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Simultaneous determination of 11 phytoestrogens in human serum using a 2 min liquid chromatography/tandem mass spectrometry method

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ABSTRACT

A rapid 2 min liquid chromatography–tandem mass spectrometry (LC–MS/MS) method operating in multiple reaction ion monitoring mode was developed and validated that allows for the characterization and simultaneous quantification of 11 phytoestrogen metabolites with mass transitions *m/z* 241/119 (equol), 253/132 (daidzein), 255/149 (dihydrodaidzein), 257/108 (*O*-desmethylangolesin), 269/133 (genistein), 283/184 (glycitein), 267/191 (formononetin), 289/109 (biochanin A), 267/91 (coumestrol), enterodiol (301/253), and enterolactone (297/253). The method was demonstrated to be specific and sensitive, and a linear response for each phytoestrogen was observed over a range of 1–5000 ng/mL in human serum with the exception of dihydrodaidzein, whose lower limit of quantification was 2 ng/mL. The separation was carried out on a Synergi Polar-RP 2.5 micron (50 mm × 2.0 mm i.d.) column at 50 °C with water and acetonitrile (both containing 10 mM ammonium acetate) as the mobile phase under gradient conditions at a flow rate of 0.75 mL/min. This LC–MS/MS method is very useful for high-throughput analysis of phytoestrogens and proved to be simple, sensitive, reproducible, and reliable.

1. Introduction

Phytoestrogens are structurally diverse diphenolic compounds that are present in plants and may impact human health, at least in part, by virtue of their estrogenic effects. Isoflavones, coumestanes and lignans are the major biologically active phytoestrogens. Most phytoestrogens bind to estrogen receptors (α and β) and may function as estrogen agonists and antagonists. The potential role(s) of dietary phytoestrogens in the prevention of chronic diseases (e.g., cancer, heart disease, neurodegeneration and osteoporosis) is of substantial interest [1–8]. Epidemiological evidence suggests that consumption of phytoestrogens is associated with a lower risk of breast cancer [9,10] and animal studies have demonstrated that early life exposure to the soy isoflavones reduces the number of mammary tumors induced by carcinogens [11,12]. On the other hand, there are reports suggesting that phytoestrogens may increase the growth of pre-existing breast cancer [13,14]. As for steroid hormones, most phytoestrogens have an enterohepatic circulation and are metabolized by phase II enzymes to glucuronide and sulfate conjugates.

We initially developed a specific, quantitative LC–MS/MS method for these compounds in 1996 [15] and subsequently improved it in 2004 [16]. However, research on phytoestrogens requires an ever-increasing number of samples to be analyzed. Thus, analytical methods that are sensitive, reproducible and high throughput are needed to evaluate the beneficial or adverse effects of phytoestrogens in numerous pre-clinical and clinical studies. There are several reports on analytical techniques used in quantification of phytoestrogens in biological samples [17–19]. HPLC with UV or coulometric array detection has been used by several investigators for quantification of phytoestrogens [20–22]. However, these methods suffer from poor sensitivity and selectivity [23]. Starkey et al. [24] used capillary electrochromatography to determine trace isoflavone phytoestrogens in biological samples. Gas chromatography–mass spectrometry (GC–MS) has been used as a sensitive analytical technique for phytoestrogen analysis [25–28]. However, the large sample volume, multi-step sample preparation procedures and requirement of derivatization prior to analysis for polar and involatile compounds limit the usefulness of GC–MS analysis. LC–MS/MS has become the method of choice due to its high sensitivity, selectivity and easy sample preparation [29–31]. Grace et al. [31] reported quantification of phytoestrogens

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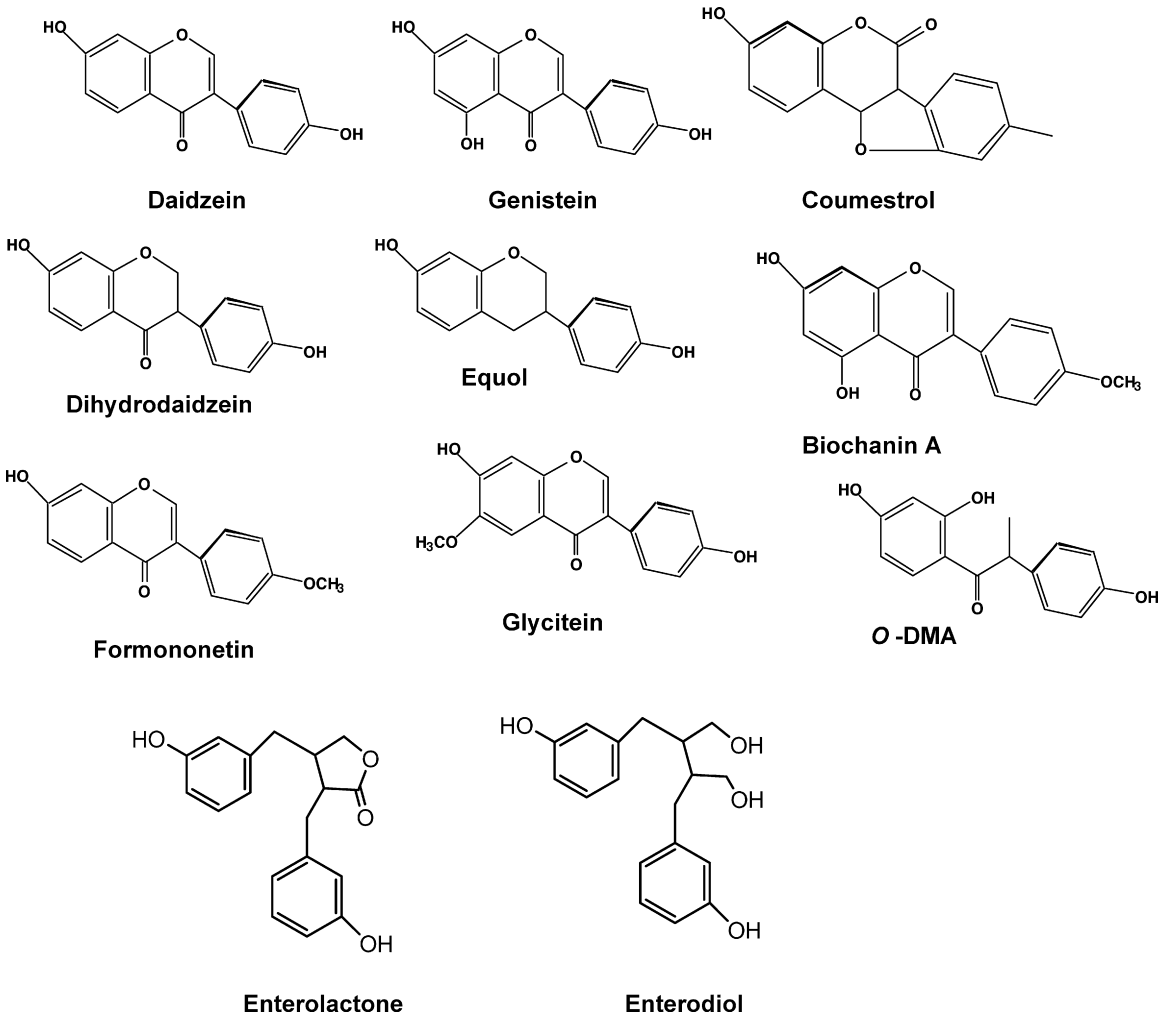

Fig. 1. Chemical structures of standard phytoestrogens.

in human urine and serum using LC–MS/MS. However, the method involves a 10 min chromatographic run time for analysis of nine phytoestrogens. Another LC–MS/MS method for the determination of urinary phytoestrogens has been reported [32]. However, the method suffers from a relatively long chromatographic run time (9 min) and small number of phytoestrogens.

The aim of this study was to develop and validate a multi-target, high-throughput LC–MS/MS method for the quantification of phytoestrogens in serum. We now describe a rapid 2 min LC–MS/MS method for simultaneous quantification of 11 phytoestrogens in serum (Fig. 1), thus making the assay suitable for high-throughput analysis required for analysis of large number of samples in phytoestrogen research.

2. Experimental

2.1. Chemicals

Genistein, daidzein, dihydrodaidzein (DHD), equol, *O*-desmethylangolesin (*O*-DMA), glycitein, biochanin A, coumestrol, enterodiol, enterolactone, and chrysin were purchased from LC-laboratories (Woburn, MA). All HPLC solvents and reagents were purchased from Fisher Scientific Co. (Norcross, GA) and were of HPLC grade. Phenolphthalein β-glucuronide, 4-methylumbelliferone sulfate and β-glucuronidase/sulfatase from *Helix pomatia* was purchased from Aldrich–Sigma Chemical Co. (St, Louis, MO).

2.2. Sample preparation

We previously showed that the average recovery of isoflavones as a group using diethyl ether was ~90%, whereas their recoveries using Sep-pak C_{18} cartridge was only 82% [15]. We therefore, used liquid–liquid extraction of phytoestrogens in serum with diethyl ether for sample preparation. Blank serum samples were obtained from Millipore Corporation (Temecula, CA). Stock solutions of individual phytoestrogens were prepared in DMSO and then diluted with methanol–water (80:20, v/v) to obtain appropriate working solution containing all phytoestrogens and the internal standard (IS, chrysin; 200 ng/mL). For calibration standards and quality control (QC) samples, each blank serum sample (200 μL) was spiked with appropriate concentration of working solution of phytoestrogens. Since hydrolysis is an important step that converts the conjugated forms into the aglycone forms of phytoestrogens, enzymatic hydrolysis of the phytoestrogen glucuronides and sulfates was used for the total phytoestrogen determination in biological samples. We, therefore, followed the extraction procedure that mimics the extraction of conjugated metabolites. Spiked serum samples were hydrolyzed by incubating with β-glucuronidase/sulfatase at 37 °C overnight and extracted into diethyl ether using our reported method [15]. Phenolphthalein β-glucuronide, 4-methylumbelliferone sulfate were added to monitor completeness of hydrolysis with β-glucuronidase/sulfatase. After concentrating samples to dryness, they were dissolved in methanol–water (80:20, v/v) prior to LC–MS/MS analysis.

Table 1
MS/MS parameters optimized for phytoestrogens and internal standards.

Analyte	Q1/Q3	Dwell (ms)	DP (V)	CE (eV)	CXP (V)
Equol	314/119	50	−65	−30	−5
Daidzein	253/132	50	−65	−55	−10
Dihydrodaizein	255/149	50	−50	−30	−9
O-DMA	257/108	50	−70	−40	−5
Genistein	269/133	50	−75	−40	−5
Glycitein	283/184	50	−65	−45	−5
Formononetin	267/251	50	−75	−35	−5
Coumestrol	267/91	50	−50	−50	−2
Biochanin A	283/268	50	−70	−30	−5
Enterolactone	297/253	50	−80	−30	−10
Enterodiol	301/253	50	−70	−30	−9
Phenophthalein	317/93	50	−50	−20	−5
4-MU	175/119	50	−50	−38	−4
Chrysin	253/143	50	−50	−50	−5

DP: declustering potential; CE: collision energy; CXP: cell exit potential.

2.2.1. Validation study

The analytical method was validated to demonstrate the specificity, recovery, lower limit of quantification (LLOQ), accuracy, and precision of measurements. Specificity was established by the lack of interference peaks at the retention time for the internal standards and phytoestrogens. Linearity was tested at seven levels of concentrations covering a range of 1–5000 ng/mL. The regression parameters of slope, intercept and correlation coefficient were calculated by linear least-square regression ($1/x^2$ weighting).

The recovery of the method was determined by comparing the peak areas obtained from the blank serum samples spiked prior to extraction with the peak areas obtained from spiked post-extraction serum samples. The matrix effect was evaluated for all analytes and the internal standard. For this, blank serum samples were processed according to the liquid–liquid extraction method described above and then spiked with phytoestrogens and IS at the final concentration after extraction. The matrix effect for each phytoestrogens was calculated by comparison of mean peak area ($n = 6$) obtained for blank serum samples spiked with phytoestrogens and IS after extraction and peak area of standards in methanol–water (80:20, v/v) at concentrations 50, 500 and 2000 ng/mL. The matrix effect results obtained in this study were calculated as follows:

$$\text{matrix effect} = \left[1 - \frac{Y}{X}\right] \times 100\%$$

where X and Y represent the mean peak areas of phytoestrogen standards in methanol–water (80:20, v/v) and spiked post-extraction at corresponding concentrations, respectively.

The accuracy and precision (presented as %CV) of this analytical method were determined using quality control (QC) samples in four to six replicates of 50, 500, and 2000 ng/mL of phytoestrogens in serum. Accuracy was determined by comparing the calculated concentration using calibration curves to nominal concentration. The limit of quantification (LOQ) was defined as precision and accuracy within 20%. The LLOQ was defined as the smallest amount of the analyte that could be measured in a sample with sufficient precision and accuracy (within 20% for both parameters) and was chosen as the lowest concentration on the calibration curve.

2.2.2. Liquid chromatography–mass spectrometry

LC–MS/MS analyses of serum samples were performed using a system consisting of a Shimadzu Prominence HPLC with a refrigerated auto sampler (Shimadzu Scientific Instruments, Inc., Columbia, MD), and an API 4000 (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada) triple quadrupole mass spectrometer. Chromatography was performed on a Synergi 2.5 micron Polar-RP 100A column (50 mm × 2.0 mm i.d. column with a 5 mm × 2.0 mm i.d. guard) at a flow rate of 0.75 mL/min. The mobile phase consisted of (A) 10 mM ammonium acetate and (B) acetonitrile containing 10 mM ammonium acetate, using a gradient elution of 25–70% B at 0.75 min and return to 25% B at 1.0 min. The temperature of the column oven was 50 °C. The column effluent was introduced into the mass spectrometer using an electrospray ionization interface operating in the negative ion mode. The source temperature was set at 400 °C and ion spray voltage of 4 kV. Nitrogen was used as nebulizer, secondary and curtain gas. The tandem mass spectrometer was tuned in the multiple reaction monitoring mode to monitor mass transitions m/z 241/119 (equol), 253/132 (daidzein), 255/149 (dihydrodaidzein), 257/108

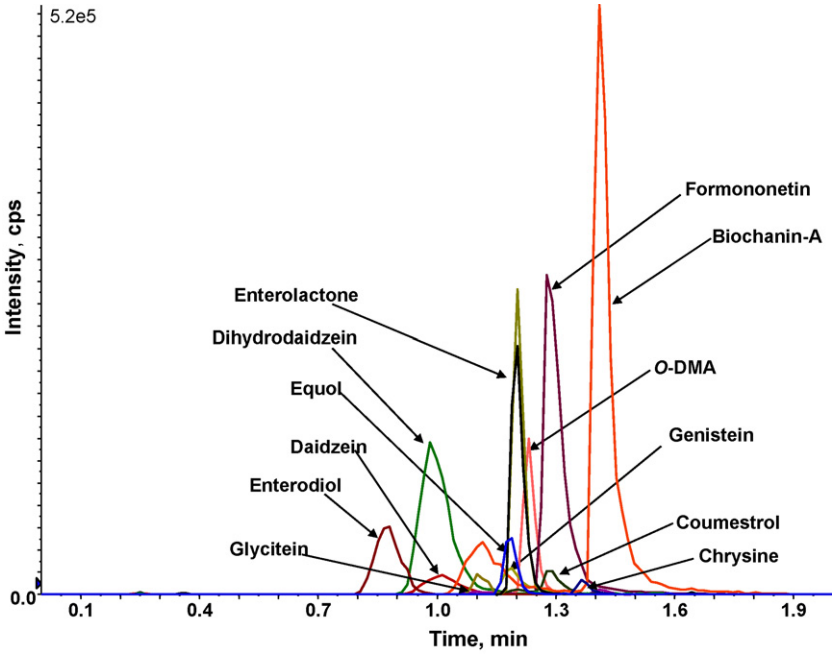

Fig. 2. MRM chromatogram showing separation of 11 phytoestrogens using a 2 min run time.

Table 2
Comparison of LC–MS/MS methods with 15, 5 and 2 min run times for phytoestrogen analysis.

15 min method	5 min method	2 min method
Mobile phase		
10 mM NH_4OH (solvent A)	10 mM NH_4OH (solvent A)	10 mM NH_4OH (solvent A)
MeCN with 10 mM	MeCN with 10 mM	MeCN with 10 mM
NH_4OH (solvent B)	NH_4OH (solvent B)	NH_4OH (solvent B)
Column		
Phenomenex phenyl-hexyl	Phenomenex phenyl-hexyl	Phenomenex Polar-RP
2.0 × 150 mm with guard	2.0 × 30 with guard	2.0 × 50 mm guard
Particle size 3 μm	Particle size 3 μm	Particle size 2.5 μm
Column temperature		
Ambient	40 °C	50 °C
Flow rate		
0.2 mL	0.4 mL	0.75 mL
HPLC pump		
Shimadzu LC-10 AD	Shimadzu LC-10 AD	Shimadzu LC-20 AD
Gradient		
0 min = 20% B	0 min = 25% B	0 min = 25% B
10 min = 70% B	2 min = 80% B	0.75 min = 70% B
11 min = 20% B	2.5 min = 25% B	1.0 min = 25% B
15 min = stop	5 min = stop	2 min = stop

(*O*-desmethylangolesin), 269/133 (genistein), 283/184 (glycitein), 267/191 (formononetin), 289/109 (biochanin A), 267/91 (coumestrol), 301/253 (enterodiol), 297/253 (enterolactone), internal standards 175/119 (4-methylumbelliferone), 317/93 (phenolphthalein) and 253/143 (chrysin). The optimized MS/MS operating parameters used in this study are listed in Table 1. Sample injections of 10 μL were carried out and the autosampler temperature was set at 4 °C.

Table 3
Precision and accuracy of quality control samples.

Analyte	Nominal concentration (ng/mL)	Accuracy (%)			Precision (%CV)			Inter-day
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	
Equol	50	100.42	90.13	96.60	2.01	4.33	5.11	3.74
	500	103.30	99.85	114.66	2.31	5.61	1.93	2.97
	2000	97.60	89.90	103.96	6.11	10.61	10.13	8.34
Daidzein	50	99.98	102.73	94.04	4.35	6.44	8.23	6.62
	500	101.48	98.31	97.73	3.14	5.44	7.42	5.38
	2000	92.50	87.41	86.03	2.88	3.61	3.96	3.58
Dihydrodaidzein	50	103.00	100.15	101.66	3.94	1.43	4.99	3.63
	500	103.79	95.20	106.00	3.96	6.44	3.35	4.34
	2000	91.70	90.40	96.33	1.68	5.80	6.60	2.82
O-DMA	50	104.00	93.72	96.51	5.16	4.71	5.80	5.32
	500	105.67	93.78	102.33	3.22	9.42	5.54	5.84
	2000	101.20	93.57	100.93	5.53	5.37	6.53	3.63
Genistein	50	107.66	106.83	99.08	3.97	3.37	6.65	4.86
	500	97.50	88.90	91.36	5.40	3.61	5.60	4.96
	2000	95.13	92.28	93.38	2.63	3.97	4.17	3.59
Glycitein	50	97.00	80.00	92.13	11.64	7.77	7.52	8.59
	500	100.66	91.96	99.96	6.76	5.09	5.24	5.58
	2000	96.20	92.70	100.21	2.34	5.87	5.19	4.29
Formononetin	50	95.43	98.65	98.50	4.28	7.69	4.93	5.59
	500	101.53	96.60	108.66	4.95	6.09	2.99	4.35
	2000	92.72	94.08	101.90	2.09	7.80	2.26	3.78
Coumestrol	50	92.97	89.24	99.42	8.66	9.15	6.69	7.66
	500	93.86	93.95	96.03	9.05	7.78	5.34	7.26
	2000	100.45	89.95	84.12	7.83	7.34	3.66	6.95
Biochanin A	50	104.23	103.13	107.50	6.82	3.88	3.26	4.48
	500	89.95	94.01	98.41	4.46	6.43	5.24	5.15
	2000	98.88	95.00	93.68	2.91	3.10	5.47	3.90
Enterodiol	50	93.35	88.63	96.95	9.22	7.46	5.36	7.11
	500	104.95	98.95	112.16	7.81	4.10	4.12	4.98
	2000	96.50	87.40	94.73	3.98	6.05	7.94	5.86
Enterolactone	50	104.15	102.20	108.83	9.23	5.71	2.50	5.57
	500	103.30	92.26	104.00	5.43	8.33	1.98	5.03
	2000	84.68	83.33	91.90	1.52	0.35	7.56	3.09

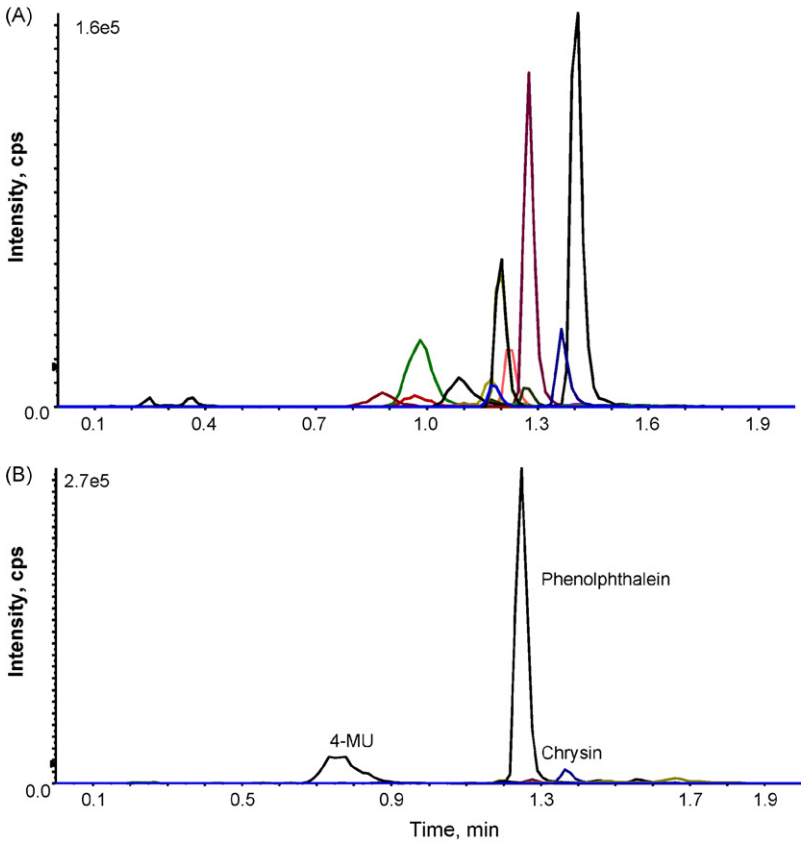

Fig. 3. Representative MRM chromatograms of serum spiked with phytoestrogens (50 ng/mL) (A); internal standards 4-MU, phenolphthalein, and chrysin (B).

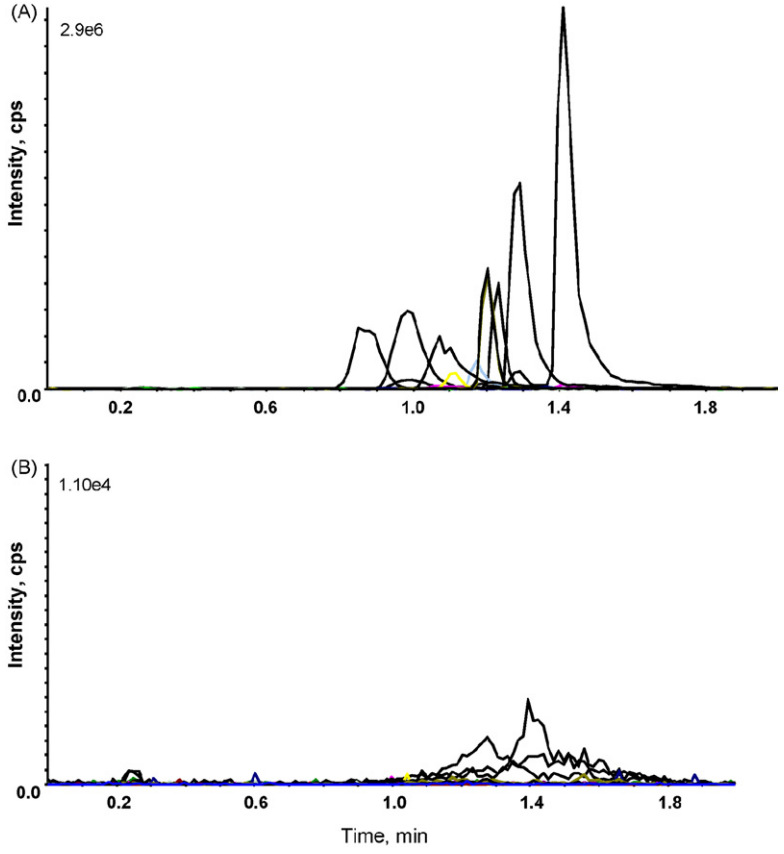

Fig. 4. Representative MRM chromatograms for phytoestrogens in plasma (5000 ng/mL) (A); 10 μL methanol blank (B).

3. Results and discussion

3.1. Chromatography and mass spectrometry

Fast chromatographic separation of 11 phytoestrogens with acceptable resolution for LC–MS/MS was a major challenge. In order to optimize the separation and detection of phytoestrogens, variables such as column type, column temperature, gradient, and flow rate were investigated. Our original LC–MS/MS method involved a gradient analysis that had a chromatographic run time of 15 min per sample. Raising the column temperature to 40 °C, increasing the flow rate from 0.2 mL/min to 0.4 mL/min and the shortening the column length from 150 mm to 30 mm, reduced the run time to 5 min. We subsequently found that the phytoestrogens were stable at 50 °C in the mobile phase, a temperature that allowed the flow rate to be further increased to 0.75 mL/min. Phenyl-hexyl columns with different particle sizes were tested for better resolution. By decreasing the column particle size to 2.5 μm while extending the column length to 50 mm, an acceptable chromatographic separation was obtained with a run time of 2 min (Fig. 2). Since the injection takes about 0.2 min for each consecutive injection, a total of 27 samples can be analyzed using this method per hour. Table 2 summarizes experimental parameters for detection of phytoestrogens in 15, 5 and 2 min LC–MS/MS runs.

Deprotonated molecular ions $[M-H]^-$ of all phytoestrogen standards were induced to fragment in the collision cell, and after optimization, the most abundant product ions among the resulting product ion spectra were chosen for quantitative MRM of all phytoestrogens (Table 1).

3.2. Method validation

The methods were validated in accordance with the FDA guidance for bioanalytical method validation and also based on the paper of Shah et al. [33,34]. A full validation was performed for rat serum based on the following criteria.

3.2.1. Linearity and dynamic range

The most desirable analytical method typically combines an efficient chromatographic separation with short run time and required sensitivity. An epidemiological study shows that the plasma concentrations of phytoestrogens such as daidzein (20 μg/L) and genistein (40 μg/L) vary 5–50 times among participants [35]. It is therefore, desirable to develop a LC–MS/MS assay method with a wide dynamic range. This assay was linear for each of the analytes over a range of 1–5000 ng/mL in serum with correlation coefficients >0.99, except for dihydrodaizein, whose lower limit of quantification (LLOQ) was 2 ng/mL. At the LLOQ of phytoestrogens accuracy ranged from 95.53 to 114.83% with precision in the range of 3.08–13.49%. At all concentrations, accuracy ranged from 87.53 to 114.83% and precision ranged from 0.83 to 14.85%. These results indicated a linear relationship between the peak areas and concentrations of all standard phytoestrogens in this developed assay.

The accuracy and precision (presented as %CV) of this analytical method were determined using QC samples at concentrations 50, 500 and 2000 ng/mL in five to six replicates. The performance characteristics of the method were established by validation procedures employing assays with standard solutions, sample blanks and spiked samples. The intra- and inter-batch precision and accuracy were found to be well within acceptable limits as described above (Table 3). At the lowest quality control (50 ng/mL), the intra-day precisions for the phytoestrogens were less than 15% and the accuracies were between 80 and 114.66%. The inter-day precision all concentrations ranged from 2.82 to 8.59%. This ultra-fast method is very useful for high-throughput analysis of phytoestrogens and proved to be reproducible, reliable and the results were comparable to those obtained by regular flow LC/MS/MS analysis.

3.2.2. Specificity and selectivity

Representative MRM chromatograms of extracted blank serum and blank serum spiked with phytoestrogens (50 ng/mL) and internal standards (IS) are shown in Fig. 3. The LC–MS/MS method demonstrated high specificity because only ions derived from phytoestrogens and the internal standards were observed, indicating that there was no endogenous substance in serum interfering in the analysis of phytoestrogens.

3.2.3. Carry over

Carry over is one of the most commonly encountered problems in the quantification of metabolites in biological samples by LC/MS/MS. As can be seen in Fig. 4, when a methanol blank was injected right after the highest level of phytoestrogens spiked serum sample (5000 ng/mL), little or no detectable carryover was observed on the column. Note that full-scale for the methanol blank was 270 times smaller than for the phytoestrogen standards. Since the peak intensities were also much lower, the carryover was estimated to be less than 0.2%.

3.2.4. Matrix effects and recovery

Evaluating the matrix effect (ion suppression or enhancement) has become an integral part of method development and valida-

Table 4
Calculated % matrix effects of serum on the peak area response of phytoestrogens and internal standard.

Analyte	Concentration (ng/mL)	Matrix effect (%)
Equol	50	2.78
	500	18.28
	2000	4.55
Daidzein	50	16.38
	500	9.08
	2000	2.51
Dihydrodaidzein	50	13.22
	500	15.21
	2000	3.00
O-DMA	50	1.22
	500	14.52
	2000	−0.41
Genistein	50	30.99
	500	28.51
	2000	3.84
Glycitein	50	21.01
	500	25.33
	2000	−0.55
Formononetin	50	34.95
	500	28.18
	2000	4.50
Coumestrol	50	31.90
	500	20.57
	2000	−2.42
Biochanin A	50	23.82
	500	14.85
	2000	20.62
Enterodiol	50	15.15
	500	9.82
	2000	−12.03
Enterolactone	50	−13.66
	500	1.65
	2000	−12.29
Chrysin (IS)	200	25.09%

Table 5
Stability of quality control samples.

Compound	Nominal concentration (ng/mL)	Mean measured concentration (ng/mL)	
		Autosampler at 4 °C, 72 h	Long storage −20 °C, 2 months
Equol	50	43.35 ± 2.50	45.68 ± 3.98
	500	487.80 ± 9.20	475.66 ± 30.16
	2000	1793.33 ± 67.42	1921.66 ± 94.74
Daidzein	50	47.03 ± 2.50	50.83 ± 1.87
	500	534.20 ± 21.05	491.66 ± 7.17
	2000	1848.33 ± 72.77	1861.66 ± 71.67
Dihydrodaidzein	50	45.55 ± 1.97	47.52 ± 5.23
	500	485.83 ± 26.35	219.20 ± 15.90
	2000	1738.33 ± 85.18	828.50 ± 27.01
O-DMA	50	48.31 ± 3.75	54.80 ± 5.67
	500	469.16 ± 24.01	534.66 ± 28.57
	2000	1861.66 ± 114.61	2151.66 ± 110.89
Genistein	50	50.90 ± 3.19	51.16 ± 3.34
	500	487.33 ± 33.15	497.33 ± 37.59
	2000	1875.00 ± 116.40	2190.00 ± 11.83
Glycitein	50	44.31 ± 2.44	40.15 ± 1.98
	500	481.00 ± 39.11	489.50 ± 28.26
	2000	1886.66 ± 87.10	2045.00 ± 191.91
Formononetin	50	47.36 ± 4.16	47.58 ± 3.22
	500	512.33 ± 26.41	507.66 ± 27.82
	2000	2018.33 ± 106.09	1925.00 ± 167.06
Coumestrol	50	46.26 ± 6.68	56.80 ± 2.37
	500	549.33 ± 36.74	498.00 ± 26.1
	2000	2120.00 ± 104.30	1905.00 ± 128.17
Biochanin A	50	52.47 ± 2.27	56.10 ± 1.49
	500	444.00 ± 29.81	523.00 ± 23.34
	2000	1893.33 ± 202.06	2130.00 ± 88.31
Enterodiol	50	44.96 ± 3.45	46.84 ± 2.47
	500	488.16 ± 13.04	489.83 ± 20.79
	2000	1906.66 ± 68.89	1963.33 ± 119.27
Enterolactone	50	52.20 ± 3.27	48.73 ± 6.55
	500	495.50 ± 16.92	484.66 ± 34.44
	2000	1801.66 ± 75.70	1678.00 ± 66.55

tion [36,37]. Blank serum samples were extracted, then spiked and analyzed for potential interferences by endogenous matrix components. The matrix effects from serum on each phytoestrogen analytes are listed in Table 4. At 5, 500 and 2000 ng/mL concentration levels, the analytes had matrix effects within the range of −13 to 34%. These results indicated that there were minor to moderate matrix effects for phytoestrogens in serum [38].

Recovery studies of phytoestrogens were performed at concentrations of 50, 500 and 5000 ng/mL by comparing the peak areas of the extracted plasma samples with those of post-spiked samples. Overall mean extraction recoveries at these concentrations for equol, daidzein, dihydrodaidzein, *O*-DMA, genistein, glycitein, formononetin, coumestrol, biochanin A, enterodiol, enterolactone, were 83.20, 82.05, 88.43, 75.89, 57.48, 86.20, 85.15, 77.09, 65.59, 86.66 and 81.43%, respectively.

3.2.5. Stability

The stability of QC samples was investigated thoroughly to evaluate their stability in serum samples under different conditions (autosampler and long term storage stability). The stability data are summarized in Table 5. Phytoestrogens are stable in serum for at least 72 h of storage in the autosampler. The autosampler stability was evaluated at 4 °C over 72 h and precision of QC samples ranged from 1.88 to 14.44, indicating that they are stable for at least 72 h in human serum when stored at 4 °C.

Repeated freeze and thawing of plasma samples spiked with phytoestrogens did not affect their stability. The longer term (over 2 months) stability of phytoestrogens in human serum was evaluated by analyzing frozen QC samples in six replicates. All compounds showed acceptable range of concentration except for dihydrodaidzein at concentrations 500 and 2000 ng/mL (Table 5). It is possible that dihydrodaidzein may decompose when stored over 2 months in human serum.

3.3. Analysis of phytoestrogens in serum samples

The method described was successfully applied for quantification of phytoestrogens in serum samples collected from a study of dietary and serum phytoestrogens and women's health conditions in midlife (Table 6). The samples were analyzed in duplicate.

Table 6
Mean concentration, standard deviation and %CV for phytoestrogen levels in human serum samples (ng/mL).

	Equol	Daidzein	DHD	*O*-DMA	Genistein	Glycitein	Formononetin	Biochanin A	Enterodiol	Enterolactone
Mean	24.17	27.43	31.61	19.24	26.83	12.49	0.26	1.05	5.26	9.99
STDV	0.72	0.72	1.27	0.51	1.00	1.29	0.02	0.06	0.23	0.15
%CV	2.98	2.63	4.00	2.65	3.72	10.30	8.00	6.03	4.43	1.49

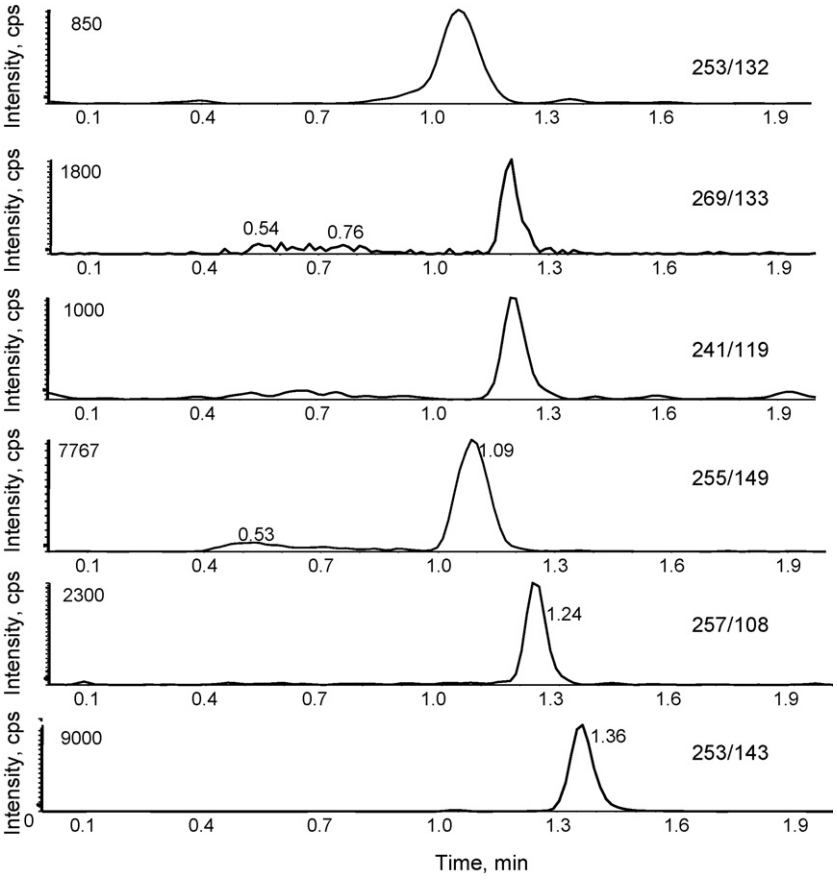

Fig. 5. MRM chromatograms from a human serum sample showing daidzein (27.94 ng/mL), genistein (27.54 ng/mL), equol (24.68 ng/mL), DHD (32.51 ng/mL), O-DMA (19.60 ng/mL) and IS (200 ng/mL).

MRM chromatograms showing detection of various phytoestrogens in a serum sample are shown in Fig. 5. The most abundant phytoestrogens were dihydrodaidzein (32.51 ng/mL), followed by daidzein (27.94 ng/mL) and genistein (27.54 ng/mL). No coumestrol was detected from the sample. It indicates that isoflavones daidzein and genistein are the major components in their diet. These data show the applicability of this assay in quantification of phytoestrogens in large population based studies.

4. Conclusion

An accurate, reproducible and ultra-fast LC–MS/MS assay for the quantification of phytoestrogens in human plasma has been developed and validated. The assay permits analysis over a range of 1–5000 ng/mL of phytoestrogens using 200 μL of aliquots of human plasma with precise and accurate quantification. Using this method, we have now analyzed over 2000 serum samples, indicating that this assay has adequate sensitivity and specificity to perform high-throughput analysis of a large number of samples obtained from epidemiological studies.

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