Development of a high-throughput LC/APCI-MS method for the determination of thirteen phytoestrogens including gut microbial metabolites in human urine and serum

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ABSTRACT

The investigation into the potential usefulness of phytoestrogens in the treatment of menopausal symptoms requires large-scale clinical trials that involve rapid, validated assays for the characterization and quantification of the phytoestrogenic precursors and their metabolites in biological matrices, as large interindividual differences in metabolism and bioavailability have been reported. Consequently, a new sensitive high-performance liquid chromatography-mass spectrometry method (HPLC–MS) for the quantitative determination of thirteen phytoestrogens including their most important gut microbial metabolites (genistein, daidzein, equol, dihydrodaidzein, *O*-desmethylangolensin, coumestrol, secoisolariciresinol, matairesinol, enterodiol, enterolactone, isoxanthohumol, xanthohumol and 8-prenylnaringenin) in human urine and serum within one single analytical run was developed. The method uses a simple sample preparation procedure consisting of enzymatic deconjugation followed by liquid–liquid extraction (LLE) or solid-phase extraction (SPE) for urine or serum, respectively. The phytoestrogens and their metabolites are detected with a single quadrupole mass spectrometer using atmospheric pressure chemical ionization (APCI), operating both in the positive and the negative mode. This bioanalytical method has been fully validated and proved to allow an accurate and precise quantification of the targeted phytoestrogens and their metabolites covering the lower parts-per-billion range for the measurement of relevant urine and serum levels following ingestion of phytoestrogen-rich dietary supplements.

1. Introduction

Phytoestrogens typically are non-steroidal plant-derived polyphenolic chemical entities that can mimic the effects of natural estrogens, either through binding with the estrogen receptors (ER) or by influencing other cellular and molecular mechanisms [1,2]. These compounds are indicated as the active substances of herbal extracts used in dietary supplements for the relief of menopausal symptoms [3]. Based on their chemical structures, the biologically most active phytoestrogens can be divided into four main groups, isoflavonoids, lignans, coumestans and prenylflavonoids (Fig. 1).

Isoflavonoids, mainly soy-derived, are the most widely used and studied phytoestrogens. The most abundant soy phytoestrogens, genistein (GEN) and daidzein (DAID), both exert relatively weak estrogenic effects via ER-alpha. On the other hand, genistein

binds ER-beta almost as efficient as 17 beta-estradiol [4]. Furthermore, the metabolism of daidzein by human intestinal microbiota results in its reduction to dihydrodaidzein [5], which can be further converted to *O*-desmethylangolensin (*O*-DMA) [6] and/or equol (EQ) [7], which is a more potent phytoestrogen than daidzein with respect to both ER-alpha and ER-beta [4]. Approximately only one-third to one-half of the human population is subject to the exclusive gut bacterial transformation of daidzein into *S*-equol, which may affect the health outcomes of soy consumption regarding its potency towards both estrogen receptor subtypes (with potency ER-beta > potency ER-alpha) [8]. Lignans, consisting as dimers of phenylpropane linked by β–β bonds, are found in most fibre-rich foods, such as whole grains, seeds, fruit, vegetables, and particularly in flaxseed. The non-estrogenic plant lignans secoisolariciresinol (SECO) and matairesinol (MAT) can readily be metabolized by intestinal bacteria into the mammalian lignans enterodiol (END) and enterolactone (ENL) [9,10]. To date, 8-prenylnaringenin (8-PN), a prenylflavonoid derived from hops is regarded as one of the most potent phytoestrogens known, at least with respect to ER-alpha [11–13]. Xanthohumol (X), quantitatively the most important
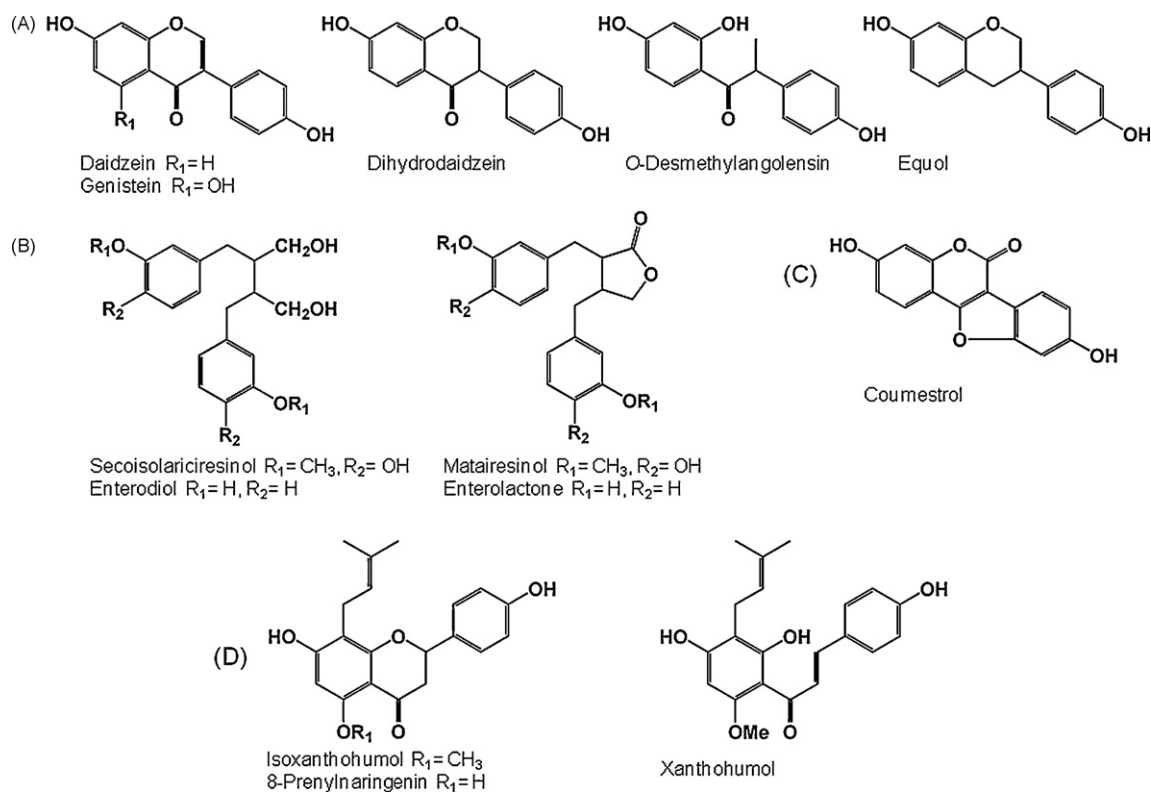
Fig. 1. Chemical structures of phytoestrogens (including precursors and metabolites) under investigation: (A) isoflavonoids (from soy); (B) lignans (from flax); (C) a coumestan (from red clover and alfalfa); (D) prenylflavonoids (from hops).

prenylchalcone in hops, does not act as a modulator of the ER, but has been described as a very promising anti-cancer agent with a large diversity of mechanisms of action. It also possesses anti-oxidant, anti-viral, and anti-inflammatory properties [14,15]. Recent studies have shown that intestinal microbial metabolism plays a significant role in the bio-activation of prenylflavonoids. During beer brewing, xanthohumol is isomerized into isoxantho-humol (IX), the most abundant prenylflavonoid in beer. In addition, herbal extracts of hops may contain significant amounts of iso-xanthohumol. Isoxanthohumol, which has no estrogenic properties itself, can be converted into 8-prenylnaringenin via an enzymatic O-demethylation, most likely the result of intestinal bacterial activity [16]. Finally, coumestrol, probably the less commonly occurring phytoestrogen, has mainly been isolated from clover sprouts and alfalfa. Although an increasing number of studies are dedicated to determine biological effects of coumestrol in humans and animals, the dietary exposure to coumestrol is very low and no considerable physiological levels have been reported yet [17,18].

The use of phytoestrogen supplements for the management of menopausal symptoms has grown exponentially since the negative reports on hormone-replacement therapy (HRT) resulting from the outcome of the Women's Health Initiative (WHI) [19] and Million Women Study [20]. However, clinical trials do not consistently show clinical benefits of the use of phytoestrogens. One of the factors that may contribute to the highly variable results is the large interindividual variability in metabolism and bioavailability. Therefore, future clinical trials on the efficacy and safety of phytoestrogens should include an estimation of the final exposure to the bioactive metabolites upon phytoestrogen intake. Thus, rapid, reliable and accurate analytical methods for their determination in biological matrices are required to support clinical investigations.

Since the beginning of research on phytoestrogens and their metabolites in a great diversity of matrices, from plants to human biological fluids and tissues, a wide range of analytical techniques for their determination and quantification have been reported. As reviewed by several authors [21,22] the techniques used can be classified into chromatographic and non-chromatographic, with high-performance liquid chromatography (HPLC) and gas chromatography (GC) as main tools in the former group and immunoassay techniques based on antibodies in the latter group. Generally, chromatographic separation is coupled to detection techniques like ultraviolet (UV) and mass spectrometry (MS). Most reported chromatographic methods cover detection and quantification of a limited number of phytoestrogens and/or their intestinal metabolites in biological fluids. Moreover, the greater part of the techniques require expensive equipment like a tandem mass spectrometer and analyses are often preceded by an intensive multi-step clean-up and complex sample preparation procedures such as derivatization to increase compound volatility for GC [18,23,24].

Here, we present a new chromatographic method for the simultaneous determination of thirteen phytoestrogens and their most important precursors/metabolites (genistein, daidzein, equol, dihydrodaidzein, O-desmethylangolensin, coumestrol, secoisolariciresinol, matairesinol, enterodiol, enterolactone, isoxanthohumol, xanthohumol and 8-prenylnaringenin) in human urine and serum. This simple method consists of enzymatic hydrolysis using *Helix pomatia* β-glucuronidase/sulfatase, followed by organic solvent extraction or solid-phase extraction, for urine and serum, respectively, and separation and detection using HPLC-UV-atmospheric pressure chemical ionization-mass spectrometry. This method was developed for the analysis of samples collected in a clinical trial conducted by the same laboratory in collaboration with the Ghent University Hospital, but can also serve as a guiding principle for other research groups in the domain of phytoestrogens.

2. Experimental

2.1. Chemicals and reagents

Isoxanthohumol, xanthohumol and 8-prenylnaringenin were available in our laboratory [16]. Daidzein en genistein were purchased from Acros Organics (Morris Plains, NJ, USA), equol from Extrasynthèse (Genay, France) and dihydrodaidzein and O-desmethylangolensin from Plantech UK (Reading, UK). Sec-oisolariciresinol, matairesinol, enterodiol, enterolactone, coumestrol, the internal standard 4-hydroxybenzophenone and β-glucuronidase/sulfatase from Helix pomatia (Type H1) were obtained from Sigma–Aldrich (St. Louis, MO, USA). All reference standards had a minimum HPLC purity of 95%, except for matairesinol (85%). Sodium acetate was obtained from UCB (Leuven, Belgium). Methanol, diethyl ether and n-hexane used during sample preparation were from ChemLab (Zedelgem, Belgium). Formic acid was purchased from Acros Organics. Water, methanol and acetonitrile used for chromatography were of LC–MS grade (Biosolve, Valkenswaard, The Netherlands).

2.2. Standards and samples

Primary stock solutions of standards and internal standard were prepared in methanol at a concentration of 1.0 mg/ml and 0.2 mg/ml, respectively. From these, a working solution containing each of the thirteen standards was made at a concentration of 10 μg/ml for IX, X, 8-PN and COUM, and 100 μg/ml for DAID, GEN, DHD, O-DMA, EQ, SECO, MAT, END and ENL. This mixture and further dilutions were used for spiking of urine samples, whereas a 2:5 dilution was used for serum samples. All the above mentioned solutions were kept at −20 °C.

For method development and validation, pooled human blank urine was collected from volunteers in the laboratory and pooled human blank serum was purchased from Innovative Research (Novi, MI, USA). Blank urine and serum from different individuals were obtained from the dietary intervention trial (FYTOES) for which the method was developed. All samples were stored at −20 °C until analysis. Prior to extraction, urine samples were centrifuged at 3000 rpm for 10 min to remove solids and the serum was defatted using n-hexane (1:1; v/v).

2.3. Preparation of calibration standards and quality control samples

Calibration standards (calibrators), except blanks, were prepared by spiking with the standard mixture to give concentrations for IX, X, 8-PN and COUM ranging from 0.1 ng/ml to 400.0 ng/ml in urine, and 0.04 ng/ml to 100.0 ng/ml in serum; for DAID, GEN, DHD, O-DMA, EQ, SECO, MAT, END and ENL, final concentrations in urine ranged from 1.0 ng/ml to 4000 ng/ml and in serum from 0.4 ng/ml to 1000 ng/ml. The added volume was less than 5% of the total volume of the samples in order to maintain the integrity of matrix. Quality control (QC) samples were freshly and independently prepared at three levels (low, medium and high). Moreover, DAID, GEN, DHD, O-DMA, EQ, SECO, MAT, END and ENL were spiked in urine at 800 ng/ml for low QC level, at 2000 ng/ml for medium QC level, and at 3000 ng/ml for high QC level, and IX, X, 8-PN and COUM were spiked at 80 ng/ml for low QC level, at 200 ng/ml for medium QC level, and at 300 ng/ml for high QC level. In serum, a 1:5 dilution of the three QC level spike solutions was used. All these prepared solutions were aliquoted and stored at −20 °C until required.

2.4. Preparation of samples

2.4.1. Urine

Urine sample preparation was based on a simple protocol previously published by Bolca et al. [25]. Prior to extraction, thawed urine samples (2.0 ml) were diluted with an equal volume of sodium acetate buffer (pH 5.0; 0.1 M), spiked with 100 μl internal standard (20 μg/ml) and 30 μl of deconjugation mixture (Helix pomatia 33 mg/ml in sodium acetate buffer (pH 5.0; 0.1 M); β-glucuronidase activity: 300 U/mg and sulfatase activity: 15.3 U/mg) and then incubated for 1 h at 37 °C. After incubation, a liquid–liquid extraction (LLE) was carried out (twice) using 5 ml diethyl ether and subsequent vortex mixing (30 s). The collected organic solvent layers (5 ml (2 × 2.5 ml) in total) were dried under a gentle nitrogen stream, dissolved in 100 μl injection solvent and transferred into a vial for LC–MS analysis. The injection volume was set at 25 μl.

2.4.2. Serum

For the extraction of the target compounds from the serum samples, a standard protocol that makes use of solid-phase extraction (SPE) was evaluated and optimized. The final method is summarized as follows: 800 μl of each sample was transferred to an eppendorf tube, diluted with an equal volume of deconjugation mixture, and spiked with 50 μl internal standard and then incubated at 37 °C overnight. Bond Elut C18 SPE cartridges were preconditioned with 3 ml methanol and 3 ml sodium acetate buffer prior to use. The sample was applied and after washing with 3 ml aqueous methanol (5%) and 5 min drying of the sorbent under vacuum, the target compounds were eluted with 3 ml methanol. This was followed by drying of the eluents under a gentle stream of nitrogen and dissolving in the injection solvent. An injection volume of 25 μl or 50 μl was applied.

2.5. Liquid chromatography-mass spectrometry

Method development, optimization and validation were conducted on an Agilent Technologies (AT, Santa Clara, CA, USA) 1200 series LC, equipped with a binary gradient pump, autosampler, thermostatted column oven and photodiode array UV detector. Chromatographic separation of all compounds in one single run was performed with a Waters XBridge C18 reversed phase (3.5 μm) column (3.0 mm id × 150 mm column) connected to a 3.0 mm id × 20 mm C18 guard column (3.5 μm) and maintained at a temperature of 55 °C. The mobile phase consisted of H_2O (eluent A) and MeOH/MeCN (80:20, w/w) (eluent B), both acidified with 0.025% (v/v) formic acid. The mobile phase was degassed by the integrated AT 1200 series vacuum degasser. The gradient and flow rate were programmed as follows: 0–3.5 min: 35–45% B (flow: 0.6 ml/min), 3.5–5.5 min: isocratic 45% B (flow: 0.6 ml/min), 5.5–13 min: 45–100% B (flow: 0.6 ml/min), 13–16 min: isocratic 100% B (flow: 0.8 ml/min), 16–16.10 min: 100–35% B (flow: 0.6 ml/min) and 3.9 min for re-equilibration of the column.

MS analysis was performed using an AT multimode ionization source coupled to a single quadrupole detector (MSD), SL version. A standard APPI/APCI calibration mix was used for daily tuning of the MSD and processing of the data was carried out using the LC/MSD Chemstation software (Rev. B.02.01). All analytes were ionized using atmospheric pressure chemical ionization (APCI), either in the positive or in the negative mode. For quantification in the SIM mode, four MSD signals were set, three in the APCI positive mode and one in the APCI negative mode with optimized fragmentor voltages and gain values for all analytes (Table 1). The HPLC effluent entered the ionization chamber only in the time window between 3.5 min and 14.0 min and two different time

Table 1
MSD signal groups with target ion values (m/z), gain values, fragmentor voltages and cycle times.

MSD signals	Time windows & analytes	Ions (m/z)	Gain value	Fragmentor voltage (V)	Cycle time (%)
MSD 1 (APCI+)	3.5–10.4 min		1.0		10.0
	IS	199		125	
	DHD	255		175	
	DAID	257		125	
	GEN	271		200	
	10.41–14.0 min		2.0		100.0
	8-PN	341		150	
	IX, X	355		200	
MSD 2 (APCI+)	3.5–10.4 min		3.0		30.0
	EQ	243		100	
	O-DMA	259		125	
	COUM	269		175	
MSD 3 (APCI+)	3.5–10.4 min		3.0		30.0
	SECO	327		120	
	MAT	359		125	
MSD 4 (APCI−)	3.5–10.4 min		3.0		
	ENL	297		125	30.0
	END	301		175	

windows were applied for optimal sensitivity as also given in Table 1.

2.6. Method validation

The HPLC/APCI-MS method was fully validated following the guidance for industry of the US Food and Drug Administration (FDA) for the Validation of Bioanalytical Methods [26], with emphasis on the key elements described by Bansal et al. [27]. The following parameters were evaluated: selectivity, linearity, sensitivity, accuracy, inter- and intra-assay precision and stability. Additionally, recovery for the analytes of interest was calculated and an assessment of matrix effects was made in correspondence with the strategy applied by Matuszewski et al. [28]. SPSS for Windows version 17.0 was used to carry out all statistical analyses.

2.6.1. Selectivity

The selectivity, i.e., the ability of an analytical method to differentiate and quantify the analytes in the presence of other components in the sample, was assessed by analyzing five different batches of blank urine and serum. Samples (blank and zero) were processed by the appropriate extraction procedure and the resulting MS chromatograms were evaluated for the presence of interferences at the retention times of the analytes and the internal standard.

2.6.2. Linearity and sensitivity

Calibration curves were constructed over individually specified ranges using eight non-zero standard points in six replicates. In addition, a blank (non-spiked sample) and a zero sample (only spiked with IS) were run to exclude the presence of interferences. Peak area ratios between the analytes and the internal standard were plotted against the nominal concentrations of the calibration standards. Unweighted linear regression analysis generated calibration curves with the standard equation: $y = ax + b$, where y is the peak area ratio, x the concentration, a the slope, and b the intercept of the regression line. The adequacy of the assumed model and the linearity of the calibration curves was evaluated by observing correlation coefficients (r^2) and by visual inspection of the plots of the residuals (assumption of homoscedasticity). In addition, linearity was also assumed if the relative residuals did not exceed 15% and when at least 75% of the calibration standards met the stated accep-

tance criteria. Daily prepared calibration curves were constructed by injecting calibration standards at the beginning and at the end of each batch of samples.

The limit of detection (LOD) and the limit of quantification (LOQ) of the method were defined as the lowest concentration with a signal-to-noise ratio of 3 and 10, respectively, in spiked samples, where the noise data were taken from the analysis of blank matrices.

2.6.3. Accuracy and precision

The accuracy and the precision of the method were evaluated by analyzing QC samples at three levels (low, medium and high) ($n = 6$) according to the procedure described above on four consecutive days. The accuracy (%RE) was calculated for each analyte in terms of correspondence of the measured mean value to the nominal concentration value. Analysis of variance (ANOVA) was used to calculate inter- and intra-assay precision for each QC level. To determine inter-assay variation (intermediate precision), the QC samples were analyzed at the three levels on four different days. The intra-assay variation (repeatability) was expressed for each QC level as the coefficients of variation (%CV) of measurements on the same day. The accuracy and intra-assay precision values should be less than 15% and should not exceed 20% for the inter-assay variation.

2.6.4. Stability

Two types of short-term stability experiments were performed at the low and high QC level. The autosampler stability was estimated on processed samples ($n = 3$) at room temperature before and after an interval of approximately 15 h. The stability of the compounds was also examined during three freeze–thaw cycles at −20 °C. Three replicates of spiked urine and serum samples were allowed to thaw at ambient temperature and were then refrozen for minimum 12 h. Aliquots of all samples were quantified at the end of the third freeze–thaw cycle. The concentration of each compound was compared to that of fresh samples and expressed in terms of degradation.

2.6.5. Recovery and matrix effect

For the assessment of recoveries of the analytes and matrix effects caused by constituents in urine and serum, a strategy was applied which has previously been described by Matuszewski et al.

[28]. The recovery was calculated as the ratio $((C/B) \times 100)$ between the responses of the QC samples in real blank matrix (low and high level, $n = 3$) spiked after extraction (B) and before extraction (C) and is therefore not influenced by effects of the sample matrix. The absolute matrix effect (ME%) can be defined as the ratio of the responses between samples spiked after extraction (B) and pure standards in the mobile phase (A). When the value of this ratio $((B/A) \times 100)$ is <100% there is signal suppression and signal enhancement is noted if the value is >100%. Taking into account the heterogeneity of this effect between different sample batches, experiments were performed in six independent batches of blank urine and serum.

3. Results and discussion

3.1. Chromatographic and mass spectrometric method development

First, the reversed chromatographic separation of the thirteen analytes of interest and the internal standard was optimized. Although complete resolution of EQ, GEN and ENL on UV detection was not accomplished with the optimized LC method, subsequent mass selective detection allowed full separation of these compounds. The interface and MS parameters were optimized individually for each component and are listed in Table 1. Atmospheric pressure chemical ionization (APCI) in the positive mode was selected, except for END and for ENL which gave the highest sensitivity with APCI in the negative mode. Pseudomolecular ions were predominant, with $[M+H]^+$ for the positive and $[M-H]^-$ for the negative mode. However, for SECO, the fragment ion $[M+H-2H_2O]^+$ was found to constitute the base peak (i.e., 100% relative intensity of the ion), in agreement with the observations by Bambagiotti-Alberti et al. [29] and Schmidt et al. [30]. Formation of this fragment ion is probably due to protonation of the γ-hydroxyl groups of SECO and subsequent loss of water from the unstable oxonium ions. Finally, using single-ion monitoring (SIM) and operating the MS in the chosen ionization method, the fragmentor voltage, a component-dependent parameter in MS sensitivity and possible initiator of collision-induced dissociation (CID), was varied from 50 V to 400 V. Gain values and MS cycle times were individually defined per MS signal to obtain maximum sensitivity. Moreover, two time groups were assigned to the first of four MSD signals, in order to benefit the detection (i.e., 100% cycle time) of the hop-derived compounds X, IX and 8-PN, which elute at the end of the solvent gradient. In Fig. 2, merged, normalized MS chromatograms of the standard mixture in solvent are illustrated at medium level of the calibration range.

3.2. Method validation results

3.2.1. Specificity

Only a few small peaks were observed in the mass chromatograms of the different blank and zero urine and serum samples (resolution between target and interfering peak always between 0.5 and 1.0). Since the present method also makes use of extracted ions for quantification, the background is further reduced and the sensitivity as well as specificity are increased. Moreover, simultaneous recording of characteristic retention times and UV spectra favoured accurate identification and quantitation of the target compounds. During validation, appropriate blanks were included to correct for any possible interference problems.

3.2.2. Assessment of linearity and sensitivity

The linearity was tested in urine for the range of concentrations 0.1–400.0 ng/ml for IX, 8-PN, X and COUM and 1.0–4000.0 ng/ml for DAID, GEN, DHD, O-DMA, EQ, SECO, MAT, END and ENL. In serum,
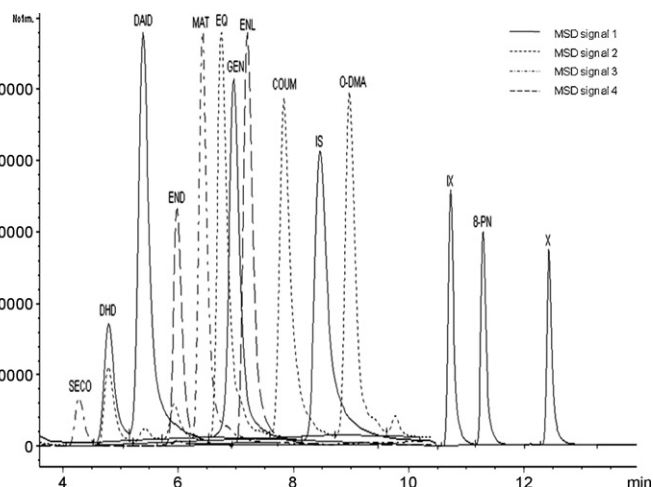

Fig. 2. Normalized and merged MS chromatograms of the four MSD signals (pure standard mixture at medium range of calibration). Note: the appearance of some peaks in multiple signals are due to overlay of the window of m/z values and/or small impurities in the standards; peak tailing is due to the solvent strength of the injection solvent (100% MeOH).

the concentrations ranged from 0.04 ng/ml to 100.0 ng/ml for IX, 8-PN, X and COUM, and 0.4 ng/ml to 1000.0 ng/ml for the other compounds. Calibration curves showed linear responses for all analytes over the dynamic ranges and the corresponding regression correlation coefficients (r^2) were all >0.995. Weighting was not considered since the linearity of all thirteen compounds in both urine and serum was satisfactory. Indeed, visual inspection of residual plots confirmed the hypothesis of homogeneity of variance (randomly scattered). In addition, the residuals were in accordance with the stated acceptance criteria, as the %RRE (% relative residual error, i.e., percent relative difference of the residual from the nominal value at a certain calibration point) was for all standards ≤15% at each calibration point. The limits of detection (S/N = 3) for all compounds, both in urine and serum, are listed in Table 2. In urine, the LODs ranged from 0.2 ng/ml to 7.7 ng/ml, and in serum, from 1.4 ng/ml to 20.4 ng/ml, except for SECO which gave LODs in urine and serum of 65.1 ng/ml and 132.6 ng/ml, respectively. If necessary, the target compounds, the levels of which are assumed to be below the corresponding LOD in real samples, can be reanalyzed with only one MSD signal active to further improve the sensitivity of detection. The limits of quantification were set as the lowest point of the calibration curves with a signal-to-noise ratio of at least 10.

3.2.3. Accuracy and precision

The accuracy and precision of the method were determined by spiking blank urine and serum samples at low, medium and high

Table 2
Limits of detection (LOD) in urine and serum.

	Urine LOD (ng/ml)	Serum LOD (ng/ml)
Dihydrodaidzein	2.4	7.9
Daidzein	1.0	1.9
Genistein	0.8	2.2
Isoxanthohumol	0.2	1.5
8-Prenylnaringenin	0.4	1.5
Xanthohumol	0.6	1.4
Enterodiol	2.7	3.1
Enterolactone	2.5	2.4
Equol	5.8	13.8
Coumestrol	0.4	6.2
O-DMA	7.7	20.4
Secoisolariciresinol	65.1	132.6
Matairesinol	7.5	17.8

Table 3
Accuracy (%) in urine and serum for three different QC levels ($n = 6$).

	Urine			Serum		
	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
DHD	4.7	0.5	0.7	3.8	6.3	0.4
DAID	1.6	10.3	11.4	4.0	0.7	4.9
GEN	1.8	13.1	13.4	3.1	0.08	7.5
IX	7.1	2.6	0.2	11.8	14.0	6.0
8-PN	10.5	11.1	8.6	6.6	6.4	9.2
X	12.0	4.5	14.6	6.4	9.0	0.01
END	6.9	5.2	8.5	15.0	8.3	16.7
ENL	11.0	5.6	7.3	17.3	4.6	14.4
EQ	2.7	1.1	1.4	1.7	6.7	6.5
COUM	0.9	0.7	1.5	14.4	3.0	18.7
O-DMA	5.2	9.2	3.2	8.2	12.7	8.4
SECO	10.4	6.2	0.2	14.8	4.9	4.5
MAT	4.7	0.2	6.9	12.9	12.8	14.5

Table 5
Absolute recovery (%) in urine and serum at high QC level ($n = 6$) (%RSD).

	Urine	Serum
DHD	97.3 (14.4)	89.2 (7.8)
DAID	119.0 (11.4)	95.2 (6.2)
GEN	99.6 (10.6)	90.6 (8.5)
IX	91.9 (16.9)	46.8 (9.4)
8-PN	78.7 (14.9)	88.3 (12.2)
X	74.1 (16.1)	58.8 (16.1)
END	99.8 (9.7)	95.6 (9.4)
ENL	115.5 (10.1)	95.0 (7.1)
EQ	91.1 (11.9)	95.5 (6.2)
COUM	89.7 (16.4)	19.7 (14.0)
O-DMA	91.7 (15.2)	83.2 (9.3)
SECO	67.5 (15.9)	95.8 (7.8)
MAT	87.8 (14.6)	98.0 (7.2)

quality control levels, with six replicates on four consecutive days. The accuracy, expressed as the percentage relative error (%RE), was within the acceptable ranges of 20% for all compounds at all concentrations. In urine, the accuracy ranged from 0.2% to 14.6% and, in serum, from 0.01% to 18.7% (Table 3). The precision of the method is reflected in the variance of quality control samples over time. Inter- and intra-assay variations in urine and serum were thoroughly investigated for the three QC levels and were expressed as %CV (Table 4). The %CV for the inter-assay precision in urine and serum did not exceed the acceptable 20% and ranged from 4.3% to 18.8% and 4.4% and 15.7% in urine and serum, respectively. The intra-assay precision in urine was overall better than 10% (%CV < 15% as required by the acceptance criteria), except for END, for which, at medium level was 14.0%. In serum, the intra-assay precision results were also confirm with the stated criteria and with the exception of DAID and END, at respectively high and low QC level, were also below 10%.

3.2.4. Stability

The chemical stability of the analytes was assessed in the target matrix under specific conditions of time and temperature. In particular, the short-term stability was investigated in urine and serum. In urine, all components were stable in the processed samples after a period of 15 h in the autosampler (room temperature), except for END and ENL. At both low and high levels a significant ($P < 0.05$) and consistent increase in concentration was found for END and ENL (i.e., 54.5% increase at low and 37.8% increase at high level for END and 54.0% at low and 31.5% at high level for ENL). With the exception of a significant ($P < 0.05$) average decrease of 26.1% in concentration of COUM at low QC level, the stability of processed serum samples after 15 h in the autosampler was confirmed. Fur-

thermore, in urine, following three repeated cycles of freezing and thawing, only IX and 8-PN showed degradation in the low QC samples (i.e., >20% difference in concentration between reference and stability samples). At both low and high levels, an acceptable but slight degradation was noticed for END and ENL (>15% and <20% difference). Finally, an instability of MAT, estimated as an average of 42.0% (±4.8%) loss in concentration of MAT at low and high levels was observed after three freeze and thaw cycles.

3.2.5. Recovery and matrix effect

The recoveries (IS-normalized) in urine for all analytes were greater than 67%, both for low and high concentrations. In serum samples, the average recoveries were found to be higher than 83% for most analytes, except for IX, X and COUM. The lower recoveries in serum for IX and X (i.e., 40.6% and 46.8% for IX and 69.1% and 58.8% for X at low and high level, respectively) and especially for COUM, which had an average recovery of 24.1% (±5.0%), can be explained by strong retention on the SPE sorbent due to their relatively non-polar character and the low solubility of COUM in the elution solvent (MeOH). All recoveries at high level are listed in Table 5.

In this study, matrix effects (ME%) in urine and serum were evaluated by analyzing low and high QC samples (only the matrix effects of the high QC samples in urine and serum are shown here in Table 6). In urine, an overall ion enhancement was observed, especially for GEN and COUM, with an average of 43% and 49% increase of the signal due to matrix effects, respectively. Serum matrix components resulted for SECO in more than a twofold increase of the observed signal. The high matrix effect observed with SECO may be attributed to co-elution in the beginning of the gradient with retained matrix components that lead to a strong enhancement of the ionization of SECO and subsequent overestimation of measured concentrations. A relative overall ion enhancement was noticed in

Table 4
Inter- and intra-assay precision (%CV) for three different QC levels ($n = 6$) in urine and serum.

	Inter-assay (urine/serum)			Intra-assay (urine/serum)		
	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
DHD	14.3/13.4	10.8/7.6	14.6/11.5	6.9/2.9	6.6/6.8	5.6/5.9
DAID	17.8/12.8	10.5/6.1	14.4/11.3	6.9/9.1	5.0/5.1	6.4/10.8
GEN	11.7/13.7	8.4/5.3	13.0/13.2	7.4/3.0	5.0/3.7	4.1/5.6
IX	18.7/11.1	9.2/15.0	4.3/11.2	7.2/3.3	5.4/5.7	3.1/7.9
8-PN	15.6/11.2	12.6/7.5	15.0/10.4	8.7/3.1	6.1/1.2	3.3/7.4
X	16.0/10.5	15.3/7.8	17.6/11.9	9.3/3.4	8.1/3.7	3.8/8.4
END	10.7/11.2	15.8/5.2	10.9/6.5	8.1/10.7	14.0/7.8	5.4/6.4
ENL	14.0/10.5	14.4/7.8	10.4/15.4	9.4/3.2	5.6/3.2	4.5/3.5
EQ	14.3/11.0	15.2/7.5	12.1/13.4	5.6/3.3	6.5/1.4	8.2/6.8
COUM	13.3/13.1	16.8/4.4	15.1/14.1	7.3/9.3	5.1/1.3	3.3/10.0
O-DMA	18.8/10.0	17.8/6.4	6.6/12.5	6.0/3.0	10.0/5.2	5.7/5.8
SECO	14.8/15.7	13.4/11.9	16.5/15.5	7.4/2.8	3.8/5.1	6.9/8.4
MAT	12.3/13.4	14.7/7.1	17.6/9.5	7.1/2.9	4.1/6.2	7.0/7.5

Table 6
Matrix effects (ME% (RSD%)) in urine and serum at high QC level in 6 different matrix blanks ($n = 3$).

	A	B	C	D	E	F
Urine						
DHD	95.2(7)	111.9(4)	111.3(6)	113.8(7)	104.0(7)	106.6(3)
DAID	104.9(8)	115.5(13)	111.3(7)	117.7(10)	134.0(3)	132.4(1)
GEN	131.0(13)	136.0(6)	146.9(9)	159.8(14)	150.7(7)	133.4(3)
IX	109.7(3)	116.6(5)	125.1(4)	148.6(5)	107.8(7)	120.5(6)
8-PN	112.4(7)	115.8(5)	109.0(2)	120.3(15)	102.7(6)	105.2(2)
X	107.5(12)	108.8(10)	91.2(3)	102.2(6)	85.6(8)	82.5(6)
END	98.1(11)	123.6(4)	127.2(3)	124.9(5)	101.1(7)	85.5(6)
ENL	86.6(11)	106.7(4)	110.3(7)	117.9(5)	101.3(6)	66.6(8)
EQ	86.6(10)	122.5(9)	93.6(5)	111.0(3)	94.3(12)	93.3(7)
COUM	149.2(13)	145.4(9)	165.1(9)	127.4(12)	161.6(10)	147.1(1)
O-DMA	108.1(6)	102.2(7)	99.9(6)	102.8(8)	98.8(7)	103.4(9)
SECO	95.6(6)	128.4(12)	138.6(11)	116.8(7)	105.3(13)	109.1(5)
MAT	99.1(5)	121.2(8)	113.3(5)	127.9(9)	101.8(6)	108.2(2)
Serum						
DHD	107.3(4)	116.9(4)	112.0(5)	113.0(6)	101.1(7)	83.9(6)
DAID	113.0(4)	116.3(3)	118.4(4)	120.3(5)	124.2(3)	93.3(2)
GEN	131.3(5)	135.6(3)	136.2(2)	137.2(5)	131.7(8)	93.2(1)
IX	119.6(6)	126.4(1)	117.4(2)	123.6(4)	121.9(7)	91.2(2)
8-PN	104.5(8)	111.8(7)	91.1(11)	103.4(6)	99.8(1)	70.9(11)
X	135.8(3)	136.4(1)	181.0(8)	162.8(3)	139.5(14)	96.8(3)
END	106.8(6)	131.6(5)	150.7(3)	158.8(3)	133.4(5)	101.7(2)
ENL	98.1(4)	112.5(1)	141.3(4)	144.5(2)	124.3(6)	101.6(2)
EQ	114.2(8)	113.5(2)	108.2(7)	110.5(7)	109.4(15)	88.6(6)
COUM	131.4(4)	132.3(5)	127.8(3)	129.3(3)	125.3(3)	99.4(4)
O-DMA	126.6(2)	123.9(7)	130.0(3)	123.5(4)	113.4(11)	85.6(4)
SECO	253.4(10)	235.3(9)	261.9(7)	281.6(4)	214.8(3)	150.2(5)
MAT	130.9(9)	127.4(5)	119.3(4)	121.7(8)	128.7(3)	92.7(5)

serum, although more stabilized matrix effects for SECO and the other components were observed in one of the six batches of serum (serum F). In that particular case, the serum was obtained from a totally different source (see Section 2.2, purchased pooled human blank serum) and did not represent the average type of serum sample that is generally obtained during the clinical trials this method was developed for. Moreover, the variation of the overall matrix effect in urine and serum samples was typically less than 15% (urine (A–F): 3.5–14.8% and serum (A–E): 2.0–15.7%) and considered relatively stable. This observation concerning matrix effects again highlights the need for multiple source relative matrix effect evaluation instead of assessments based on single source biological fluid measurements. As a conclusion, these findings of the matrix effects caused by urine and serum factors have clearly shown the relevance of a profound and extensive investigation during LC/MS method development and validation but were not the initial intention of this project.

4. Conclusions

Our HPLC/APCI-MS methodology represents a new quantitative assay for the simultaneous determination of thirteen phytoestrogens, including their most important precursors and gut microbial metabolites, in human urine and serum. The method applies a relatively short and low-cost sample preparation procedure. Validation parameters revealed that accuracy, intermediate precision and repeatability are within the general ranges for acceptance and furthermore the sensitivity is in the lower ppb-range. Moreover, the detection system can be easily integrated in a standard small-scale analytical laboratory. This validated method was successfully applied for the analyses of various urine and serum samples collected in a dietary intervention trial with food supplements combining different classes of phytoestrogens, as reported in detail by Bolca et al. [31]. The strength of our method, as mentioned above, is the ability to measure a wide panel of analytes in complex biological matrices, even after supplementation of mixtures of phytoestrogen-rich supplements. It can be concluded that

the present method, covering the detection of a wide and representative range of dietary phytoestrogens should provide a framework for extensive analysis in a (pre)clinical environment, whenever efficiency studies are impeded by interindividual differences in exposure levels.

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