JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Targeted LC-MS/MS Method for the Quantitation of Plant Lignans and Enterolignans in Biofluids from Humans and Pigs

Natalja P. Nørskov,*[,†] Anja Olsen,[§] Anne Tjønneland,[§] Anne Katrine Bolvig,[†] Helle Nygaard Lærke,[†] and Knud Erik Bach Knudsen[†]

[†]Department of Animal Science, Aarhus University, AU-Foulum, Blichers Alle 20, P.O. Box 50, DK-8830 Tjele, Denmark

[§]Danish Cancer Society Research Center, Strandboulevarden 49, DK-2100 Copenhagen, Denmark

ABSTRACT: Lignans have gained nutritional interest due to their promising role in the prevention of lifestyle diseases. However, epidemiological studies are in need of more evidence to link the intake of lignans to this promising role. In this context, it is necessary to study large population groups to obtain sufficient statistical power. Therefore, there is a demand for fast, sensitive, and accurate methods for quantitation with high throughput of samples. This paper presents a validated LC-MS/MS method for the quantitation of eight plant lignans (matairesinol, hydroxymatairesinol, secoisolariciresinol, lariciresinol, isolariciresinol, syringaresinol, medioresinol, and pinoresinol) and two enterolignans (enterodiol and enterolactone) in both human and pig plasma and urine. The method showed high selectivity and sensitivity allowing quantitation of lignans in the range of 0.024−100 ng/mL and with a run time of only 4.8 min per sample. The method was successfully applied to quantitate lignans in biofluids from ongoing studies with humans and pigs.

KEYWORDS: *plant lignans, enterolignans, LC-MS/MS, plasma, urine, pigs, humans*

INTRODUCTION

Plant lignans are present in a variety of foods consumed by mammals: cereals, vegetables, and fruits.[1] When ingested, plant lignans are released from the food matrix by intestinal esterases in the mucosa or, the major part, by the microbial esterases in the colon and then converted by the colonic microflora to enterolignans, enterolactone and enterodiol.[2−4] The presence of plant lignans and enterolignans in blood and urine is positively correlated with the consumption of lignan-rich foods.[5] Epidemiological studies have linked the consumption of lignan-rich foods and the presence of enterolignans in blood with their possible protective role against several diseases, for example, breast, prostate, and colon cancer, as well as type 2 diabetes and cardiovascular diseases.[5,6] The potential of these compounds to affect human health has led to the need to develop fast and sensitive methods for the quantitation of lignans in biofluids such as plasma and urine with the high throughput of samples needed for epidemiological studies.

LC-MS/MS methods for the determination of plant and enterolignans in human plasma, serum, and urine have been reported before.[7−9] However, these methods were developed more than 10 years ago and have high run time and low sensitivity. Newer methods developed for the determination of phytoestrogens in plasma[10] and urine[11] include only two enterolignans and no plant lignans. The aim of this work was to simplify and extend these previously described methods to enable quantitation of both plant and enterolignans in biofluids in one combined method with a low run time and high sensitivity using state-of-the-art LC-MS/MS equipment. The new method was developed for the simultaneous quantitation of lignans in both human and pig plasma and urine. Moreover, this paper, to our knowledge, is the first paper to contain method validation for both plasma and urine from two species—humans and pigs.

MATERIALS AND METHODS

Materials. Acetonitrile and methanol were obtained from Fluka/Sigma-Aldrich (St. Louis, MO, USA). The following standards were used: enterolactone, enterodiol, matairesinol, hydroxymatairesinol, secoisolariciresinol, lariciresinol, isolariciresinol, syringaresinol, medioresinol, and pinoresinol from Plantech (Berksher, UK). The purity of enterolactone, enterodiol, matairesinol, secoisolariciresinol, lariciresinol, syringaresinol, and medioresinol was 96%, and 95% was the purity of pinoresinol, hydroxymatairesinol, and isolariciresinol. Internal standard (IS) was glycocholic acid (glycine-1^{13}C) from Sigma-Aldrich (St. Louis, MO, USA). For the enzymatic hydrolysis β-glucuronidase type H-1 from *Helix pomatia* was purchased from Sigma-Aldrich. Sodium acetate was obtained from Merck (Darmstadt, Germany), and glacial acetic acid and formic acid were from Fluka/Sigma-Aldrich. All solvents used were of HPLC grade.

Preparation and Storage of Standards. All standards were dissolved in 100% acetonitrile and kept at −80 °C. Then the working solution containing all lignan standards in the concentration of 400 ng/mL was prepared and used for the preparation of standard curves and spiking of plasma and urine. The working solution and the standard curves were kept at −80 °C at all times. The stability of the internal standard was tested in water. Moreover, we used standard curves to test freeze and thaw and short- and long-term stability.

Plasma and Urine Samples. The human plasma and urine used for optimization and validation of the method were donated by a volunteer at Aarhus University after 3 days on a low-lignan diet. The pig plasma and urine were collected from pigs kept on low- and high-lignan diets for 17 days. Urinary creatinine concentration was measured according to the Jaffe reaction.

Test Plasma. Unhydrolyzed plasma from both humans and pigs contained no or only traces of lignans. The concentration of lignans in

Received: March 11, 2015
Revised: June 15, 2015
Accepted: June 16, 2015
Published: June 16, 2015

DOI: 10.1021/acs.jafc.5b01275
J. Agric. Food Chem. 2015, 63, 6283−6292

human hydrolyzed plasma was <67 nM for enterolignans and <1 nM for plant lignans. The concentration of lignans in hydrolyzed pig plasma was <2 nM for enterolactone and close to zero for plant lignans and enterodiol.

Test Urine. The concentrations of lignans in unhydrolyzed and hydrolyzed human urine were <3.4 and 100 nM for enterolignans and <0.05 and 17 nM for plant lignans, respectively. Unhydrolyzed urine from pigs contained only traces of lignans. The concentration of lignans in hydrolyzed pig urine was <6.7 nM for enterolactone and <0.2 nM for enterodiol and plant lignans.

Sample Hydrolysis. Plasma samples of 0.5 mL were treated with 1 mL of freshly dissolved β-glucuronidase (≥300000 units/g solid)/sulfatase (≥10000 units/g solid) (2 mg/mL in 50 mM sodium acetate buffer, pH 5) and incubated in a shaker at 37 °C in 19 h according to the method of Smeds et al.[9] Urine samples of 0.25 mL used for the quantitation of plant lignans and 2.5 μL for the quantitation of enterolignans were also treated with 1 mL of β-glucuronidase (2 mg/mL in 50 mM sodium acetate buffer, pH 5) and incubated in a shaker at 37 °C in 19 h. The optimal hydrolysis time for urine samples was determined by incubating samples for longer and shorter periods of time, where 19 h was the time at which lignans reached their maximal concentration. After incubation, 1 mL of 0.2% of formic acid was added, and the samples were centrifuged for 15 min at 4 °C at 60*g* and then ready for solid phase extraction (SPE).

SPE Cleanup of Plasma and Urine. Samples were cleaned up using SPE C18-E columns or SPE C18-E 96-well plates from Phenomenex (Torrance, CA, USA). The SPE method included conditioning, washing, and elution of lignans. The C18-E column or 96-well plate was conditioned with acetonitrile and then with water. Afterward, the samples were loaded and allowed to elute slowly through the C18 material. The column or plate was then washed with 0.5 mL of 5% methanol for plasma or with 20% methanol for urine. Lignans from plasma and urine were eluted with 150 and 300 μL of 100% acetonitrile, respectively. The eluted samples were then diluted to 25% acetonitrile with 450 and 900 μL of water containing glycocholic acid (glycine-1^{13}C) as IS. A final concentration of IS was 20 ng/mL. IS was used for autosampler correction and matrix effects validation. Samples were spun at 10 °C for 5 min prior to LC-MS/MS measurements.

LC-MS/MS Equipment and Method. The LC-MS/MS measurements were performed on a microLC 200 series from Eksigent/AB Sciex (Redwood City, CA, USA) and QTrap 5500 mass spectrometer from AB Sciex (Framingham, MA, USA). The mass spectrometer was equipped with an ESI source. The microLC was equipped with an Ascentis C18 column, 100 mm × 1.0 mm with 3.0 μm partical size from Sigma-Aldrich. The LC conditions were as follows: Eluent A consisted of water with 0.1% formic acid and eluent B was acetonitrile with 0.1% formic acid. The column was equilibrated for 3 min. The gradient started at 75% eluent A and 25% eluent B, was held constant for 1 min, then increased to 85% eluent B during 3.3 min and kept constant for 0.5 min. The total run time was 7.8 min. The autosampler racks were kept at 10 °C, and the column oven was set to 23 °C. Negative mode was used for ionization. The flow injection analysis (FIA) was performed to optimize the turbo V source of the instrument, where curtain gas was set to 20 psig, nebulizer gas (gas1) to 50 psig, heater gas (gas2) to 60 psig, and temperature to 300 °C and ionization spray was operated at −4500 eV. Deprotonated molecules were detected in MRM mode. The compound-dependent parameters were optimized for each lignan by syringe infusion of pure standard and are shown in Table 1. Data analysis was performed in Analyst software 1.6 from AB Sciex.

Calibration Curves. Calibration curves had 7−12 points depending on the analyte. The mixture of pure solvents was prepared in 25% acetonitrile in the range of 0.0244−100 ng/mL. IS was added to the calibration curves at a final concentration of 20 ng/mL. The analyte/internal standard concentration ratio was plotted against the analyte/internal standard peak area ratio as a linear regression curve with $1/x$ weighting.

Accuracy, Precision, and Recovery. The accuracy, precision, and recovery of the lignans were tested at three concentrations, low, medium, and high, using five measurements per concentration. Plasma samples were tested at 0.0977, 1.56, and 50 ng/mL, respectively. Urine samples were tested at 0.39, 1.56, and 50 ng/mL, respectively. For determination of the accuracy and precision at low and medium concentrations, the unhydrolyzed plasma and urine were spiked with standards after SPE extraction, whereas at high concentration hydrolyzed plasma and urine were used. Recoveries of the lignans were determined by spiking plasma and urine with standards prior to enzymatic hydrolysis and SPE using unhydrolyzed plasma and urine for low and medium concentrations and hydrolyzed plasma and urine for high concentration. Blank hydrolyzed or unhydrolyzed plasma and urine were used to subtract the background during the calculations. Quality control (QC) samples were used to validate the interbatch variation. Ten QC samples were used for each run.

Table 1. Compound-Dependent LC-MS/MS Parameters: Declustering Potential (DP), Entrance Potential (EP), Collision Energy (CE), and Cell Exit Potential (CEP)

	Q1 mass (m/z)	Q3 mass (m/z)	DP (V)	EP (V)	CE (eV)	CEP (V)
enterolactone	297.1	189.1	−140	−10	−26	−21
enterodiol	301.1	253.1	−140	−10	−32	−19
matairesinol	357.2	82.9	−145	−10	−26	−7
hydroxymatairesinol	373.1	217.1	−115	−10	−32	−13
secoisolariciresinol	361.2	165.0	−150	−10	−34	−11
lariciresinol	359.1	329.0	−40	−10	−16	−21
isolariciresinol	359.2	344.0	−165	−10	−26	−31
pinoresinol	357.2	151.0	−155	−10	−24	−11
syringaresinol	417.1	181.0	−170	−10	−26	−13
medioresinol	387.2	151.0	−15	−10	−26	−25

Statistics. The accuracy describes the percentage deviation of the mean from the true value. Accuracy was calculated as relative error (ER) of replicated measurements, with acceptance criteria that it should not deviate by >±20% at low concentration or by >±15% at medium and high concentrations. The precision describes the closeness of individual measurements of an analyte applied to the multiple samples. Precision was calculated as relative standard deviation (RSD), where it should not exceed by >20% at low concentration or by >15% at medium and high concentrations. The recovery of the analyte represents the amount of the analyte recovered after the sample preparation procedure. Recovery was calculated as a percentage of the analyte recovered after the SPE procedure. The interbatch precision was validated using QC samples, which can provide the bases of accepting or rejecting the run. The acceptance criterion was that at least 67% of QC samples were within an RSD of 15%. Matrix effects were investigated by calculating the matrix factor (MF) for lignan and IS as the ratio between the peak area in the presence of matrix and in the absence of matrix. If the ratio was close to 100%, no ion suppression had occurred. The IS-normalized MF was calculated by dividing the MF of the analyte with the MF of the IS. The RSD of the IS-normalized MF should not exceed >15%.

Method Validation. The method was validated according to the guidelines of the U.S. Food and Drug Administration (FDA)[12] and European Medicines Agency of Science Medicines Health.[13]

RESULTS

SPE Cleanup. The sample cleanup procedure presented in this paper was developed and optimized for both low and high numbers of plasma and urine samples. Therefore, both single SPE C18 columns and SPE C18 plates can be used. The sample cleanup was kept as simple as possible to minimize transfer losses and labor time involved. A simplified sample cleanup procedure is far more amenable for a high throughput of samples.

Chromatographic Analysis. Chromatographic parameters were optimized to achieve high resolution and efficiency at

DOI: 10.1021/acs.jafc.5b01275
J. Agric. Food Chem. 2015, 63, 6283−6292

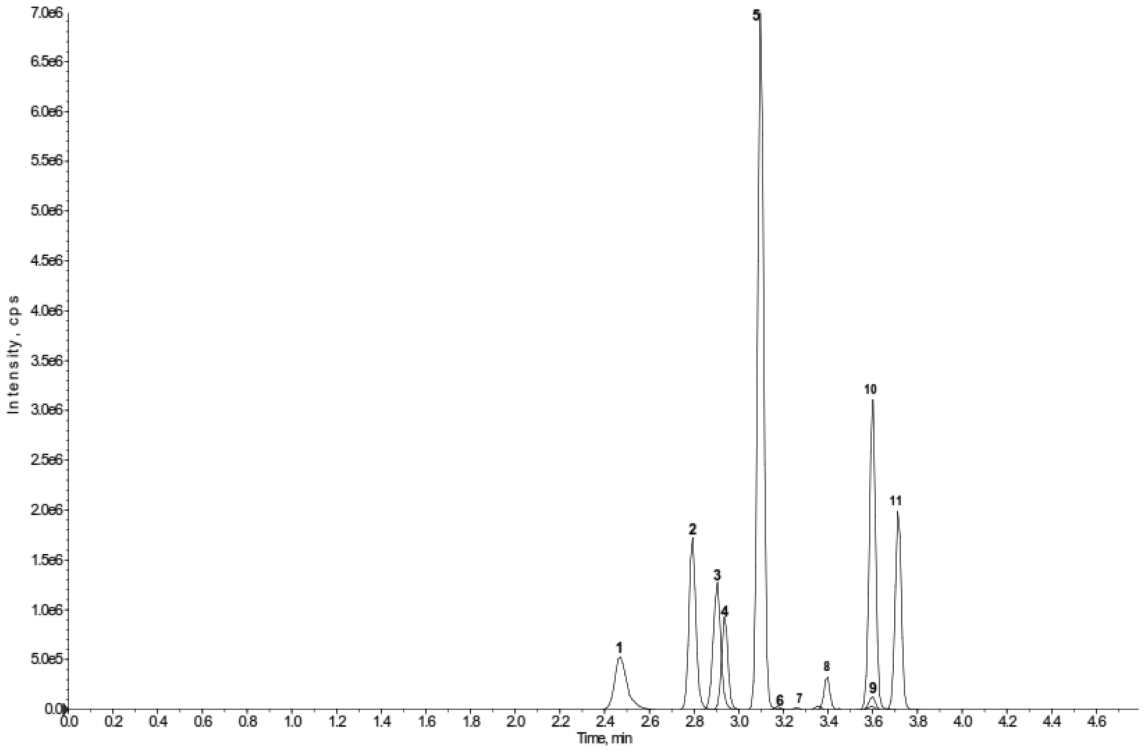

Figure 1. Typical MRM chromatogram of lignan standards at 50 ng/mL. Peaks: 1, isolariciresinol, RT 2.47 min; 2, secoisolariciresinol, RT 2.79 min; 3, hydroxymatairesinol, RT 2.90 min; 4, lariciresinol, RT 2.94 min; 5, enterodiol, RT 3.10 min; 6, syringaresinol, RT 3.17 min; 7, medioresinol, RT 3.26 min; 8, internal standard, RT 3.40 min; 9, pinoresinol, RT 3.60 min; 10, matairesinol, RT 3.60 min; 11, enterolactone, RT 3.71 min.

minimum run time. Good chromatographic separation was achieved at a short run time of 4.8 min, which is desirable when the number of samples is high. Only 2 of 10 lignans had similar retention times (RT) (Figure 1). Isolariciresinol had the lowest affinity to the C18 column, as indicated by the broader peak shape compared to the other lignans. Isolariciresinol was also the first lignan, eluting at 2.47 min, followed by secoisolariciresinol with RT of 2.79 min (Figure 1). Variation in the RT was negligible, with interbatch variation of <6 s. However, column-to-column variation of 10 s was observed. The chromatographic method was optimized using the standards prepared for the standard curve. These series of standards were also used to ensure the reproducibility of the chromatographic method and MS performance and were run each intra- and interbatch. No carry-over effect was observed when running blank samples containing only solvent and zero samples containing the solvent and the internal standard (data not shown). Zero samples also ensured the reproducibility of the IS.

Method Validation. *Quantitative Validation.* Calibration curves of pure standards were used to quantitate unknown concentrations of lignans in plasma and urine. The calibration curves generally consisted of 11 or 12 points; however, in the case of pinoresinol, syringaresinol, and medioresinol, they consisted of 7−10 points. The lower limit of quantitation (LLOQ) was accepted as the lowest standard on the calibration curve if the analyte response was at least 5 times the response of the blank sample. The highest standard defined the upper limit of quantitation (ULOQ). All calibration curves showed good linearity throughout the used range of concentration with LLOQ accuracy varying from 85 to 97% and precision below 20% and ULOQ accuracy from 90 to 105% and precision below 15%. The LLOQ and ULOQ and the corresponding regression coefficients for each lignan are listed in Table 2.

Table 2. Lower Limit of Quantitation (LLOQ) and Upper Limit of Quantitation (ULOQ) and Their Corresponding Regression Coefficients (r)

	LLOQ, nM (ng/mL)	ULOQ, nM (ng/mL)	r
enterolactone	0.16 (0.048)	167.6 (50)	0.9993
enterodiol	0.081 (0.024)	165.4 (50)	0.9997
matairesinol	0.068 (0.024)	139.5 (50)	0.9997
hydroxymatairesinol	0.13 (0.048)	133.6 (50)	0.9992
secoisolariciresinol	0.067 (0.024)	138.0 (50)	0.9997
lariciresinol	0.14 (0.048)	277.5 (100)	0.9996
isolariciresinol	0.068 (0.024)	277.5 (100)	0.9998
pinoresinol	0.27 (0.0975)	279.9 (100)	0.9994
syringaresinol	1.9 (0.78)	239.0 (100)	0.9977
medioresinol	4.0 (1.56)	257.5 (100)	0.998

Matrix Effect Validation. Ion suppression of ESI in MS instruments is a well-known problem. Several methods can be used for the validation of ion suppression due to matrix effects. Because both hydrolyzed and unhydrolyzed plasma and urine contained lignans in different concentrations, which would complicate the standard curve calculations, it was decided to use standards of three concentrations spiked into the pure solvent and SPE extracted plasma and urine. This experiment established that any matrix effect on the analyte response was corrected by a corresponding decrease in the IS response and thus permitted the use of calibration curves in pure solvent. Even though the IS corrected for possible matrix effects, the matrix factor (MF) was calculated for each lignan and IS (data not shown). Furthermore, the average MF was calculated for IS of human and pig plasma and urine and used as a measure indicating the influence of the matrix on the ionization of

DOI: 10.1021/acs.jafc.5b01275
J. Agric. Food Chem. 2015, 63, 6283−6292

lignans. The MFs for human and pig plasma were 99 and 98%, respectively, showing that no ion suppression had occurred. The MF of hydrolyzed versus unhydrolyzed plasma was also compared, but no differences were observed. In the case of enterolignans in urine the MF was close to 100%, whereas it was more variable when plant lignans were measured. Because 10 times more sample volume was used for measuring the plant lignans, variation in MF between the human and pig urine and unhydrolyzed versus hydrolyzed urine was observed. The MFs of the human hydrolyzed and unhydrolyzed urine were 90 and 69%, respectively. The MFs of the pig hydrolyzed and unhydrolyzed urine were 82 and 72%, respectively. The RSD of the IS normalized MF was then calculated for each of the three concentrations of human and pig urine and found to be not greater than 15%.

Accuracy, Precision, and Recovery. The accuracy, precision, and recovery were the main parameters used for the validation of the method. They were tested by the addition of the standards at three concentrations, low, medium, and high (Tables 3 and 4). The low concentration of plasma was chosen as 3 times the LLOQ of the standard curve, and in the case of urine 5 times the LLOQ was chosen, due to higher concentrations of lignans in the test urine. Because the ULOQ was set to 100 ng/mL, the high concentration was selected as the second highest concentration on the standard curve. In the case of precision and accuracy the standards were added to plasma and urine after the SPE procedure to be able to test the matrix effects and overall instrument performance. With regard to recovery of the lignans, the standards were added before the enzymatic hydrolysis and SPE procedure to test the affinity of the lignans to the SPE sorbent. In general, the results in Tables 3 and 4 show good or acceptable accuracy, precision, and recovery for all of the lignans tested in this study.

In the case of plasma (Table 3), the accuracy varied from −2.4% for hydroxymatairesinol to 12.7 and 8.9% for enterolactone and enterodiol, respectively, for human plasma at low concentration. This was opposite for pig plasma, where enterodiol and enterolactone showed the best accuracies of 1.6 and 4.7%, respectively, whereas secoisolariciresinol and hydroxymatairesinol had accuracies of 20 and 16.9%, respectively. At medium and high concentrations the method showed good or acceptable accuracy and precision, which was lower than the acceptance criteria of ±15%. Also, high or acceptable recoveries were observed for all of the lignans for human and pig plasma at all three concentrations.

In the case of urine (Table 4), the precision and accuracy of plant lignans, when corrected by the IS, were acceptable even though some ion suppression due to matrix effect had occurred. The concentration of enterolactone in human urine was too high to be able to calculate accuracy, precision, and recovery at low concentration. In general, the recoveries for enterolactone and enterodiol were high at all three concentrations, whereas in the case of plant lignans recoveries at high concentration were slightly lower compared to low and medium concentrations.

Selectivity and Sensitivity. Panels A and B of Figure 2 show the selectivity of the method in both human test plasma and urine spiked with low concentration of standards for all lignans except pinoresinol, syringaresinol, and medioresinol, where spiked concentrations were medium and high, respectively. A high selectivity of the method was demonstrated for plasma samples, where low background with no interfering peaks was observed (Figure 2A). Only a few additional peaks were visible for isolariciresinol, hydroxymatairesinol, lariciresinol, and

Table 3. Accuracy, Precision, and Recovery of Human and Pig Plasma Lignans at Low (L) = 0.0977 ng/mL, Medium (M) = 1.56 ng/mL, and High (H) = 50 ng/mL Concentrations, n = 5

	human plasma									pig plasma								
	accuracy (RE %)			precision (±RSD %)			recovery (%)			accuracy (RE %)			precision (±RSD %)			recovery (%)		
	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
enterolactone	12.7	5.9	1.4	20.0	7.6	2.6	99.4 ± 13.2	101.9 ± 8.9	95.4 ± 4.4	4.7	1.5	5.4	10.7	4.6	2.2	102.7 ± 12.3	104.2 ± 12.9	95.7 ± 3.8
enterodiol	8.9	4.9	1.5	16.5	4.2	2.2	104.4 ± 4.5	93.0 ± 2.4	96.2 ± 2.2	1.6	6.9	−1.5	4.7	7.3	2.7	80.0 ± 8.9	80.0 ± 9.3	103.5 ± 6.7
matairesinol	−4.0	2.9	0.1	6.4	2.8	1.4	93.7 ± 10.2	96.5 ± 2.5	82.8 ± 2.6	14.7	8.7	5.7	5.2	7.3	2.7	89.7 ± 12.5	89.1 ± 11.5	92.5 ± 4.8
hydroxymatairesinol	−2.4	0.6	−2.2	6.6	4.7	1.7	63.9 ± 5.5	76.3 ± 5.2	94.6 ± 4.4	−16.9	−12.4	−3.6	17.4	7.1	3.0	65.2 ± 14.1	66.5 ± 7.0	79.6 ± 5.1
secoisolariciresinol	−4.4	−2.1	−2.0	13.6	3.0	1.5	96.7 ± 16.7	86.3 ± 5.2	94.0 ± 3.7	20.0	13.5	1.7	5.4	3.8	2.0	98.7 ± 9.0	80.2 ± 3.8	83.2 ± 5.7
lariciresinol	8.3	0.5	4.0	16.3	3.3	2.5	102.6 ± 9.9	83.6 ± 5.2	91.2 ± 2.2	−17.2	−10.0	−1.5	15.2	6.3	1.4	83.8 ± 9.6	95.7 ± 3.8	87.6 ± 5.9
isolariciresinol	−8.7	0.2	−0.1	19.3	1.2	2.1	107.7 ± 3.4	88.8 ± 1.8	76.7 ± 3.5	13.5	3.3	1.1	9.5	10.1	3.9	88.3 ± 8.2	81.1 ± 3.8	87.6 ± 5.8
pinoresinol	8.1	0.6	−4.0	10.3	4.2	2.1	76.5 ± 4.5	93.4 ± 10.1	83.9 ± 4.9	13.2	−4.8	3.6	9.6	0.9	2.5	96.8 ± 5.5	85.5 ± 7.6	92.9 ± 4.1
syringaresinol	nq[a]	3.7	−4.7	nq	10.8	1.7	nq	107.6 ± 11.0	84.4 ± 4.3	nq	0.9	3.8	nq	10.5	5.5	nq	99.1 ± 3.2	79.4 ± 4.6
medioresinol	nq	nq	−1.6	nq	nq	4.3	nq	nq	97.3 ± 5.9	nq	nq	−2.5	nq	nq	4.4	nq	nq	81.8 ± 1.7

[a]Not quantitated.

DOI: 10.1021/acs.jafc.5b01275
J. Agric. Food Chem. 2015, 63, 6283−6292

Table 4. Accuracy, Precision, and Recovery of Human and Pig Urine Lignans at Low (L) = 0.0977 ng/mL, Medium (M) = 1.56 ng/mL, and High (H) = 50 ng/mL Concentrations, $n = 5$

	human urine									pig urine								
	accuracy (RE %)			precision (±RSD %)			recovery (%)			accuracy (RE %)			precision (±RSD %)			recovery (%)		
	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
enterolactone	nq	14.3	11.5	nq	3.3	0.4	nq	100.3 ± 15.3	94.0 ± 5.0	20.0	15.0	11.5	3.6	3.3	0.4	92.9 ± 2.6	102.9 ± 20.7	94.3 ± 5.8
enterodiol	14.1	5.6	- 3.9	4.5	2.8	0.9	101.3 ± 6.0	103.3 ± 3.2	96.7 ± 3.0	10.3	5.6	−3.9	4.4	2.9	0.9	88.6 ± 3.3	100.6 ± 19.8	84.6 ± 5.5
matairesinol	−5.2	−11.8	−10.1	5.7	3.9	1.2	97.3 ± 10.7	108.9 ± 3.7	79.8 ± 2.3	7.4	−11.9	−6.6	11.4	1.3	3.4	91.5 ± 12.5	86.2 ± 5.6	82.1 ± 1.7
hydroxymatairesinol	−9.6	−12.9	−9.4	9.0	9.3	2.8	95.8 ± 6.8	63.3 ± 9.0	48.8 ± 2.3	−9.5	−12.9	−10.7	6.9	4.9	4.0	94.7 ± 14.1	118.5 ± 24.8	49.4 ± 4.1
secoisolariciresinol	−3.5	−5.9	−8.0	5.8	3.4	0.3	87.0 ± 12.1	102.2 ± 8.4	68.0 ± 4.0	2.6	4.1	4.6	4.5	3.3	3.3	93.5 ± 6.0	97.7 ± 10.0	68.4 ± 3.7
lariciresinol	11.0	5.6	−8.5	8.3	10.0	2.7	101.1 ± 9.9	103.6 ± 5.5	83.4 ± 3.9	14.7	−10.8	−6.4	12.0	3.7	4.6	85.8 ± 9.8	86.9 ± 11.9	73.1 ± 3.4
isolariciresinol	3.5	−4.5	−4.3	6.0	7.6	1.4	81.7 ± 11.2	92.7 ± 9.3	91.6 ± 5.6	14.1	−3.5	−5.1	3.0	9.3	7.4	91.2 ± 5.1	85.7 ± 12.1	89.6 ± 5.7
pinoresinol	−1.7	0.5	−6.0	3.1	9.3	3.3	60.9 ± 11.8	78.4 ± 5.8	72.1 ± 5.6	7.6	2.7	−4.3	10.0	12.0	1.3	78.9 ± 15.5	106.3 ± 10.2	96.7 ± 6.6
syringaresinol	nq[a]	10.4	−6.2	nq	13.5	3.4	nq	104.4 ± 7.7	78.8 ± 5.5	nq	6.5	7.6	nq	8.1	10.4	nq	86.5 ± 12.6	86.0 ± 7.8
medioresinol	nq	nq	−5.1	nq	nq	10.4	nq	nq	82.9 ± 4.2	nq	nq	5.8	nq	nq	8.4	nq	nq	93.3 ± 6.6

[a]Not quantitated.

syringaresinol. Similar high selectivity was observed for pig plasma (data not shown). In the case of urine higher background and more additional peaks were visible on the chromatograms compared to plasma (Figure 2B). However, no peaks interfered with the signal of the analytes. Moreover, a very low noise level at around 100−700 cps in intensity for plasma and around 200−1500 cps in intensity in the case of urine (Figure 3) increased the sensitivity of the method, allowing quantitation at low concentrations, as described in the quantitative validation section. No difference in noise level or number of additional/interfering peaks was observed for unhydrolyzed versus hydrolyzed plasma (data not shown).

Standard Stability. Repeated analyses of the standard curve over a 3 month period showed that the standards were stable for at least 1 month. Short-term stability of IS in water was also tested by comparison of the IS areas from 4 to 24 h. The result indicated that IS was stable for at least 1 day.

Real Sample Analyses. The applicability of the developed LC-MS/MS method was verified by measuring plant lignans and enterolignans in human and pig plasma and urine from two ongoing studies. Human plasma and urine were obtained from a Nordic lifestyle intervention study involving 24 men with early-stage prostate cancer, where 16 male participants were prescribed a high-lignan diet (HLD, based on whole grain rye) and 8 were used as control and were provided a low-lignan diet (LLD, based on refined wheat) during 6 months. Participants in the HLD group were prescribed 1200 g of whole grain rye per week. The detailed explanation of the intervention is described elsewhere.[14,15] The pig plasma and urine were collected from 10 pigs kept on HLD (based on whole grain rye) and 10 pigs kept on LLD (based on refined wheat) for 3 weeks and 1 week of wash-out LLD in the beginning of the study. Details of this study will be presented elsewhere. The samples from these two studies were run in batches together with 10 QC samples for each batch. Interbatch precision of pig plasma and urine was measured using QC samples of low and high concentrations, whereas in the case of human plasma and urine only one concentration was used (Table 5). The acceptance criterion of RSD of 15% was fulfilled in all cases except one; hydroxymatairesinol, at low concentration, had an RSD of 24.2%. However, this is in agreement with the low recovery and high deviation for this lignan in pig plasma at low concentration previously described under Method Validation. The results of human and pig studies are presented in Table 6. Furthermore, Figures 4 and 5 demonstrate MRM chromatograms of lignans in one of the plasma and urine samples from humans and pigs.

DISCUSSION

SPE Cleanup. One of the advantages of our developed cleanup procedure is the possibility to prepare both urine and plasma samples on the same SPE plate because the procedures are similar. Another simplification of the developed cleanup procedure is that no evaporation or resuspension of the extracted sample is necessary prior to LC-MS/MS, compared to the previously reported SPE methods.[7−9,16,17] After extraction of the analytes from the SPE plate or column, the samples can be diluted and analyzed directly on the LC-MS/MS instrument. Evaporation of the sample is usually time-consuming, and resuspension can contribute to the losses of the analytes.

Chromatographic Analysis. The separation of lignans was achieved with a run time of only 4.8 min, the lowest run time reported so far.[7−9,18] For comparison, Smeds et al.[8,9] reported

DOI: 10.1021/acs.jafc.5b01275
J. Agric. Food Chem. 2015, 63, 6283−6292

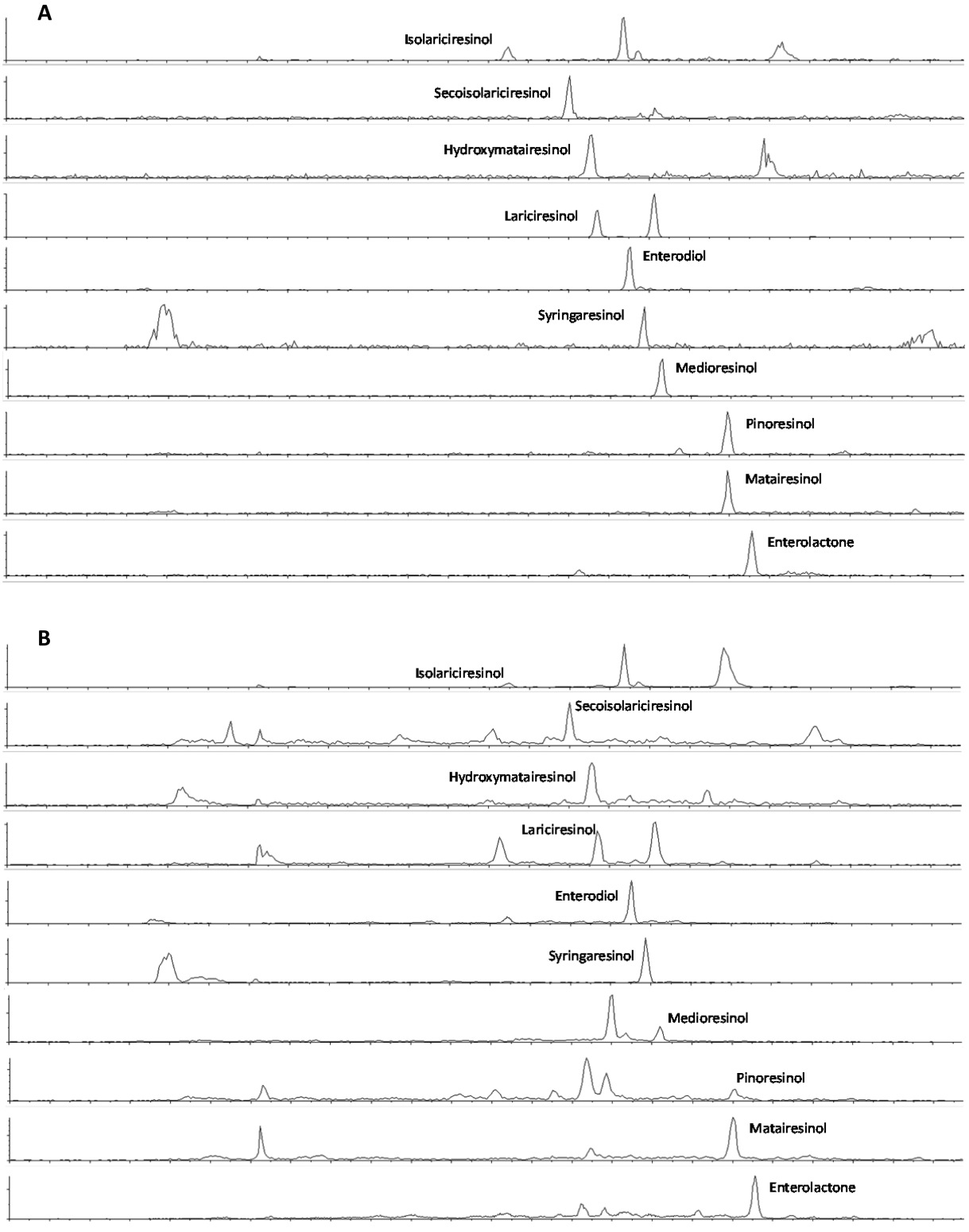

Figure 2. MRM chromatogram of lignans in test plasma spiked with 0.0977 ng/mL of standards for all lignans except pinoresinol, syringaresinol, and medioresinol, where spiked concentrations were 1.56 and 50 ng/mL respectively (A). Extracted ion MRM chromatogram of lignans in test urine spiked with 0.39 ng/mL of standards for all lignans except pinoresinol, syringaresinol, and medioresinol, where spiked concentrations were 1.56 and 50 ng/mL, respectively (B).

run times of 17 and 18 min to separate similar numbers of lignans. However, shorter run times were reported for separation of phytoestrogens in human serum[10] and urine,[11] where only enterolignans were included. The low run time is favorable when large numbers of samples are analyzed, which can effectively minimize the analytical time and therefore decrease the cost of analyses.

Method Validation. *Quantitative Validation.* The sensitivity of the method was demonstrated by low LLOQ and low noise level of the blank test plasma and urine. The sensitivity

DOI: 10.1021/acs.jafc.5b01275
J. Agric. Food Chem. 2015, 63, 6283−6292

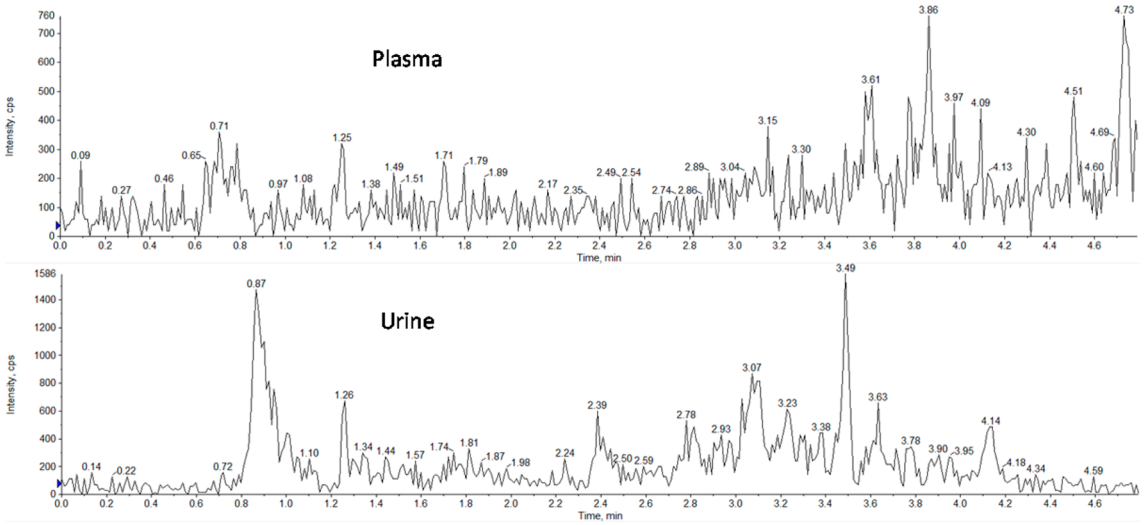

Figure 3. Typical MRM chromatogram of noise level in plasma and urine.

Table 5. Interbatch Precision of Human and Pig Plasma and Urine, Represented as ±RSD, n = 10 (Quality Control Samples (QC) at Low (L) and High (H) Concentration of Lignans)

	human plasma	human urine	pig plasma		pig urine	
	H	H	L	H	L	H
enterolactone	4.9	5.6	8.1	6.4	4.2	2.9
enterodiol	5.9	7.1	nq	6.5	6.4	3.4
matairesinol	10.8	5.3	nq	10.8	2.5	10.4
hydroxymatairesinol	nq[a]	8.8	24.2	8.2	4.0	6.6
secoisolariciresinol	6.2	12.0	8.0	4.9	8.6	5.7
lariciresinol	6.4	7.4	13.0	9.6	4.7	8.6
isolariciresinol	12.6	8.0	14.2	6.8	6.3	3.2
pinoresinol	14.0	5.2	nq	nq	5.5	6.0
syringaresinol	nq	12.2	nq	nq	9.7	4.8
medioresinol	nq	nq	nq	nq	nq	nq

[a]Not quantitated.

was 10−100 times higher depending on the lignan analyzed compared to the previously reported LC-MS/MS method of Smeds et al.[9] This was achieved without the need for concentrating the sample during the SPE procedure; on the contrary, the samples were diluted four times. This improved sensitivity is probably due to the use of the most recent LC-MS/MS system in our laboratory compared to the methods reported nearly 10 years ago. However, newer LC-MS/MS methods recently reported for the determination of phytoestrogens in human serum and urine did not show similar sensitivity either.[10,11] Prasain et al.[10] measured enterolignans in serum at LLOQ of 1 ng/mL, which is >10 times higher than LLOQ of our method. Moreover, to our knowledge no LC-MS/MS method has been published before on the quantitation of pinoresinol, syringaresinol, and medioresinol in plasma and urine. However, the LLOQ for syringaresinol and medioresinol was much higher compared to that of other plant lignans, and therefore syringaresinol was only possible to measure in urine, whereas medioresinol could not be measured in plasma or in urine.

Matrix Effects and Selectivity. Matrix-based and solvent-based calibration curves can be constructed to analyze the matrix effects on the instrument response[7,19] or to analyze the standards at three concentrations both dissolved in solvent and spiked into SPE extracted plasma and urine.[9,20] On the basis of the results of the spiking experiment, no matrix effects were detected for hydrolyzed or unhydrolyzed human and pig plasma, which is in agreement with previous analyses of human plasma reported by Smeds et al.[8,9] The selectivity was not

Table 6. Real Sample Analyses of Human Plasma Presented As Averaged (nM ± SD) and Urine As Averaged (μM/mM* or nM/mM of Creatinine ± SD), Low-Lignan Diet (LLD) following a High-Lignan Diet (HLD)[a]

	human plasma		human urine		pig plasma		pig urine	
	LLD	HLD	LLD	HLD	LLD	HLD	LLD	HLD
enterolactone	41.9 ± 35.7	59.6 ± 60.5	0.8* ± 0.7	1.4* ± 2.2	5.1 ± 1.8	150.5 ± 28.4	0.09* ± 0.03	4.8* ± 1.1
enterodiol	5.5 ± 10.8	10.0 ± 21.1	0.1* ± 0.1	0.2* ± 0.3	0.1 ± 0.07	3.1 ± 1.1	3.7 ± 1.7	55.9 ± 26.4
matairesinol	0.9 ± 0.6	0.6 ± 0.3	13.2 ± 6.0	15.3 ± 3.3	nq[b]	0.6 ± 0.3	0.9 ± 0.3	15.4 ± 9.7
hydroxymatairesinol	0.1 ± 0.1	0.2 ± 0.2	0.6 ± 0.4	1.1 ± 0.9	0.8 ± 0.3	0.9 ± 0.3	5.5 ± 2.6	3.4 ± 2.2
secoisolariciresinol	5.5 ± 5.8	3.1 ± 3.8	3.2 ± 2.5	4.3 ± 9.5	0.4 ± 0.1	1.1 ± 0.5	1.2 ± 0.5	10.5 ± 5.0
lariciresinol	0.9 ± 0.6	0.7 ± 0.4	18.3 ± 9.8	16.1 ± 7.3	0.5 ± 0.1	2.4 ± 0.5	2.0 ± 0.5.0	25.2 ± 11.6
isolariciresinol	3.5 ± 3.5	2.5 ± 3.5	73.9 ± 54.6	64.9 ± 43.2	0.3 ± 0.1	0.6 ± 0.3	0.9 ± 0.5	2.0 ± 0.4
pinoresinol	2.2 ± 2.5	2.0 ± 3.0	60.0 ± 32.8	75.8 ± 28.6	nq	nq	1.3 ± 1.1	16.5 ± 9.3
syringaresinol	nq	nq	5.3 ± 1.4	8.6 ± 4.4	nq	nq	1.6 ± 0.3	31.4 ± 14.2
medioresinol	nq	nq	nq	nq	nq	nq	nq	nq

[a]The numbers of samples for human plasma and urine of LLD were n = 8 and HLD n = 16 and for pig plasma and urine of both LLD and HLD were n = 10. [b]Not quantitated.

DOI: 10.1021/acs.jafc.5b01275
J. Agric. Food Chem. 2015, 63, 6283−6292

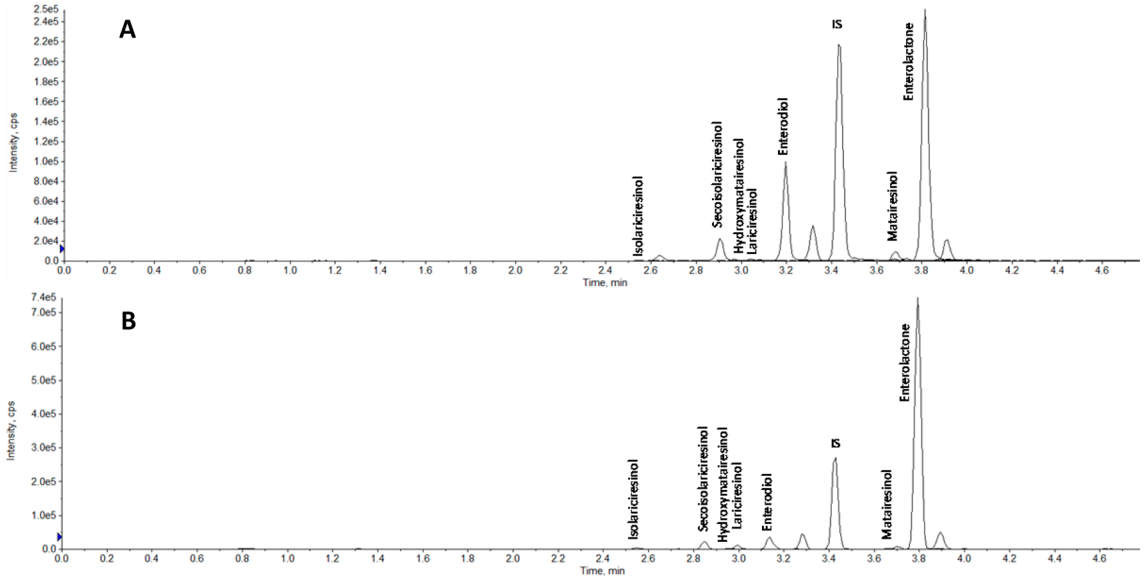

Figure 4. MRM chromatogram of lignans in one human plasma sample (A) and one pig plasma sample (B) from real sample analyses.

affected by hydrolysis of the plasma, where no additional or interfering peaks were observed. Prasain et al.[10] had detected minor to moderate matrix effects when analyzing phytoestrogens in serum. In our study, we measured minor to moderate matrix effects when analyzing plant lignans in urine. Moreover, a difference in matrix effects between hydrolyzed and unhydrolyzed urine was also measured. Our result showed that less ion suppression occurred after hydrolysis of the urine sample. However, no difference in the noise level or selectivity was observed for hydrolyzed/unhydrolyzed urine. To minimize the matrix effects in urine when plant lignans are measured, it is possible to use less sample volume.

Accuracy, Precision, and Recovery. In general, the accuracy and precision of this method for plasma and urine at low, medium, and high concentrations were good or acceptable. As expected, at the low concentration the accuracy and precision had higher deviation compared to the medium and high concentrations. Valentin-Blasini et al.[7] have also reported that the accuracy of their method increased with increasing analyte concentration.

In the case of plasma, at high concentration the accuracy was very good with an ER of only 0.1−4.7% in human plasma and 1.1−5.7% in pig plasma. Similarly, precision was also high at high concentration. At low concentration higher variation was observed among the lignans; also, the differences between human and pig plasma were greater. For example, in the case of enterodiol and enterolactone, the difference between the accuracy of these lignans in human versus pig plasma can probably be explained by the fact that no enterodiol and enterolactone were detected in unhydrolyzed test pig plasma, whereas traces of this compound were detected in human unhydrolyzed test plasma. The opposite situation was observed for hydroxymatairesinol, where low concentration of this compound was detected in both hydrolyzed and unhydrolyzed test pig plasma and no hydroxymatairesinol was detected in human test plasma.

Recoveries of enterolactone and enterodiol were high, not lower than 90% for human plasma and 80% for pig plasma. These data are comparable to previously reported recoveries for enterolignans by others using GC-MS, LC-MS, and LC with coulometric electrode array detector.[7−9,18,19,21] Lower recoveries of 68−91% for enterolignans in human plasma were reported by Nurmi et al.[22] using LC with coulometric electrode array detector and in serum by Prasain et al.[10] Recoveries for plant lignans in plasma were more varied, with the lowest recovery for hydroxymatairesinol, 63.9−94.6% for human plasma and 65.2−79.6% for pig plasma. Similarly low recoveries of 65−69% for hydroxymatairesinol were reported by Smeds et al.[8] Penalvo et al.[18] reported recoveries varying from 77 to 86% for plant lignans in human plasma. Our recoveries for other plant lignans except hydroxymatairesinol in human plasma varied from 76.5 to 107.7%, which is comparable to recoveries reported by Penalvo et al.[18]

In the case of urine, the recoveries for enterolactone and enterodiol were high at all three concentrations and therefore in accordance with recoveries reported elsewhere.[7,16,20] Plant lignans have shown high recoveries at low and medium concentrations, whereas recoveries were slightly lower at high concentration, except pinoresinol. Because our test urine when hydrolyzed contained higher concentrations of lignans compared to unhydrolyzed urine, this could have influenced the result.

Test Plasma and Urine. The increased concentration of lignans after hydrolysis of the plasma and urine was expected because lignans and especially enterolactone and enterodiol have been detected almost exclusively as conjugates of glucuronic acid and sulfate in human serum and urine.[23,24] The results of this study show that pig plasma and urine, similarly to human plasma and urine, contain lignans mainly in conjugated form.

Real Sample Analyses. As consumption of diet containing a high level of lignans is associated with a higher concentration of lignans in the blood and urine compared to a diet containing a low level of lignans, we have tested this newly developed method on real samples from two studies, with humans and with pigs. The analyses of human plasma and urine showed large interindividual variation in the concentration of lignans, which was expected because absorption and bioconversion of lignans depend greatly on the microbiota of the individual. Large interindividual variation in humans was also observed in other studies.[9,25,26] Therefore, the difference between participants consuming HLD and LLD was not so pronounced compared to the pig study, where dietary intake is under more strict control.

DOI: 10.1021/acs.jafc.5b01275
J. Agric. Food Chem. 2015, 63, 6283−6292

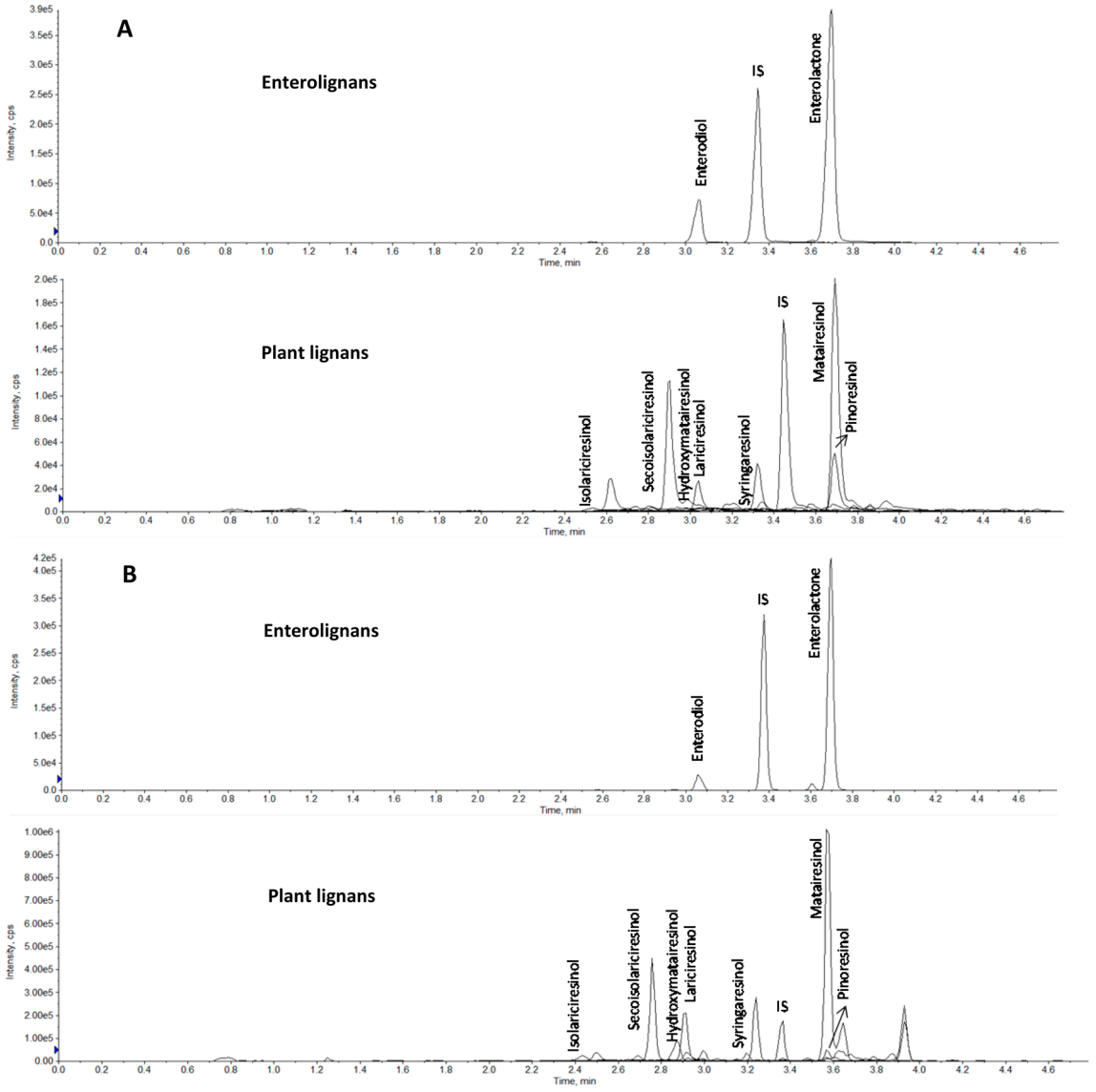

Figure 5. Extracted ion MRM chromatogram of lignans in one human urine sample (A) and one pig urine sample (B) from real sample analyses.

The difference between HLD and LLD in the pig study was large, which was as expected. The two types of diets, used in this pig study, have previously been used in model experiments with pigs, where large differences in lignan concentration between HLD and LLD in the plasma and urine were observed.[27,28] Moreover, interindividual variation among the pigs is much lower compared to humans, because the pigs genetically are more homogeneous and compliant to the experimental diets. It can be noted that the ratio between enterodiol and enterolactone in human plasma and urine was 3−5 times higher compared to pig plasma and urine. This indicates that pigs have a higher capacity to convert enterodiol to enterolactone compared to humans, probably due to a different microbiota. Moreover, the ratio between plant lignans and enterolignans in human plasma and urine after consumption of HLD was also 3−5 times higher compared to pig plasma and urine after consumption of the HLD. In contrast, when the two species consumed a LLD, the ratios between plant lignans and enterolignans in both human and pig plasma and urine were similar. This again can indicate differences in the absorption and bioconversion of lignans between humans and pigs and that the pig's conversion of plant lignans to enterolignans is more efficient. In general, the concentration of enterolignans in plasma and urine from both humans and pigs was higher compared to plant lignans, which is in accordance with our current knowledge that the majority of plant lignans are converted to enterolignans prior to uptake from the gut.[1]

The current paper presents a fast and easy (around 200 samples per day) LC-MS/MS method to quantitate two enterolignans, enterolactone and enterodiol, and eight plant lignans, matairesinol, hydroxymatairesinol, secoisolariciresinol, lariciresinol, isolariciresinol, syringaresinol, medioresinol, and pinoresinol, in biofluids from humans and pigs. The merit of our approach was to simplify the SPE cleanup and to shorten the analytical time used per sample without compromising the analytical performances of our method in terms of accuracy, precision, reproducibility, and sensitivity. Although we were successful in simplifying the SPE cleanup, some matrix effects were observed when plant lignans in urine samples were measured. This problem can be minimized by using a lower sample volume, because the concentration of plant lignans in urine is quite high. Furthermore, the method was successfully applied to quantitate lignans after the

DOI: 10.1021/acs.jafc.5b01275
J. Agric. Food Chem. 2015, 63, 6283−6292

consumption of HLD and LLD in plasma and urine. The lignans could be quantitated at pico- to nanomolar concentrations with good accuracy and precision. Moreover, the method is applicable for large numbers of samples and allows simultaneous analyses of plasma and urine from humans and pigs with a wide range of lignan concentrations.

AUTHOR INFORMATION

Corresponding Author

*(N.P.N.) Phone: +45 8715 7724. Fax: +45 8715 4249. E-mail: Natalja.Norskov@anis.au.dk.

Funding

We thank the Danish Council for Strategic Research for financing the project "The effects of enterolignans in chronic diseases − ELIN" (11964).

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Axelson, M.; Sjovall, J.; Gustafsson, B. E.; Setchell, K. D. R. Origin of lignans in mammals and identification of a precursor from plants. *Nature* **1982**, *298*, 659−660.

(2) Setchell, K. D. R.; Lawson, A. M.; Mitchell, F. L.; Adlercreutz, H.; Kirk, D. N.; Axelson, M. Lignans in man and in animal species. *Nature* **1980**, *287*, 740−742.

(3) Setchell, K. D. R.; Lawson, A. M.; Borriello, S. P.; Harkness, R.; Gordon, H.; Morgan, D. M. L.; Kirk, D. N.; Adlercreutz, H.; Anderson, L. C.; Axelson, M. Lignan formation in man − microbial involvement and possible roles in relation to cancer. *Lancet* **1981**, *318*, 4−7.

(4) Saarinen, N. M.; Smeds, A.; Makela, S. I.; Ammala, J.; Hakala, K.; Pihlava, J. M.; Ryhanen, E. L.; Sjoholm, R.; Santti, R. Structural determinants of plant lignans for the formation of enterolactone in vivo. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2002**, *777*, 311−319.

(5) Adlercreutz, H. Lignans and human health. *Crit. Rev. Clin. Lab. Sci.* **2007**, *44*, 483−525.

(6) Peterson, J.; Dwyer, J.; Adlercreutz, H.; Scalbert, A.; Jacques, P.; McCullough, M. L. Dietary lignans: physiology and potential for cardiovascular disease risk reduction. *Nutr. Rev.* **2010**, *68*, 571−603.

(7) Valentin-Blasini, L.; Blount, B. C.; Rogers, H. S.; Needham, L. L. HPLC-MS/MS method for the measurement of seven phytoestrogens in human serum and urine. *J. Exposure Anal. Environ. Epidemiol.* **2000**, *10*, 799−807.

(8) Smeds, A.; Hakala, K. Liquid chromatographic-tandem mass spectrometric method for the plant lignan 7-hydroxymatairesinol and its potential metabolites in human plasma. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2003**, *793*, 297−308.

(9) Smeds, A. I.; Hakala, K.; Hurmerinta, T. T.; Kortela, L.; Saarinen, N. M.; Makela, S. I. Determination of plant and enterolignans in human serum by high-performance liquid chromatography with tandem mass spectrometric detection. *J. Pharm. Biomed. Anal.* **2006**, *41*, 898−905.

(10) Prasain, J. K.; Arabshahi, A.; Moore, D. R.; Greendale, G. A.; Wyss, J. M.; Barnes, S. Simultaneous determination of 11 phytoestrogens in human serum using a 2 min liquid chromatography/tandem mass spectrometry method. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2010**, *878*, 994−1002.

(11) Parker, D. L.; Rybak, M. E.; Pfeiffer, C. M. Phytoestrogen biomonitoring: an extractionless lc-ms/ms method for measuring urinary isoflavones and lignans by use of atmospheric pressure photoionization (appi). *Anal. Bioanal. Chem.* **2012**, *402*, 1123−1136.

(12) Guidance for Industry: Bioanalytical Method Validation; U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center of Veterinary Medicine (CVM), May 2001; available at http://www.Fda.Gov/drugs/guidancecomplianceregulatoryinformation/guidances/default.Htm.

(13) Guideline on Bioanalytical Method Validation. Eurpean Medicines Agency of Science Medicine Health. Committee for Medicinal Products for Human Use (CHMP), February 2012; available at http://www.Ema.Europa.Eu/docs/en_gb/document_library/scientific_guideline/2011/08/wc500109686.Pdf.

(14) Landberg, R.; Andersson, S. O.; Zhang, J. X.; Johansson, J. E.; Stenman, U. H.; Adlercreutz, H.; Kamal-Eldin, A.; Aman, P.; Hallmans, G. Rye whole grain and bran intake compared with refined wheat decreases urinary c-peptide, plasma insulin, and prostate specific antigen in men with prostate cancer. *J. Nutr.* **2010**, *140*, 2180−2186.

(15) Moazzami, A. A.; Zhang, J. X.; Kamal-Eldin, A.; Aman, P.; Hallmans, G.; Johansson, J. E.; Andersson, S. O. Nuclear magnetic resonance-based metabolomics enable detection of the effects of a whole grain rye and rye bran diet on the metabolic profile of plasma in prostate cancer patients. *J. Nutr.* **2011**, *141*, 2126−2132.

(16) Grace, P. B.; Taylor, J. I.; Botting, N. P.; Fryatt, T.; Oldfield, M. F.; Bingham, S. A. Quantification of isoflavones and lignans in urine using gas chromatography/mass spectrometry. *Anal. Biochem.* **2003**, *315*, 114−121.

(17) Jacobs, E.; Kulling, S. E.; Metzler, M. Novel metabolites of the mammalian lignans enterolactone and enterodiol in human urine. *J. Steroid Biochem. Mol. Biol.* **1999**, *68*, 211−218.

(18) Penalvo, J. L.; Nurmi, T.; Haajanen, K.; Al-Maharik, N.; Botting, N.; Adlercreutz, H. Determination of lignans in human plasma by liquid chromatography with coulometric electrode array detection. *Anal. Biochem.* **2004**, *332*, 384−393.

(19) Kuijsten, A.; Buijsman, M.; Arts, L. C. W.; Mulder, P. P. J.; Hollman, P. C. H. A validated method for the quantification of enterodiol and enterolactone in plasma using isotope dilution liquid chromatography with tandem mass spectrometry. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2005**, *822*, 178−184.

(20) Nurmi, T.; Voutilainen, S.; Nyyssonen, K.; Adlercreutz, H.; Salonen, J. T. Liquid chromatography method for plant and mammalian lignans in human urine. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2003**, *798*, 101−110.

(21) Edel, A. L.; Aliani, M.; Pierce, G. N. Supported liquid extraction in the quantitation of plasma enterolignans using isotope dilution GC/MS with application to flaxseed consumption in healthy adults. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2013**, *912*, 24−32.

(22) Nurmi, T.; Adlercreutz, H. Sensitive high-performance liquid chromatographic method for profiling phytoestrogens using coulometric electrode array detection: application to plasma analysis. *Anal. Biochem.* **1999**, *274*, 110−117.

(23) Adlercreutz, H.; Vanderwildt, J.; Kinzel, J.; Attalla, H.; Wahala, K.; Makela, T.; Hase, T.; Fotsis, T. Lignan and isoflavonoid conjugates in human urine. *J. Steroid Biochem. Mol. Biol.* **1995**, *52*, 97−103.

(24) Adlercreutz, H.; Fotsis, T.; Lampe, J.; Wahala, K.; Makela, T.; Brunow, G.; Hase, T. Quantitative-determination of lignans and isoflavonoids in plasma of omnivorous and vegetarian women by isotope-dilution gas-chromatography mass-spectrometry. *Scand. J. Clin. Lab. Invest.* **1993**, *53*, 5−18.

(25) Zeleniuch-Jacquotte, A.; Adlercreutz, H.; Akhmedkhanov, A.; Toniolo, P. Reliability of serum measurements of lignans and isoflavonoid phytoestrogens over a two-year period. *Cancer Epidemiol. Biomarkers Prev.* **1998**, *7*, 885−889.

(26) Morton, M. S.; Chan, P. S. F.; Cheng, C.; Blacklock, N.; MatosFerreira, A.; AbranchesMonteiro, L.; Correia, R.; Lloyd, S.; Griffiths, K. Lignans and isoflavonoids in plasma and prostatic fluid in men: samples from Portugal, Hong Kong, And the United Kingdom. *Prostate* **1997**, *32*, 122−128.

(27) Laerke, H. N.; Mortensen, M. A.; Hedemann, M. S.; Knudsen, K. E.; Bach; Penalvo, J. L.; Adlercreutz, H. Quantitative aspects of the metabolism of lignans in pigs fed fibre-enriched rye and wheat bread. *Br. J. Nutr.* **2009**, *102*, 985−994.

(28) Knudsen, K. E. B.; Serena, A.; Kjaer, A. K. B.; Tetens, I.; Heinonen, S. M.; Nurmi, T.; Adlercreutz, H. Rye bread in the diet of pigs enhances the formation of enterolactone and increases its levels in plasma, urine and feces. *J. Nutr.* **2003**, *133*, 1368−1375.

DOI: 10.1021/acs.jafc.5b01275
J. Agric. Food Chem. 2015, 63, 6283−6292