applied sciences

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A Comprehensive HPLC-DAD-ESI-MS Validated Method for the Quantification of 16 Phytoestrogens in Food, Serum and Urine

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Received: 8 October 2020; Accepted: 12 November 2020; Published: 17 November 2020

Abstract: There has been increased interest in phytoestrogens due to their potential effect in reducing the risk of developing cancer and cardiovascular disease. To evaluate phytoestrogens' exposure, sensitive and accurate methods should be developed for their quantification in food and human matrices. The present study aimed to validate a comprehensive liquid chromatography-mass spectrometry (LC-MS) method for the quantification of 16 phytoestrogens: Biochanin A, secoisolariciresinol, matairesinol, enterodiol, enterolactone, equol, quercetin, genistein, glycitein, luteolin, naringenin, kaempferol, formononetin, daidzein, resveratrol and coumestrol, in food, serum and urine. Phytoestrogen extraction was performed by solid-phase extraction (food and serum) and liquid-liquid extraction (urine), and analyzed by LC diode-array detector (DAD) coupled with a single quadrupole MS with electrospray ionization (ESI) in negative mode. Validation included selectivity, sensibility, recovery, accuracy and precision. The method was proved to be specific, with a linear response ($r^2 \geq 0.97$). Limits of quantification were 0.008–3.541 ng/mL for food, 0.01–1.77 ng/mL for serum and 0.003–0.251 ng/mL for urine. Recoveries were 66–113% for food, 63–104% for serum and 76–111% for urine. Accuracy and precision were below 15% (except for enterodiol in food with 18% and resveratrol in urine with 15.71%). The method is suitable for the quantification of a wide number of phytoestrogens in food, serum and urine. The method was successfully applied in highly consumed food items ($n = 6$) from North Mexico and biofluids from healthy women ($n = 10$).

Keywords: phytoestrogens; food metabolites; biofluids; flavonoids; lignans; coumestrol; resveratrol

1. Introduction

Phytoestrogens are diphenolic plant metabolites that have been associated with a lower risk of cancer and cardiovascular diseases [1,2]. Phytoestrogens can induce biological activity through estrogenic mechanisms, by mimicking the action of 17β-oestradiol [3], and non-estrogenic mechanisms,

by regulating enzyme and antioxidant activities [4,5]. Despite these beneficial effects observed on in vitro and animal studies, there is still no clear evidence on the effects of phytoestrogens on human health.

Epidemiological studies, investigating the association between phytoestrogens and health, have been limited due to the difficulty determining phytoestrogen exposure. Traditionally, a food frequency questionnaire or a 24 h recall are used to accomplish this goal. The information obtained from these questionnaires is translated into quantitative records through a food composition database and an individual's consumption is estimated [6,7]. However, food databases with the content of phytoestrogens are incomplete, focused on a small group of phytoestrogens (i.e., isoflavones) and, in some cases, are unavailable [8,9]. In Mexico, foreign databases have been used to estimate phytoestrogens consumption with the inclusion of a limited number of food items [8]. These adapted food tables do not provide the phytoestrogens content of regional foods. These regional foods are characteristic of a typical Mexican diet and could represent a valuable source of phytoestrogens for the population. Phytoestrogen levels in serum and urine have been used to determine phytoestrogen intake; however, this is not always feasible, especially in large epidemiological studies.

A proper analytical method for the quantification of phytoestrogens depends not only on the matrix to be analyzed and the phytoestrogens (and other compounds) contained in the matrix but also on the processing of the matrix and the capabilities of the analytical technique [10]. Improvement on analytical techniques in the last decade has driven phytoestrogens structural characterization, identification and quantification in different matrices [11]. Liquid chromatography (LC) and time-resolved fluoroimmunoassay (TR-FIA) have been developed to analyze phytoestrogens in food [12] and biological samples [13]; however, some of them have low sensitivity and selectivity [14,15]. Gas chromatography-mass spectrometry (GC–MS) is a technique with high sensitivity and selectivity but requires several clean-up steps, complex sample preparation and derivatization [14,16,17]. LC–MS is the analytical method preferred due to a simple sample preparation, high sensitivity and specificity [18–20]. Recently, advances on LC separation along with MS^n structural information and resolving power have improved identification and quantification of small molecules in complex matrices in the lower ppb-level. The relatively low cost of LC-MS instruments has contributed to their widespread use in the analysis of phytoestrogens with single quadrupole (Q) that is more economically available in Latin America. MS instruments, such as Q, time-of-flight (TOF), Q-TOF, triple quadrupole (QqQ) and others, have ensured more known and unknown phytoestrogens being accurately identified. However, most LC-MS methods have focused on specific phytoestrogens to be analyzed per run [10,11]. Furthermore, a validated LC-MS method for the simultaneous quantification of phytoestrogens in food and biofluids has not been established. Even though these are complex and different matrices, with different phytoestrogen metabolites and with a wide variation in concentrations (i.e., serum levels lower compared to food and urine), quantification can be achieved with a comprehensive LC-MS method.

In the present study, we established and validated a high-performance liquid chromatography diode-array detector electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS) method for the quantification of sixteen phytoestrogens (biochanin A, secoisolariciresinol, matairesinol, enterodiol, enterolactone, equol, quercetin, genistein, glycitein, luteolin, naringenin, kaempferol, formononetin, daidzein, resveratrol and coumestrol), suitable for the analysis of food, serum and urine. The novelty of this method is the inclusion of a wide variety of phytoestrogens with high oestrogen-like activity present in food, beverages and related metabolites in human biofluids. The method was successfully applied to commonly eaten food items from North Mexico, as well as to urine and serum samples from healthy women. This method is flexible and suitable for phytoestrogens' quantification, and could be used to analyze human samples in large-scale epidemiological studies investigating the association between phytoestrogen exposure and human health. This study will contribute to the development of food databases in Latin-American countries with the inclusion of regional food items, which could represent an important dietary source of phytoestrogens.

2. Materials and Methods

2.1. Materials

Biochanin A, secoisolariciresinol, matairesinol, enterodiol, enterolactone, coumestrol, equol, genistein, glycitein, kaempferol, formononetin, daidzein, resveratrol, 4-methylumbelliferyl sulfate potassium salt (4MUS), phenolphthalein β-D-glucuronide (PhP-β-glu), 4-hydroxybenzophenone (IS), sodium acetate, formic acid, dimethyl sulfoxide (DMSO), β-glucuronidase/sulfatase (from Helix pomatia; type H1, β-glucuronidase activity: 300 U/mg and sulfates activity: 15.3 U/mg) and cellulose (from Trichoderma reesei, ≥1 U/mg) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Quercetin, luteolin, and naringenin were purchased from INDOFINE Chemical Company Inc. (Hillsborough, NJ, USA). All standards had a minimum HPLC purity of 85% or thin layer chromatography (TLC) purity of 99%. Methanol, acetonitrile, acetic acid and n-hexane were purchased from J. T. Baker (Philipsburg, NJ, USA). Water, DMSO, acetic acid, methanol and acetonitrile were HPLC grade. Sodium acetate and formic acid were of reagent grade (99 and 95% purity, respectively). Pooled human blank serum was purchased from Innovative Research (Novi, MI, USA).

2.2. Standards Preparation

Standards stock solutions were prepared at a concentration of 1 mg/mL in methanol (biochanin A in ethanol, 20 µL of DMSO was added to glycitein and coumestrol to increase their solubility in methanol). From these, a working solution containing the sixteen phytoestrogens was made at a concentration of 24,000 ng/mL. This mixture was used to make further dilutions, spike samples and quality controls. The internal standard was prepared in methanol at a concentration of 20,000 ng/mL. Deconjugation standards, 4-methylumbelliferyl sulfate potassium salt and phenolphthalein β-D-glucuronide, were prepared at a concentration of 1 mg/mL in water and methanol, respectively. From these, a working solution containing both deconjugation standards was made for spiking the samples (serum; 330,000 ng/mL and 400,000 ng/mL, urine; 24,000 ng/mL and 20,000 ng/mL, respectively).

2.3. Phytoestrogen Extraction from Food

Food matrices with low levels of phytoestrogens (i.e., boiled rice and potatoes) according to the Phenol-Explorer 3.6 database and U.S. Department of Agriculture (USDA) database [21,22] were used for validation analysis. Food matrix was homogenized, frozen (−70 °C), and lyophilized (−50 °C) under vacuum conditions before extraction. The dried sample (100 mg) was diluted with 10% methanol in sodium acetate buffer (1 mL, pH 5.0, 0.1 M) and sonicated for 30 min. The sample was centrifuged (30 min, 2500× *g*), and the supernatant was transferred to a vial. The precipitate was washed and centrifuged twice. β-glucuronidase/sulfatase and cellulose mixture (3 mL, 10 U/mL) was added to the supernatant (2 mL) and incubated for 37 °C for 16 h. Strata C18 solid-phase extraction (SPE) cartridges (50 mg) were preconditioned with methanol (1 mL) and 5% methanol (1 mL). The sample was applied to the cartridges and washed with 5% methanol (2 mL). Phytoestrogens were eluted with methanol (3 mL) and dried under a gentle stream of nitrogen at 37 °C. Additionally, methanol (500 µL) was added to the vial and evaporated to increase recoveries. Finally, phytoestrogens were resuspended in methanol (80 µL) and initial mobile phase composition (120 µL, 65% Solvent A, 35% Solvent B). Regional food items (n = 6, North Mexico, Table 4) were selected for analysis as part of the validation process based on their frequency of consumption [23], form of consumption (raw or cooked) and possible content of phytoestrogens.

2.4. Phytoestrogen Extraction from Serum

Methanol (200 µL) and n-hexane (1 mL) were added to serum (1 mL) followed by centrifugation to remove proteins and lipids. The remaining (800 µL) was spiked with internal standard (50 µL, 20 µg/mL) and deconjugation standards (10 µL), and was vortexed for 10 s. Conjugated phytoestrogens

were hydrolyzed by the addition of β-glucuronidase/sulfatase mixture (800 µL, 33 mg/mL in sodium acetate buffer; pH 5.0; 0.1 M) and were incubated at 37 °C overnight. Bond Elut C18 SPE cartridges (Agilent, 3 mL, 100 mg) were preconditioned with methanol (3 mL) and sodium acetate buffer (3 mL). The samples were centrifuged (10 min, 7200× *g*) and then applied to the cartridges. The matrix interferences were washed with methanol—2% acetic acid (3 mL, *v*/*v*, 5/95). Phytoestrogens were eluted with methanol (3 mL), followed by acetonitrile (2 mL). The eluate was dried under a gentle stream of nitrogen at 37 °C and was resuspended in methanol (80 µL) and initial mobile phase composition (120 µL). Serum samples were collected and analyzed from healthy women (n = 10), who gave written consent before they participated in the study, as part of the validation process. This research was conducted in accordance with the Declaration of Helsinki and the protocol was reviewed and approved by the Ethical Committee of Centro de Investigación en Alimentación y Desarrollo A.C. (registration number CE/005/08).

2.5. Phytoestrogens Extraction from Urine

Pooled urine samples were obtained from subjects following a low phytoestrogens diet (intake < 40 µg/day) and used as matrix blanks for validation analysis. Urine (2 mL) was centrifuged and diluted with sodium acetate buffer (2 mL, pH 5.0; 0.1 M). Internal standard (100 µL) and deconjugation standards (4 µL) were added to the sample. Phytoestrogens were hydrolyzed by the addition of β-glucuronidase/sulfatase mixture (30 µL, 5.14 mg/mL in sodium acetate buffer; pH 5.0; 0.1 M) and were incubated at 37 °C for 4 h. Liquid-liquid extraction was done by adding diethyl ether (5 mL, twice) to the urine. The organic layer (approx. 10 mL) was transferred and evaporated under a gentle stream of nitrogen at 37 °C. The sample was resuspended in methanol (80 µL) and initial mobile phase composition (120 µL). Urine samples (12 h, n = 10) were collected, corrected for creatinine and analyzed as part of the validation process from healthy women.

2.6. Liquid Chromatography/Mass Spectrometry

The method was validated on an Agilent HP 1100 series G1946A LC-MSD (model VL, Chemstation software rev B.03.02), equipped with a degasser, quaternary pump, autosampler, thermostated column oven, photodiode array ultraviolet (UV) detector (DAD), coupled to a single quadrupole (Agilent Technologies, Inc., Palo Alto, CA, USA). An injection volume of 50 µL was applied to the HPLC-MS. Phytoestrogens separation was performed using a Water XBridge C18 reversed-phase (3.0 mm id × 150 mm, 3.5 µm) column with a C18 guard column (3.0 mm id × 20 mm, 3.5 µm), at 52 °C. The mobile phase consisted of water (Solvent A) and methanol/acetonitrile (80:20, *v*/*v*, Solvent B), both with formic acid (0.025%, *v*/*v*), at a flow rate of 0.6 mL/min. The gradient elution was programmed as follows: 35–40% B at 5 min, 100% B at 16 min, returned to 35% B at 19 min and re-equilibrated for 3 min. DAD was set at 290, 340, 260, 216 and 280 nm. Phytoestrogens were analyzed using electron spray ionization (ESI) interface in negative ion mode, with capillary voltage at 3.5 kV, drying gas flow of 11 L/min and 350°C, and nebulizer gas pressure of 60 psi. Ion confirmation was done using the four mass-selective detector (MSD) signals set in single ion monitoring (SIM) quantification.

2.7. Method Validation

The analytical method was evaluated in terms of selectivity, linearity, sensitivity, accuracy, precision and recovery. Internal standard and deconjugation standards were included during the validation process. Analytical controls were included to identify any interference in the samples. Every analysis included a glassware blank (water processed without internal standard), reagent blank (reagents and solvents without matrix processed with internal standard), matrix blank (matrix low/without phytoestrogens, see Materials Section 2.1 for serum, Section 2.3 for food and Section 2.5 for urine, processed with internal standard), spiked matrix blank, and a solution containing the 16 phytoestrogens. A random real sample (women's biofluids and regional food) was processed in duplicate. Statistical analysis was carried on with NCSS 2001 statistical program.

2.7.1. Selectivity

Blank samples were tested for interferences at the retention time of the phytoestrogens and deconjugation standards in the four MSD signals.

2.7.2. Linearity and Sensitivity

System and method linearity were tested for all phytoestrogens covering a wide range, from 0.16 to 16,000 ng/mL, due to the different concentrations expected in food, serum and urine samples. The final concentration of the standards ranged from 0.008 to 1200 ng/mL in food and urine and 0.02 to 2000 ng/mL in serum. Linearity was performed three times, and each level of concentration was injected in triplicate. Area, phytoestrogens concentrations and correlation coefficients were obtained from the generated chromatograms. Peak area of the phytoestrogens and standards were plotted against the nominal concentrations of the calibration standards. Linear regression analysis generated calibration curves with the equation:

$$y = mx + b, \quad (1)$$

where y is the peak area, x the concentration of the targeted phytoestrogen, m the slope and b the intercept of the regression line. Linearity was evaluated by observing correlation coefficients (r^2) for the system and method. The limits of detection (LOD) and quantification (LOQ) of the method were defined as the lowest concentration with a signal-to-noise ratio of 3 and 10, respectively.

2.7.3. Recovery

Recovery ($RE\%$) was performed by comparing the analytical results of spiked samples (food at 100 ng/mL, serum at 16 and 40 ng/mL and urine at 200 ng/mL) before extraction (B) with the standards (A) which represented 100% recovery using the equation:

$$RE\% = (B/A) \times 100, \quad (2)$$

RE% was performed in independent batches of blank samples due to the heterogeneity of the samples and was considered acceptable between 60% and 120 %.

2.7.4. Accuracy and Precision

Accuracy and precision were determined by analyzing spiked samples (quality controls for food: 100 ng/mL, serum: 16 and 40 ng/mL and urine: 200 ng/mL) on three different days in independent batches of blank samples. The relative error (%) corresponded to the accuracy, which is calculated for each analyte, comparing the calculated mean result to the nominal concentration of the analyte. Precision was divided into within-run (intra-assay) and between-run (inter-assay). Precision and accuracy at each concentration level should not exceed 15% of the coefficient of variation (CV).

3. Results and Discussion

3.1. Chromatography and Mass Spectrometry Optimization

In order to optimize the separation and detection of the 16 phytoestrogens of interest, variables such as column length, column temperature, mobile phase, gradient and flow were evaluated. The final parameters presented in Section 2.6 Liquid chromatography/mass spectrometry (column description, column temperature and mobile phase gradient flow) allowed the resolution of the phytoestrogens by HPLC-MS. Phytoestrogens identification was achieved by retention time, UV and mass spectra (Figure 1). From the absorption spectra, flavonoids presented their two maximum wavelengths of absorbance in the range of 305 to 375 nm (band I) and 200 to 280 nm (band II) [24]. The wavelengths of maximum absorbance for resveratrol were found at 215 and 310 nm, which are characteristic of its alkene and m-dihydroxibencenalkene [25]. For lignans, the maximum wavelengths were at

200, 220 and 280 nm, which represent the furan, bencil and phenilaril structures, characteristic of these compounds [26].

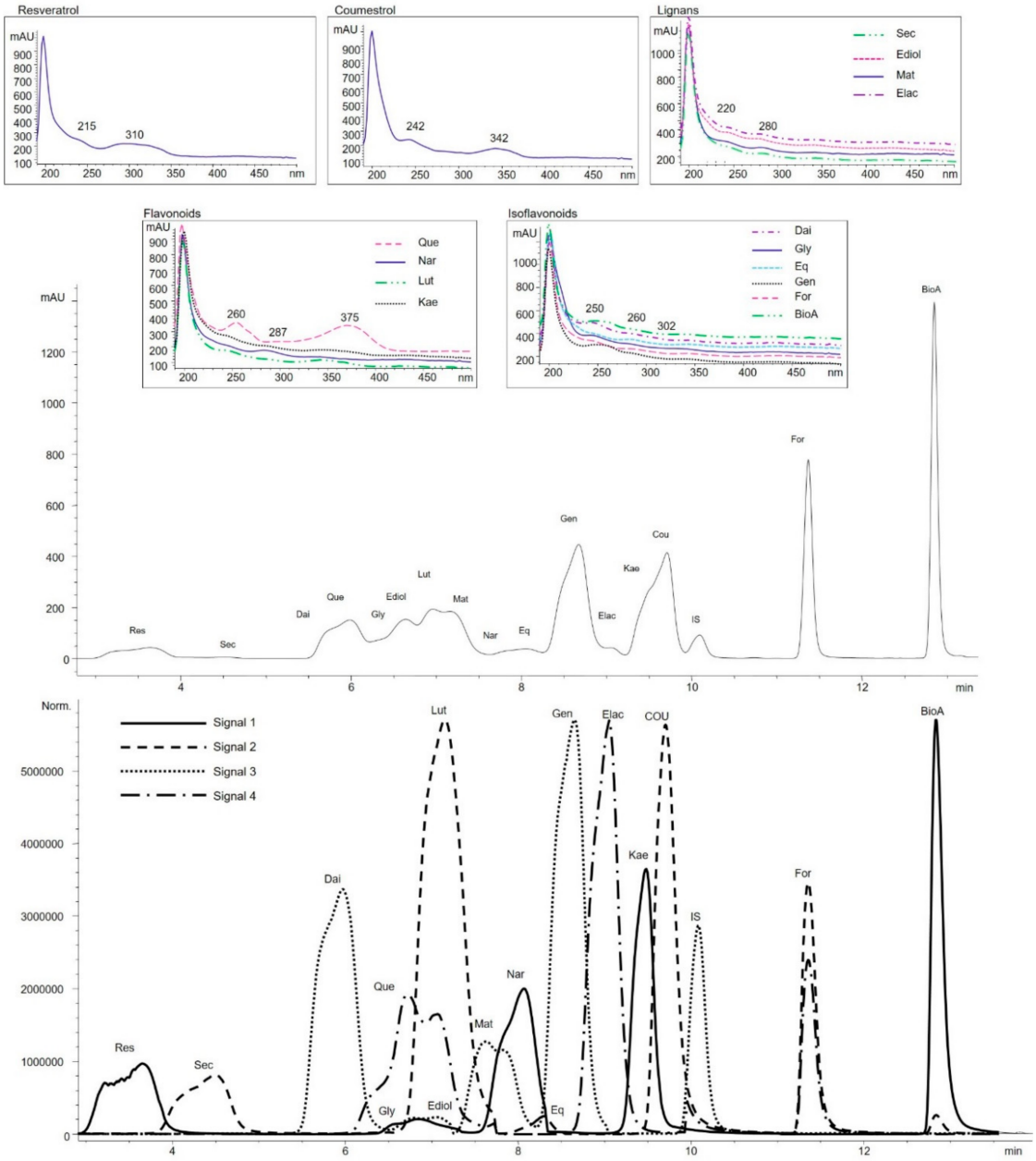

Figure 1. Ultraviolet (UV) spectra, high-performance liquid chromatography (HPLC) and normalised-mass spectrometry (MS) chromatograms of the four mass-selective detector (MSD) signals of pure phytoestrogens standards (160 ng/mL) and internal standard (IS) in the injection solvent (methanol and initial mobile phase; *v*/*v*, 40/60). Bio A, biochanin A; Cou, coumestrol; Dai, daidzein; Ediol, enterodiol; Elac, enterolactone, Eq, equol; For, formononetin; Gen, genistein; Gly, glycitein; Kae, kaempferol; Lut, luteolin; Mat, matairesinol; Nar, naringenin, Que, quercetin; Sec, secoisolariciresinol; and Res, resveratrol. The overlapping peaks are due to overlay of the m/z values used in multiple MSD signals and the different ion types of the phytoestrogens. Distortion of peaks is due to the solvent strength of the injection solvent. The ion type, *m*/*z* and MSD used for quantification are describe in Table 1.

Table 1. Target ion (*m*/*z*), retention time (min) and fragmentor voltage (V) for phytoestrogens quantification in the four MSD signals.

MSD ESI (-)	Analyte	Retention Time (min)	Ion (*m*/*z*)	Fragmentor Voltage (V)
1	Resveratrol	3.7	227.1	180
	Glycitein	6.9	283.0	160
	Naringenin	8.0	271.0	180
	Kaempferol	9.5	285.0	200
	Biochanin A	12.8	283.1	180
2	Secoisolariciresinol	4.5	361.1	100
	Luteolin	7.2	285.1	180
	Equol	8.3	241.1	160
	Coumetrol	9.7	267.1	200
3	PhP β-glu ‡	4.7	493.1	100
	Daidzein	5.9	253.1	180
	Enterodiol §	7.0	253.1	180
	Matairesinol	7.8	357.0	130
	Genistein	8.6	269.0	180
	PhP ‡	9.2	317.1	100
	IS *	10.0	197.1	100
4	4MU ‡	4.0	175.1	100
	Quercetin §	6.6	151.1	200
	Enterolactone	9.0	297.1	180
	Formononetin	11.3	267.1	200
	4MUS ‡	13.8	293.2	100

* Internal standard (IS); 4-hydroxybenzophenone and ‡ deconjugation standards; 4-methylumbelliferyl sulfate (4MUS), 4-methylumbelliferone (4MU), phenolphthalein β-D-glucuronide (PhP-β-glu) and phenolphthalein (PhP). The $[M-H]^-$ were chosen for quantification except for § quercetin and enterodiol. ESI, electrospray ionization; MSD, mass-selective detector.

Different mass parameters, such as fragmentation voltage were tested to increase predominant ions, to reduce noise and to increase the sensitivity of the method. Spray chamber parameters were set according to the column flow with a gas temperature of 350 °C, nebulizer pressure of 60 psi and drying gas flow of 11.0 L/min. Phytoestrogens were evaluated in positive and negative ion mode using ESI. Negative mode was selected due to high sensitivity, minor noise, and lower fragmentation. A scan analysis was undertaken to all the phytoestrogen standards to identify and confirm each phytoestrogen chemical structure. The predominant ions were $[M-H]^-$, while fragment ion formation presented a low relative abundance (by Retro-Diels–Alder, neutral and radical fragmentation). Each ionization pattern ion was characteristic for each phytoestrogen which has been observed previously, using different interfaces and masses [17,24,27,28]. The $[M-H]^-$ were chosen for quantification in SIM mode (Table 1). Since quercetin and enterodiol present the similar molar masses (302 g/mol) and a close elution, the ions with *m*/*z* of 151.1 and 253.1 were selected for their quantification, respectively. Phytoestrogens were analyzed using the four MSD signals.

3.2. Selectivity

Only a few peaks were observed in the reagent blanks and in the matrix blanks but did not interfere with the analysis (Figure 2). These peaks were due to the enzyme *Helix pomatia*, which has been reported to contain levels of some phytoestrogens. The enzyme H1 contained naringenin (0.47 ng/mg), kaempferol (0.02 ng/mg), biochanin A (0.01 ng/mg), and luteolin (0.517 ng/mg) but not secoisolariciresinol and genistein (as previously reported for the HP-2 type enzyme [29,30]). Grace et al. 2006 [30] showed that *Helix pomatia* could be purified to remove phytoestrogen contamination by SPE. However, this additional purification step could affect the enzymatic activity, remove or reduce components involved in the deconjugation process, and lower the levels of free phytoestrogens. In particular, the concentration of coumestrol has been found to be reduced (by 17%) when the

purified enzyme was used compared to the crude enzymatic solution. In serum, changing the enzyme concentration [29] could reduce interferences, however, complete hydrolysis should be ensured and awaits evaluation. The low content of phytoestrogens in the enzyme were corrected for the presence of phytoestrogens in each sample analyzed using the reagent blank [31]. Coleution of secoisolariciresinol with matrix components at the beginning of the gradient can interfere with the analysis in serum [32]. However, solid-phase extraction and liquid-liquid extraction conditions reduced endogenous substances in serum, urine and food. Additionally, changes in the chromatographic method and using single ion monitoring allowed the reduction of interferences and maximized selectivity.

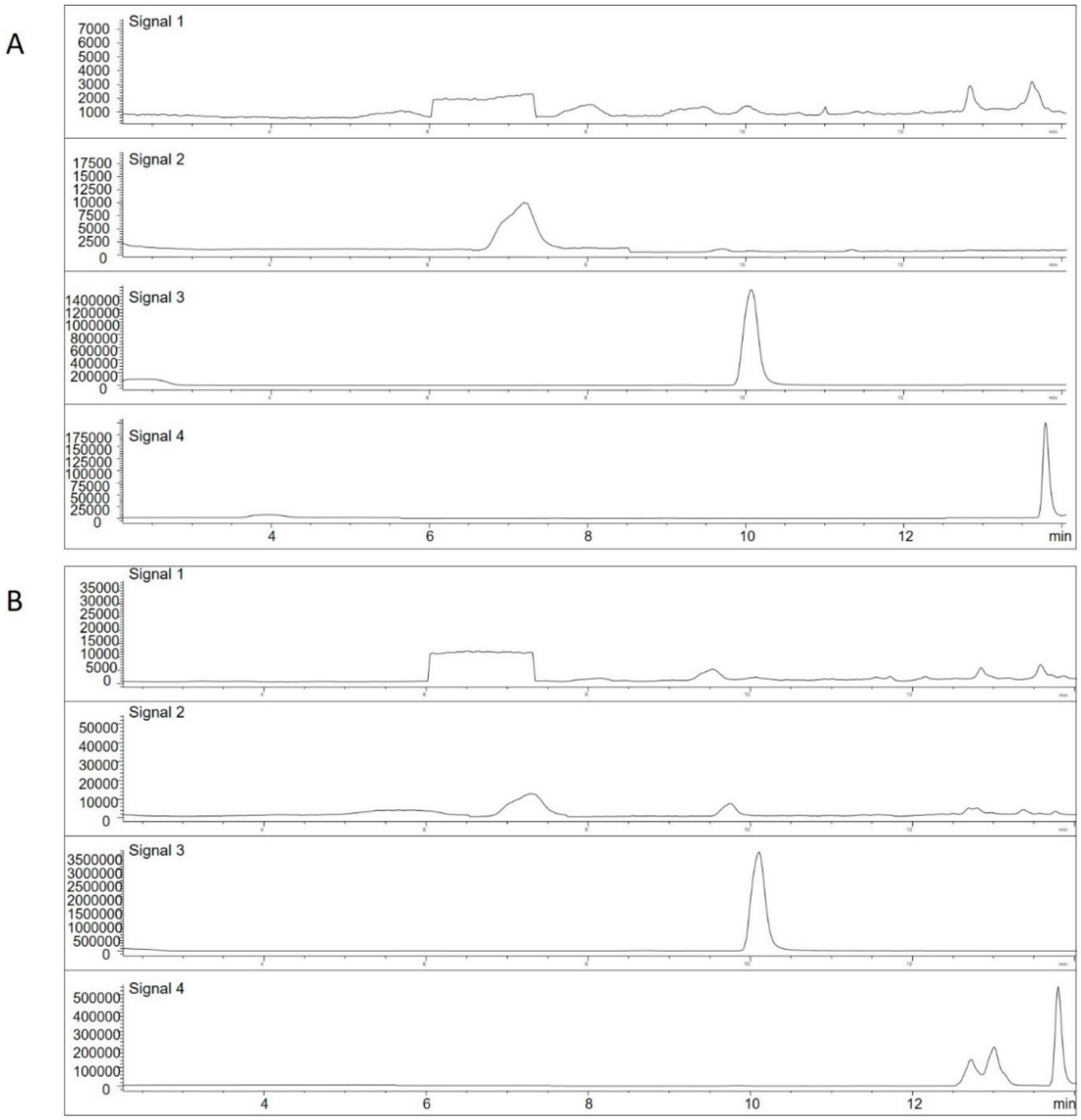

Figure 2. Representative MS chromatogram of the four MSD signals of a reagent blank (**A**) and a human blank serum (**B**) with internal standard.

3.3. Linearity and Sensitivity

Phytoestrogen levels present a wide variation between food and biofluids of individuals. Phytoestrogens quantification must be ensured below the part per billion, mainly when the method will be applied to individuals with a low intake of phytoestrogens. When human samples and food are meant to be analyzed at the same time, a dynamic range is the best way to ensure the flexibility of the analytical method. Calibration curves showed a linear response for all the 16 phytoestrogens.

Linearity of the LC-MS system response was tested at a range of 0.16 to 16,000 ng/mL for phytoestrogens in solution, which showed correlation coefficients of ≥0.98. The analytical method demonstrated a linear response in the range of 0.02 to 2000 ng/mL for serum, and 0.008 to 1200 ng/mL for food and urine, with correlation coefficients of >0.97 in the samples, except for enterodiol (r^2 = 0.95) in food, biochanin A (r^2 = 0.95) and coumestrol (r^2 = 0.96) in urine (Table 2).

Table 2. Limits of detection (LOD), limits of quantification (LOQ), method linearity (r^2) and recoveries (RE%) of phytoestrogens in food, serum and urine.

	Food				Serum					Urine			
				RE%				RE%					RE%
Phytoestrogen	**LOD**	**LOQ**	**r^2**	**100 ng/mL**	**LOD**	**LOQ**	**r^2**	**16 ng/mL**	**40 ng/mL**	**LOD**	**LOQ**	**r^2**	**200 ng/mL**
Resveratrol	0.025	0.084	0.994	44	0.04	0.12	0.992	92	86	0.018	0.06	0.998	50
Biochanin A	0.006	0.022	0.979	74	0.01	0.04	0.977	77	68	0.002	0.008	0.951	97
Secoisolariciresinol	0.023	0.078	0.993	66	0.07	0.25	0.987	99	99	0.008	0.027	0.993	59
Luteolin	0.009	0.033	0.995	20	0.01	0.02	0.998	82	72	0.028	0.096	0.982	80
Coumestrol	0.003	0.117	0.977	88	0.01	0.05	0.971	30	29	0.001	0.003	0.960	101
Formononetin	0.007	0.023	0.998	78	0.04	0.13	0.978	73	65	0.003	0.011	0.970	92
Daidzein	0.011	0.037	0.987	67	0.001	0.003	0.972	102	94	0.042	0.141	0.984	76
Enterodiol	0.043	0.144	0.948	72	0.06	0.20	0.981	98	88	0.039	0.133	0.983	103
Equol	1.061	3.541	0.992	94	0.53	1.77	0.968	104	96	0.068	0.227	0.981	90
Kaempferol	0.008	0.029	0.991	15	0.05	0.16	0.986	76	63	0.003	0.012	0.989	76
Glycitein	0.212	0.709	0.968	92	0.13	0.45	0.980	90	88	0.075	0.251	0.969	111
Matairesinol	0.012	0.041	0.968	87	0.01	0.04	0.984	93	91	0.059	0.198	0.980	98
Naringenin	0.014	0.041	0.988	70	0.01	0.04	0.997	84	71	0.005	0.019	0.985	83
Quercetin	0.011	0.036	0.989	80	0.06	0.21	0.984	83	76	0.061	0.206	0.986	84
Enterolactone	0.002	0.008	0.978	85	0.01	0.03	0.979	80	74	0.001	0.003	0.976	110
Genistein	0.006	0.021	0.981	113	0.003	0.01	0.988	101	89	0.001	0.006	0.975	87

Limits of detection (LOD) and quantification (LOQ) were set as the lowest final concentration in the sample, with a signal-to-noise ratio of 3 a 10, respectively, expressed in ng/mL. Linearity was performed three times, and each level of concentration was injected in triplicate. The linearity of the method was evaluated in the range of 0.02 to 2000 ng/mL for serum, and 0.008 to 1200 ng/mL for food and urine. Mean recoveries were calculated as the ratio of the peak area of phytoestrogen spiked before extraction to the peak area of the standard multiplied by 100 in blank samples on three different days.

Limits of detection and quantification are listed in Table 2. A comparative table with relevant methods is available as supplementary material for food, serum and urine (Supplementary Tables S1–S3). In food, the LODs and LOQs were lower for enterolactone (0.002 ng/mL and 0.008 ng/mL, respectively) and higher for equol (1.061 ng/mL and 3.541 ng/mL, respectively). The LODs were lower than the method reported by Milder et al. (2004) with 2.3 ng/mL (0.2–10 µg/100 g, in bread) [33] and Kuhnle et al. (2007) with 1 ng/mL (1.5 µg/100 g) [28] but comparable with Vila-Donat et al. (2015) with 0.03–0.3 ng/mL (legumes, LOQ of 0.01 ng/mL for biochanin A) [34]. The LODs correspond to 0.001 to 0.52 µg/100 g of food (final concentration depends on humidity), suitable for the analysis of food with low levels of phytoestrogens, and it is a useful method to develop a food database where phytoestrogens intake is characterized by a wide variety of food with low phytoestrogen levels. In serum, the LODs ranged from 0.001 to 0.53 ng/mL and LOQs from 0.003 to 1.8 ng/mL. These limits were lower than previous methods using different detectors, with LODs from 0.01 to 132.6 ng/mL [14,17,27,32,35–37], and comparable with formononetin (0.02 ng/mL), enterodiol (<0.01–0.06 ng/mL,) and glyciteina (0.01 ng/mL) [38,39]. Therefore, the method presents an advantage for the analysis of serum in the ppb-levels. In urine, the LODs for the method ranged from 0.001 to 0.075 ng/mL, and LOQs from 0.003 to 0.25 ng/mL. Previous methods reported LODs between 0.05 and 65.1 ng/mL [14,32,40,41], with similar limits for enterodiol (0.04 ng/mL), equol (0.06 ng/mL) and glycitein (0.0007 ng/mL) [40,42]. The results indicate that the liquid-liquid extraction and HPLC-DAD-ESI-MS presented a high sensibility for the analysis of phytoestrogens in human urine. The sensitivity of the method could be explained by the addition of formic acid from the mobile phase in the injection solvent that improved the ionization efficiency, as well as the concentration of the sample during extraction and the injection volume (60% to 10-fold larger).

3.4. Recovery

Recoveries (REs) of phytoestrogens (Table 2) in food were in the range of 66 to 113%, except for kaempferol, luteolin and resveratrol (15 to 44%). Previous methods (Supplementary Table S1) have reported recoveries between 70 to 110% and others as low as 30% [12,28,33,34,43]. Recovery in food could vary between samples due to the intrinsic composition of the food matrix. Potential interfering substances, such as proteins, lipids, carbohydrates and fiber could result in decreased hydrolysis and RE% of phytoestrogens [28]. Also, the matrix effect is challenging to establish in food and could differ significantly due to the variety of matrices and the lack of quality control for foodstuff samples (as opposed to single matrix sample like serum) [28].

REs in serum were from 63 to 104% except for coumestrol (30%). Compared to previous methods (Supplementary Table S2, an improvement in recovery was observed for equol (88%) [37], coumestrol (20%) [32], and secoisolariciresinol (86–96%) [32,44], while the rest presented comparable recoveries [14,27,36]. Despite the advances in the method extraction, RE of coumestrol was below 60%. Coumestrol with a relatively non-polar characteristic may yield low recoveries, which enhances high retention on the sorbent, low solubility in the elution solvent and low RE% [32]. Although a higher recovery can be achieved by liquid-liquid extraction (77%) [36], the present method represents an advance in coumestrol analysis given that other methods excluded its analysis in serum [14,36]. A minor matrix effect was observed in pooled blank serum samples, with ion suppression for biochanin A, coumestrol and formononetin and enhanced ionization for matairesinol and secoisolariciresinol (Supplementary Table S4).

For urine, liquid-liquid extraction presented REs within the expected levels. The REs of the spiked samples were between 76 to 111%, except for resveratrol (50%) and secoisolariciresinol (59%). Acceptable recoveries of phytoestrogens in urine have been observed in previous methods (Supplementary Table S3) but these have focused mainly on isoflavones (65–110%) [14,32,37,40,41,44]. Matrix effect was not considered as urine could be described as a relatively "clean" sample (as opposed to serum) [29].

Acceptable RE% for all compounds is a difficult task when a wide variety of phytoestrogens are being analyzed per chromatographic run. The pretreatment of the sample and the washing solution used in the solid-phase extraction resulted in an overall improvement in recovery of kaempferol, biochanin A and luteolin (increasing up to 30–50%, data not shown). The recoveries of equol and glycitein were lower when using 100% methanol as the injection solvent (data not shown). In the same way, REs decreased for some of the phytoestrogens when changing the solvent to the mobile phase. The injection solvent was varied to increases the solubility of the phytoestrogens while maintaining the strength of the mobile phase from methanol to methanol/initial mobile phase (*v*/*v*, 40/60). Increasing the methanol percentage in the injection solvent and maintaining a percentage of mobile phase allowed acceptable recoveries (60–110%). Cao et al. (2010) and Prasain et al. (2010) presented high RE (65–105%) using methanol/mobile phase as the injection solvent [35,36]. Although aqueous injection solvent could be used [14,17], it should be mentioned that the present study analyzes a wide variety of phytoestrogens, with different polarities.

3.5. Accuracy

Accuracy (Table 3) was determined by using quality control samples in replicates at different levels (16 and, 40 ng/mL) for serum and one level for food (100 ng/mL) and urine (200 ng/mL). Relative errors were between 1.0 to 13% for food, 0.3 to 15% for serum and 2 to 10% for urine. Only the accuracy of enterodiol in food was above the value recommended by the Food and Drug Administration (FDA) (<15% CV other than lower LOQ [45]) with 18%. Previous methods (Supplementary Tables S1–S3) have presented similar acceptable accuracy levels for food (bread [44]), serum [32,36,37,44] and urine [32,40,44].

Table 3. Accuracy * (%), inter- [a] and intra- [b] precision (%) of phytoestrogens in blank serums on three different days.

	Food			Serum						Urine		
	100 ng/mL			16 ng/mL			40 ng/mL			200 ng/mL		
Phytoestrogen	Accuracy	Inter-Assay	Intra-Assay	Accuracy	Inter-Assay	Intra-Assay	Accuracy	Inter-Assay	Intra-Assay	Accuracy	Inter-Assay	Intra-Assay
Resveratrol	4	8.36	7.11	5	9.43	6.67	11	12.27	1.88	10	15.71	5.82
Biochanin A	7	3.13	1.97	11	8.50	3.87	9	14.30	3.57	2	8.01	2.19
Secoisolariciresinol	6	1.50	2.90	4	3.73	2.05	3	3.54	4.39	5	4.63	4.70
Luteolin	5	5.53	11.56	6	10.55	5.92	0.6	6.87	6.86	5	12.99	3.65
Coumestrol	10	9.83	5.99	13	14.46	2.33	1	4.87	3.65	2	3.74	1.97
Formononetin	1	7.24	4.08	12	11.03	3.20	5	6.57	5.30	2	10.24	1.89
Daidzein	12	2.86	6.93	0.3	11.79	11.31	8	12.48	13.32	6	3.31	6.61
Enterodiol	18	3.12	5.96	9	6.09	3.73	7	14.73	3.45	6	1.32	5.22
Equol	6	8.47	6.34	1	4.11	0.88	1	6.17	7.41	7	3.28	4.07
Kaempferol	9	11.16	13.16	10	14.25	3.90	5	1.46	2.96	3	11.49	3.98
Glycitein	9	10.91	6.94	12	14.84	2.72	3	9.10	2.95	4	2.43	4.25
Matairesinol	13	11.21	5.61	1	4.14	1.27	6	10.30	2.84	3	11.59	3.33
Naringenin	10	4.06	9.19	2	12.21	4.75	1	2.22	4.12	5	5.18	4.41
Quercetin	8	10.08	11.21	15	14.87	3.18	6	14.19	7.25	5	8.10	2.38
Enterolactone	8	2.72	5.70	0.5	6.08	5.61	3	8.49	4.73	4	3.47	3.54
Genistein	8	0.44	4.44	2	14.05	3.74	10	14.29	6.78	4	6.25	2.88

* Value expressed as the observed mean concentration to the nominal concentration of each analyte. Precision is presented as [a] inter-assay (between-runs) and [b] intra-assay (within-runs). Accuracy and precision at each level should not exceed 15% of the coefficient of variation.

3.6. Precision

Inter-assay precision (Table 3) in food was between 0.44 (genistein) and 11.21% (matairesinol), and intra-assay precision between 1.97 (biochanin A) and 13.16% (kaempferol). Serum inter-assay precision was between 1.46 (kaempferol at 40 ng/mL) and 14.87% (quercetin at 16 ng/mL). The intra-assay precision for serum did not exceed 15% and was found between 0.85 and 13.32%, with the highest values for daidzein (11.31% at 16 ng/mL and 13.32% at 40 ng/mL). While urine inter-assay precision was within acceptable levels (1.32–13%), except for resveratrol with 15.71%. Intra-assay precision for urine was below 7%. Acceptable values have been previously reported (Supplementary Tables S1–S3) for food [12,28], serum [32,36,37,44] and urine [17,32,37,41,44].

Accuracy and precision values indicate that the HPLC-DAD-ESI-MS method is useful for the analysis of the 16 phytoestrogens and proved to be exact, reproducible and reliable at the evaluated levels. As only one quality control level for urine and food, and two levels for serum were included in the analysis, this could be considered as a limitation of the study. Nonetheless, analytical controls such as glassware blank, reagent blank, matrix blank, and phytoestrogens solution were included in every run to ensure the quality of the analytical test.

3.7. Analysis of Phytoestrogens in Food, Serum and Urine

As part of the validation process, regional commercial food (n = 6, Table 4), urine (12 h) and serum samples from healthy women (n = 10, Table 5) were analyzed. The CVs of samples processed in duplicate ranged from 2–13% for food, 2–8% for serum and 1–13% for urine (except for resveratrol with 20%), thus the proposed method presents adequate repeatability. A representative MS chromatogram of a commercial food sample (turkey ham), serum and urine from a healthy woman is illustrated in Figure 3.

Phytoestrogens concentrations were below the LOD for most of the foodstuff. However, daidzein, kaempferol, naringenin and genistein content in turkey ham, beans, mandarins and tomatoes products were high. Although some selected types of foodstuff have been analyzed previously, only a few phytoestrogens have been included in the analysis (mostly daidzein, quercetin, genistein and some lignans). Comparable levels were observed with previous studies (Supplementary Table S5) [22,28,46,47] for tomato products (<0.01–14 μg/100 g) and lettuce (not detected–1.63 μg/100 g), except for naringenin in tomato (27.1 μg/100 g [46]). These comparable levels highlight the suitability for the method to quantify phytoestrogens in food. Higher phytoestrogens content was observed for citrus and beans

compared to previous studies (0.02–10.02 µg/100 g [22,46] and not detected–66 µg/100 g [22,28,46], respectively). The high levels of naringenin in beans could be explained by the use of chili, onion, tomato and other vegetables as condiments. The high levels in turkey ham could be explained by the use of soy as an additive to reduce the price of processed food while adjusting protein levels, with isoflavones content similar to soy-based meat products (465–4430 µg/100 g) [48,49]. The Mexican consumer ombudsman has recently published notifications that some ham products in Mexico do not comply with the percentage of meat advertised, omit the added soy on their labels, and could represent a risk as allergens for consumers [50]. Our study reported the first comprehensive phytoestrogens content of highly consumed Mexican food items that are a source of wide types of phytoestrogen.

Table 4. Phytoestrogen content (µg/100 g of wet weight) of regional food from North Mexico.

	Lettuce, Raw	Beefsteak Tomato, Cooked	Tomato, Puree	Mandarin, Raw	Pinto Beans, Cooked	Turkey Ham
Resveratrol	-	-	-	-	-	-
Biochanin A	-	-	-	0.06	0.04	-
Secoisolariciresionol	0.64	-	-	2.51	14.24	-
Luteolin	-	0.15	0.6	0.1	-	-
Coumestrol	-	0.18	0.05	0.02	0.59	-
Formononetin	-	0.41	0.36	0.04	-	-
Daidzein	0.04	-	0.7	-	3.5	395.24
Enterodiol	0.21	-	-	4.87	-	-
Equol	-	-	-	11.6	-	-
Kaempferol	0.01	0.07	1.95	-	406.41	0.64
Glycitein	-	-	-	0.53	-	172.39
Matairesinol	-	1.32	1.91	0.02	-	-
Naringenin	0.06	140.6	322.39	83.9	81.97	13.17
Quercetin	0.02	0.33	16.5	0.99	1	-
Enterolactone	-	-	0.02	0.03	-	0.09
Genistein	0.03	0.24	0.89	-	1.96	356.24

Table 5. Serum and urinary levels in Northern Mexican women (n = 10).

Phytoestrogen	Serum (ng/mL)	Urine	
		(ng/mL)	(ng/mmol Creatinine)
Resveratrol	0.28 ± 0.36	1.53 ± 1.63	0.20 ± 0.21
Biochanin A	0.53 ± 0.38	1.15 ± 0.75	0.15 ± 0.10
Secoisolarisiresinol	<LOD	1.81 ± 1.96	0.24 ± 0.26
Luteolin	7.08 ± 1.54	2.51 ± 1.59	0.33 ± 0.21
Coumestrol	0.65 ± 0.44	1.01 ± 1.54	0.13 ± 0.20
Formononetin	0.03 ± 0.09	1.74 ±1.87	0.23 ± 0.24
Daidzein	4.50 ± 4.80	148.77 ± 193.00	19.51 ± 25.31
Enterodiol	0.07 ± 0.20	0.91 ± 2.04	0.04 ± 0.08
Equol	8.51 ± 4.55	36.38 ± 37.60	4.77 ± 4.93
Kaempferol	20.14 ± 6.43	48.61 ± 97.48	6.37 ± 12.78
Glycitein	0.56 ± 1.44	52.71 ± 71.2	6.91 ± 9.35
Matairesinol	<LOD	1.58 ± 4.15	0.21 ± 0.54
Naringenin	1.76 ± 0.68	78.43 ± 89.57	10.28 ± 11.75
Quercetin	5.53 ± 3.29	1.40 ± 1.17	0.18 ± 0.15
Eneterolactone	0.61 ± 0.47	36.03 ± 27.31	4.72 ± 3.58
Genistein	4.85 ± 5.12	56.52 ± 76.27	7.41 ± 10.00

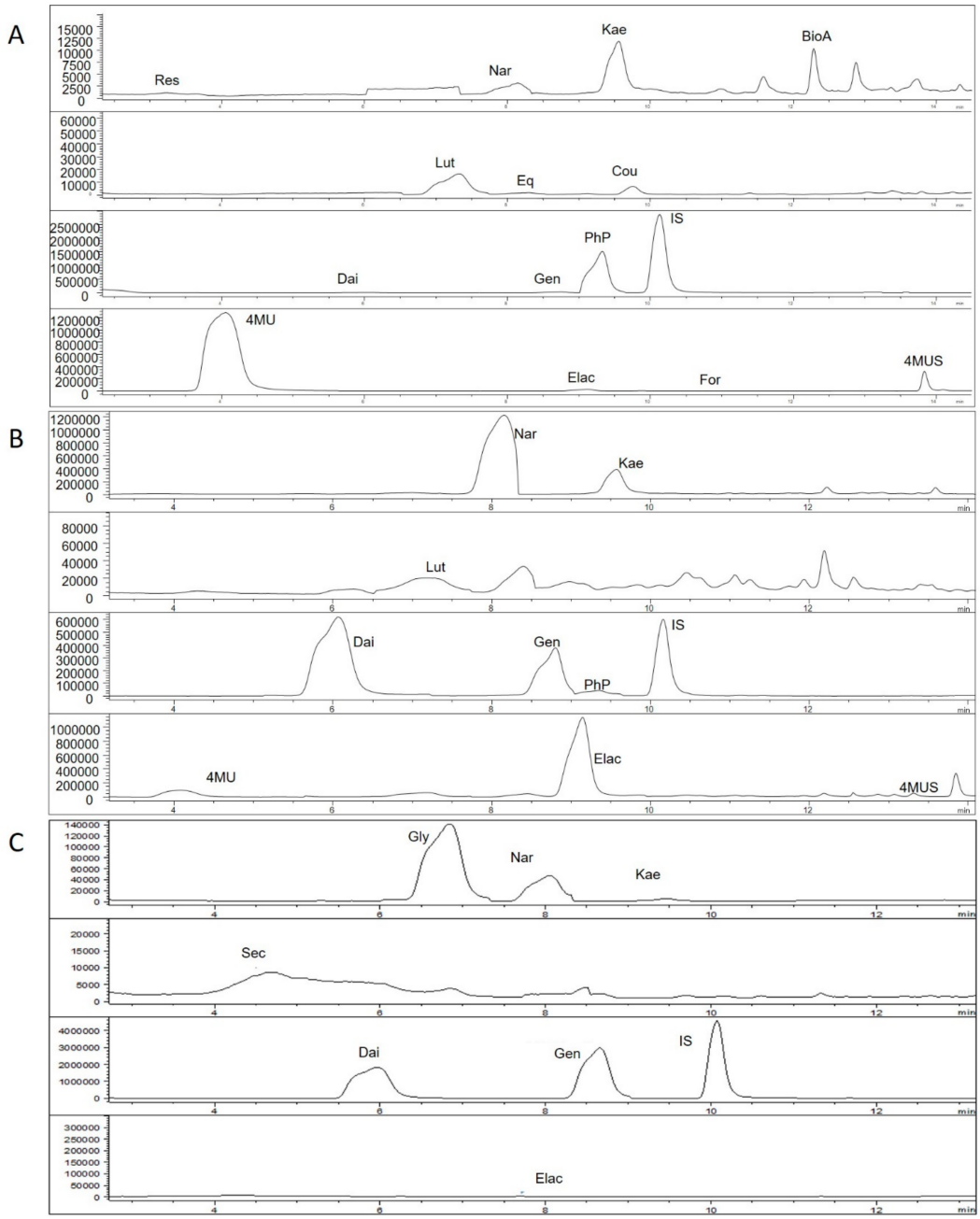

Figure 3. Representative MS chromatogram of the four MSD signals of real serum (**A**) and urine (**B**) samples from a healthy woman, and commercial food (**C**, turkey ham) with internal standard. 4MU, 4-methylumbelliferone; 4MUS, 4-methylumbelliferyl sulfate; Bio A, biochanin A; Cou, coumestrol; Dai, daidzein; Elac, enterolactone, Eq, equol; For, formononetin; Gen, genistein; IS, 4-hydroxybenzophenone (internal standard); Gly, glycitein; Kae, kaempferol; Lut, luteolin; Nar, naringenin, PhP, phenolphthalein; Sec, secoisolariciresinol; and Res, resveratrol.

For biofluids, phytoestrogens mean concentrations were below the LOD to 20.14 ng/mL for serum and 0.91 to 148.77 ng/mL for urine. Higher levels of flavonoids in serum and urine could be related to the sampling period (winter and spring), characterized by an increased intake of fruits and vegetables [51]. The lower levels for isoflavones, resveratrol, coumestrol and lignans could be due to the limited intake of wine/grapes, soy products and cereals in the North region of Mexico. These phytoestrogens levels are consistent with the daily median intake of (poly)phenols in adult Mexican women from the Mexican Teachers' Cohort (MTC), with flavonoids as a main dietary source (33.5%) and stilbenes and lignans as

minor sources (0.1%) of (poly)phenols. Fruit (oranges, mandarins and orange juice) contributed to ~26% of (poly)phenols intake in the MTC, followed by cereals and tubercules with 10% and vegetables and legumes with 9% [52]. Serum and urinary levels of equol were higher compared to Asian infants (11.36 ng/mL urine) [20] and adults (<0.54–0.63 ng/mL urine) [41], and adults supplemented with isoflavones (not detected plasma levels after 123 mg soy supplementation) [53].

4. Conclusions

We described a comprehensive HPLC-MS validated method for phytoestrogen analysis in human samples and foodstuffs, which represents an advance in the quantification of phytoestrogens. The method allowed the simultaneous quantification of 16 phytoestrogens, expanding the number of phytoestrogens analyzed by chromatographic run in a short time. The evaluated parameters demonstrated that linearity, selectivity, accuracy, precision and recovery are within the acceptable range, with high sensitivity in the lower ppb-level. The method was successfully applied to food, urine and serum samples. Therefore, this method is flexible and suitable for phytoestrogens quantification that could be used to analyze human samples in large-scale epidemiological studies investigating the association between phytoestrogen exposure and human health. The method described here could be used to determine phytoestrogens' content in regional food and create a phytoestrogens database in (North) Mexico.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/22/8147/s1, Supplementary Table S1. Comparison of relevant methods for the quantification of phytoestrogens in food, including matrix (g), system (injection volume), extraction (reconstitution) used, phytoestrogens analyzed, limits of detection/limits of quantification (LOD/LOQ, ng/mL), recoveries (RE%), accuracy (%), and inter/intra- precision (%). Supplementary Table S2. Comparison of relevant methods for the quantification of phytoestrogens in serum, including matrix (g), system (injection volume), extraction (reconstitution) used, phytoestrogens analyzed, limits of detection/limits of quantification (LOD/LOQ, ng/mL), recoveries (RE%), accuracy (%), and inter/intra-precision (%). Supplementary Table S3. Comparison of relevant methods for the quantification of phytoestrogens in urine, including matrix (g), system (injection volume), extraction (reconstitution) used, phytoestrogens analyzed, limits of detection/limits of quantification (LOD/LOQ, ng/mL), recoveries (RE%), accuracy (%), and inter/intra-precision (%). Supplementary Table S4. Matrix Effect (%) at low and high quality control level in four different blank serums. Supplementary Table S5. Comparison of phytoestrogens content (µg/100 g) found in this study with relevant previously studies and public databases.

Author Contributions: Conceptualization, G.C.-J. and A.I.V.-Q.; methodology, G.C.-J., A.I.V.-Q., S.A.P.-D., K.M.C.-S., M.M.C.-S., M.d.C.B.-A. and M.d.R.R.-B.; validation, G.C.-J., A.I.V.-Q., S.A.P.-D., K.M.C.-S., M.M.C.-S., M.d.C.B.-A. and M.d.R.R.-B.; formal analysis, S.A.P.-D., K.M.C.-S., M.M.C.-S. and P.G.-C.; investigation, S.A.P.-D., K.M.C.-S., M.M.C.-S., M.d.S.S.-T., G.C.-J., A.I.V.-Q. and P.G.-C.; data curation, G.C.-J., S.A.P.-D., M.d.S.S.-T., M.d.C.B.-A., M.d.R.R.-B., M.L.G.-C. and A.I.V.-Q.; writing—original draft preparation, S.A.P.-D.; review and editing, S.A.P.-D., G.C.-J., M.d.R.R.-B., M.L.G.-C., and A.I.V.-Q.; supervision, G.C.-J. and A.I.V.-Q.; project administration, G.C.-J.; funding acquisition, G.C.-J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the National Council of Science and Technology (CONACyT) in Mexico and was conducted with register grant number #CB-2008-01-106028.

Acknowledgments: The authors would like to thank the women for their time and contribution to the research and for providing samples for analysis. We thank Imperial College London Open Access Fund for covering the open access publication fees and Agilent Technologies for providing the mass spectrometer. The authors would like to thank Angel Ojeda-Contreras, Heber Castillo, Gerardo Martinez and Gabriel Fuente for their technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

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