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High throughput quantification of phytoestrogens in human urine and serum using liquid chromatography/tandem mass spectrometry (LC–MS/MS)

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Abstract

Phytoestrogens are currently the subject of intense study owing to their potential protective effects against a number of complex diseases. However, in order to investigate the interactions between phytoestrogens and disease state effectively, it is necessary to have analytical methods which are sensitive, reproducible, and require low sample volumes. We report an assay for three isoflavones (daidzein, genistein, and glycitein), two metabolites of daidzein (equol and O-desmethylangolensin), three lignans (secoisolariciresinol, enterodiol, and enterolactone), and one flavanone (naringenin) in human urine and serum. A high throughput of samples has been achieved via the use of 96-well plate sample extraction and liquid chromatography/tandem mass spectrometry (LC–MS/MS) analysis incorporating column switching, thus making the assay suitable for use on large sample numbers, such as those found in epidemiological studies. The robustness of the assay was proven via the comparison of data generated on two different LC–MS/MS systems, with and without column switching.

Keywords: Phytoestrogens; Isoflavones; Lignans; LC–MS/MS; Urine; Serum; Column switching

1. Introduction

Phytoestrogens are diphenolic compounds that naturally occur in a variety of plants, some of which subsequently enter the human food chain. They can be further divided into several sub-groups, the most studied of which are the isoflavones and lignans. Isoflavones occur in high concentrations in soy foods, where they exist mainly as glycosides, whereas lignans are rather more widespread, occurring in a variety of grains and seeds, and in particularly high concentrations in linseed. Phytoestrogens are structurally similar to 17β-estradiol and exhibit both estrogenic and anti-estrogenic activity in the body.

Interest in these compounds stems from the belief that they may offer protective effects against a number of complex diseases, such as certain types of cancer [1–7], cardiovascular disease [8–10], osteoporosis [11–15], and menopausal symptoms [16,17], and can also affect brain function [18]. In populations where phytoestrogen consumption is high, such as Japan, the incidence of many of these diseases is much lower than in the West, where consumption of phytoestrogens is not as common. However, it is known that endogenous sex hormones are associated with an increased risk of breast cancer in women, even at low levels [19], and so the fact that phytoestrogens may exhibit an estrogenic, and therefore potentially a detrimental, effect on human health is also of concern. For example, a recent study of women in the UK found that exposure to isoflavones was associated with increased breast cancer risk, significantly so for equol and daidzein, although the number of cases used was relatively small [20].

In order to probe more efficiently the potential benefits, or adverse effects, of phytoestrogen consumption, it is necessary to develop analytical methodologies which are capable of the sensitive and accurate quantification of a number of analytes in low volumes of human biofluids and which are also capable of

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a high throughput of samples. A range of analytical techniques has been used to quantify phytoestrogens in urine, plasma, and serum. HPLC, with UV or coulometric array detection, has the advantages of low cost and ease of use but can suffer from poor sensitivity and selectivity [21–25]. A technique that has been extensively used for large studies is time-resolved fluoroimmunoassay (TR-FIA), with methods developed for the analysis of enterolactone [26,27], genistein [28], daidzein [28], O-desmethylangolensin (O-DMA) [29], and equol [30]. TR-FIA has the advantages of high sensitivity and rapid analysis. However, the specificity of TR-FIA is lower than that offered by techniques such as mass spectrometric detection. This reduced specificity can lead to measurement errors if compounds with similar structures to the analyte of interest are present [30]. A further limitation is that, unlike chromatographic assays, only one analyte can be measured per assay.

Mass spectrometry, coupled with chromatography, has often been used as a means to detecting phytoestrogens owing to its intrinsic sensitivity and selectivity. Gas chromatography–mass spectrometry (GC–MS) has been used for some time for the analysis of phytoestrogens in biological fluids [31–38]. Major drawbacks to many GC–MS assays are the fact that large sample volumes are required, and that sample preparation techniques are commonly complex and laborious, often involving multi-stage purification procedures. A further consideration for GC–MS analysis is that phytoestrogens are polar, involatile compounds and so they require derivatisation prior to analysis. A procedure has been described to analyse low volumes of human urine using a simple one-stage sample purification, however, the sensitivity using selected ion monitoring (SIM) was not sufficient for the analysis of plasma or serum from subjects consuming a normal Western diet, which is low in phytoestrogens [32].

Recent years have seen a sharp increase in the number of assays developed for phytoestrogens using liquid chromatography–mass spectrometry (LC–MS) [39–46]. Both electrospray and atmospheric pressure chemical ionisation (APCI) have been used to ionise analytes in both positive and negative ion modes and various types of mass spectrometer have been employed including ion traps, single quadrupole, and triple quadrupole instruments. The greatest sensitivity and selectivity is achieved using liquid chromatography/tandem mass spectrometry (LC–MS/MS) via the use of triple quadrupole mass spectrometers in selected reaction monitoring (SRM) mode. An advantage of LC–MS/MS is that sample preparation is normally simplified and, unlike GC–MS, sample extracts do not require derivatisation prior to analysis.

In order to be suitable for the analysis of samples from large, epidemiological studies, an assay must have sufficient sensitivity and work with a low volume of sample. However, in order to achieve a suitable throughput, the time taken for sample extraction and analysis should also be as rapid as possible. We now describe a technique for the analysis of nine phytoestrogens (genistein, daidzein, equol, O-DMA, glycitein, enterodiol, enterolactone, naringenin, and secoisolariciresinol) (Fig. 1) using, for the first time, solid-phase extraction in 96-well plate format. Sample analysis time has been significantly reduced using a column switching technique without any deterioration

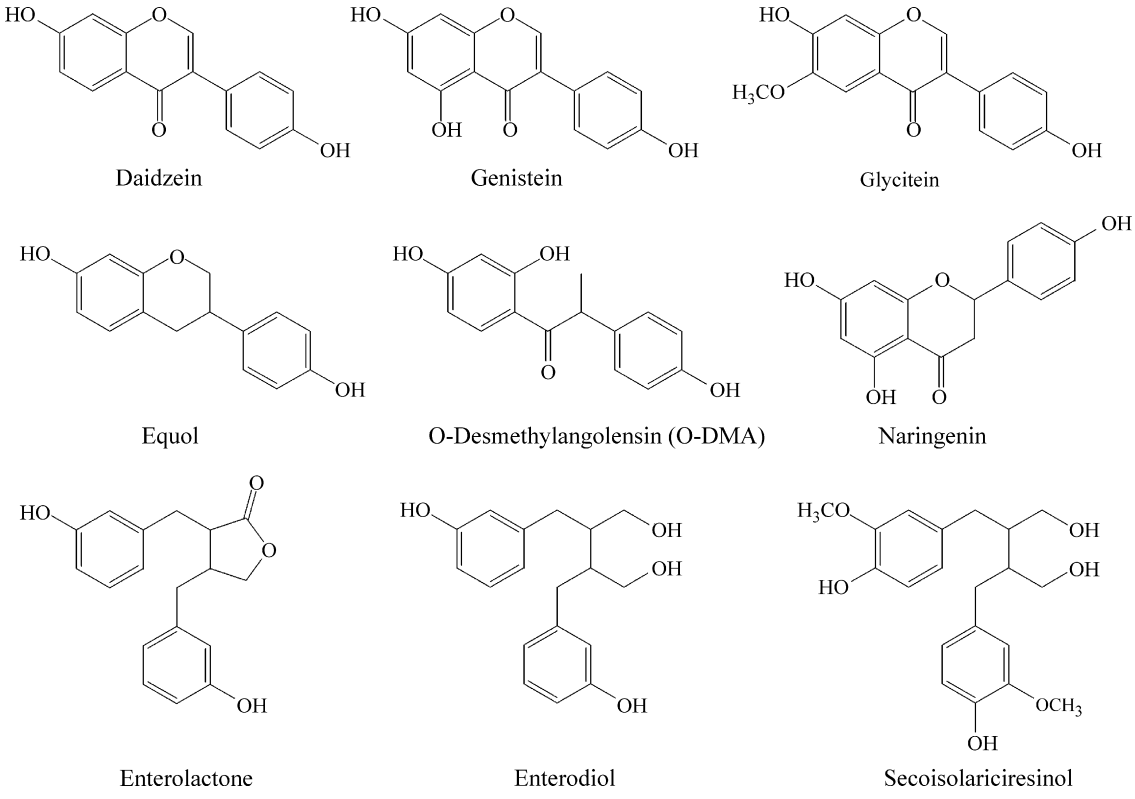

Fig. 1. Structures of the phytoestrogens analysed.

in performance. The assay has also been cross-validated on two completely different LC–MS/MS systems, with and without column switching.

2. Experimental

2.1. Chemicals

Daidzein, O-desmethylangolensin, enterodiol, enterolactone, equol, genistein, glycitein, naringenin, and secoisolariciresinol were purchased from Plantech UK (Reading, Berkshire, UK). [$^{13}C_3$]Daidzein, [$^{13}C_3$]O-desmethylangolensin, [$^{13}C_3$]enterodiol, [$^{13}C_3$]enterolactone, [$^{13}C_3$]equol, [$^{13}C_3$]genistein, and [$^{13}C_3$]glycitein were purchased from Dr. Nigel Botting (University of St. Andrews, Fife, UK). β-Glucuronidase from *Helix pomatia*, type HP-2, was purchased from Sigma (Poole, Dorset, UK).

2.2. Samples

Urine samples were taken from women who had been diagnosed with invasive breast cancer one year earlier, this was after their treatments had finished and they had returned to a more normal diet, although frequently adding self-prescribed supplements. Their treatment was simple surgical excision followed by local radiotherapy. Hormonal treatments were not used as these patients had early disease, and phytoestrogen data were not used to modify their treatment or dietary advice. Within an hour of urine collection, 20 mL aliquots of urine were centrifuged at 3000 × *g* for 30 min in an unrefrigerated centrifuge to remove cells and debris. Urine samples were then transferred to polypropylene tubes and stored in a −80 °C freezer. Samples were shipped between laboratories still frozen on cardice where they were stored at −80 °C prior to analysis.

Urine samples were collected from healthy male and female volunteers from the UK ($n = 27$). Following characterisation of these urine samples for their phytoestrogen content, quality control samples were generated by mixing the urine samples such that suitable natural levels of phytoestrogens were present in the resulting pooled urine samples.

2.3. Purification of Helix pomatia digestive juice

β-Glucuronidase from *Helix pomatia* digestive juice, which also contains aryl sulfatase activity, is commonly used to convert glucuronic acid and sulphate conjugates to the aglycone form. However, it has been shown that this enzyme mixture can contain appreciable levels of some phytoestrogens [47]. Therefore, the enzyme was purified to remove phytoestrogen contaminants as described previously [47]. Briefly, a Strata-X solid-phase extraction cartridge (1 g, 20 mL, Phenomenex, Macclesfield, Cheshire, UK) was conditioned with methanol and sodium acetate buffer (140 mM, pH 5). A 10% (v/v) solution of *Helix pomatia* digestive juice in sodium acetate buffer (140 mM, pH 5) was then passed directly through the cartridge and the purified enzyme solution collected in a clean tube.

2.4. Sample preparation

Urine (200 μL) was allowed to thaw to room temperature before adding 240 μL of purified β-glucuronidase/aryl sulfatase from *Helix pomatia*, 10% (v/v) in sodium acetate buffer (140 mM, pH 5), and 10 μL of a mixture of the seven [$^{13}C_3$]-labelled internal standards in methanol (1 μg/mL). Conjugates were allowed to hydrolyse to the aglycones by heating at 37 °C overnight.

Aglycones were extracted from the hydrosylate via solid-phase extraction (SPE) on 96-well Strata-X plates (30 mg, Phenomenex) using a Multiprobe II HT EX (Perkin Elmer, Boston, MA, USA) robotic liquid handling system. Prior to SPE, methanol was added to each sample (200 μL). Plates were conditioned with methanol (700 μL) and 30% methanol (700 μL) before loading the samples on to the plate. The plate was then washed with 40% methanol (600 μL) before elution of the aglycones in 1:1 acetonitrile/methanol (600 μL). The eluates were dried under nitrogen at 40 °C and reconstituted in 40% methanol (100 μL).

2.5. LC–MS/MS

Two different instruments were used for LC–MS/MS analysis. The first was a Quattro Premier triple quadrupole mass spectrometer (Waters, Manchester, UK), fitted with an electrospray probe. This instrument was interfaced to a 1525μ HPLC pump and 2777 autosampler (Waters). A Rheos 2000 HPLC pump (Flux Instruments, Basel, Switzerland) was used as an auxiliary pump for column switching applications. The second system was a Sciex 4000 QTrap (Applied Biosystems, Warrington, UK), fitted with a TurboIonSpray probe. The instrument was interfaced to a LC-10ADVPμ HPLC pump and a SIL-HT autosampler (Shimadzu, Milton Keynes, UK).

2.5.1. Quattro Premier LC–MS/MS operating conditions

A binary solvent system was used consisting of 60% of 0.1% ammonium acetate in water (pH 4.8) plus 40% methanol as solvent A, and methanol as solvent B. Targa C_{18} columns were used for the analysis (2.1 mm × 150 mm, 3 μm; Higgins Analytical, CA, USA). A gradient program was used, at 200 μL/min, as follows: solvent B was increased from 0 to 25% over 1 min and then from 25 to 60% over the next 7 min. The flow rate was then increased to 250 μL/min and the column flushed with 100% solvent B for 1 min. The flow rate was then returned to 200 μL/min and the solvent composition changed to 100% solvent A for 1 min (total run time 10 min). During the 10 min gradient, an auxiliary pump was equilibrating a second, identical HPLC column by pumping 100% solvent A at 200 μL/min through the column, via an electronically operated model E36 10-port switching valve (Valco Instruments Company Inc., Houston, TX, USA). At 9.8 min into the run, the position of the 10-port switching valve was switched via a signal from the 1525μ pump, programmed through the controlling software, MassLynx 4.0 (Waters). This allowed one column to be re-equilibrated whilst a sample was analysed via gradient chromatography on the second column (see Fig. 2).

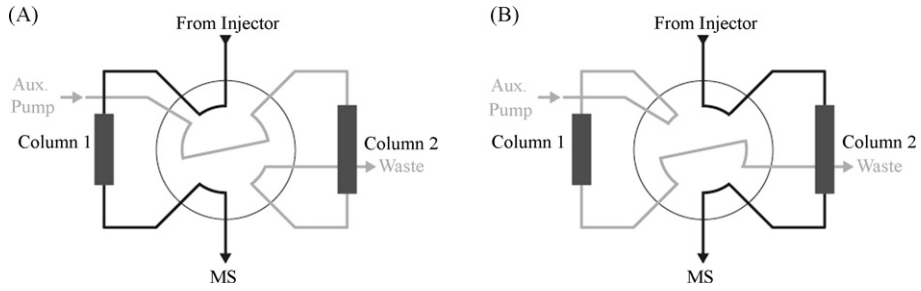

Fig. 2. Schematic representation of the column switching process, showing the solvent flow paths in the two different positions of the 10-port valve.

The flow from the column running the gradient program was fed directly into the electrospray probe of the Quattro Premier.

All analyses were carried out in negative ion electrospray mode, with the capillary voltage at 2.5 kV. The source temperature was maintained at 120 °C and the desolvation temperature at 350 °C. Nitrogen was used as both desolvation and cone gas at 500 and 25 L/h, respectively. Cone voltages and collision energies were optimised for each analyte individually and the most abundant product ion chosen for the selected reaction monitoring (SRM) transition. Argon was used as collision gas at a pressure of 2.5×10^{-3} mbar. The instrument was monitored in SRM mode, with the following transitions monitored for each analyte: *m/z* 253 → 224 for daidzein, 256 → 227 for [$^{13}C_3$]daidzein, 257 → 109 for O-DMA, 260 → 137 for [$^{13}C_3$]O-DMA, 301 → 253 for enterodiol, 304 → 255 for [$^{13}C_3$]enterodiol, 297 → 253 for enterolactone, 300 → 255 for [$^{13}C_3$]enterolactone, 241 → 121 for equol, 244 → 123 for [$^{13}C_3$]equol, 269 → 133 for genistein, 272 → 183 for [$^{13}C_3$] genistein, 283 → 268 for glycitein, 286 → 271 for [$^{13}C_3$]glycitein, 271 → 119 for naringenin, and 361 → 165 for secoisolariciresinol. The dwell time was 50 ms for each transition. The system was controlled using MassLynx 4.0 software (Waters).

2.5.2. 4000 QTrap LC–MS/MS operating conditions

The same binary solvent system and HPLC column were used as described in Section 2.5.1, however, only a single column was used in this case. A gradient program was used, at 200 μL/min, as follows: solvent B was increased from 0 to 25% over 1 min and then from 25 to 60% over the next 7 min. The flow rate was then increased to 250 μL/min and the column flushed with 100% solvent B for 1 min. The column was then re-equilibrated with 100% solvent A for 5 min before returning the flow rate to 200 μL/min (total run time 14 min). The flow from the column was fed directly into the TurboIonSpray probe of the 4000 QTrap.

All analyses were carried out in negative ion electrospray mode, with a capillary voltage of 4.3 kV. The source temperature was 600 °C. Nitrogen was used as both curtain gas (10 arbitrary units) and collision gas (set to "high"). The declustering potential, collision energy, and collision cell exit potential were optimised for each analyte individually. SRM transitions were as described in Section 2.5.1. The dwell time was 50 ms for each transition. The system was controlled using Analyst 1.4 software (Applied Biosystems).

2.6. Calibration curves

Analytes were quantified by means of calibration curves formed from known concentrations of mixtures of analyte standards, with constant levels of internal standards. These standards were spiked in to synthetic urine and subjected to the normal sample preparation procedures. Nine calibration levels were used (0, 0.1, 0.25, 0.5, 1, 10, 100, 500, and 2000 ng/mL). Calibration standards were injected at the beginning and the end of each batch and the final calibration curves constructed from both sets of points. [$^{13}C_3$]-Labelled phytoestrogens were used as internal standards for the analogous compounds. [$^{13}C_3$]Genistein was used as internal standard for naringenin and [$^{13}C_3$]enterodiol was used as internal standard for secoisolariciresinol.

3. Results and discussion

3.1. Extraction of aglycones from the matrix

Following hydrolysis of the phytoestrogen glucuronides and sulfates to the aglycone forms, it is then necessary to selectively extract these compounds from any interferences that may be present in the sample matrix. Many published methods for phytoestrogen extraction from biological fluids involve complex sample preparation techniques, involving multiple extraction steps. However, simple, one-step solid-phase extraction procedures have recently been introduced, using C_{18} cartridges [32,40]. Whilst this has increased the throughput of the sample preparation technique, using cartridges is still time consuming, even with robotic sample processors. In order to develop a faster sample extraction procedure, the use of SPE in 96-well plate format, using a polymeric sorbent, was investigated.

As all human urine samples tested contained appreciable levels of at least some of the analytes of interest, recovery studies were carried out using synthetic urine spiked with a mixture of phytoestrogens at three different levels, 10, 50, and 250 ng/mL ($n = 3$ for each). Anthraflavic acid was added as external standard during dissolution of samples prior to LC–MS/MS analysis. Relative responses were obtained by dividing the peak areas for the analytes by the peak area for anthraflavic acid and recoveries calculated by comparing the relative responses of samples spiked both before and after SPE. The recoveries (Table 1) were high for all analytes across all concentrations tested.

To ensure accurate quantification, [$^{13}C_3$]-labelled phytoestrogens were used as internal standards for all analytes

Table 1
Mean recoveries (%) for the SPE procedure ± standard deviation

	Daidzein	O-DMA	Enterodiol	Enterolactone	Equol	Genistein	Glycitein	Naringenin	Secoisolariciresinol
2 ng (10 ng/mL) spike	93 ± 3	88 ± 12	99 ± 3	103 ± 6	96 ± 4	82 ± 8	93 ± 8	87 ± 10	103 ± 10
10 ng (50 ng/mL) spike	93 ± 2	93 ± 3	99 ± 0	98 ± 1	95 ± 6	90 ± 6	92 ± 5	92 ± 4	95 ± 5
50 ng (250 ng/mL) spike	92 ± 7	97 ± 2	89 ± 5	95 ± 4	91 ± 2	93 ± 3	92 ± 2	95 ± 5	89 ± 8

except naringenin and secoisolariciresinol, for which isotopically labelled compounds were not available. These internal standards take account of any losses during the extraction procedure. However, for high sensitivity analyses, it is important that the recoveries are high. [$^{13}C_3$]Genistein was chosen as an internal standard for naringenin, and [$^{13}C_3$]enterodiol as internal standard for secoisolariciresinol. These internal standards were chosen owing to the structural similarity between the internal standard and analyte, and similar SPE recoveries.

The extraction procedure described for urine has also been successfully applied to serum, with the only addition being that serum samples are centrifuged after the addition of methanol, which takes place after hydrolysis and prior to SPE.

3.2. LC–MS/MS analysis

3.2.1. Column switching

Whilst the use of 96-well SPE significantly raises throughput in terms of sample preparation, this is of no benefit unless the sample analysis time is also rapid. Due to the structural similarity of the analytes, and the possibility of false positives from matrix interferences if analytes are eluted too quickly, a gradient HPLC method was necessary. The method used was based upon a shallow gradient over 8 min, which allowed separation of the analytes of interest. This was then followed by a column flushing stage which took 1 min and removed any remaining hydrophobic material from the HPLC column. Following the flush, the column was re-equilibrated back to the starting mobile phase conditions over a 5-min period.

In order to reduce the overall analysis time, a column switching HPLC procedure was introduced which allowed one column to run a gradient, followed by column flush, whilst a second, identical, column was equilibrated using an auxiliary pump. The procedure is shown schematically in Fig. 2. The column switching system consisted of one binary gradient HPLC pump, one isocratic HPLC pump, one electronically controlled 10-port switching valve, and two HPLC columns. All components were connected via the 10-port valve as shown in Fig. 2. In the first switching state (Fig. 2A) the gradient pump was connected, via the injector, to column 1. When the injection was made, the sample passed through column 1 and was eluted via a solvent gradient as normal. During this time, column 2 was connected to the auxiliary pump which was pumping 100% solvent A (starting mobile phase conditions) through column 2. After 9.8 min, a signal was sent to the 10-port switching valve, causing the 10-port valve to switch to the second position (Fig. 2B). In this position, the flow from the auxiliary pump was then diverted to column 1, thus allowing column 1 to be re-equilibrated. The flow from the gradient pump was then diverted to column 2. As column 2 had previously been equilibrated during the gradient analysis on column 1, an injection could be made immediately, thereby reducing the overall analysis time from 14 to 10 min.

3.2.2. Sensitivity and dynamic range

Both LC–MS/MS systems were found to be capable of quantification at the lowest calibration point of 0.1 ng/mL, which was set as the target limit of quantification for both systems. Fig. 3 shows the extracted SRM chromatogram for 0.1 ng/mL enterolactone on both the 4000 QTrap and Quattro Premier. The signal-to-noise ratio was found to be two times higher on the 4000 QTrap (27:1 versus 13:1) at this level.

As the concentrations of phytoestrogens vary considerably between individual subjects, it was important that the assay performed over a wide dynamic range. The desired dynamic range was from 0.1 to 2000 ng/mL. Both instruments performed well over the required range, with coefficients of determination (R^2) > 0.99, however, there were differences in the type of regression that had to be used for the two instruments.

The Quattro Premier exhibited a linear response for all analytes over the concentration range 0.1–2000 ng/mL, as shown in Fig. 4 for O-DMA. The 4000 QTrap required a quadratic regression for all analytes as indicated in Fig. 5. However, this regression also produced $R^2 > 0.99$ and exhibited reproducibility akin to that of the Quattro Premier (see Section 3.3). The range up to 2000 ng/mL covered the majority of phytoestrogen concentrations found in real urine samples. The analyte which is most likely to be found above this level is enterolactone. As concentrations above 2000 ng/mL started to approach the level at which the signal would saturate, an additional SRM transition was included for enterolactone (297 → 119) which gave a lower response than the primary transition (297 → 253). This allowed the use of an additional calibration point at 5000 ng/mL for enterolactone, thus increasing the number of samples that can be quantified for this analyte without dilution and re-analysis.

3.3. Quality control and reproducibility

In order to ensure the quality of data over time it was important to include quality control (QC) samples within each batch that represented the upper, median, and lower concentration ranges for all of the analytes. Ideally these samples should be natural samples, rather than samples that have been spiked with phytoestrogen aglycones, as phytoestrogens in the natural samples would be present in conjugated forms. A sample that has merely been spiked with aglycones would not allow for quality control of the hydrolysis component of the sample preparation procedure. Obtaining natural samples with suitable levels of all of the analytes is extremely difficult and so, in order to obtain

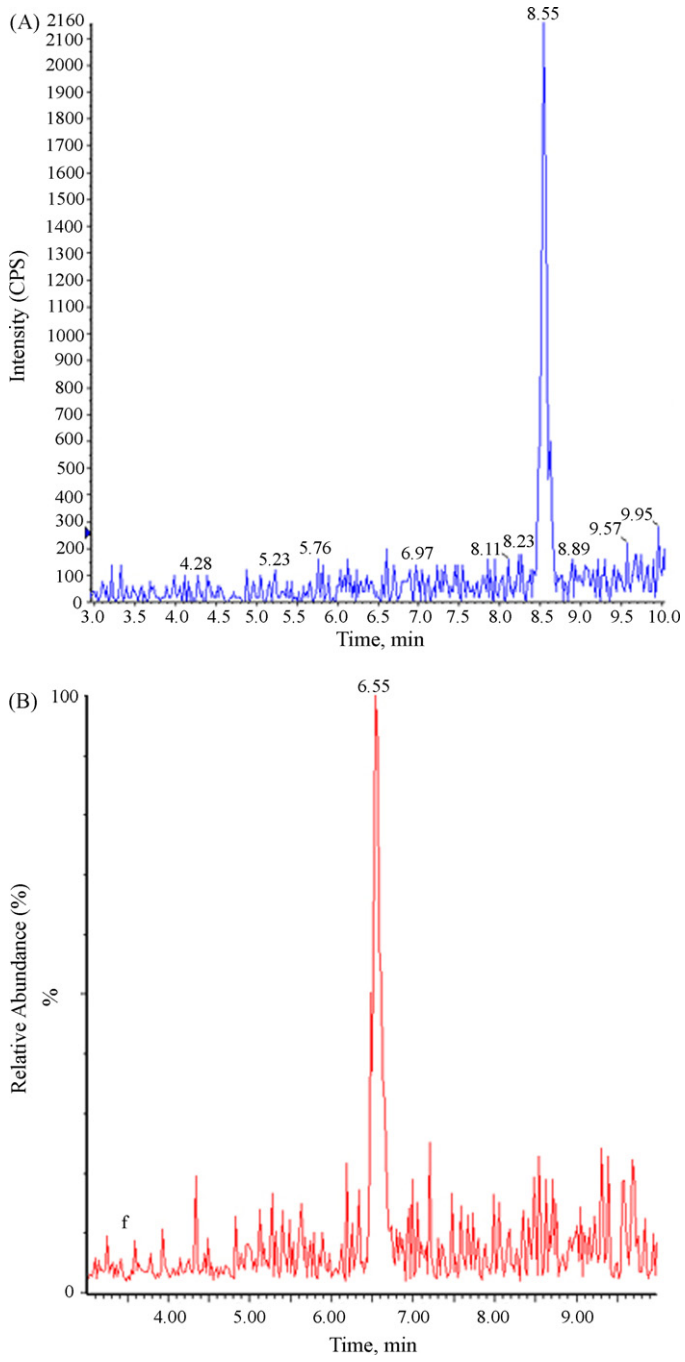

Fig. 3. Extracted SRM chromatogram for 0.1 ng/mL enterolactone on: (A) 4000 QTrap and (B) Quattro Premier.

suitable quality control samples, a number of urine samples were collected from volunteers and the phytoestrogen concentrations measured. Suitable amounts of various urine samples were then mixed such that, after extensive characterisation, the resulting urine mixtures were suitable as low, medium, and high QC samples. Each QC sample was analysed in duplicate in each batch. For the QC samples to pass, the calculated concentrations of each analyte were allowed to differ by no more than 15% from the target value.

The reproducibility of the assay was assessed by analysing the 3 quality control samples over 10 batches of samples. The

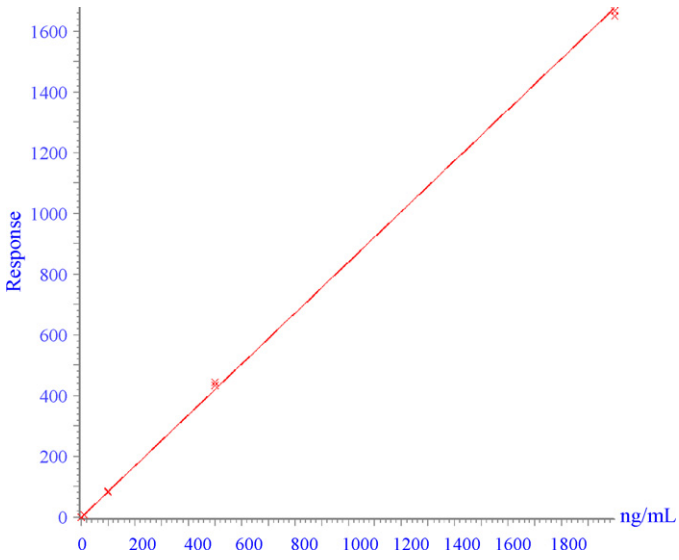

Fig. 4. Calibration curve for O-DMA on the Quattro Premier (linear regression, 1/*x* weighting).

average intra-assay coefficients of variation (CVs) for each analyte for the three quality control samples are shown in Table 2 and the inter-assay CVs shown in Table 3. Results are shown for both of the instruments used.

Intra-assay CVs were comparable for both instruments, with a mean CV of 3.5% across all analytes for the Quattro Premier and 3.0% for the 4000 QTrap, indicating a high degree of repeatability. Inter-assay CVs were also very low for the majority of analytes and again mean values were comparable for the two instruments used, at 6.8% for both the Quattro Premier and the QTrap. The highest inter-assay CV was found in the low QC sample for naringenin, which is an analyte without a [$^{13}C_3$]-labelled analogue as internal standard.

Reproducibility and repeatability for the analysis of serum samples was also investigated. Low, medium, and high concen-

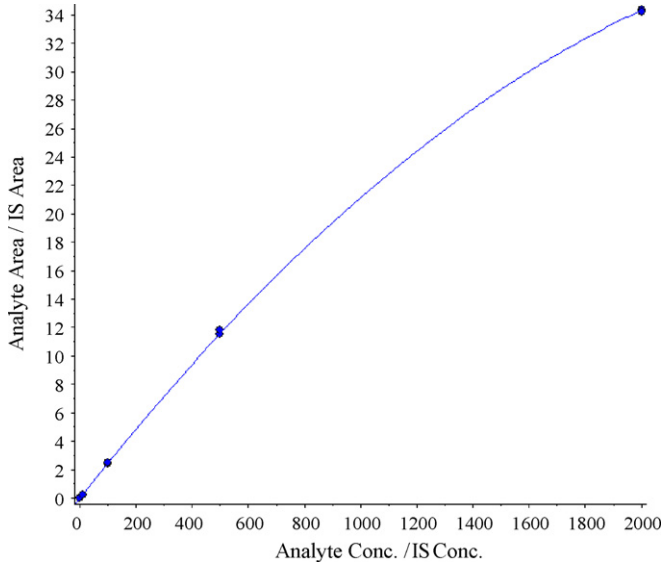

Fig. 5. Calibration curve for O-DMA on the 4000 QTrap (quadratic regression, 1/*x* weighting).

Table 2
Mean intra-assay CVs for three different QC samples on two different instruments

	Low QC		Medium QC		High QC	
	Quattro	4000 QTrap	Quattro	4000 QTrap	Quattro	4000 QTrap
Daidzein	3.3	2.7	1.7	1.1	2.5	2.4
O-DMA	5.3	4.7	2.8	2.2	2.1	1.3
Enterodiol	2.1	2.7	2.7	2.1	2.5	2.3
Enterolactone	1.3	3.0	2.0	2.6	2.6	2.2
Equol	5.4	6.3	5.5	3.4	3.7	1.7
Genistein	4.2	3.9	3.6	3.8	3.0	2.4
Glycitein	7.1	5.2	1.7	2.9	2.6	1.3
Naringenin	5.4	3.6	7.0	4.6	3.9	4.7
Secoisolariciresinol	4.3	3.1	3.6	3.3	3.3	2.0

Table 3
Inter-assay CVs for three different QC samples on two different instruments

	Low QC		Medium QC		High QC	
	Quattro	4000 QTrap	Quattro	4000 QTrap	Quattro	4000 QTrap
Daidzein	8.4	7.3	6.4	5.2	5.5	6.6
O-DMA	5.9	7.1	5.8	6.7	4.6	8.1
Enterodiol	6.5	5.0	5.5	6.1	4.2	5.8
Enterolactone	8.9	6.8	5.0	5.5	4.3	6.2
Equol	6.2	6.7	7.8	5.4	4.7	6.6
Genistein	8.2	8.4	6.0	5.6	6.1	6.6
Glycitein	7.6	5.7	4.8	5.8	4.4	6.7
Naringenin	14.1	13.9	8.6	8.6	7.3	5.9
Secoisolariciresinol	9.0	7.0	9.8	8.7	7.9	6.1

tration serum QC samples were analysed in duplicate across four batches of samples on the 4000 QTrap and found to be comparable to those found for urine samples. The mean inter-assay CV across all analytes was 5.5% (range 0.7–9.8%) and the mean intra-assay CV was 3.6% (range 1.2–6.5%).

3.4. Comparison of data generated on two different systems

In order to assess the robustness of the assay, three quality control urine samples, of low, medium, and high concentrations, were analysed on both the Quattro Premier with column switching and the 4000 QTrap without column switching. Data were collected over ten batches of samples, with two replicates of each quality control sample in each batch. The data from both instruments were compared using a *t*-test to establish whether there was any statistical difference in the data generated on the two instruments. The results are shown in Table 4 for all analytes in each of the three quality control samples. The data from both instruments were considered to be statistically equivalent if $p > 0.05$ for a two-tailed *t*-test. The only *p*-value found to be <0.05 was for enterolactone in the high QC sample. All other analytes gave statistically identical results on both instruments. Although the difference in enterolactone concentrations between instruments at high concentration was statistically significant, the difference only amounted to a deviation of 10% in the mean values, or a mean inter-instrument CV across ten batches of 7.8%.

Table 4
p-Values from a two-tailed *t*-test to compare data generated on two different instruments

Analyte	Low QC	Medium QC	High QC
Daidzein	0.322	0.206	0.930
O-DMA	0.398	0.384	0.468
Enterodiol	0.341	0.068	0.163
Enterolactone	0.341	0.211	3.9×10^{-7}
Equol	0.058	0.670	0.960
Genistein	0.321	0.964	0.847
Glycitein	0.369	0.495	0.051
Naringenin	0.128	0.053	0.113
Secoisolariciresinol	0.075	0.332	0.415

These data indicate that, whilst it is obviously preferable to run an entire study on a single instrument, the assay described is sufficiently robust that data produced on two different instruments, from different manufacturers, and using different HPLC procedures (column switching and conventional HPLC) could be pooled. The data also indicate that there are no disadvantages in terms of the quality of the data, in using column switching to increase throughput.

3.5. Levels of phytoestrogens in urine samples

Table 5 shows the levels of phytoestrogens found in 200 urine samples from women who had been diagnosed with invasive breast cancer one year earlier. The range in excretion between individuals was very large, with wide confidence intervals for

Table 5
Mean, median, standard deviation, range, and 95% confidence intervals for phytoestrogen levels in 200 urine samples (ng/mL)

	Equol	Daidzein	O-DMA	Genistein	Naringenin	Glycitein	Enterolactone	Enterodiol	Secoisolariciresinol
Mean	52	469	72	283	520	45	1691	234	124
Median	5	119	7	50	182	9	1047	62	69
Standard deviation	350	1177	269	815	1008	134	2029	553	273
Range	0–3495	1–8200	0–2521	0–6110	0–7596	0–1037	2–13156	0–4337	0.7–3200
95% confidence interval	4–101	306–632	35–109	170–396	381–660	26–63	1410–1972	157–311	86–162

all analytes. As indicated in the ranges shown, several very high concentrations were observed in individual samples for some analytes. The effect of several of these high concentration samples was to skew the mean concentration to a higher value. Therefore, median concentrations are also shown in Table 5.

The most abundant phytoestrogen was the lignan enterolactone. The sum of the median concentrations for the lignans was found to be over six times higher than the sum of the median concentrations for the isoflavones, and their metabolites. This reflects the fact that lignans are much more widespread in the UK diet than isoflavones.

Mean concentrations of equol, daidzein, O-DMA, genistein, glycitein, enterolactone, and enterodiol were higher than those previously found in a study of healthy women taking part in a prospective study of diet and cancer [32]. However, all samples are analysed blind and so it is impossible to assess whether the higher concentrations of phytoestrogens found in this sub-set of samples can be attributed to differences in the subjects used in the two studies, or their diets, until the study and further data analysis, is completed.

4. Conclusions

The LC–MS/MS methodology described here allows the analysis of sub-ppb concentrations of nine phytoestrogens in both human urine and serum with a very high degree of reproducibility. The fact that sample preparation takes place in 96-well plate format, and that the analysis time per sample can be reduced to 10 min through the use of column switching, means that the methodology is well-suited to the analysis of large numbers of samples, such as those found in epidemiological studies. The successful cross-validation of the assay on two different LC–MS/MS systems indicated that the methodology is sufficiently robust that data generated on different instruments could be pooled if necessary.

References

[1] H. Adlercreutz, Lancet Oncol. 3 (2002) 364.
[2] J. Wietrzyk, G. Grynkiewicz, A. Opolski, Anticancer Res. 25 (2005) 2357.
[3] J.L. Limer, V. Speirs, Breast Cancer Res. 6 (2004) 119.
[4] P.D. Gikas, K. Mokbel, Int. J. Fertil. Womens Med. 50 (2005) 250.
[5] D. Lechner, E. Kallay, H.S. Cross, Vitam. Horm. 70 (2005) 169.
[6] J.M. Holzbeierlein, J. McIntosh, J.B. Thrasher, Curr. Opin. Urol. 15 (2005) 17.
[7] M.B. Schabath, L.M. Hernandez, X. Wu, P.C. Pillow, M.R. Spitz, J. Am. Med. Assoc. 294 (2005) 1493.
[8] T.B. Clarkson, M.S. Anthony, Bailliere Clin. Endocrinol. 12 (1998) 589.
[9] M.S. Anthony, Arterioscler. Thromb. Vasc. Biol. 22 (2002) 1245.
[10] F.M. Sacks, Circulation 111 (2005) 385.
[11] Z.C. Dang, C. Lowik, Trends Endocrinol Metab. 16 (2005) 207.
[12] K.D.R. Setchell, E. Lydeking-Olsen, Am. J. Clin. Nutr. 78 (2003) 593S.
[13] M.J. Yamaguchi, Health Sci. 48 (2002) 209.
[14] B.H. Arjmandi, B.J. Smith, J. Nutr. Biochem. 13 (2002) 130.
[15] J.J.B. Anderson, S.C. Garner, Nutr. Res. 17 (1997) 1617.
[16] E.E. Krebs, K.E. Ensrud, R. MacDonald, T.J. Wilt, Obstet. Gynecol. 104 (2004) 824.
[17] J. Eden, Bailliere Clin. Endocrinol. 12 (1998) 581.
[18] E.D. Lephart, T.W. West, K.S. Weber, R.W. Rhees, K.D.R. Setchell, H. Adlercreutz, T.D. Lund, Neurotoxicol. Teratol. 24 (2002) 5.
[19] Endogenous Hormones and Breast Cancer Collaborative Group, J. Natl. Cancer. Inst. 94 (2002) 606.
[20] P.B. Grace, J.I. Taylor, Y.L. Low, R.N. Luben, A.A. Mulligan, N.P. Botting, M. Dowsett, A.A. Welch, K.T. Khaw, N.J. Wareham, N.E. Day, S.A. Bingham, Cancer Epidemiol. Biomarkers Prev. 13 (2004) 698.
[21] A.A. Franke, L.J. Custer, W. Wang, C.Y. Shi, Proc. Soc. Exp. Biol. Med. 217 (1998) 263.
[22] B.F. Thomas, S.H. Zeisel, M.G. Busby, J.M. Hill, R.A. Mitchell, N.M. Scheffler, S.S. Brown, L.T. Bloeden, J.J. Dix, A.R. Jeffcoat, J. Chromatogr. B 760 (2001) 191.
[23] T. Nurmi, H. Adlercreutz, Anal. Biochem. 274 (1999) 110.
[24] P.H. Gamache, I.N. Acworth, Proc. Soc. Exp. Biol. Med. 217 (1998) 274.
[25] K. Ouchi, P. Gamache, I. Acworth, S. Watanabe, Biofactors 22 (2004) 353.
[26] H. Adlercreutz, G.J. Wang, O. Lapcík, R. Hampl, K. Wähälä, T. Mäkelä, K. Lusa, M. Talme, H. Mikola, Anal. Biochem. 265 (1998) 208.
[27] K. Stumpf, M. Uehara, T. Nurmi, H. Adlercreutz, Anal. Biochem. 284 (2000) 153.
[28] G.J. Wang, O. Lapcík, R. Hampl, M. Uehara, N. Al-Maharik, K. Stumpf, H. Mikola, K. Wähälä, H. Adlercreutz, Steroids 65 (2000) 339.
[29] R. L'homme, E. Brouwers, N. Al-Maharik, O. Lapcík, R. Hampl, H. Mikola, K. Wähälä, H. Adlercreutz, J. Steroid Biochem. Mol. Biol. 81 (2002) 353.
[30] E. Brouwers, R. L'homme, N. Al-Maharik, O. Lapcík, R. Hampl, K. Wähälä, H. Mikola, H. Adlercreutz, J. Steroid Biochem. Mol. Biol. 84 (2003) 577.
[31] H. Adlercreutz, P. Kiuru, S. Rasku, K. Wähälä, T. Fotsis, J. Steroid Biochem. Mol. Biol. 92 (2004) 399.
[32] P.B. Grace, J.I. Taylor, N.P. Botting, T. Fryatt, M.F. Oldfield, S.A. Bingham, Anal. Biochem. 315 (2003) 114.
[33] G.E. Kelly, C. Nelson, M.A. Waring, G.E. Joannou, A.Y. Reeder, Clin. Chim. Acta 223 (1993) 9.
[34] K.D.R. Setchell, A.M. Lawson, L.M. McLaughlin, S. Patel, D.N. Kirk, M. Axelson, Biomed. Mass Spectrom. 10 (1983) 227.
[35] H. Adlercreutz, J. van der Wildt, J. Kinzel, H. Attalla, K. Wähälä, T. Mäkelä, T. Hase, T. Fotsis, J. Steroid Biochem. Mol. Biol. 52 (1995) 97.
[36] J. Tekel, E. Daeseleire, A. Heeremans, C. van Peteghem, J. Agric. Food Chem. 47 (1999) 3489.
[37] S.L. Pumford, M.M. Morton, A. Turkes, K. Griffiths, Ann. Clin. Biochem. 39 (2002) 281.
[38] M. Morton, O. Arisaka, A. Miyake, B. Evans, Environ. Toxicol. Pharm. 7 (1999) 221.

[39] D. Locati, S. Morandi, A. Cupisti, L. Ghiadoni, A. Arnoldi, Rapid Commun. Mass Spectrom. 19 (2005) 3473.
[40] P.B. Grace, J.I. Taylor, N.P. Botting, T. Fryatt, M.F. Oldfield, N. Al-Maharik, S.A. Bingham, Rapid Commun. Mass Spectrom. 17 (2003) 1350.
[41] D.B. Clarke, A.S. Lloyd, N.P. Botting, M.F. Oldfield, P.W. Needs, H. Wiseman, Anal. Biochem. 309 (2002) 158.
[42] A.A. Franke, L.J. Custer, L.R. Wilkens, L. Le Marchand, A.M.Y. Nomura, M.T. Goodman, L.N. Kolonel, J. Chromatogr. B 777 (2002) 45.
[43] L. Valentin-Blasini, B.C. Blount, H.S. Rogers, L.L. Needham, J. Exp. Anal. Environ. Epidemiol. 10 (2000) 799.
[44] C.O. Cimino, S.R. Shelnutt, M.J.J. Ronis, T.M. Badger, Clin. Chim. Acta 287 (1999) 69.
[45] S. Barnes, L. Coward, M. Kirk, J. Sfakianos, Proc. Soc. Exp. Biol. Med. 217 (1998) 254.
[46] L. Coward, M. Kirk, N. Albin, S. Barnes, Clin. Chim. Acta 247 (1996) 121.
[47] P.B. Grace, P. Teale, J. Chromatogr. B 832 (2006) 158.