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Liquid chromatography method for plant and mammalian lignans in human urine

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Abstract

Recently new mammalian lignan precursors were identified but no analysis methods are available for assay of those compounds in human urine. Previously published methods were developed for GC–MS about only two plant lignans were included. Consequently, a method for HPLC equipped with a coulometric electrode array detector was developed to measure plant and mammalian lignans in human urine. The plant lignans, secoisolariciresinol (Seco), matairesinol (Mat), lariciresinol (Lar), pinoresinol (Pin), syringaresinol (Syr) and isolariciresinol (IsoL) were included into the new method together with two mammalian lignans, enterolactone (Enl) and enterodiol (End). Validation of the method demonstrated that it could be applied to normal urine containing low amounts of plant lignans and moderate amounts of mammalian lignans, but the method was also applicable for samples from study subjects in supplementation studies, i.e. sample with very high concentrations of mammalian lignans. The method was found to be a useful tool for studies on plant lignan intake and the activity of micro flora in the metabolism of plant lignans.

Keywords: Lignans; Enterolactone

1. Introduction

The mammalian lignans, enterolactone (Enl) and enterodiol (End), were first identified over 20 years ago [1] and especially high serum enterolactone concentrations have been recently associated with a decreased risk of certain diseases such as coronary heart disease [2] and breast cancer [3,4].

Very few analysis methods for lignans in urine have been published, almost all originating from the same method development work [5]. The original method has been modified over the years [6–9], but the modified methods are still rather laborious, with several purifications steps and derivatization before the GC–MS analysis [10]. One simpler method has been developed for sample pretreatment, but the analysis of lignans was still carried out with GC–MS after derivatization [11]. Quick and simple screening method utilizing time-resolved fluoroimmunoassay has been published, but the method included only one lignan, enterolactone [12].

Urinary concentrations of the plant lignan, matairesinol (Mat), have been analysed in one larger study [3] though more data is available for urinary plant lignans from smaller-scale studies [13,14] or from the method development work [10]. Some qualitative studies have also been carried out to identify urinary plant lignans, matairesinol, lariciresinol (Lar) and isolariciresinol (IsoL) [15,16]. The plant lignans, secoisolariciresinol (Seco) and matairesinol, have long been recognized as mammalian lignan precursors [17] but recently also other precursors of mammalian lignans have been identified [18]. It was found that pinoresinol (Pin) and lariciresinol are extensively metabolized, syringaresinol (Syr) is only partly metabolized and isolariciresinol is not at all metabolized to the mammalian lignans, enterolactone and enterodiol.

The aim of this study was to develop a method to permit the convenient analysis of urinary lignan profiles, since urinary plant lignans can provide information about dietary

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lignan intake and the activity of the gut micro flora. Urinary plant lignans may also provide new data about the dietary lignan sources, though little is known on the levels of pinoresinol, lariciresinol, syringaresinol and isolariciresinol values present in foods. The method for urinary lignans will be applied in the cohort of the Kuopio Ischemic Heart Disease Risk Factor Study (KIHD). The same method for urinary lignans will be applied also in clinical studies when health effects of dietary components are being studied.

2. Experimental

2.1. Chemicals

Standards for the mammalian lignans, enterolactone and enterodiol, were synthesized in the Laboratory of Organic Chemistry, Department of Organic Chemistry, the University of Helsinki [19]. The following plant lignans, secoisolariciresinol, matairesinol, pinoresinol, lariciresinol, syringaresinol and isolariciresinol, were purchased from Plantech (Reading, UK). The structures of the lignans and the proposed metabolic pathway are presented in Fig. 1. The weighed amount of each standard was dissolved in MeOH. The quantification standard was a mixture of all plant and mammalian lignans.

MeOH was purchased from LabScan (Dublin, Ireland), ACN from Merck (Darmstad, Germany) both of HPLC grade. Sodium acetate and glacial acetic acid were p.a. quality (Riedel-de-Haën, Seelze, Germany). The buffer stock solution for mobile phase and for sample pretreatment was prepared from sodium acetate salt and glacial acetic acid with purified water (Millipore Corporation Waters, Bedford, USA). The enzyme mixture *Helix pomatia* was purchased from BioSepra (France), pure β-glucuronidase from Boehringer GmBH (Mannheim, Germany), pure sulphatase from Sigma, HCl and diethyl ether from Riedel-de-Haën (Seelze, Germany). QAE-Sephadex A-25 ion exchange gel was purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). The gel was used in its acetate form as earlier described by Adlercreutz et al. [13].

2.2. Apparatus

The analyses were carried out with HPLC using coulometric electrode array detection (CEAD) (ESA Inc., Chelmsford, MA, USA). The HPLC system consisted of two pumps, an autosampler with cooled sample tray and an electrode array detector with eight electrode pairs, which were placed together with the columns into a thermostated chamber at 37 °C. The detector consisted of two detector cells each containing four measuring channels and one reference electrode to measure the background signals. Different potentials to oxidize the analytes under interest were set on each channel and measuring potentials were 180, 300, 420, 470, 490, 640, 700 and 720 mV. The mammalian lignans Enl and End were detected on channel 6 at 640 mV, plant lignans Seco and Syr were detected on channel 2 at 300 mV and Lar, Pin, Mat and IsoL were detected on channel 3 at 420 mV. The potentials were optimized to obtain the maximum response for all lignans on their quantification channel. Hydrodynamic voltammograms were used to determine the potentials oxidizing most effectively the various analytes.

2.3. Chromatographic conditions

Lignans were separated with gradient elution using the same mobile phase, which has been earlier applied for plasma lignans [20]. The mobile phase consisted of eluent (A) 50 mM sodium acetate buffer pH 5/MeOH 80/20 (v/v) and (B) 50 mM sodium acetate buffer pH 5/MeOH/ACN 40/40/20 (v/v/v). The total flow rate was 0.3 ml/min with the gradient profile presented in Fig. 2 giving the percentages amount of eluent B in the total flow. The analytical column was Inertsil ODS-3 (GL Sciences Inc., Japan) 150 mm × 3 mm packed with 3 μm end-capped particles and the precolumn was Inertsil ODS-2 (GL Sciences Inc., Japan) 10 mm × 3 mm and it was packed with 5 μm C18 particles. The total run time was 87 min, which included a stabilization period of 18 min. The injection volume was 10 μl. The performance of the HPLC and detector was evaluated by determining the signal linearities, the limits of detection (LOD), resolution values, and variation of the retention times and detector responses. The resolution values were calculated with the following formula: $R = (1/4)(\alpha - 1) \times \sqrt{N} \times k/(k + 1)$ where N is plate number, α is separation factor and k is retention factor [21].

2.4. Optimization of the sample pretreatment

Sample pretreatment consisted of three crucial steps, which were hydrolysis, extraction and purification of the sample. The lignans are present as glucuronide and sulphate conjugates in urine and therefore hydrolysis is necessary [13]. *H. pomatia*, which is the digestive juice of a snail, contains both β-glucuronidase and sulphatase and it has been widely used in the hydrolysis of urine samples containing lignans [5,10,22].

H. pomatia is an effective hydrolyzing agent, but it also contains plant-derived compounds, and this has to be taken into account in any assay. In the GC–MS method for food lignans, *H. pomatia* was purified with active charcoal to reduce the background [23]. The effects of the purification of the enzyme mixture on the analysis results were verified. In order to obtain a convenient method, it is desirable that there should be as few purification steps as possible. Therefore, also a combination of pure β-glucuronidase and sulphatase was tested. In addition to the purification and type of the enzymes, the conditions for enzyme hydrolysis, i.e. the amount of active enzyme units, incubation time, temperature, buffer pH, dilution of the sample, need of ascorbic acid were tested. Ascorbic acid has been

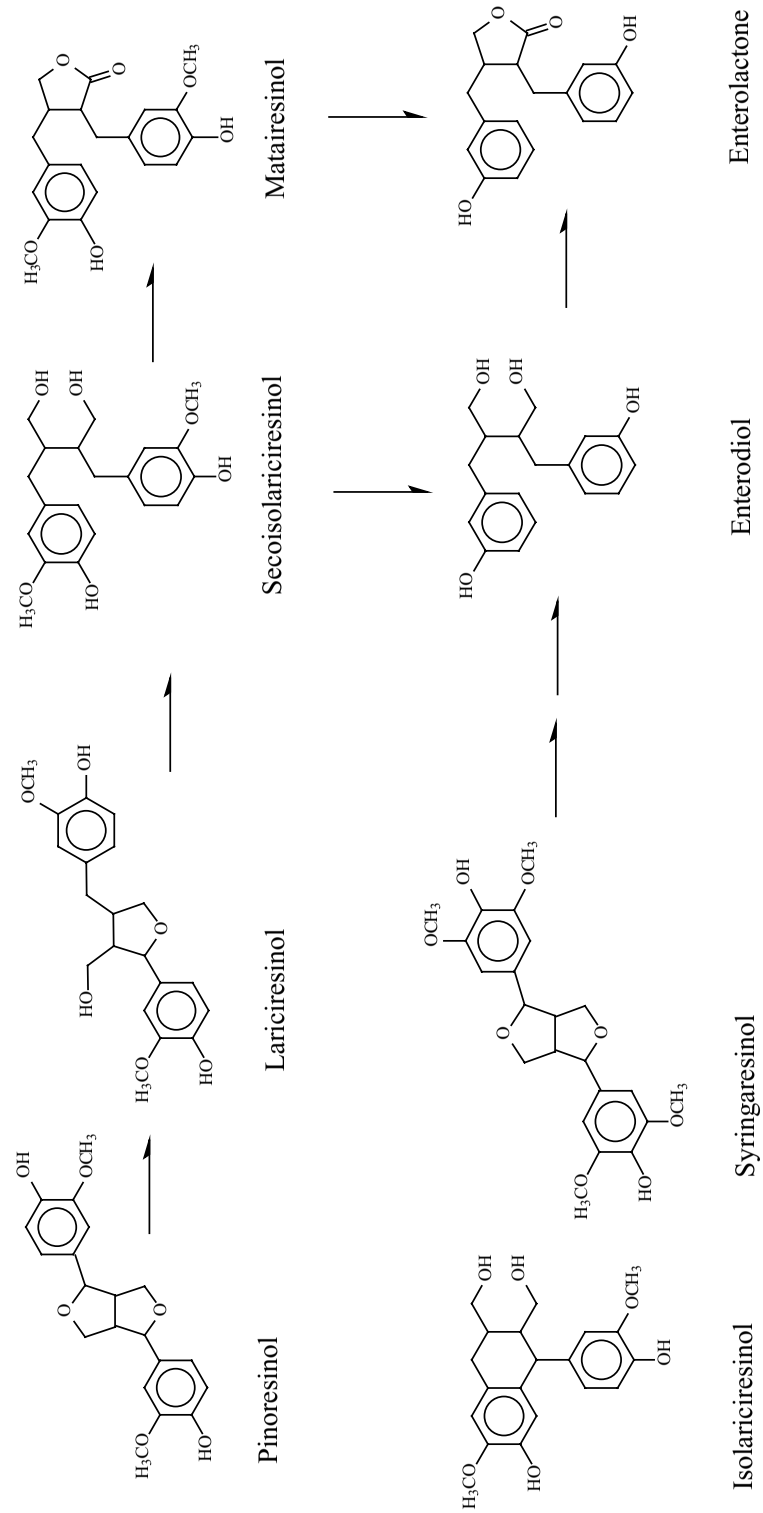

Fig. 1. Structures and metabolism of the lignans.

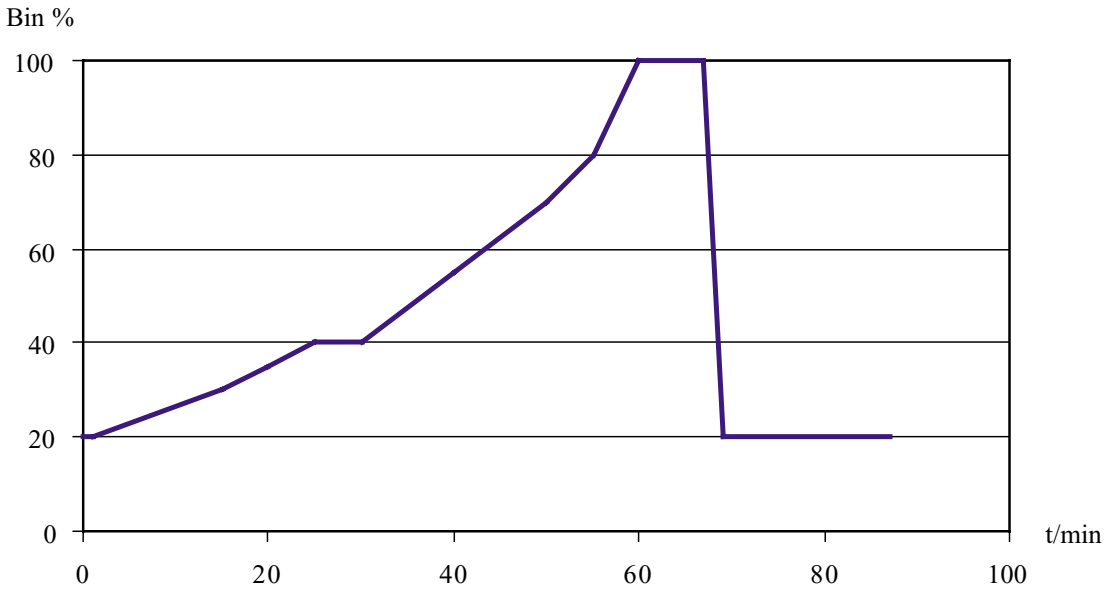

Fig. 2. Gradient profile. Total flow rate was 0.3 ml/min of which the amount of eluent B is given in percentage values.

often used to stabilize phenolic analytes during hydrolysis [10,22,24].

Diethyl ether was chosen for the extraction of the hydrolyzed conjugates, because ether has been earlier shown to extract lignans from different matrices [20,23] and furthermore, it can be readily evaporated after the extraction. The efficiency of the extraction was evaluated with the recovery of the lignan standards. The effect of lowering the pH on the extraction efficiency was also tested. Hydrochloric acid was used to decrease the pH of the samples before extraction.

In previously published methods for urinary lignans, the samples were purified with different types of ion exchange chromatography. The aim of this new method was to have only a single purification step and the chosen technique was ion exchange chromatography with QAE-Sephadex gel in its acetate form. The samples were loaded onto a 0.5 cm × 3 cm purification column and eluted with 4 ml of MeOH. More polar compounds than lignans were retained on the column. The recovery of the lignans in the purification step was evaluated with standards.

2.5. Precision and accuracy

Precision was determined with control urine samples, which contained low, moderate, high and very high amounts of mammalian lignans. The samples were separately collected from the subjects consuming their habitual diet. It was not possible to have samples with levels of plant lignans to cover a wide range of concentrations, because the levels were usually quite low. The intra-assay precision was determined by analyzing each control ten times. Inter-assay precision was determined with results obtained on different days.

The accuracy of the method was determined with two different urine matrices. The standard mixture containing all of the included plant and mammalian lignans was added into triplicate samples. The lowest added amounts of plant lignans were 60–70 nmol/l and the highest amounts were 2700–3000 nmol/l. The corresponding values of the mammalian lignans were 100–120 and 4000–5000 nmol/l, respectively. These concentration levels corresponded to a range from over 10 ng to several hundreds nanograms per sample. Samples with standard additions were analyzed applying the method and the results were calculated after subtracting the background produced by the enzyme and the urine matrix itself. Standard additions covered a linear range and correlations for the linearities were therefore determined. Furthermore variations for the triplicates at each added level were calculated.

3. Results

3.1. Chromatographic parameters

The chromatographic parameters are presented in Table 1. Variation in the retention times was negligible, with inter-assay values less than 30 s. The column can be considered as completely stabilized if the variation of the retention time is under 0.5% [21] and that criterion was fulfilled for the assays. The inter-assay variation of the retention times slightly exceeded 0.5%. The total run time was long, but that cannot be avoided when several analytes are to be measured. Also a long stabilization period after each run was necessary to guarantee the repeatability of the separation. The intra-assay variation of the detector response was negligible, but some variation occurred between the assays

Table 1
Chromatographic parameters for the mammalian and plant lignans

Analyte	RT			Detection potential (mV)	Detector response inter-assay (CV%)[c]	R[d]	R (CV%)[e]
	Minutes	Intra-assay (CV%)/s[a]	Inter-assay (CV%)/s[b]				
Isolariciresinol	20.35	0.202/2.5	1.24/15	420	2.10	20.2	3.19
Lariciresinol	31.22	0.130/2.4	1.06/20	420	2.84	1.02	2.77
Secoisolariciresinol	31.85	0.117/2.2	1.12/21	300	8.72	17.0	0.28
Syringaresinol	41.40	0.098/2.4	1.01/25	300	4.59	2.42	0.96
Pinoresinol	42.88	0.092/2.0	0.95/24	420	3.44	5.64	1.08
Enterodiol	45.13	0.077/2.1	0.97/26	640	2.35	11.0	2.19
Matairesinol	46.51	0.076/2.1	1.00/28	420	2.68	8.78	0.31
Enterolactone	51.51	0.069/2.1	0.80/25	640	3.01		

[a] $N = 12$.
[b] $N = 6$.
[c] $N = 6$.
[d] Resolution values were calculated from the formula: $R = (1/4)(\alpha - 1) \times \sqrt{N} \times k/(k + 1)$.
[e] $N = 3$.

(Table 1). Coefficients of variation ranged from 2.1 to 8.7% and therefore the system was calibrated during the each analysis assay. Adjacent peaks have baseline separation if the resolution value is 1.5 for peaks of similar size [21]. One critical peak pair in the run was lariciresinol and secoisolariciresinol. Even though the resolution value was under 1.5 there was no problem in their quantification for these two lignans, because their detection potentials differed such that the quantification signals were obtained from different channels. This can be clearly seen from the standard chromatogram presented in Fig. 3. Resolution values were calculated from three different assays and variations were negligible, which can be seen also from the very stable retention times.

3.2. Quantification parameters

The quantification parameters are presented in Table 2. Signal linearities were excellent for all of the lignans. The full range of linearities was not determined, because the studied range was sufficient for plant lignans and a wider range of linearity for mammalian lignans has earlier been presented [20]. Quantification limits (LOQ) were calculated from the limits of detections with the LOQ being determined as five times the LOD. The quantification limits ranged from 3.05 ng/ml (End) to 7.05 ng/ml(Mat). Since the enzyme mixture already contained some plant lignans, only occasional values below the quantification limits were seen. Those val-

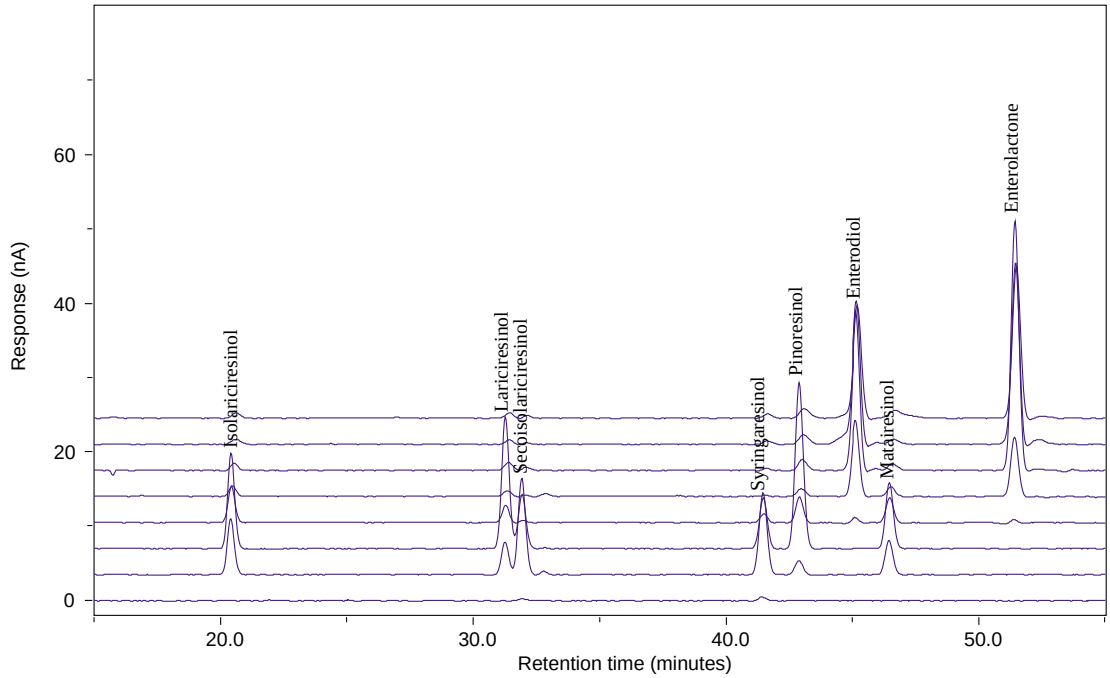

Fig. 3. Standard chromatogram.

Table 2
Quantification parameters for the mammalian and plant lignans

Analyte	LOD ((ng/ml)/(nmol/l))	Linearity[a] ((ng/ml)/(nmol/l))	*r*	LOQ[b] ((ng/ml)/(nmol/l))	Mean recovery STD[c]
Isolariciresinol	0.97/2.7	484/1344	0.9994	4.85/13.5	83.9/6.2
Lariciresinol	1.04/2.9	520/1444	1.0000	5.20/14.4	95.7/7.0
Secoisolariciresinol	1.26/3.5	504/1392	1.0000	6.30/17.4	86.8/6.5
Syringaresinol	1.14/2.7	456/1091	1.0000	5.70/13.6	81.8/8.1
Pinoresinol	0.67/1.9	536/1497	0.9998	3.37/9.41	97.7/7.1
Enterodiol	0.61/2.0	614/2033	0.9999	3.05/10.1	94.1/7.0
Matairesinol	1.41/3.9	494/1380	0.9998	7.05/19.7	102/7.5
Enterolactone	0.74/2.5	736/2470	0.9996	3.70/12.4	96.9/8.0

[a] A wider range can be achieved using solutions containing higher amounts of analytes.
[b] LOQ = 5LOD.
[c] $N = 10$, values describe the recovery of the analytes in QAE-Ac^- purification.

ues were denoted as not quantified as the values were below the LOQ after subtraction of the background.

3.3. Sample pretreatment

The final sample pretreatment method is presented in Fig. 4. This section contains a summary of the findings which were taken into account during the method development. Different amounts of active units of pure β-glucuronidase together with pure sulphatase were tested, but their hydrolysis efficiency was lower compared to *H. pomatia*. Purifying *H. pomatia* with active charcoal reduced the activity of the enzyme mixture and it was decided to use the enzyme mixture as such even though subtracting the background was then needed. The hydrolysis reagent was 0.3 M sodium acetate buffer pH 5 containing 2500 U/ml of β-glucuronidase from *H. pomatia*. Buffer pH 5 improved the hydrolysis efficiency compared to buffer pH 4. Increasing the amount of enzyme units did not improve the hydrolysis efficiency and a lower amount of enzyme did not provide sufficient hydrolysis activity. Ascorbic acid was added to the vials before the enzyme hydrolysis, because that resulted in higher measured values. Even though the recovery standards of the analysis assays were not taken through the enzyme hydrolysis, the results in general did indicate that some protection for the analytes would be advisable.

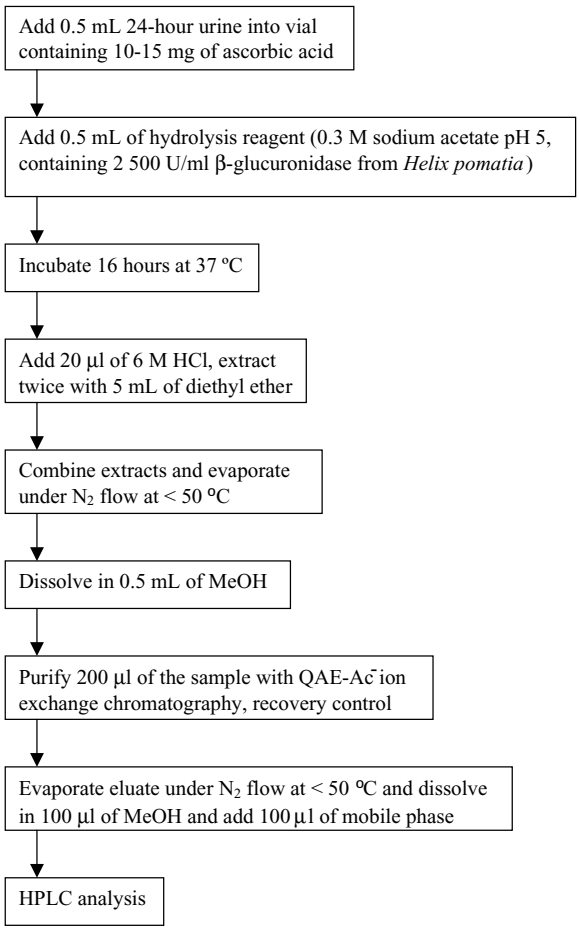

Fig. 4. Flow diagram of the sample pretreatment.

An incubation time of 16 h was chosen, because that simplifies the assay arrangement. When overnight hydrolysis was used, extractions could be performed during the next day and that enabled the handling of a larger number of the samples in one assay. If only a few samples are to be analyzed, a hydrolysis time of 2 h can be utilized without loss of hydrolysis efficiency. The hydrolysis temperature of 37 °C was chosen, because higher temperatures led to slight losses of Lar and Pin. The same temperature can be applied for the 2 and 16 h hydrolysis and the same results are achieved.

Samples were extracted twice with 5 ml of diethyl ether. The extraction efficiency was increased when a small amount of hydrochloric acid was added into cool samples prior to extraction. Since the recoveries of the extraction step were good and diethyl ether was quickly evaporated, no other extraction solvents were considered. Extracts were combined and evaporated under N_2 flow to speed up the evaporation and to protect the samples from oxygen. The samples were kept in a heated block during the evaporation and the temperature was kept under 50 °C. Higher temperatures were observed to cause loss of the plant lignans Lar and Pin due to their decomposition. Dry samples were dissolved into 0.5 ml of MeOH and 200 µl of the samples were further purified for lignan analysis and the rest of the samples were stored until the analyses were completed.

Table 3
Precision of the method presented with four different control samples

Analyte	Control 1 (low)			Control 2 (medium)			Control 3 (high)			Control 4 (extra high)		
	nmol/l[a]	CV%[b]	CV%[c]	nmol/l[e]	CV%[b]	CV%[c]	nmol/l[a]	CV%[b]	CV%[c]	nmol/l[a]	CV%[b]	CV%[c]
Isolariciresinol	55	9.9	25	46	17	–	94	21	23	–	–	–
Lariciresinol	52	5.3	14	–	–	–	120	9.4	13	–	–	–
Secoisolariciresinol	45	16	36	–	–	–	97	11	23	64	7.5	23
Syringaresinol	–[d]	–	–	19	21	–	41[e]	34	–	28[e]	29	–
Pinoresinol	–	–	–	–	–	–	14[e]	14	–	–	–	–
Enterodiol	137	4.0	12	305	5.9	–	934	9.4	12	7298	5.2	13
Matairesinol	19	16	19	–	–	–	35	11	11	–	–	–
Enterolactone	88	4.9	12	803	4.8	–	1880	9.6	12	1866	5.5	12

[a] Inter-assay mean value, $N = 18$.
[b] Intra-assay precision, $N = 10$.
[c] Inter-assay precision.
[d] Not detected.
[e] Intra-assay mean value, $N = 10$.

Samples were purified with QAE-Sephadex ion exchange gel in its acetate form and MeOH eluates were evaporated under N_2 flow. Dry samples were dissolved into 100 μl of MeOH after which 100 μl of mobile phase was added to prevent peak distortion during the HPLC separation. Samples containing very high amounts of lignans were diluted more to confirm that the results were obtained within the linear range.

Reagent blank was prepared in each assay to subtract the effects of hydrolysis reagent, which increased the measured concentrations from 3 to 20 ng/ml for different plant lignans. The amount of plant lignans in the reagent blank varied depending on the enzyme batch in use.

3.4. Recovery of the analytes

Recovery of the analytes was studied in the different steps of the sample pretreatment. Recoveries covering the enzyme hydrolysis, extraction and purification distorted the final results, obtained when the accuracy of the method was determined. The recovery in the extraction step was around 90%. Even though some loss of analytes seemed to occur in the extraction step, using recoveries for the purification step resulted in the most accurate results and that technique was used in the final method. Mean recoveries ranged from 82% (Syr) to 102% (Mat) and standard deviation for the recovery values ranged 6.2 (IsoL) to 8.1 (Syr). Individual values for

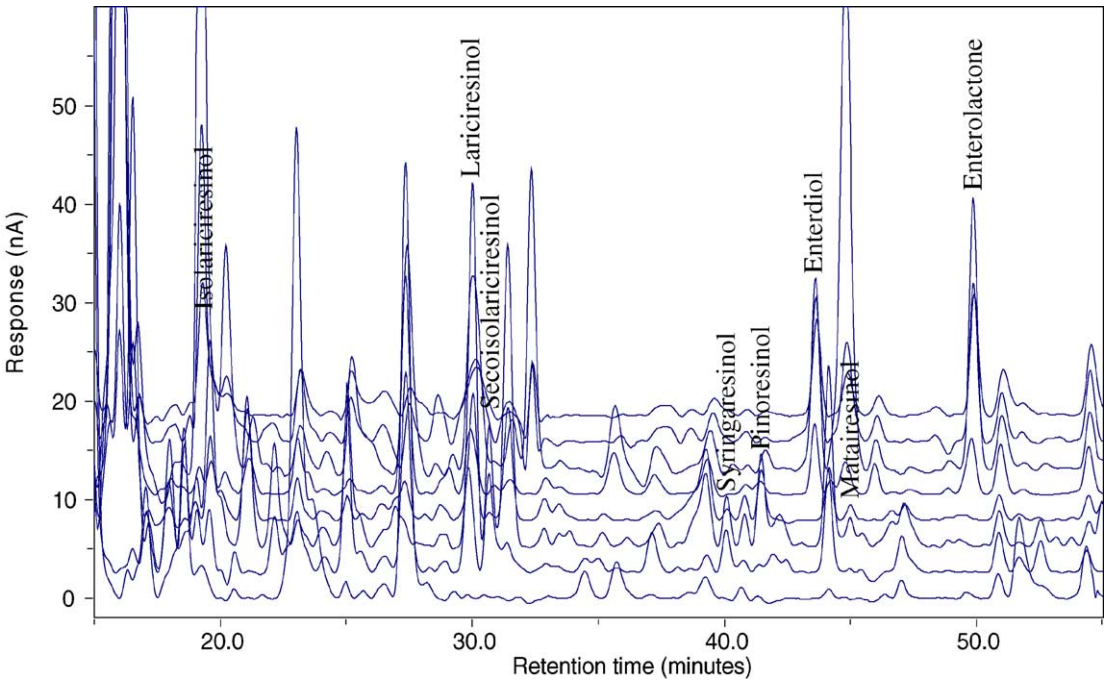

Fig. 5. Lignan profile of human urine.

each analyte are presented in Table 2. Recovery values were determined in duplicate for every batch of 20 samples. The average of the duplicates was used for calculating the final results.

3.5. Precision of the method

The precision results for the control samples are presented in Table 3. The concentrations of the analytes in the control samples are given as well as intra- and inter-assay CVs. The assay range was from 137 to 7298 nmol/l and from 88 to 1866 nmol/l for enterodiol and enterolactone, respectively. The intra-assay variations of mammalian lignans were very similar throughout the range, the lowest value being 4.0% and the highest 9.6%. Between assay variation was slightly higher and CV (%) values ranged from 12 to 13%. The concentrations of the plant lignans were very low in all controls. It was also possible to measure reliably values corresponding to or close to LOQ (Mat in control 1 and Pin in control 3). Controls 1, 3 and 4 have been used to control the analysis assays and control 2 was analyzed only in one assay. Three control samples were analyzed with every batch of 20 samples.

3.6. Accuracy of the method

The results for the accuracy determination are presented in Table 4. The accuracy of the method was excellent throughout the studied range and recoveries of the added compounds were close to 100% for each step except at the lowest added amount. The recovery of lariciresinol was slightly lower than that of the other compounds. The results of the accuracy determination were calculated using the recovery of the pure compounds in the purification step and subtracting the background. Coefficients of variations were low and linearity was complete throughout the studied range. The results for Pin represent only one matrix, because the other matrix contained some compound which disturbed the analysis. LOQ values for all lignans were lower than the lowest point of the added standard. The range from the lowest added amount to LOQ values can be included into the method, but the precision may vary as can be seen from the results of the control samples presented in Table 3, this being especially the case for the plant lignans. The mammalian lignans showed acceptable accuracy and recovery also with the lowest added amount of standards. The lowest added amount of mammalian lignans was similar to the amounts quantified in control 1 (Table 3).

Table 4
Accuracy of the method

Analyte							r^a
Isolariciresinol							
ng[b]	12.1	24.2	48.4	121	242	484	0.9999
%[c]	99.8	101	98.5	99.7	102	100	
CV%[d]	23.4	16.0	14.9	5.31	8.51	6.07	
Lariciresinol							
ng[b]	13.0	26.0	52.0	130	260	520	0.9991
%[c]	56.5	72.3	73.7	75.2	73.4	80.5	
CV%[d]	11.9	8.38	10.9	6.79	6.90	4.22	
Secoisolariciresinol							
ng[b]	12.6	25.2	50.4	126	252	504	0.9998
%[c]	66.9	88.0	89.4	91.3	93.9	97.8	
CV%[d]	37.3	11.9	7.21	2.25	4.73	4.52	
Syringaresinol							
ng[b]	14.1	28.2	56.4	141	282	564	0.9999
%[c]	69.8	98.4	104	108	108	108	
CV%[d]	42.4	10.5	9.57	3.18	5.07	4.79	
Pinoresinol[e]							
ng[b]	13.4	26.8	53.6	134	268	536	0.9998
%[c]	80.4	96.9	98.6	100	105	106	
CV%[d]	19.5	5.76	6.13	2.33	4.98	3.84	
Enterodiol							
ng[b]	15.4	30.7	61.4	123	307	614	0.9998
%[c]	101	99.8	98.9	100	100	102	
CV%[d]	13.6	13.2	7.52	5.66	7.33	5.78	
Matairesinol							
ng[b]	12.4	24.7	49.4	124	247	494	0.9999
%[c]	63.7	89.6	92.8	99.7	103	108	
CV%[d]	23.7	13.6	8.42	4.31	4.95	4.88	
Enterolactone							
ng[b]	18.4	36.8	73.6	184	368	736	0.9998
%[c]	95.9	105	107	108	109	109	
CV%[d]	13.8	14.4	7.32	4.63	6.44	5.79	

[a] Correlation between added and measured amount.
[b] Added amount nanogram per sample.
[c] Percentage recovery of the analyte in two different matrices; $N = 6$.
[d] CVs for triplicate additions in two matrices.
[e] Only one assay was taken into account. Unknown peak disturbed quantification of pinoresinol in the other matrix.

3.7. Mammalian and plant lignans in human urine

A chromatogram of the urinary lignans is presented in Fig. 5. A great many of compounds eluted at the beginning of the chromatogram, but the retention time of the first lignan was approximately 20 min and the major part of the impurities had eluated before that time point. The presented chromatogram of human urine sample is not a typical profile of the urinary lignans, but this sample was chosen as it is possible to see all of the plant lignans. Furthermore the concentration of the mammalian lignans was quite low in relation to the plant lignans and the amount of enterodiol was quite high in relation to enterolactone. The excretion of the urinary mammalian lignans from 10 subjects consuming their habitual omnivorous diet is presented in Fig. 6 to show the variation in the excretion of the mammalian lignans. The excretion of the plant lignans for the same subjects is presented in Fig. 7. The excretion of the mammalian lignans was the sum of enterolactone and enterodiol. All of the subjects, except number 1, excreted both compounds with dif-

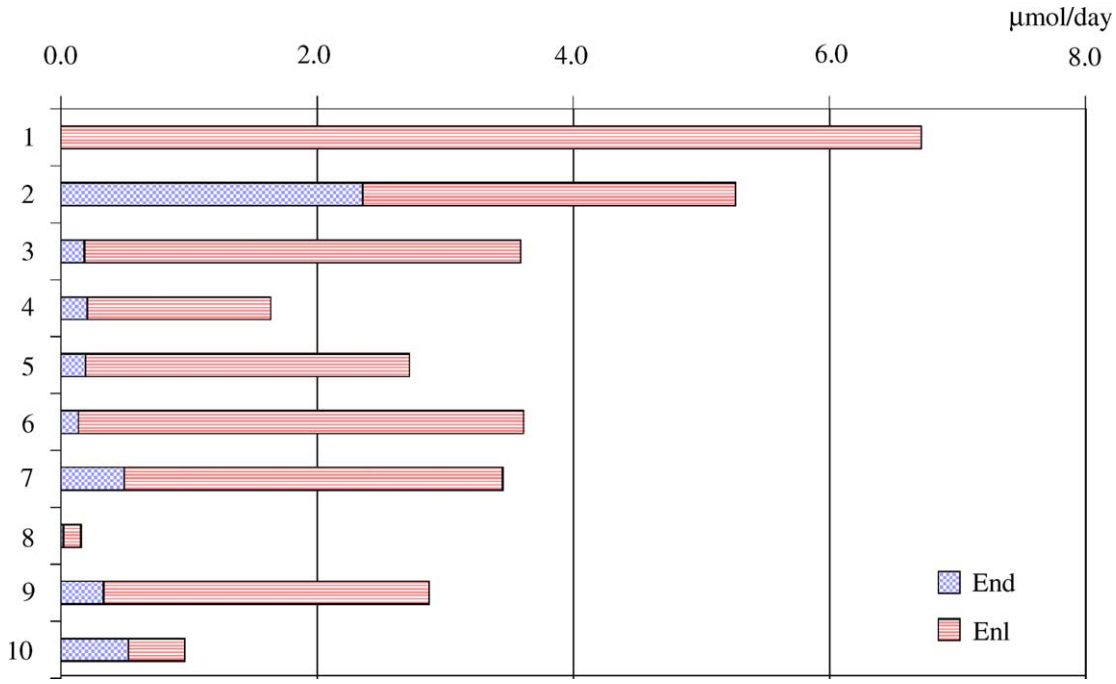

Fig. 6. Urinary excretion of two mammalian lignans in 10 subjects consuming their habitual diet.

ferent ratios. The subject, excreting only enterolactone, had the highest daily excretion of both mammalian and plant lignans. Subjects 5–7 excreted very low amounts of plant lignans, but their mammalian lignan excretion was normal. Subjects 8 and 10 excreted plant lignans even though their mammalian lignan excretion was low. All subjects except subject 9 excreted isolariciresinol, which is not at all metabolized to the mammalian lignans [18].

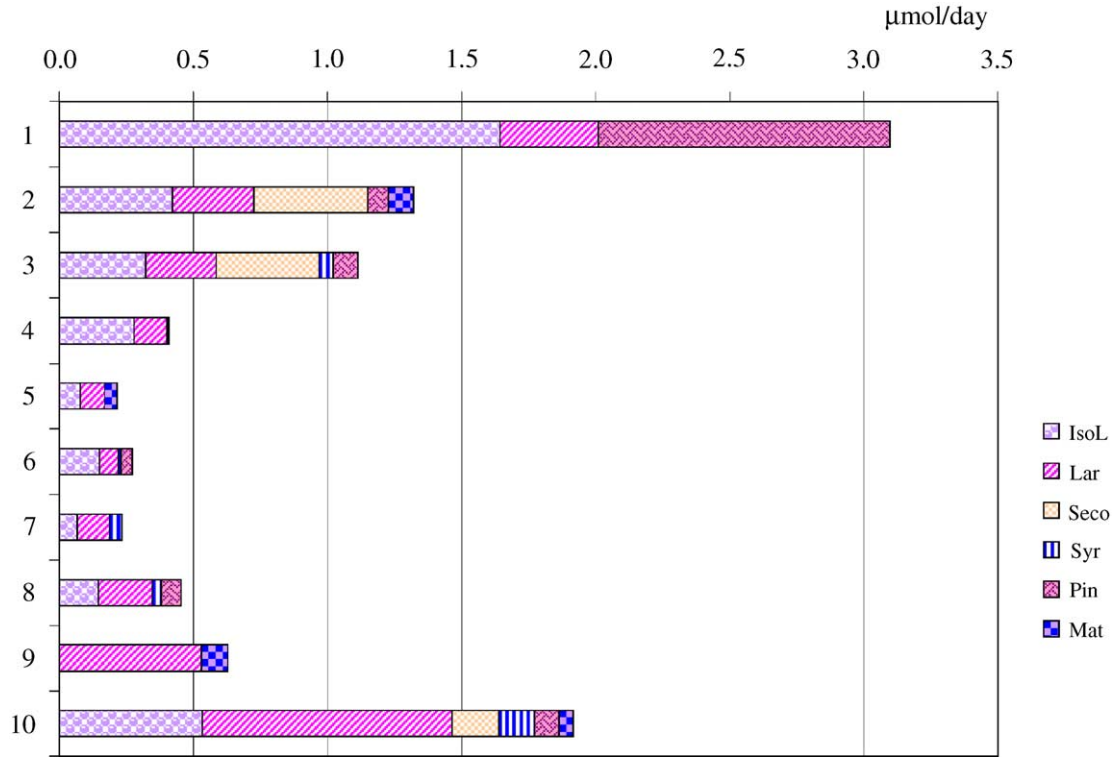

Fig. 7. Urinary excretion of six plant lignans in 10 subjects consuming their habitual diet. Subjects are presented in the same order in Figs. 6 and 7.

4. Discussion

A new HPLC-CEAD method for urinary mammalian and plant lignans was developed. This method for the first time makes it possible to quantify the new mammalian lignan precursors pinoresinol, lariciresinol and syringaresinol as well as the plant lignan, isolariciresinol in human urine. The coulometric electrode array detector has been often used to measure phenolic compounds in biological samples [20,25–28], but only a few applications have been developed for human urine [29,30]. Chromatographic and quantification parameters showed that the method development was successful and the performance of the method was reliable. The chromatographic conditions were repeatable and the method was very sensitive and linear. The wide variety of control sample analyses proved that the method was precise and standard additions to urine samples showed satisfactory accuracy. The only disadvantage was the long run time. Very often, when several compounds have to analyze, it is not possible to keep the run time short and this has simply to be accepted.

The examples shown in Figs. 6 and 7 reveal the wide variations in urinary excretion of the lignans. That has been known for a long time [10,14], but now it is possible to determine if the lignan intake has been low, or if the low excretion of the mammalian lignans is due to an inactive micro flora in the gut.

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