volume injected (10 μL), $V_e$ = volume of the analyte without the precipitation (160 μL), $V_a$ = volume of the aliquot (100 μL), and $V_p$ = volume of the plasma used at the beginning of extraction (75 μL).

### *Method Validation Study*

*Accuracy.*—To demonstrate the accuracy of the method, individual standards were subjected to the complete extraction and analytical processes, and measured values compared to calculated values. The closeness of agreement between the calculated value and the measured value was determined for each standard sample using 6 different concentrations of isoflavones added to a control rat plasma matrix (control rat plasma containing undetectable amounts of isoflavones). Plasma samples were injected 3 times on 3 different days. Results were reported as relative error. Accuracy of the standard samples was <10% relative error (Table 4).

*Precision.*—The precision of the method was determined by preparing and assaying 4 replicates each of 6 different concentrations of isoflavones. Table 5 demonstrates single laboratory relative standard deviation (RSD) values between 0.1 and 2.6% for the 6 concentrations: 10.0–2410.0 nM for daidzein, genistein, and glycitein, and 30.0–6360.0 nM for equol.

**Table 5. Precision evaluation using isoflavone standard solutions as unknown samples**

| Isoflavone | Mean of observed concn[a], nM | Relative standard deviations from the mean, % |
|---|---|---|
| Daidzein[b] | 10.0 | 1.7 |
| | 50.0 | 1.6 |
| | 680.0 | 2.6 |
| | 1450.0 | 0.6 |
| | 2410.0 | 0.2 |
| Genistein | 10.0 | 1.7 |
| | 50.0 | 0.1 |
| | 250.0 | 1.2 |
| | 700.0 | 0.9 |
| | 1450.0 | 0.6 |
| | 2410.0 | 0.8 |
| Glycitein | 10.0 | 1.7 |
| | 50.0 | 0.8 |
| | 230.0 | 1.2 |
| | 700.0 | 1.5 |
| | 1450.0 | 0.7 |
| | 2410.0 | 0.2 |
| Equol | 30.0 | 2.4 |
| | 130.0 | 0.7 |
| | 630.0 | 1.4 |
| | 1870.0 | 1.3 |
| | 3870.0 | 0.5 |
| | 6360.0 | 1.2 |

[a] $n = 4$ for all injections.
[b] Five levels of concentrations were used for daidzein.

*Recovery of isoflavones from rat plasma*.—To measure an analyte in a complex sample matrix (plasma), a spike recovery method was performed. Because other components of the matrix may interfere with the separation, detection, or accurate quantitation of the analyte, potential effects from matrix components were investigated. The analyte reference standard was added to a blank matrix (75 μL of plasma) at various levels. Plasma samples were run through the entire analytical procedure from sample preparation through final analysis. A minimum of 3 replicate measurements were performed at each level on different days and by 2 different technicians. Recoveries were determined by comparing the peak areas for the isoflavones in the extracts of spiked samples with the peak areas observed by injecting the standards directly into the LC/MS instrument (external standards). Also, an injection of the blank matrix was made to determine the matrix background. Recovery results are shown in Table 6.

*Repeatability*.—Homogeneous plasma samples prepared with isoflavones at 5 different concentrations and containing constant amounts of 4-methylumbelliferone sulfate and phenolphthalein β-D glucuronide as internal standards were analyzed in triplicate on 3 different days (Table 6). These samples were subjected to the entire analytical procedure, from sample preparation through final analysis.

*LOD and limit of quantification (LOQ)*.—The detection limits for isoflavones were estimated by making solutions with known amounts of isoflavones and a constant amount of internal standard (800 nM), using the lowest concentration of the standards and defining the LOD as a signal-to-noise ratio (S/N) of 3 : 1 and the LOQ as a S/N of 10 : 1. Table 7 shows the LODs and LOQs of isoflavones.

*System stability*.—The stability of the system was checked by injecting a known concentration of the standard 20 times in one day.

## Results and Discussion

In this study, a rapid, accurate method based on HPLC/MS combined with a PDA has been developed that permits the sensitive measurement of isoflavones and their metabolites in rat plasma samples. As far as we are aware, this is the first report of a method to determine the plasma concentration of aglycone isoflavones and metabolites of daidzein and genistein in one fast run of 16.0 min with well separated peaks using a Zorbax SB-CN reversed-phase column. This method builds on a previously published technique (10) and results in improved selectivity, sensitivity, and precision, as well as shorter run time for the detection of a full spectrum of isoflavones in rat plasma. Figures 1–3 present representative HPLC/MS chromatograms of isoflavones. Figure 2 shows a representative chromatogram for the standard isoflavone glucosides (daidzin, glycitin, and genistin), aglycones (daidzein, genistein, and glycitein), biochanin A, equol, and 3 internal standards (apigenin, phenolphthalein, β-D glucoronide, and 4-methylumbelliferyl sulfate). The retention times were for daidzin, 2.9 min; glycitin, 3.3 min; genistin, 5.2 min; 4-methylumbelliferone, 5.3 min; daidzein, 9.7 min; glycitein, 10.0 min; equol, 11.3 min; genistein, 11.7 min; apigenin, 12.6 min; phenolphthalein, 13.0 min; and biochanin A, 14.9 min. Genistin and 4-methylumbelliferone coeluted with very close retention times of 5.2 and 5.3 min, respectively, however, further analysis with MS showed no mass interference between those 2 compounds. Isoflavone concentrations in rat plasma samples were analyzed with and without enzyme hydrolysis (Figures 1 and 3, respectively).

Quantitative measurements of isoflavones and their metabolites using chromatographic methods required appropriate internal standards to correct for unknown losses during the procedure used. These standards range from $^{2}H$- and $^{13}C$-labeled stable isotope forms of the isoflavones of interest, or compounds with similar chemical structure and properties that are not naturally present in the sample to be studied (3). For LC-based techniques, apigenin, 4-methylumbelliferyl-sulfate, and phenolphthalein β-D-glucuronide have been used to correct for extraction and incomplete hydrolysis (9, 13, 14). The use of isotopically

**Table 6. Recovery and repeatability of isoflavones from plasma after enzyme hydrolysis as determined by LC/MS and calculated against external calibration curve analyzed on 3 different days**

| | | Recovery | | | |
|---|---|---|---|---|---|
| | Concn, nM | Day 1 | Day 2 | Day 3 | Mean ± SD |
| Daidzein | 2.0 | 96.5 | 93.0 | 93.6 | 94.4 ± 1.9 |
| | 10.0 | 108.7 | 105.2 | 108.2 | 107.3 ± 1.8 |
| | 20.0 | 95.1 | 87.8 | 91.8 | 91.5 ± 3.6 |
| | 240.0 | 91.6 | 93.4 | 104.4 | 96.4 ± 6.9 |
| | 480.0 | 92.5 | 90.6 | 90.4 | 91.1 ± 1.1 |
| | 960.0 | 88.7 | 85.3 | 99.0 | 91.0 ± 7.1 |
| Genistein | 2.0 | 104.5 | 100.3 | 100.9 | 101.9 ± 2.2 |
| | 10.0 | 94.0 | 97.7 | 100.4 | 97.3 ± 3.2 |
| | 20.0 | 99.4 | 92.7 | 88.1 | 93.4 ± 5.6 |
| | 240.0 | 80.5 | 83.6 | 94.2 | 86.1 ± 7.1 |
| | 480.0 | 87.6 | 85.0 | 89.0 | 87.2 ± 2.0 |
| | 960.0 | 85.1 | 90.3 | 91.8 | 89.1 ± 3.5 |
| Glycitein | 10.0 | 99.1 | 101.8 | 103.3 | 101.4 ± 2.1 |
| | 20.0 | 99.9 | 101.7 | 101.3 | 100.9 ± 0.9 |
| | 240.0 | 96.4 | 98.7 | 105.7 | 100.2 ± 4.8 |
| | 480.0 | 97.0 | 101.6 | 93.7 | 97.4 ± 3.9 |
| | 960.0 | 96.6 | 101 | 98.3 | 98.6 ± 2.2 |
| Equol | 30.0 | 100.7 | 110.3 | 95.8 | 102.2 ± 7.3 |
| | 60.0 | 101.4 | 98.9 | 95.8 | 98.7 ± 2.8 |
| | 600.0 | 85.7 | 92.4 | 102.6 | 93.5 ± 8.5 |
| | 1200.0 | 90.0 | 94.6 | 94.2 | 92.9 ± 2.5 |
| | 2400.0 | 99.9 | 97.7 | 96.8 | 98.1 ± 1.6 |
| 4MUF[a] | 800.0 | 101.5 | 100.0 | 99.9 | 100.4 ± 0.7 |
| Phenolphthalein | 800.0 | 90.9 | 95.6 | 89.5 | 92.0 ± 2.6 |
| Apigenin | 800.0 | 82.2 | 89.2 | 86.2 | 86.0 ± 2.8 |

[a] 4-Methylumbelliferone.

labeled compounds ($^2H$ or $^{13}C$) as internal standards was mostly reported in GC/MS methodologies; because of their extensive set of workup steps, these methods have the greatest need for stable internal standard (3).

A choice of apigenin as an internal standard was first introduced by Barnes et al. (13) for LC/MS analysis of isoflavones; however, apigenin did not resolve from genistein using an isocratic HPLC separation. Apigenin was later separated from genistein by Griffith and Collison (9) with a long run of 55.0 min. In the gradient LC/MS method reported here, genistein and apigenin are well resolved with retention times of 11.7 and 12.6 min, respectively (Figure 2). Furthermore, apigenin is commercially available, and it has a chemical structure very similar to the isoflavone aglycones (analog of genistein, 5, 7, 4′-trihydroxyflavone) and is more hydrophobic than genistein. This chemical property of apigenin is useful for detection of any hydrophobic loss of the isoflavones during the extraction and analysis (9).

Two other internal standards were added at the beginning of the extraction (4-methylumbelliferyl-sulfate and phenolphthalein β-D-glucuronide) to check for the efficiency of enzymatic hydrolysis in each sample. Detection of unconjugated phenolphthalein and 4-methylumbelliferone and the disappearance of phenolphthalein β-D-glucuronide and 4-methylumbelliferyl-sulfate was an indication that the enzyme hydrolysis was efficient. Recovery [mean (%) ± standard deviation (SD)] of isoflavones from plasma after enzyme hydrolysis and liquid-phase extraction, as determined by LC/MS and calculated against external calibration curve analyzed on 3 different days, are reported in Table 6. Average recovery of 6 different concentrations of daidzein, genistein, glycitein, and equol were 94.9 ± 7.3, 90.6 ± 5.4, 102.5 ± 9.2, and 94.1 ± 6.5, respectively. It was demonstrated that this extraction procedure was 86–100% efficient in recovery of isoflavone aglycones, which was higher in comparison to previously reported data (10). However, for the biochanin A, a

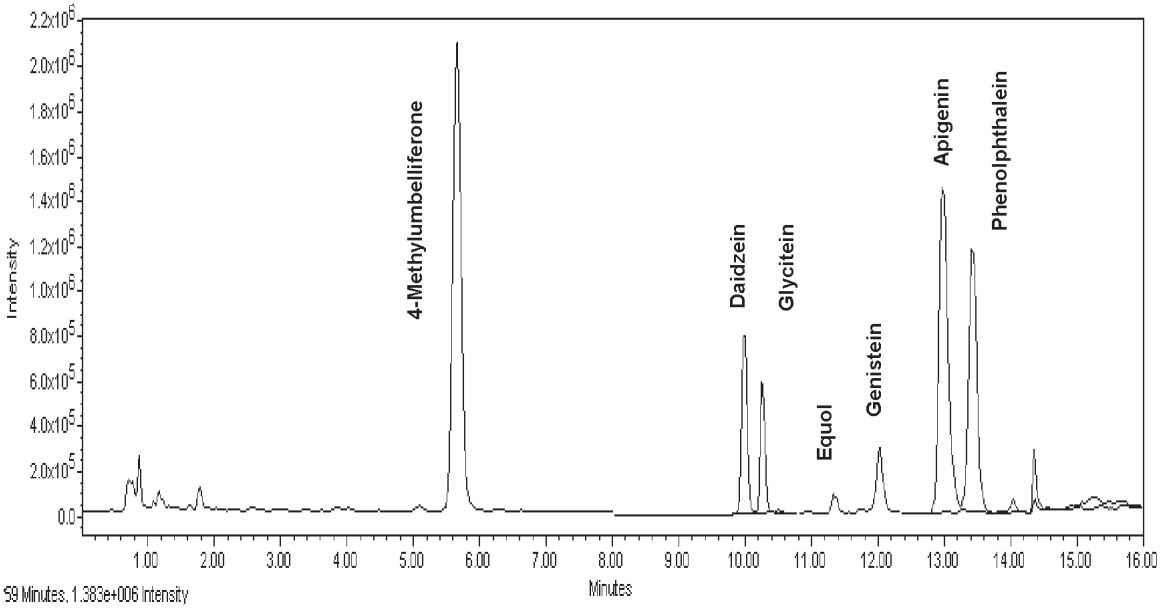


**Figure 3. A representative HPLC/MS chromatogram of isoflavone in rat plasma sample without enzyme hydrolysis.**

low recovery (75–85%) was obtained at high concentrations due to the chemical nature of this compound. Biochanin A is the most hydrophobic compound of interest, and low recovery is due to the hydrophobic loss of this compound at high concentration (results not shown).

LODs obtained from authentic standards ranged between 0.2 and 20.0 nM and LOQs ranged between 0.5 and 60.0 nM, allowing for sensitive quantitation of isoflavones (Table 7).

Soy isoflavones circulate in blood in several molecular forms, including glucuronide and sulfate conjugates (95%), freely circulating aglycones, and protein-bound aglycones (5%; 13, 15). There are 2 conjugation sites on genistein, daidzein, and glycitein, and each of these sites can be sulfated or glucuronidated. Thus, there are monoglucuronides, monosulfates, diglucuronides, disulfates, and mixed conjugates with one site glucuronidated and one site sulfated (16). In order to obtain the total isoflavone concentration in rat plasma samples, there is a need to use enzymatic digestion of the isoflavone conjugates with subsequent detection of aglycones. Accurate quantification of the isoflavone concentration depends on the complete hydrolysis of their conjugated forms. Therefore, a time-course experiment was conducted from 0 to 24 h (0, 15, 30, 45, 60, 120, 180, and 240 min, and 24 h) incubation time. Maximum hydrolysis was monitored by the disappearance of the conjugated forms of the internal standards [phenolphthalein β-D-glucuronide (*m/z* 495.5) and 4-methylumbelliferyl sulfate (*m/z* 257.0)] and the appearance of the deconjugated forms [phenolphthalein (*m/z* 319.2) and 4-methylumbelliferone (*m/z* 177.1)], monitored by LC/MS in the positive ion mode. The time course of isoflavone hydrolysis by commercial hydrolytic enzymes was previously determined to be maximal at

**Table 7. Limits of detection and quantitation for isoflavones analyzed with the proposed LC/MS method**

| Analyte | Limit of detection, nM | Limit of quantitation, nM |
|---|---|---|
| Daidzein | 0.3 | 0.8 |
| Genistein | 0.5 | 1.5 |
| Glycitein | 0.2 | 0.5 |
| Daidzin | 0.4 | 1.2 |
| Genistin | 1.5 | 5.0 |
| Glycitin | 1.5 | 5.0 |
| 4-Methylumbelliferone | 1.0 | 3.0 |
| Biochanin A | 0.3 | 1.0 |
| Equol | 20.0 | 60.0 |

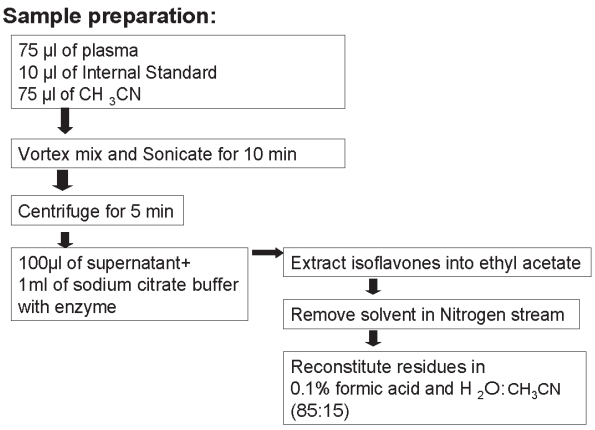


**Figure 4. Sample preparation and extraction for isoflavone conjugates in rat plasma.**

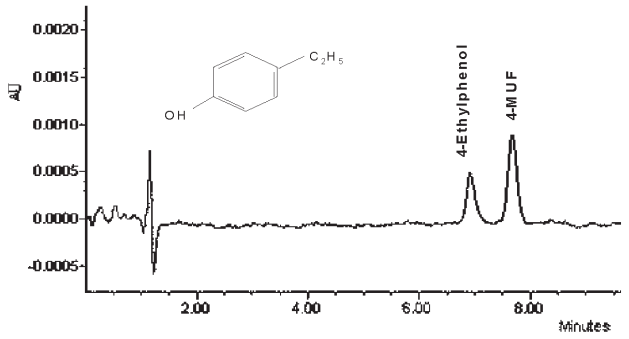


**Figure 5. Structure and chromatogram detected by UV of 4-ethyl phenol and 4-methylumbelliferone (4-MUF; internal standard).**

30 min (10). In this study, maximum hydrolysis was obtained after 45 min incubation at 37°C. Incomplete incubation time would result in incomplete hydrolysis of conjugated isoflavones and a low result. Failure to detect unconjugated phenolphthalein and 4-methylumbelliferone in the expected amounts identified insufficient enzyme hydrolysis by this method. Deconjugation of 4-methylumbelliferyl-sulfate to 4-methylumbelliferone was very rapid, while for phenolphthalein β-D-glucuronide, quantitative formation phenolphthalein occurred after 45 min. In this study, enzyme solution was prepared daily, however according to Twaddle et al. (17), enzymatic activity is stable for at least 5 freeze-thaw cycles, and stable in the frozen state for at least 2 weeks.

The resulting unconjugated isoflavones were then extracted by liquid–liquid extraction using ethyl acetate. Liquid–liquid extraction has the advantage over solid-phase extraction (SPE) in that most of the electrolytes in the plasma sample ($Na^+$, $K^+$, and phosphate) are left behind in the aqueous phase (13). Because more-hydrophilic compounds prefer the polar aqueous phase, whereas more-hydrophobic compounds will be found mainly in the organic solvent (18), polar solvents have been recommended for efficient extractions of isoflavones (15, 19). In our laboratory, the best extraction efficiency was found using 3 extractions each with 2 mL ethyl acetate. A fourth extraction with 2 mL solvent improved the recovery of the isoflavones by less than 2% and was, therefore, not utilized. The presented method for extraction of isoflavones and their metabolites in rat plasma is shown in Figure 4.

In this study, in addition to liquid-phase extraction, SPE with Strata-X [Phenomenex (Torrance, CA), 30 mg, 1 mL, 33 μm particle size, 85 Å pore size, and 800 $m^2$/g surface area] was performed for standard isoflavone aglycones (daidzein, genistein, and glycitein); equol; biochanin A; phenolphthalein; and 4-methylumbelliferone. Potential interferences were removed by the addition of a wash solution [1 mL, ACN-methanol (50 + 50)]. Recovery of the standards was above 90%, except for biochanin A and phenolphthalein (75.1 and 75.3%, respectively). Twaddle et al. (17) demonstrated and validated a reversed-phase SPE method with Isolute ENV+, 25 mg columns (Jones Chromatography, Lakewood, CO) for serum samples containing genistein, daidzein, and equol in a 96-well format for subsequent LC/ESI-MS/MS or LC/ESI-MS analysis.

A Zorbax SB-CN column in a combination with an ACN–formic acid (0.1% in water) elution system exhibited the best selectivity, recovery, and peak shape, and the shortest retention time for all of the analytes of interest compared with other HPLC columns (Phenomenex, Luna C18, 150 × 2.1 mm, 3 μm and Phenomenex Ultracarb, 150 × 2.0 mm, 5 μm) with different mobile phases and different acidity levels. The choice of the more hydrophilic column (silica-based packing with cyanopropyl stationary phase; Zorbax SB-CN) compared with C18, allows better peak separation for equol from genistein, phenolphthalein, and 4-methylumbelliferone and from the rest of the compounds. The chromatographic run time with this column was reduced from 55.0 min with the C18 column to16.0 min.

4-Ethyl phenol (a metabolite of genistein with the lowest molecular weight of all compounds of interest, Mw = 122.1) was detected by running standard and the internal standard (4-methylumbelliferone) through the PDA detector. Because of the chemical structure of this compound and the lack of extra electron pair donation from nitrogen groups for protonation in the MS detector, we were able to detect it in the same run with the PDA detector by Tee-splitting the flow of the effluent, 75% to PDA and 25% to MS detectors. Monitoring was carried out at 276.4 nm to achieve sensitive detection at or very near the absorption maximum of 4-ethyl phenol, which had a retention time of 7.6 min (Figure 5). In plasma, no detectable level of 4-ethyl phenol was obtained.

In conclusion, the HPLC/MS combined with a PDA detection method described in this study allowed the measurement of isoflavones, including aglycones as well as the metabolites of daidzein and genistein, in a short run time of 16.0 min. It also required fewer steps for sample preparation, extraction, and less technician time compared with the previously published methods. Recently, further advances in LC, termed ultra-performance (UPLC), offer significant theoretical advantages in resolution, speed, and sensitivity for analytical determinations, particularly when coupled with mass spectrometers capable of higher-speed acquisitions compared with LC/MS (20).

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(4.6 × 75 mm, 3.5 μm particle size) was a kind gift of Agilent Technologies.

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