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Improved high sensitivity analysis of polyphenols and their metabolites by nano-liquid chromatography-mass spectrometry

Landon Wilson^b, Ali Arabshahi^b, Brigitte Simons^d, Jeevan Prasain^{a,b,c}, and Stephen Barnes^{a,b,c,*}

^aDepartment of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, Alabama, USA

^bTargeted Metabolomics and Proteomics Laboratory, University of Alabama at Birmingham, Birmingham, Alabama, USA

^cO'Brien Acute Kidney Injury Center, University of Alabama at Birmingham, Birmingham, Alabama, USA

^dAB Sciex, Concord, Ontario, Canada

Abstract

This study was conducted to assess the value of a high resolution, high mass accuracy time-of-flight analyzer in combination with nanoliquid chromatography for the analysis of polyphenols and their metabolites. The goal was to create a method that utilizes small volumes of biological fluids and provides a significant improvement in sensitivity compared with existing methods. Accordingly, nanoLC-MS and nanoLC-pseudo-multiple reaction monitoring (MRM) methods were developed that had a lower limit of quantification of 0.5 nM for several polyphenols and were linear over 2–3 orders of magnitude ($R^2 > 0.999$). Using urine samples, the ability to observe and quantify polyphenols in such a complex biological fluid depended on much narrower mass windows (0.050 amu or less) on a TOF analyzer than those used on a quadrupole analyzer (0.7 amu). Although a greater selectivity was possible with the low mass resolution of a triple quadrupole instrument using the MRM approach, for the daidzein metabolite O-DMA, a chromatographically resolvable second peak could only be substantially reduced by using a 0.01 amu mass window. The advantage of a TOF analyzer for product ion data is that the whole MSMS spectrum is collected at high mass accuracy and MRM experiments are conducted *in silico* after the analysis.

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*Author for correspondence: Stephen Barnes, PhD, Department of Pharmacology and Toxicology, McCallum Research Building, Room 452, University of Alabama at Birmingham, 1918 University Boulevard, Birmingham, AL 35294, Tel # (205) 934-7117; Fax # (205) 934-6944; sbarnes@uab.edu.

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Keywords

Polyphenols; nanoliquid chromatography-mass spectrometry; sample volumes; sensitivity; quantification; mass accuracy

Introduction

Two forces are driving improvements in the current methods of liquid chromatography-mass spectrometry (LC-MS) analysis of polyphenols and their metabolites: the need to work with initial sample volumes of 1 μ l or less from small animal models (mice and zebrafish) and a method that is sensitive enough to measure concentrations that are at or below the disassociation constants of target receptors, i.e., subnanomolar (nM). We have previously reported several LC-MS procedures for the analysis of isoflavones and catechins (1–14). Each was based on use of reverse-phase LC separation of these analytes, formation of analyte ions by electrospray ionization (ESI) or atmospheric chemical ionization (APCI) and their analysis by multiple reaction ion monitoring (MRM) mass spectrometry using a triple quadrupole mass spectrometer.

MRM-MS is a procedure where, one at a time, specific precursor ions ($[M+H]^+$ or $[M-H]^-$) are filtered by the first quadrupole. These ions are then accelerated and collided with nitrogen gas in the second quadrupole, the resulting product ions filtered by the third quadrupole, and the number of unique product ions for an individual analyte recorded by a detector. Each one of these collection periods may last 25–50 msec. Therefore, during each second, 20–40 different ion transitions can be monitored and for chromatographic peaks wider than 10 sec, sufficient information is available to accurately determine the area under the peaks. Used this way, the LC-MRM-MS procedure is regarded as the “gold standard” for not only isoflavones and other polyphenols, but also a wide range of small molecules (15).

However, a limitation of the MRM procedure is the low mass resolution of the quadrupole filter. It has an effective band pass of 0.7 m/z for both the precursor and product ions. This means that its robustness is offset by poor mass selectivity since ions with m/z values within 0.35 m/z of the chosen ion may also be filtered, at least in part, by the quadrupole.

An alternative tandem mass spectrometer combination is the quadrupole-orthogonal-time-of-flight (Q-tof) instrument. It offers two advantages – first, high mass resolution and high mass accuracy spectra for the precursor ions can be recorded at the beginning of each duty cycle (typically for 100 msec). Then product ion MSMS spectra from selected precursor ions are recorded (typically for 100 msec for each precursor ion). This has the advantage over triple quadrupole analysis in that the product ion MSMS spectra also have high mass resolution and high mass accuracy. Also, unlike the MRM on a triple quadrupole instrument where multiple data collections are needed for each selected product ion, the whole mass spectrum is recorded at the same time. Selection of the product ions to validate the identity of a compound can be carried out AFTER data collection. The collected data can therefore be regarded as a library to be searched at a later time.

To address the issue of the sensitivity of LC-MS assays, it is necessary to consider the impact of flow rates. Using a 2.1 mm i.d. reverse-phase column, a mobile phase flow rate of 200 μ l/min and injected sample amount equivalent to 20 μ l of serum, the lower limit of quantitation (LLOQ) of our current LC-MRM-MS method on an AB Sciex 4000 triple quadrupole mass spectrometer is approximately 10 nM (12). This value varies according to the isoflavones and their metabolites. The goals of the present study were to explore the use of much smaller columns (with i.d. values less than 100 μ m) and lower flow rates (nl/min) that are typically used in proteomics research (16). Since both MS and MS/MS spectra were collected using a Q-tof mass spectrometer, we also assessed whether the high mass accuracy of the MS spectral data was sufficiently specific to be used to build a quantitative method in biological samples.

Materials and methods

Materials

Genistein, daidzein, dihydrodaidzein (DHD), equol, O-desmethyldangoulesin (O-DMA), glycitein, biochanin A, coumestrol, enterodiol and enterolactone were purchased from LC-laboratories (Woburn, MA) and were at least 99% pure. Dihydrogenistein was a gift from Dr. Adrian Franke, Cancer Center of Hawaii. All HPLC solvents and reagents were purchased from Fisher Scientific Co. (Norcross, GA) and were of highest HPLC grade available. Phenolphthalein β -glucuronide, 4-methylumbelliferone sulfate and β -glucuronidase/sulfatase from *Helix pomatia* were purchased from Aldrich–Sigma Chemical Co. (St. Louis, MO).

Methods

The polyphenols were made as mixed standard solutions ranging from 10 to 1000 nM in 80% aqueous methanol. These were diluted twenty-fold with water to make working solutions from 0.5 to 50 nM in 4% aqueous methanol.

Remnant urine specimens from a previous study were selected that had polyphenol concentrations that were less than 100 nM as measured by our previous method (12). Aliquots (50 μ l) of these urines were diluted with 250 μ l of 0.3 M ammonium acetate buffer, pH 5 and treated with 20 units of *Helix pomatia* β -glucuronidase/sulfatase for 16 hr at 37°C. The deconjugated polyphenols in the hydrolysate were extracted with two volumes of diethyl ether. The ether phase was taken to dryness under nitrogen and the dried residues reconstituted in 200 μ l 4% aqueous methanol with sonication. Extracts were centrifuged at 14,000 $\times$ g for 10 min to remove any particulates and diluted 20-fold with 0.1% formic acid.

LC-MS analysis

Analysis was carried out on a 15 cm $\times$ 75 μ m i.d. C₁₈ reverse-phase ChipLC column (AB Sciex, Concord, Ontario, Canada) with a 0.5 cm $\times$ 200 μ m i.d. C₁₈ reverse-phase pre-column cartridge. The ChipLC column was contained in an AB Sciex NanoFlex unit maintained at 45°C. The mobile phase flow rate was 300 nl/min provided by an AB Sciex Eksport 415 nanoLC pump. Aliquots (5 μ l) of the standard or unknown isoflavones were injected onto the column which had been equilibrated with 0.1% formic acid in water (solvent A). The

eluting solvent was 100% acetonitrile:0.1% formic acid (solvent B). After washing the column with solvent A for 5 min, the polyphenols were eluted with a 10 min linear gradient (1–99%) of solvent B in solvent A. The column was washed for a further 5 min with solvent B and then re-equilibrated in solvent A for 15 min. The eluate was passed into the nanospray III ionization interface of an AB Sciex 5600 TripleTOF mass spectrometer operating in the positive mode. The nanoESI voltage was 2300 V. In each 1.55 sec duty cycle, a high-resolution mass spectrum was collected for the first 250 msec and product ion mass spectra for the 13 individual polyphenol and standard precursor ions were collected for 100 msec each. To test for reproducibility of the measurement, each sample was injected and analyzed five times.

Ion chromatograms were generated from collected data in two ways: first, the MS data were filtered at the expected exact mass of each polyphenol metabolite precursor ion $[M+H]^+$ with mass windows of 0.7, 0.1, 0.05 and 0.01 amu. Second, the MSMS data were examined to identify product ions that were coming from the selected precursor ions. These ions were then selected on the basis of their uniqueness for each polyphenol. The mass windows of each selected product ion were set at 0.7, 0.1, 0.05 and 0.01 amu. Integrated peak areas from the MS and multiple reaction ion monitoring (MRM) using a 50 mDa mass window were used to determine the concentrations of the analytes in the urine extracts by comparing them to a series of standards.

Results

Initial experiments arose from untargeted LC-MS analysis of aliquots (50 μ l) of mouse sera sent for metabolomics analysis in the UAB-UCSD O'Brien Acute Kidney Injury Center by Drs. Sanjay Nigam and Wei Wu from UCSD. The urine samples were hydrolyzed to deconjugate β -glucuronides and sulfate esters and the resulting aglycones recovered by extraction into diethyl ether. The assay was carried out on a 15 cm $\times$ 75 μ m i.d. reverse-phase column at a flow rate of 300 nl/min, collecting MS data and data-dependent MSMS data. Initial data analysis was performed using XCMS-Online¹. Inspection of the data from these mice revealed strong signals coming from the isoflavones (daidzein, genistein) and their metabolites (dihydrodaidzein, equol and O-desmethylangolensin) accompanied by MSMS data that verified their identities. Calibration of the samples using an external isoflavone standard suggested that the observed concentrations were in the range of 10–20 nM, close to the LLOQ for these compounds in the assays previously described using triple quadrupole MRM-MS at flow rates from 200–1000 μ l/min (12). These flow rates from 200–1000 μ l/min represent the optimal linear flow velocities for 2.1 mm and 4.6 mm i.d. columns.

A feature of LC-ESI-MS is that as the flow rate is decreased, the efficiency of transfer of solutes from the liquid to the gas phase is increased by the square of the ratio of the internal diameters of the columns. This has been heavily exploited by the proteomics community who typically use flow rates in the 50–300 nl/min range for columns with internal diameters from 20–75 μ m (16). So, based on the linear flow velocities of a 2.1 mm i.d. column

¹Freely available online software for metabolomics available at <https://xcmsonline.scripps.edu/>

operating with an idealized flow rate of 200 $\mu\text{l}/\text{min}$, a 1.0 mm i.d. column should have a flow rate of 45.35 $\mu\text{l}/\text{min}$, using the change in diameter squared in making the calculation. Similarly, a 200 μm i.d. column should have a flow rate of 1.81 $\mu\text{l}/\text{min}$ and a 75 μm i.d. column should have a flow rate of 255 nl/min .

Using a 15 cm $\times$ 75 μm i.d. C_{18} reverse-phase column, we prepared a peak area-concentration curve for each of the common polyphenols and their metabolites to determine their LLOQ and the signal-to-noise at the lowest concentrations. To do this, we examined the both MS and MSMS spectra for the $[\text{M}+\text{H}]^+$ ions for each compound. These were arranged in Table 1 to show the masses of the precursor ions and product ions that were in common between these analytes and those that were different. Product ions with the same nominal mass were placed on the same line in the Table. There were several product ions that had the same exact mass (within the mass accuracy of the mass spectrometer), for example 91.0540 m/z which was in common for equol, daidzein, O-DMA and genistein. On the other hand, there were also ions with the same nominal mass, but whose exact mass was different by approximately 50 mDa. This was due to differences in the number of hydrogen and oxygen atoms within the product ion. For example, genistein and daidzein have product ions with the same nominal mass, 153 m/z ; however, their exact masses are different. A product ion for daidzein is 153.0698 m/z , whereas for genistein it is 153.0190 m/z . Using a quadrupole mass filter with a mass window of 0.7 amu, these two masses are not separable; however, with time-of-flight detectors these ions can be readily distinguished. The mass defect (accurate mass – nominal mass) can be easily predicted for compounds consisting of C, H and O since C at 12.0000 Da does not contribute to the mass defect. Mass defects for H_{1-15} and O_{1-5} can assist the investigator in the identification of the products ions coming from polyphenol precursor ions (Table 2).

Response curves – MS and MSMS data

Peak area-concentration curves were developed using precursor ions (see Table 2) for each of the polyphenols and their metabolites. Data were collected around the exact mass using a 50 mDa window. For all the polyphenols and their metabolites, the response curves were highly linear in both the linear and log-log modes with correlation coefficients >0.999 (Fig. 1). For several of the analytes acceptable signal-to-noise ($>10:1$) was observed down to 0.5 nM or 1.0 nM. Equol and coumestrol were exceptions with LLOQs of 5 and 10 nM due to their low sensitivities compared to the other polyphenols. These data are summarized in Table 3.

Method application to urine specimens

The developed method was then applied to previously analyzed urine specimens. Urine aliquots (50 μl) were hydrolyzed and the aglycones extracted into ether, evaporated to dryness and reconstituted in 200 μl 4% aqueous methanol. Initial analyses by nanoLC-ESI-MS were carried out using 5 μl aliquots (equivalent to 1.25 μl of urine). However, these resulted in peak areas that were in general ten to twenty times or more above the largest isoflavone standard (50 nM). Accordingly, all the urine extracts were diluted 20 times (equivalent to 0.0625 μl of urine) and re-analyzed by nanoLC-ESI-MS. The resulting total

ion current LC chromatogram is shown in Figure 2 for one of these urine samples. The large hump is composed of signals from many chromatographically unresolved urine metabolites.

Since MS and MSMS data were collected to form a data library, it was possible to ascertain after the LC-MS analysis had been completed whether narrowing the mass window of precursor ions enabled direct, specific analysis of the polyphenols and their metabolites. For the compounds analyzed in this study, this was indeed the case. In Fig. 3, the ion chromatograms for precursor ion $[M+H]^+$ of the daidzein metabolite DHD (m/z 257.081) are shown for a mass window of 0.7 amu (Fig. 3A), 0.1 amu (Fig. 3B), 0.05 amu (Fig. 3C) and 0.01 amu (Fig. 3D). DHD is one of several prominent peaks observed using a 0.7 amu window, whereas with a 0.05 amu window it has become by far the major peak. Further improvements were observed using a 0.01 amu window; however, there was an approximately 35% decrease in sensitivity.

For genistein (m/z 271.061), using a 0.7 amu window, it is scarcely visible in the ion chromatogram, being obscured by other compounds with similar masses (Fig. 4A). Narrowing the mass window to 0.1 amu (Fig. 4B), 0.05 amu (Fig. 4C) and 0.01 amu (Fig. 4D) substantially improved the specificity of the detection of genistein. As observed for DHD (Fig. 3D), the use of the 0.01 amu mass window led to a 35–40% decrease in sensitivity.

For O-DMA (m/z 259.097), using the 0.7 amu mass window, its peak was obscured by a co-eluting peak (Fig. 5A). In contrast to genistein and DHD, the peak for O-DMA only became clear using a mass window of 0.01 amu (Fig. 5D). Again, there was a 35–40% loss in sensitivity using this narrow mass window.

The value of MRM analysis was explored with genistein and O-DMA. For genistein, the use of the m/z 153.019 product ion led to a reasonably clean peak even using the 0.7 amu filter (Fig. 6A). The small amount of background signal decreased as the mass window was narrowed (Fig. 6B, 6C, 6D). However, the genistein peak intensity also decreased, particularly for the 0.01 amu mass window.

For O-DMA, using the m/z 137.060 product ion, its peak (R_t 13.47 min) could be clearly seen although there was a second prominent peak (R_t 12.25 min) in the selected ion chromatogram (Fig. 7A). Narrowing the mass window to 0.1 amu (Fig. 7B) and 0.05 amu (Fig. 7C) had no effect on the intensity of the second peak. However, with the 0.01 amu mass filter, it was reduced by 7-fold, albeit that the O-DMA peak also declined by 35% once again (Fig. 7D).

The three samples chosen for this study had no measurable equol in either the undiluted or diluted samples. Accordingly, equol and dihydrogenistein concentrations were < 1 nM. The other phytoestrogens were in measurable concentrations (Table 4). Reproducibility (coefficients of variation) of multiply injected samples ranged from 1.62% for the most intense peak to 9–13% for the weakest peaks. Even for the latter, the signal-to-noise ratio was only $> 10:1$ in two cases. For over half of the data reported in Table 4, the signal-to-noise ratio was $> 100:1$ (Table 4). The concentrations of daidzein, dihydrodaidzein, O-desmethylanagolensin, genistein and glycitein in the 80-fold diluted urine samples using both

the MS and MRM methods ranged from <1 to >100 nM (Table 4) and were highly correlated ($R^2 = 0.996$, slope MS versus MRM = 1.043).

Discussion

In the present study we have demonstrated (1) the value of using a high mass resolving and mass accurate analyzer for both MS and MSMS spectra of polyphenols and their metabolites and (2) the marked increase in sensitivity that can be obtained for the analysis of these compounds by employing nanoLC-MS techniques. These advances in analysis open the way for the analysis of very sample sizes (~ 1 μ l) and for many polyphenols at very low concentrations (sub-nanomolar).

Using a TOF analyzer with a mass resolution of 40,000 and a mass error of 2–3 ppm, it became possible to detect the polyphenols and their metabolites directly in an extract of hydrolyzed urine without resorting to MSMS analysis. To do this it was necessary to narrow the mass window of MS spectra to 0.05 amu or less to clearly resolve the polyphenols and their metabolites (genistein and DHD) from other compounds in the urine extract. For O-DMA, it was necessary to use an 0.01 amu mass window.

With a resolving power of 40,000, the peak widths of these analytes at half height are 0.006–0.007 amu. This in turn means that the full peak has a width at the baseline of ~0.015 amu. This explains why using a 0.010 amu window centered on the exact mass of the analyte filters out some of the real signal and accounts for the 35% decrease in intensity observed in the study compared to a 50 amu window.

For MRM analyses, the value of having the second mass filter effectively isolated the genistein peak even with the wide 0.7 amu mass window. Narrower mass windows only caused small improvements for this isoflavone. It was different in the case of the daidzein metabolite, ODMA, where a second compound eluting ~1 min earlier than the O-DMA peak was observed even as the mass window became more narrow. This suggested that MRM experiments should be routinely carried out with the 0.01 amu mass window for the product ion. The advantage of having collected what is a data library using the Q-tof method is that decisions about how to best analyze the data can be postponed until after the data have been collected.

The study also confirmed that lowering the flow rate of the LC-MS analysis from 0.2 – 1 ml/min to hundreds of nl/min increased sensitivity by 2–3 orders of magnitude. This allowed the use of much smaller amounts of starting material. In the case of the urines in this study, satisfactory analysis was performed on the equivalent of 62 nl of urine. Given the small size of the columns used for nanoLC, it is necessary to reduce the methanol content of the solution used to reconstitute dried extracts. Using 4% aqueous methanol, the polyphenols bound to the reverse-phase column and eluted as a sharp peak (Figs. 4–7). Since the analyses were carried out using nanoelectrospray ionization in the positive mode, the signal intensities for equol and coumestrol were considerably lower than for the other polyphenols. This confirms findings in our and other laboratories where negative ion electrospray

ionization was the preferred ionization method (12, 17–25), although others have used positive ionization methods (26,27).

Besides the much increased sensitivity of the nanoLC-MS approach to the analysis of isoflavones and their metabolites, it was also highly reproducible. Five replicate injections of the three test urine extracts revealed that all the measurable compounds had CoVs less than 13% with many of the more intense peaks approaching 1%. Analysis of these data also revealed there was strong concordance of the calculated concentrations of the isoflavones and their metabolites between the MS and MRM methods ($R^2=0.996$ and a slope of 1.043). Signal-to-noise ratios below 10:1 were only observed for subnanomolar concentrations of genistein.

The high concentrations of daidzein and its metabolites, dihydrodaidzein and O-desmethyldaidzein, in urine are consistent with their much higher renal clearance compared to genistein (28). Glycitein is also a major urinary isoflavonoid since it is largely excreted without metabolism.

In summary, the basis for an improved method for the analysis of polyphenols and their metabolites in urine has been established. The use of nanoLC-MS for the analysis of small molecules such as polyphenols is rare (29), although it is a widely used technique in modern proteomics (16). It holds a great potential for the analysis of polyphenols in small biological models where sample sizes are limited. Future studies will address reproducibility, linear range and matrix effects as we have previously carried out for small molecules (12, 30).

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- Separation of polyphenols and their metabolites by nanoLC-MS has been established
- Quantitative analysis of polyphenols in the matrix of a urine extract
- Use of a narrow mass window (10–50 mDa) enables direct MS analysis
- Sensitivity using nanoLC-MS allows analysis on sub-microliter volumes of urine

Figure 1. Area-concentration response curves for isoflavones and their metabolites for nanoLC-electrospray ionization-mass spectrometry. Analysis was carried out on a 15 cm $\times$ 75 μ m i.d. C₁₈ reverse-phase ChipLC column with a 0.5 cm $\times$ 200 μ m i.d. C₁₈ reverse-phase pre-column cartridge. The ChipLC column was maintained at 45°C and the mobile phase flow rate was 300 nL/min. Aliquots (5 μ L) of the isoflavones were injected onto the column which had been pre-equilibrated with 0.1% formic acid in water (solvent A). The eluting solvent was 100% acetonitrile:0.1% formic acid (solvent B). After washing the column with solvent A for 5 min, the polyphenols were eluted with a 10 min linear gradient (1–99%) of solvent B in solvent A.

Figure 2.

Total ion current chromatogram from reverse-phase nanoLC-MS analysis of an ether extract of urine that had been hydrolyzed with β -glucuronidase/sulfatase. The LC and MS conditions are described in Figure 1. The ion current was generated from summation of the ion intensities in the collected MS spectra.

Figure 3.

Selected ion chromatograms for dihydrodaidzein following nanoLC-MS analysis of urine ether extracts. The LC and MS conditions are described in Figure 1. The selected exact mass for the dihydrodaidzein $[M+H]^+$ precursor ion was 257.0814 m/z . Ion chromatograms A, B, C and D were generated with mass windows of 0.7 (A), 0.1 (B), 0.05 (C) and 0.01 amu (D), respectively.

Figure 4.

Selected ion chromatograms for genistein following nanoLC-MS analysis of urine ether extracts. The LC and MS conditions are described in Figure 1. The selected exact mass for the genistein $[M+H]^+$ precursor ion was 271.0606 m/z . Ion chromatograms A, B, C and D were generated with mass windows of 0.7 (A), 0.1 (B), 0.05 (C) and 0.01 amu (D), respectively.

Figure 5.

Selected ion chromatograms for O-desmethylangolensin following nanoLC-MS analysis of urine ether extracts. The LC and MS conditions are described in Figure 1. The selected exact mass for the O-desmethylangolensin $[M+H]^+$ precursor ion was 259.0970 m/z . Ion chromatograms A, B, C and D were generated with mass windows of 0.7 (A), 0.1 (B), 0.05 (C) and 0.01 amu (D), respectively.

Figure 6.

MRM ion chromatograms for genistein following nanoLC-MS analysis of urine ether extracts. The LC and MS conditions are described in Figure 1. The precursor ion for genistein ($271.0606\ m/z$) was selected in the first quadrupole with a mass window of 0.7 amu. Ion chromatograms A, B, C and D were generated using the product ion $153.0190\ m/z$ with mass windows of 0.7 (A), 0.1 (B), 0.05 (C) and 0.01 amu (D), respectively.

Figure 7.

MRM ion chromatograms for O-desmethylangolensin following nanoLC-MS analysis of urine ether extracts. The LC and MS conditions are described in Figure 1. The precursor ion for O-desmethylangolensin ($259.0970\ m/z$) was selected in the first quadrupole with a mass window of 0.7 amu. Ion chromatograms A, B, C and D were generated using the product ion $137.0598\ m/z$ with mass windows of 0.7 (A), 0.1 (B), 0.05 (C) and 0.01 amu (D), respectively.

Table 1

Observed major product ions (in descending order) coming from positively charged polyphenol precursors

<u>Equol</u>	<u>Daidzein</u>	<u>DHD</u>	<u>ODMA</u>	<u>Genistein</u>	<u>DHG</u>
243.1021	255.0657	257.0814		271.0606	273.0763
	227.0744				227.0709
225.1663					225.0554
				215.0703	
207.1375			213.0889		
	199.0798				181.0679
	181.0653				179.0338
179.1433				169.0630	
161.1319					
	153.0698			153.0190	153.0179
					151.0398
			149.0596		
133.0658		137.0229	137.0598	141.0712	
123.0448	127.0526				
		123.0434		123.0435	
		121.0273	121.0639		
			119.0482		
		115.0535			
107.0488		107.0483			107.0487
105.0688					
			103.0533		
95.0846		95.0484			95.0487
				93.0688	
91.0542	91.0533	91.0533	91.0534	91.0541	
	81.0328				

<u>Equol</u>	<u>Daidzein</u>	<u>DHD</u>	<u>ODMA</u>	<u>Genistein</u>	<u>DHG</u>
77.0371	77.0381	77.0377	77.0389		77.0373
75.0246				65.0378	65.0380

Table 2

Mass defects for ions containing variable number of hydrogen and oxygen atoms^a

<u>Hydrogens^b</u>	<u>0 Oxygen</u>	<u>1 Oxygen</u>	<u>2 Oxygens</u>	<u>3 Oxygens</u>	<u>4 Oxygens</u>	<u>5 Oxygens</u>
0	-	0.0022	-0.0029	-0.0080	-0.0131	-0.0182
1	0.0151	0.0100	0.0049	-0.0002	-0.0052	-0.0103
2	0.0229	0.0178	0.0128	0.0077	0.0026	-0.0025
3	0.0308	0.0257	0.0206	0.0155	0.0104	0.0053
4	0.0386	0.0335	0.0284	0.0233	0.0182	0.0131
5	0.0464	0.0413	0.0362	0.0311	0.0261	0.0210
6	0.0542	0.0491	0.0441	0.0390	0.0339	0.0288
7	0.0621	0.0570	0.0519	0.0468	0.0417	0.0366
8	0.0699	0.0648	0.0597	0.0546	0.0495	0.0444
9	0.0777	0.0726	0.0675	0.0624	0.0574	0.0523
10	0.0855	0.0804	0.0754	0.0703	0.0652	0.0601
11	0.0934	0.0883	0.0832	0.0781	0.0730	0.0679
12	0.1012	0.0961	0.0910	0.0859	0.0808	0.0757
13	0.1090	0.1039	0.0988	0.0937	0.0887	0.0836
14	0.1168	0.1117	0.1067	0.1016	0.0965	0.0914
15	0.1247	0.1196	0.1145	0.1094	0.1043	0.0992

^a Carbon atoms do not contribute to the mass defect

^b The proton is not counted as a hydrogen atom – it contributes 0.00727 to the mass defect for positive, singly charged ions

Table 3

Signal-to-noise ratios for polyphenols and metabolites using MS and MSMS spectra

Analyte	Concentration (nM)	S/N MS	S/N MSMS ^a
Equol	5.0	2.8	23.7
Daidzein	0.5	12.3	10.3
Dihydrodaidzein	1.0	13.6	8.7
O-desmethylangolensin	1.0	8.0	7.6
Genistein	5.0	32.4	23.6
Dihydrogenistein	5.0	33.9	26.9
Glycitein	0.5	7.0	11.5
Coumestrol	10.0	20.2	28.4

^aThe S/N is given for the product ion with the highest ratio

Concentrations (nM)^a, signal-to-noise ratios and Coefficients of Variation (%)^b of polyphenols and their metabolites in 3 remnant urine samples

Table 4

Measurement type	Daidzein		Dihydrodaidzein		O-Desmethylangolensin		Genistein		Glycitein	
	Peak area	CoV	Peak area	CoV	Peak area	CoV	Peak area	CoV	Peak area	CoV
<i>Sample 1</i>										
MS	6,123,200	2.81	1,051,240	4.92	33,189	12.70	65,046	8.79	999,320	4.51
S/N	417.9		63.0		10.0		18.9		80.2	
Concentration ^a	72.5		20.18		0.79		1.59		9.92	
MRM	308,340	7.24	72,904	2.80	11,486	13.00	4,501	11.32	94,042	8.11
S/N	116.5		483.1		63.8		9.2		524.7	
Concentration ^a	75.9		15.99		1.64		2.10		11.14	
<i>Sample 2</i>										
MS	3,568,200	9.61	233,960	9.71	546,960	2.46	30,138	9.30	1,008,860	4.31
S/N	652.9		31.5		121.7		23.1		96.4	
Concentration ^a	42.3		4.49		13.06		0.74		10.01	
MRM	189,660	9.84	12,915	9.48	83,740	8.90	3,372	8.90	91,640	3.03
S/N	47.2		58.8		183.3		5.6		906.0	
Concentration ^a	46.7		2.83		18.36		1.57		10.85	
<i>Sample 3</i>										
MS	8,945,200	2.52	7,288,400	1.66	474,600	8.54	90,932	4.49	3,621,800	3.59
S/N	1,836.7		2,311.2		181.2		17.4		428.7	
Concentration ^a	105.9		139.9		11.33		2.22		35.94	
MRM	428,240	1.62	584,940	3.22	79,064	4.05	5,563	4.33	331,540	2.13
S/N	579.1		698.5		221.8		50.9		3077.4	
Concentration ^a	105.4		128.3		11.31		2.59		39.27	

^aUnits – nM;

^bUnits - %