Automated Online and Off-Line Solid-Phase Extraction Methods for Measuring Isoflavones and Lignans in Urine

Zsuzsanna Kuklenyik, Xiaoyun Ye, John Adam Reich, Larry L. Needham, and **Antonia M. Calafat***
Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA 30341

Abstract

Automated online and off-line solid-phase extraction (SPE) methods coupled to isotope dilution–high-performance liquid chromatography–tandem mass spectrometry for measuring four isoflavones (daidzein, genistein, equol, and *O*-desmethylangolensin) and two lignans (enterolactone and enterodiol) in urine are developed. The SPE recoveries for the online SPE method are excellent for most analytes (83–94%) and somewhat lower for enterolactone (61%). The recoveries for all analytes with the off-line SPE method are also very good (65–80%). The limit of detection is lower for the online method (0.1–0.7 ng/mL) than for the off-line method (0.4–3.3 ng/mL). Similarly, the reproducibility is generally better for the online method [coefficient of variation (CV) of 4–12%) than for the off-line method, except for enterolactone, which has a higher CV (18–19%) that is consistent with its lower online SPE recovery. Both methods are adequate for analyzing a large number of samples for epidemiological studies to assess the prevalence of human exposure to isoflavones and lignans.

Introduction

Phytoestrogens, compounds naturally present in some plants, display estrogenic, antiestrogenic, and antiandrogenic activities in humans and animals. Generally, phytoestrogens are less potent than the endogenously produced estrogens, but depending on dietary intake, phytoestrogens can be present in humans in much higher quantities (1). The two major classes of phytoestrogens are isoflavones and lignans. Isoflavones include daidzein, genistein, *O*-desmethylangolensin (*O*-DMA), and equol (Figure 1); daidzein is metabolized to *O*-DMA and to equol by intestinal bacteria. Lignans include enterolactone and enterodiol (Figure 1); enterolactone and enterodiol are metabolized from matairesinol and secoisolariciresinol, respectively, by the gut microflora (2,3). Sources of isoflavones include legumes, such as soybeans and soy-based products; sources of lignans include whole grains, flax, and some fruits and vegetables (4,5).

The phytoestrogens are naturally found as β-glycosidic conjugates. After ingestion, the conjugated phytoestrogens are hydrolyzed to their aglycones, absorbed, and glucuronidated in the intestine (6). The major circulating forms of the isoflavones are the glucuronidated species. Glucuronidated forms are also predominant in urine (7). Urinary concentrations of isoflavones and lignans highly correlate with serum levels and can be used as biomarkers for the dietary intake of these compounds (8–11).

Ingestion of foods rich in phytoestrogens has several beneficial health outcomes: reduced risk for breast and prostate cancer, reduction of menopausal symptoms, reduced risk for coronary health disease and cardiovascular disease, and modulation of osteoporosis (12–15). However, although phytoestrogens are not

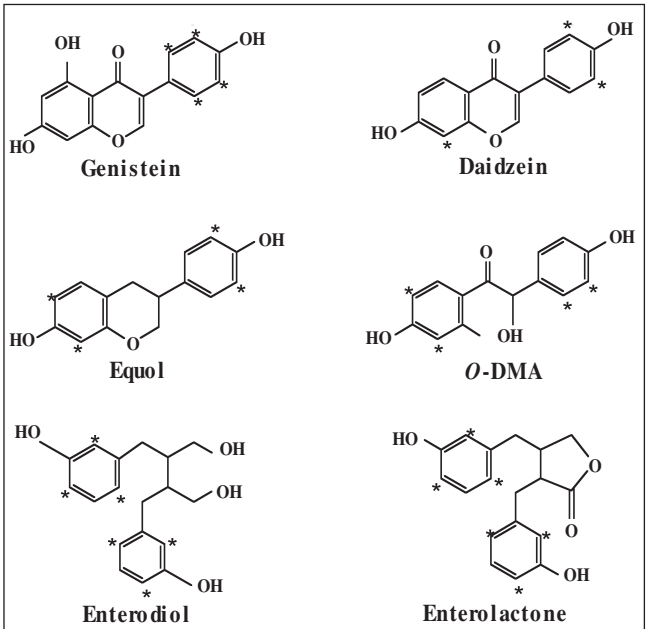

Figure 1. Chemical structures of the isoflavones daidzein, genistein, O-DMA, equol, and the lignans enterolactone and enterodiol.

* Author to whom correspondence should be addressed: email Acalafat@cdc.gov.

acutely toxic in large doses in experimental animals, phytoestrogens have caused reduced reproductive capability in animals at chronic dietary doses (16); some studies suggest adverse effects on the immune system (17,18).

The levels of daidzein, genistein, *O*-DMA, equol, enterolactone, and enterodiol have recently been determined in approximately 2500 urine samples from people in the United States aged 6 years and older who participated in the National Health and Nutrition Examination Survey in 1999 and 2000 (19). Both parent compounds (e.g., daidzein) and metabolites (e.g., equol) were chosen because previous studies showed that, even with a similar dietary intake, the metabolism of isoflavones and lignans vary greatly between people. Individual variations are greatest for equol and *O*-DMA and lower for enterolactone and enterodiol (2,20). The differences in the metabolic route among people can lead to differences in risks and benefits. For example, a high urinary excretion of 2-hydroxyestrone relative to 16α-hydroxyestrone has been associated with a reduced risk for breast cancer. Furthermore, equol excretion, but not total isoflavone excretion, correlated positively with the 2-hydroxyestrone: 16α-hydroxyestrone excretion ratio, suggesting the bacterial profile associated with equol production may also be involved in estrogen metabolism and may influence breast cancer risk (21). Therefore, development of analytical methods that allow for the simultaneous determination of both precursor compounds and their metabolic products is important.

Two automated solid-phase extraction (SPE) alternatives for high-throughput measurement of four isoflavones and two lignans in urine by isotope dilution–high-performance liquid chromatography (HPLC)–tandem mass spectrometry (MS–MS) are presented here. First, building on a manual SPE method (22), we developed an automated off-line SPE–HPLC method. Although automation of the off-line SPE procedure increased sample throughput, it still involved a substantial amount of sample handling (e.g., evaporation and reconstitution of the SPE urine extract, transfer of the extracts to autosampler vials) and produced substantial amounts of waste (e.g., individual disposable SPE cartridges, test tubes, caps, and pipette tips). To address these limitations, we developed an online SPE–HPLC method in which, after enzymatic hydrolysis, the sample was loaded onto an SPE column and the analytes were automatically transferred to the analytical HPLC column, thus eliminating the evaporation and reconstitution steps. This method has lower limits of detection (LOD) than previously reported online SPE methods for phytoestrogens (23,24) and is suitable for measuring both parent compounds and their metabolites. The online and off-line alternatives for measuring isoflavones and lignans in urine are also compared.

Experimental

Chemicals

Methanol (MeOH), acetonitrile (ACN), glacial acetic acid, ethanol (96%), dimethyl sulfoxide (DMSO), and other solvents were analytical or HPLC grade and obtained from Sigma Aldrich (St. Louis, MO). Enterolactone, enterodiol, equol, *O*-DMA, [$^2H_6$]-enterolactone, [$^2H_6$]-enterodiol, [$^2H_4$]-equol, [$^2H_4$]-*O*-DMA, and [$^2H_4$]-genistein were purchased from the University of Helsinki (Helsinki, Finland). Genistein and daidzein were purchased from Indofine Chemical (Somerville, NJ). [$^2H_3$]-daidzein was obtained from Cambridge Isotope Laboratories (Andover, MA). 4-Methylumbelliferyl glucuronide, 4-methylumbelliferyl sulfate, and β-glucuronidase/sulfatase (*Helix pomatia*, H1) were obtained from Sigma Aldrich.

Standard stock solutions were prepared by dissolving solid standards in DMSO followed by the addition of ethanol. Working standard solutions containing all analytes were prepared by serial dilutions in ethanol from the stock solutions to final concentrations that cover the linear range of the method. Internal standard stock solutions were prepared in ethanol. The spiking internal standard solution was prepared in water from the stock solutions.

4-Methylumbelliferyl sulfate and 4-methylumbelliferyl glucuronide were added to all samples and used as deconjugation standards to quantitate the extent of the enzymatic reaction. The deconjugation standard solution was prepared by dissolving 240 µg of 4-methylumbelliferyl glucuronide and 200 µg of 4-methylumbelliferyl sulfate in 10 mL of ethanol. A solution of β-glucuronidase/sulfatase enzymes at a concentration of 2 mg/mL in 1M ammonium acetate (pH 5.0) was used to hydrolyze the conjugated isoflavones and lignans.

Quality control (QC) materials were prepared by combining samples obtained from multiple anonymous donors into low- and high-concentration QC pools. QC blank samples, prepared from synthetic urine, were used to prepare the calibration standards. The three pools were dispensed in 5-mL portions in prerinsed glass vials and stored at –20°C until use.

Automated off-line SPE–HPLC

Urine (1 mL) was thawed at room temperature and spiked with deuterated internal standards (50 µL) of 4-methylumbelliferyl glucuronide/4-methylumbelliferyl sulfate solution (10 µL) and β-glucuronidase/sulfatase enzyme in ammonium acetate buffer (500 µL). The sample was gently mixed and incubated overnight (~ 17 h) at 37°C. After enzymatic deconjugation, the urine sample was placed on the Zymark RapidTrace Station (Zymark, Hopkinton, MA) for the automated off-line SPE procedure, which occurred as follows: a 60-mg/3 cc Oasis HLB cartridge (Waters, Milford, MA) was conditioned with 2 mL MeOH and 2 mL water at a controlled flow (1 mL/min). The urine sample was diluted with 1 mL of water and loaded onto the SPE cartridge at 1 mL/min. Next, the SPE column was washed with 3 mL of 0.1% NH_4OH–MeOH (90:10), 2 mL of water, and 2 mL of 70% MeOH in water. After drying the cartridges under a nitrogen flow for 1 min, the analytes were eluted with 2 mL of MeOH. Next, the urine

Table I. HPLC Gradient Program Used for the Off-Line SPE Method*

Time (min)	0	2.0	4.0	5.0	7.0	7.5	9.0
%A	65	6	50	45	5	5	65
%B	35	35	50	55	95	95	35

* Flow rate, 0.8 mL/min; mobile phase A, 10mM ammonium acetate (pH 6.5); and mobile phase B, methanol–acetonitrile (50:50).

extracts were evaporated to dryness under a stream of dry nitrogen in a TurboVap evaporator (Zymark) at 55°C, and reconstituted in 100 µL of 70% ammonium acetate (10mM, pH 6.5) and 30% ACN–MeOH (1:1) for HPLC–MS–MS analysis. Calibration standards were prepared by mixing native (50 µL) and labeled (50 µL) standard spiking solutions, synthetic urine (1 mL), 4-methylumbelliferyl glucuronide/4-methylumbelliferyl sulfate solution (10 µL), buffer (500 µL) solution, and water (390 µL) into a test tube. Calibration standards, which were not incubated, were extracted, evaporated, and reconstituted as previously indicated.

Twenty-five microliters of the reconstituted SPE extract was injected into an Agilent 1100 LC system (Agilent Technologies, Wilmington, DE) coupled with an API 4000 triple quadrupole MS (Applied Biosystems, Foster City, CA). The analytes were separated from the other compounds of the urine extract on a Prism RP column (5 µm, 50 × 3 mm) (ThermoHypersil-Keystone, Bellefonte, PA) using a nonlinear solvent gradient (Table I).

Online SPE–HPLC

Urine (500 µL) was spiked with deuterated internal standards (25 µL), 4-methylumbelliferyl glucuronide/4-methylumbelliferyl sulfate solution (5 µL), β-glucuronidase/sulfatase enzyme in ammonium acetate buffer (250 µL), and water (220 µL) in a 1.8-mL autosampler vial. The sample was gently mixed and incubated overnight (~ 17 h) at 37°C. The calibration standards were not incubated; they were prepared by mixing native (25 µL) and labeled (25 µL) standard spiking solutions, synthetic urine (500 µL), 4-methylumbelliferyl glucuronide/4-methylumbelliferyl sulfate solution (5 µL), buffer solution (250 µL), and water (195 µL).

The online SPE–HPLC–MS–MS system was built from several Agilent 1100 modules, namely two binary pumps with degassers, one autosampler, and one column compartment with a six-port switching valve, coupled with an API 4000 triple quadrupole MS (Figure 2). The two binary pumps and the autosampler were programmed and synchronized with the API 4000 MS using the Analyst software of the API 4000; both binary pumps were controlled through separate communication ports. A closed-area-network cable connected the column compartment and the second binary pump and allowed synchronization of the column switching valve with the eluent gradient program. The switching valve was programmed (Table II) using an Agilent 1100 handheld control module connected to the first binary pump (Figure 2). With the switching valve in the sample loading position (A), the first binary pump (Pump 1) was used to load in 2 min the deconjugated urine sample or calibration standard (500 µL) on a LiChrospher RP-18 ADS (25 × 4 mm, 25 µm, 60-Å pore diameter) SPE column (Merck, Darmstadt, Germany) using 20% MeOH in water at 1 mL/min. From 2 to 5 min (Figure 2), the valve automatically switched to its alternate position (B) and then back to the loading position (A), allowing the analytes to be transferred from the SPE column onto the HPLC analytical column (Prism RP; 5 µm, 50 × 3 mm) in backflush mode (0.8 mL/min) by the second binary pump (Pump 2) using a 10-min gradient program (Table II). As shown in the time line in Figure 2, during the data acquisition period, from 5 to 7 min, the SPE column was regenerated using 100% acetonitrile.

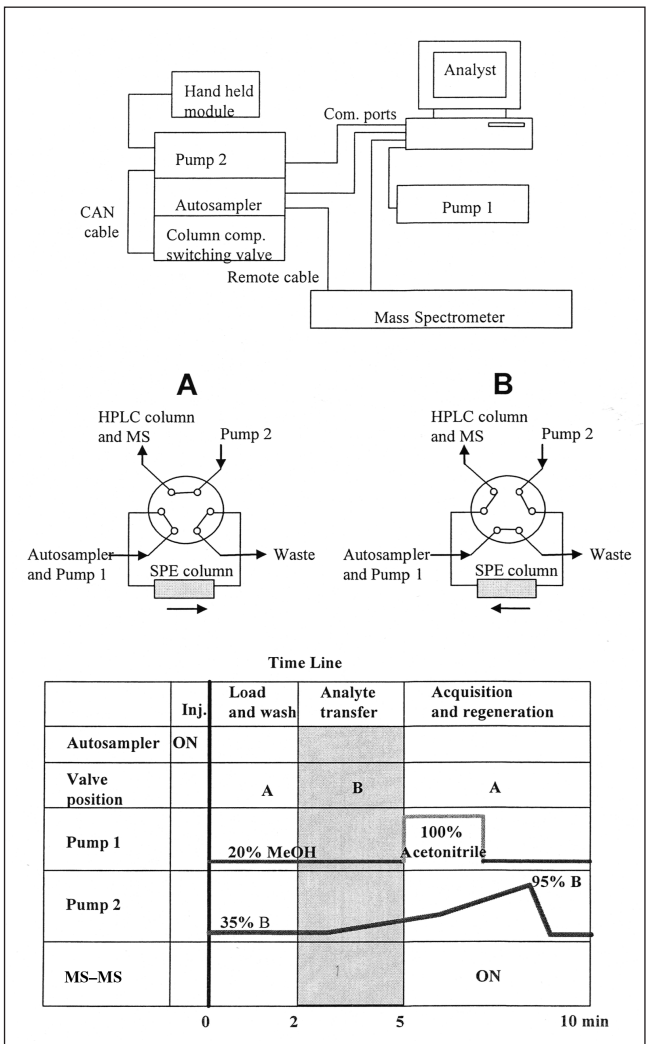

Figure 2. Connection diagram for the Agilent 1100 API 4000 online SPE–HPLC–MS–MS system (valve set up and time line).

MS–MS

Negative-ion atmospheric pressure chemical ionization (APCI) was used to generate gas-phase ions on an API 4000 triple quadrupole MS. The APCI settings were curtain gas (N_2) flow, 15 arbitrary units (au); collision gas flow, 4 au; nebulizer gas (air) flow, 40 au; nebulizing gas temperature, 500°C; and corona needle voltage, –3 V. Ionization parameters and collision cell parameters were optimized separately for each analyte. Unit resolution was used for both Q1 and Q3 quadrupoles. We monitored two molecular/fragment ion transitions for each analyte and one

Table II. HPLC Gradient Program Used for the Online SPE Method*

Time (min)	0	3.0	5.0	6.0	8.0	8.5	10.0
%A	65	65	50	45	5	65	65
%B	35	35	50	55	95	35	35

* Flow rate, 0.8 mL/min; mobile phase A, 10mM ammonium acetate (pH 6.5); and mobile phase B, methanol–acetonitrile (50:50).

for the corresponding isotopically labeled analog as follows: equol (241.1/119.1 and 241.1/135.0), [$^2H_4$]-equol (245.0/123.0); daidzein (253.1/117.1 and 253.1/223.1), [$^2H_3$]-daidzein (256.0/226.0); *O*-DMA (257.1/108.1 and 257.1/148.0), [$^2H_4$]-*O*-DMA (261.0/111.0); genistein (269.1/133.0 and 269.1/224.1), [$^2H_4$]-genistein (273.0/137.0); enterolactone (297.3/107.1 and 297.3/93.0), [$^2H_6$]-enterolactone (303.0/110.0); enterodiol (301.3/253.2 and 301.3/241.1), [$^2H_6$]-enterodiol (307.0/259.0); and 4-methylumbelliferone (174.7/133.0).

Results and Discussion

Selection of the SPE sorbents

Initially, C18 cartridges were used in the SPE method (22). For the automated off-line SPE method, Oasis HLB cartridges with *N*-methylpyrrolidone-divinylbenzene copolymer packing were used because they provided both optimal recovery of the isoflavones and lignans and removal of unwanted urine components in the extract. Specifically, the strong affinity of the analytes of interest for the polymeric sorbent facilitated the removal of other urine components with 70% MeOH, although the analytes remained on the column and eluted only with a MeOH content of 80% or higher. As a result, cleaner extracts were obtained than with the manual SPE method, allowing for injection of at least 100 samples into the HPLC system before regeneration of the HPLC column was necessary.

For the online SPE method, two precolumns were evaluated: (*i*) an Oasis HLB (20 × 5 mm, 25-µm particle size, 80-Å pore diameter) copolymer column (Waters) and (*ii*) a LiChrospher RP-18 ADS (25 × 4 mm, 25-µm particle size, 60-Å pore diameter) C18 silica column. Because of their relatively small pore size, these two columns display size-exclusion characteristics; macromolecules, such as proteins, are not able to penetrate the pores and elute with the dead volume. First, the retention of the matrix components (monitored using a UV detector) and of the analytes (monitored using MS–MS) were studied. With both columns, the matrix band, corresponding to the unretained urine components, eluted completely in 2 min at 1 mL/min. To select the optimal composition of the loading solvent, the retention of the analytes at different MeOH–water ratios were determined. For example, for enterolactone (the least retained analyte), separation from the matrix band was achieved with MeOH concentrations up to 50% (Oasis HLB) and 20% (LiChrospher RP-18 ADS).

Next, the two online SPE columns were evaluated on the basis of the resolution and peak shape of the analytes' signals obtained using the same online SPE–HPLC conditions, and the results were compared with those obtained with the off-line method. The greatest difference in peak shape was found for the latest eluting analyte, genistein (Figure 3). With the Oasis HLB column, the genistein peak was broader and showed more tailing than with the off-line SPE–HPLC method, probably because the analytes started to elute from the Oasis HLB column only when the MeOH content in the eluting solvent reached 60–80%. At this relatively high MeOH content, the retention of the analytes in the analytical HPLC column was not optimal and resulted in poorly resolved HPLC signals. In contrast, with the LiChrospher RP-18 ADS column, the shape and resolution of the analytes' chromatographic peaks were better (Figure 3). Because the LiChrospher RP-18 ADS contains a weaker SPE sorbent than the Oasis HLB, the analytes can be transferred from the SPE to the HPLC column with a relatively low MeOH content (i.e., 35%). With this organic content, the elution of the analytes on the HPLC column was slow, allowing for the analyte bands to refocus at the front end of the HPLC column, resulting in sharp and well-resolved chromatographic peaks.

The mentioned findings illustrate that, even though a strong sorbent is usually the preferred choice for an off-line SPE method, a weaker sorbent may work better for online SPE. Furthermore, elution of the analytes of interest from the SPE column has to be optimized to maximize the refocusing of the analytes' bands on the analytical HPLC column (25,26). For the reasons indicated previously, the LiChrospher RP-18 ADS column was chosen. A typical chromatogram of a urine sample including all six analytes using the online SPE method is shown in Figure 4.

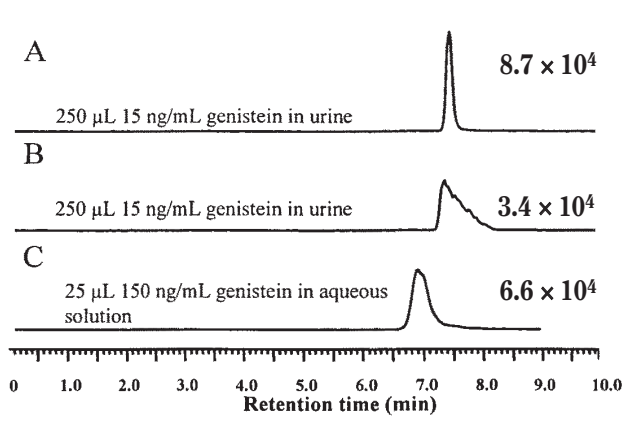

Figure 3. Comparison of the HPLC chromatograms of genistein obtained using online and off-line SPE. Online SPE using a LiChropher RP-18 ADS column (A), online SPE using an Oasis HLB column (B), and off-line SPE using an Oasis HLB column (C).

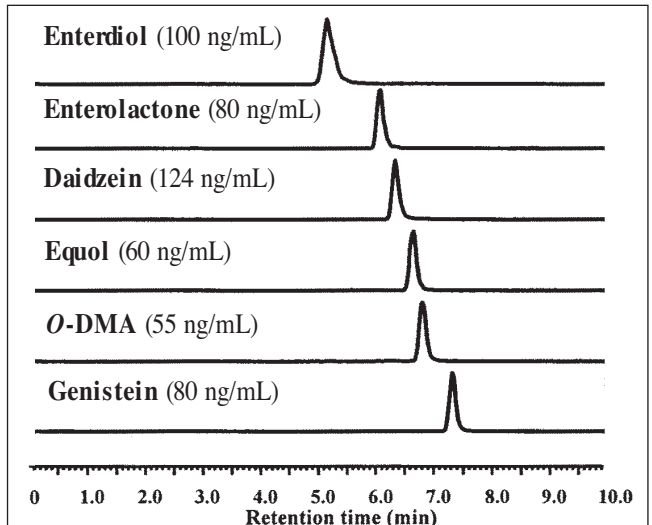

Figure 4. Chromatogram obtained using online SPE–HPLC on a LiChrospher RP-18 ADS SPE column of a standard in synthetic urine.

Comparison between the online and off-line SPE methods

Spiked urine was analyzed repeatedly to determine the SPE recoveries, accuracy, precision, and LOD of the methods. The SPE recoveries of the analytes were assessed by comparing the relative peak areas of the analytes after analyzing 500 µL of a urine sample and 25 µL of a urine extract, spiked with the same amount of analytes, by online and off-line SPE–HPLC–MS–MS, respectively. The SPE recoveries using the online SPE method were excellent (83–94%) for most analytes, and lower, but still good, for enterolactone (61%). The recoveries using the off-line SPE method were also good (65–80%) (Table III). In addition to a better extraction recovery, the judicious choice of an optimal switching valve timing operation and HPLC eluent gradient program resulted in better separation of the analytes from the rest of matrix components (i.e., fewer interferences) with the online than with the off-line SPE method.

Table III. SPE Recoveries, Spiked Standard Concentration Recoveries, and LODs with the Online SPE (and Off-Line SPE) Method

Analyte	SPE recovery (%)	*Standard concentration (ng/mL)* Spiked recovery (%)			LOD (ng/mL)
Equol		***10***	***50***	***1000***	
	90 (70)	115 (108)	93 (104)	101 (97)	0.3 (3.3)
Daidzein		***20***	***65***	***1000***	
	77 (79)	111 (121)	101 (102)	101 (98)	0.4 (1.6)
Genistein		***5***	***30***	***1000***	
	83 (65)	119 (99)	122 (112)	99 (95)	0.5 (0.8)
O-Desmethy-langolensin	85 (80)	***3*** 105 (110)	***20*** 112 (114)	***400*** 100 (102)	0.1 (0.4)
Enterodiol		***10***	***100***	***450***	
	94 (69)	111 (97)	107 (104)	99 (97)	0.4 (0.5)
Enterolactone		***25***	***60***	***1000***	
	61 (71)	117 (112)	107 (113)	101 (96)	0.7 (1.9)

Table IV. Precision of Concentration Measurements in Spiked QC Materials by Online SPE (and Off-Line SPE) Methods

Analyte	QC low		QC high	
	Mean	CV%	Mean	CV%
Equol	61.1	10 (23)	325	7 (17)
Daidzein	114	4 (11)	604	5 (7)
Genistein	61.9	5 (11)	213	5 (8)
O-DMA	14.5	6 (11)	189	6 (8)
Enterodiol	6.9	12 (16)	73.5	9 (10)
Enterolactone	43.5	19 (12)	264	18 (8)

* Mean concentrations in ng/mL. CV is the coefficient of variation.

The LODs were determined from replicate measurements of low-level calibration standards spiked into a blank synthetic urine matrix. The LOD was calculated as $3S_0$, where S_0 is the standard deviation as the concentration approaches 0 (27). The online SPE LODs (0.1–0.7 ng/mL) were lower than the off-line SPE LODs (0.4–3.3 ng/mL) (Table III) probably because of better resolution, less interferences, and better SPE recoveries with the online SPE.

Accuracy was established by determining the recovery of spiked urine samples by replicate measurements at three different concentrations. The mean recoveries in urine, expressed as a percentage of the expected value, were very good (Table III), with values ranging from 93% to 122% (online SPE) and from 96% to 121% (off-line SPE).

Precision was determined by calculating the coefficient of variation of 20 repeated measurements of the QC materials (Table IV). The reproducibility was generally better for the online method (4–12%) than for the off-line method. The precision for enterolactone by the online SPE method was not as good as for the other analytes (18–19%), consistent with its lower online SPE recovery. The greater improvement in precision with online SPE than with off-line SPE was especially significant for equol, as indicated by the decrease in the %CV from 19% to 12% (QC low) and 18% to 8% (QC high).

To prevent carryover from the autosampler needle, because of the potential large concentration differences between samples, the autosampler was programmed using the Analyst software to perform a needle-rinse cycle by dipping the needle into two different rinse vials after drawing each sample and before injecting the next. The possibility of cross-contamination from the online SPE column was also minimized. Between each online SPE separation, the SPE column was regenerated by a constant flow (1 mL/min) of ACN for 3 min while the chromatographic separation of the analytes was taking place on the HPLC analytical column. High-concentration spiked samples were injected followed by blanks, and no evidence of carryover was found. The presence of small amounts of native compounds in the blanks were rather a contribution from the labeled internal standards; a systematic error that the calibration curve intercept automatically corrected.

The online SPE–HPLC–MS–MS separation and detection was only 1 min longer than the automated off-line SPE–HPLC– MS–MS method. Therefore, similar throughputs were achieved with both methods, but the online SPE method resulted in a significant reduction in sample handling and laboratory waste than the off-line method. Moreover, with the online SPE method, over 300 samples could be injected before the replacement of the online SPE column or regeneration of the HPLC column was necessary, but with the off-line SPE method, the HPLC column had to be regenerated after every 100 samples.

Conclusion

In summary, high-throughput automated online and off-line SPE–HPLC–MS–MS methods for the simultaneous measurement of six isoflavones and lignans in urine were developed. Good sensitivity and precision were achieved with both SPE strategies, although the online method was faster and more sensitive, precise, reproducible, and cost-effective than the off-line method. Both methods are adequate for the analysis of large number of samples for epidemiological studies to assess the prevalence of human exposure to isoflavones and lignans.

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