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Determination of plant and enterolignans in human serum by high-performance liquid chromatography with tandem mass spectrometric detection

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Abstract

An HPLC-MS/MS method was validated for the determination of the plant lignans 7-hydroxymatairesinol (HMR), matairesinol (Mat), secoisolariciresinol (Seco), lariciresinol (Lar), and cyclolariciresinol (CLar) and for the enterolignans 7-hydroxyenterolactone (HEL), enterodiol (ED), and enterolactone (EL) in human serum. The method included sample enzymatic hydrolysis, solid-phase extraction, and lignan analysis using a triple quadrupole mass spectrometer with electrospray ionisation in the multiple-reaction monitoring mode. The serum lignans were quantified using deuterated Mat or EL as internal standards. The method met the validation criteria for selectivity, intra- and inter-assay precision, and accuracy. The method was applied to ten serum samples collected from healthy individuals (five men and five women) consuming their habitual Finnish diet. All lignans except HMR and Seco were found in quantifiable amounts in the samples. All serums contained EL; the average concentration was 34 nM. In three individuals, the serum concentration of plant lignans was higher than that of enterolignans. Using the method, common dietary plant lignans and their major metabolites can be reliably quantified in human serum at low-nanomolar concentrations in a simple and rapid way.
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1. Introduction

Lignans, a group of phenolic compounds that are dimers of phenylpropanes linked by β–β-bonds, are commonly found in plants as their secondary metabolites. Of edible plants, flaxseed and sesame seed are the richest sources of lignans, flaxseed containing mainly secoisolariciresinol (Seco) diglucoside, but also matairesinol (Mat) [1,2], lariciresinol (Lar) and its derivative CLar, and pinoresinol (Pin) in considerable amounts [2,4]. Sesame seed contains mainly sesamin and its derivatives, but also other lignans e.g. Pin, Lar, Seco, and Mat [2,3], 7-hydroxymatairesinol (HMR), and syringaresinol [3]. Also whole-grain bread contains relatively large amounts of Pin, Lar, and Seco and *Brassica* vegetables Pin and Lar [2].

Several plant lignans are known to be converted to so-called enterolignans (mammalian lignans), e.g. enterodiol (ED) and enterolactone (EL) by mammalian intestinal microbiota shown in numerous studies both in vivo [4–9] and in vitro [10–12]. The metabolism of plant lignans to enterolignans may have importance in disease risk modulation in humans. In epidemiological studies, high serum and urine EL has been associated with a reduced risk of breast cancer [13–15] and cardiovascular diseases [16,17]. Furthermore, some of the dietary lignans or their mammalian metabolites have been demonstrated to have anticancer properties in vivo [7–9,18–22]. Seco diglucoside and HMR as well as their metabolite EL have been demonstrated to inhibit dimethylbenz-[a]-anthracene induced mammary cancer growth in rats [7–9,18] and MCF-7 human

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breast cancer xenografts in mice [19]. Furthermore, dietary HMR has been shown to reduce the incidence of *N*-ethyl-*N*′-nitro-*N*-nitrosoguanide induced uterine adenocarcinoma [20], the number of intestinal adenomas in (Apc)^{Min} mice [21], and the growth of LNCaP human prostate cancer xenografts in athymic nude male mice [22].

Traditionally, in determining lignans in human serum, the interest has mainly focused on the analysis of enterolignans, e.g. EL and ED and their two dietary lignan precursors, Seco and Mat [23–28]. Recently, several other plant lignans, e.g. HMR, 7-oxo-Mat, α-conidendrin, Lar, CLar, Pin, and syringaresinol as well as the enterolignan 7-hydroxy-EL (HEL) have been identified in serum samples from humans consuming their habitual diet [29,30]. The methods developed for quantification of these lignans in human serum include isotope dilution GC–MS [23,24] and more recently, HPLC methods such as HPLC with coulometric electrode array detection (CEAD) [25,26,30], HPLC-photodioide array-MS [27], and HPLC-MS/MS [28,29]. In addition to these chromatographic methods, time-resolved fluoroimmunoassay (TR-FIA) has been applied extensively for analysis of EL in human serum and plasma [31–34]. Of these methods, GC–MS with SIM (selected ion monitoring), HPLC-CEAD, and HPLC-MS/MS allow determination of several compounds at a time with high sensitivity and selectivity. However, the GC–MS-SIM technique requires time-consuming multi-step sample pre-treatment, while much simpler pre-treatment is sufficient for lignan analysis with the HPLC techniques, HPLC-MS/MS requiring only a single extraction step.

We have previously described a validated method for quantification of lignans (HMR, Mat, 7-oxo-Mat, α-conidendrin, HEL, ED, and EL) in human plasma using HPLC-MS/MS with electrospray ionisation (ESI) in the multiple-reaction monitoring (MRM) mode [29]. However, this method did not include other plant lignans of dietary origin reported to be present in urine of humans consuming their habitual diet such as Seco, Lar, and CLar [1–3,35,36]. Therefore, in the present work, a validated HPLC-ESI-MS/MS method was developed for the dietary lignans HMR, Mat, Seco, Lar, and CLar and their major mammalian metabolites ED, EL, and HEL (Fig. 1). The method was applied to quantify these lignans in ten human serum samples collected from five men and five women consuming their habitual diet in order to determine the individual serum plant and enterolignan profiles.

2. Experimental

2.1. Reference compounds and chemicals

(−)-7-Hydroxymatairesinol (HMR) was isolated as a mixture of two stereoisomers from knots of Norway spruce (*Picea abies*) according to a modification of a previously described method [37]. The two diastereomers differing in the stereochemistry at C-7 were called HMR1 [(−)-*allo*-HMR] (minor isomer) and HMR2 [(−)-HMR] (major isomer) with a HMR1/HMR2 ratio of about 30/70. (+)-Lar and (−)-Seco were isolated from knots of *Pinus cembra* and *Araucaria angustifolia*, respectively, as described previously [4,38]. (+)-CLar was prepared by treating (+)-Lar with concentrated formic acid. Lar for preparation of CLar was isolated from knots of *Abies balsamea* through extraction, after which it was purified by flash chromatography and recrystallisation. (−)-Mat was prepared from (−)-HMR by modifications of a method described previously [37]. Racemic EL was purchased from VTT, Technical Research Centre of Finland (Espoo, Finland). HEL, a mixture of two isomers differing in stereochemistry at C-7, was prepared by modification of a previously described method [39]. ED was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). The internal standards Mat-d_6 and EL-d_6 were prepared according to previously described methods [40,41]. They were both isotopically pure as determined by ^{1}H NMR. All compounds were purified on silica gel and vacuum-dried. HEL, Mat-d_6, and EL-d_6 were further purified using preparative TLC. The purity of the reference compounds was determined by GC–FID by using HP 5890 Series II GC, GC–MS (HP 5890 GC coupled to an HP 5971A series MSD, Hewlett Packard, Palo Alto, CA, USA) or ^{1}H NMR (AV600 MHz NMR spectrometer, Bruker, Karlsruhe, Germany). The purities determined by GC–FID or GC–MS by dividing the peak area of the lignan with the total peak area, or by ^{1}H NMR by comparing integrated signals of EL with signals of impurities (ethanol, water) were the following: HMR and Lar 97% (GC–FID), Mat and Seco 98% (GC–FID), CLar 95% (GC–FID), HEL 92% (GC–MS), EL 96% (^{1}H NMR), Mat-d_6 100% (GC–MS), and EL-d_6 93% (GC–MS).

For the enzymatic hydrolyses, β-glucuronidase type H-1, from *Helix pomatia* was obtained from Sigma–Aldrich Co. All the solvents used were of p.a. or HPLC grade.

2.2. HPLC-MS/MS equipment

The HPLC-MS/MS analyses were conducted using an Agilent 1100 series HPLC (Agilent Technologies Inc., Palo Alto, CA, USA) system and a Quattro Micro mass spectrometer equipped with an ESI source (Micromass Ltd., Manchester, UK) according to a previously described method [4]. The HPLC column used was Agilent C18 Hypersil BDS 2.0 mm × 125 mm with a 3.0 μm particle size (Agilent Technologies Inc.). The HPLC conditions were modified by using 0.1% HAc–IPA 99:1 (v/v) as eluent A and MeOH–0.1% HAc–IPA 90:10:0.01 (v/v) as eluent B and a 16 min gradient from 22 to 73% of B and then to 95% B in 1 min. The final composition was held for 1 min followed by an equilibration time of 7 min, rendering a total analysis time of 25 min. The water used in the eluent was purified as described previously [29]. The analysis data was collected and analysed using MassLynx™ V3.5 software and the statistics calculated using QuanLynx™ V3.5 software (Micromass Ltd., Manchester, UK). Negative ions were acquired in the MRM mode and individual parameters were optimised for each lignan by syringe infusion of pure compounds.

2.3. Calibration standards and QC samples

Each reference compound was dissolved in MeOH to produce stock standard solutions. These solutions were diluted 1:9

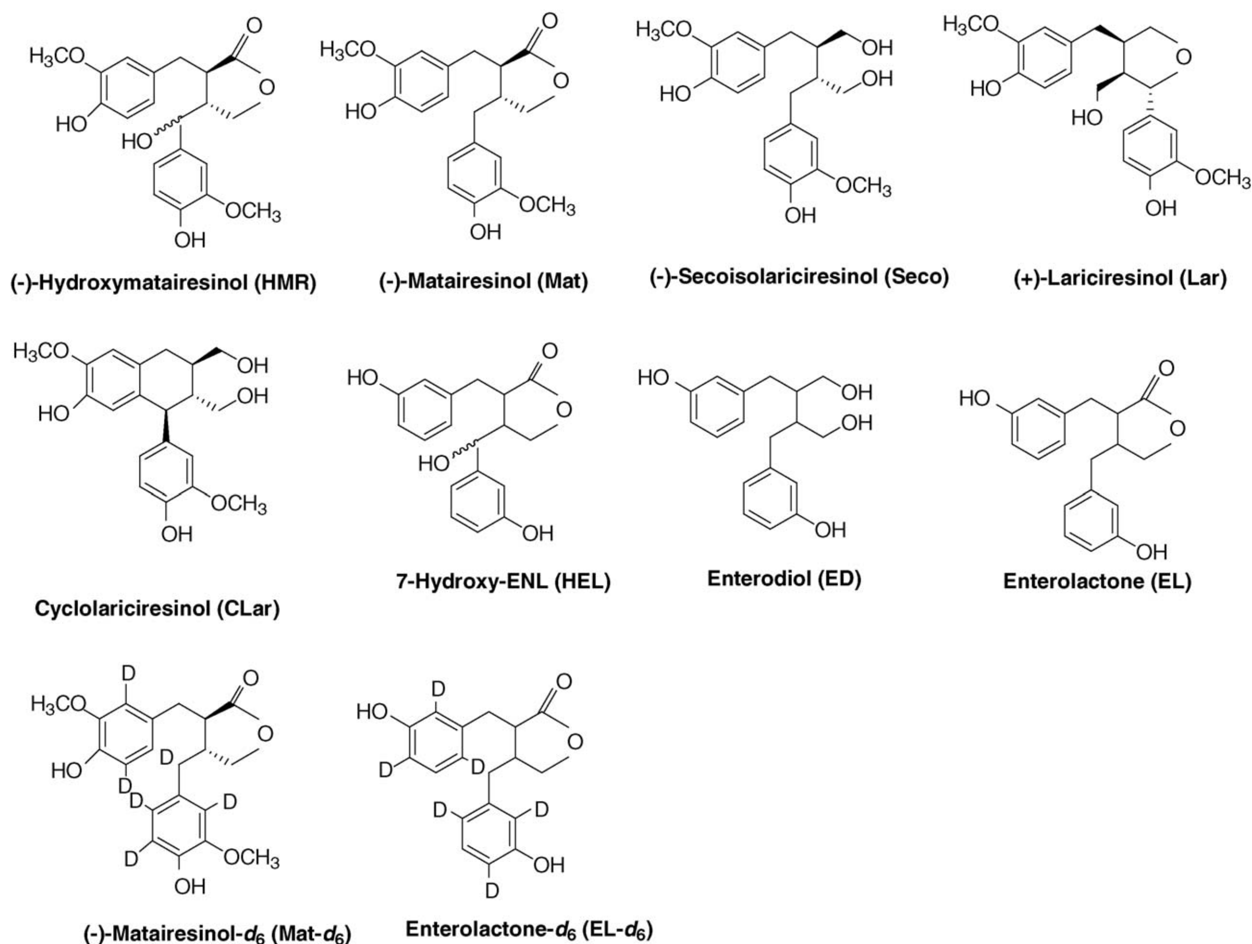

Fig. 1. Chemical structures of the analysed lignans.

(v/v) with 50% MeOH in 50 mM sodium acetate buffer (pH 4.0) to produce the most concentrated standard solution. A dilution series was prepared with 50% MeOH in acetate buffer to produce five standard solutions of descending concentration. Each of the resulting six standard solutions was further diluted with blank serum 1:99 (v/v) (see Section 2.4) to obtain calibration standards. The calibration ranges of the analytes were the following (nM): HMR 5.34–2136, Mat 6.98–558.0, Seco 1.38–552.0, Lar 8.31–664.8, CLar 1.38–554.0, HEL 5.57–445.2, ED 1.99–794.4, and EL 3.35–268.0.

The quality control (QC) samples were prepared at three concentration levels (Table 1). The high-concentration QC sample (HQC) was a 1:0.25 (v/v) dilution and the middle-concentration QC (MQC) a 1:3 (v/v) dilution of the most concentrated calibration standard. Low-concentration QCs (LQCs) corresponded to three times the concentration of the most diluted calibration standard. All solutions were stored at −20 °C.

2.4. Serum samples

For blank serum and zero samples (blank serum with internal standards added), calibration standards, and QC samples, serum from four male smokers consuming a lignan-poor diet was pooled. The serum EL concentration is likely to be lower in men than in women and in the serum of smokers than of non-smokers [32].

The internal control samples were collected from four female volunteers who had supplemented their diet with flaxseed for 3 days. This pooled serum was used for determination of intra- and inter-assay variation of the lignan concentration in hydrolysed samples and for optimisation of the duration of the enzymatic hydrolysis.

The study samples were collected from five male and five female Finnish healthy volunteers, aged 22–58 years consuming their habitual diet. All the volunteers were omnivorous except for one woman consuming a vegetarian diet. The samples were stored at −20 °C.

2.5. Sample pre-treatment

Into four aliquots of 0.6 ml of internal control serum samples, 2.0 mg of β-glucuronidase/sulphatase freshly dissolved in 1.0 ml of 50 mM sodium acetate buffer (pH 4.0) was added. The samples were incubated at 37 °C in a shaker for 1, 3, 19, and 24 h. The samples were solid-phase extracted as described below and analysed with HPLC-MS/MS. The optimal hydrolysis time of 19 h was determined in two parallel sets of samples as the time at which ED and EL reached their maximal concentration.

Table 1
Intra- and inter-assay variation of the lignans at three concentration levels, R.S.D.% of six parallel samples except if otherwise noted

Compounds	LQC			MQC			HQC		
	Concentration (nM)	Intra-assay	Inter-assay	Concentration (nM)	Intra-assay	Inter-assay	Concentration (nM)	Intra-assay	Inter-assay
HMR	16.0	10.3	13.8	534.0	6.41	9.03[a]	1709	6.79	9.74
Mat	4.17	12.9	7.63	139.5	10.2	13.1	446.4	4.84	15.0
Seco	4.14	8.21[a]	13.8	138.0	3.70	9.07	441.6	5.72	13.3
Lar	4.98	11.0	4.95	166.2	6.91[a]	9.23	531.8	5.48	10.7
CLar	4.14	14.7	8.57	138.5	1.82	11.4	443.2	10.8	12.3
HEL	3.33	10.2	13.5	111.3	9.08	12.0	356.2	5.43	6.75
ED	5.97	12.6	13.8	198.6	1.78	4.64	635.5	2.70	8.71
EL	2.01	10.3	9.01	67.0	13.0	7.67	214.4	5.35	14.6

[a] Five parallel samples.

Study samples (á 0.6 ml) and the internal control samples (for determination of intra- and inter-assay variation of lignan concentration in hydrolysed samples) were enzymatically hydrolysed for 19 h. The internal standards in MeOH (corresponding to 1.37 nmol of Mat-d_6 and 1.64 nmol of EL-d_6) were added and the samples solid-phase extracted.

The Oasis HLB 30 mg extraction cartridges (Waters Corp., Milford, MA, USA) were activated with 1.0 ml of MeOH and 1.0 ml of acetate buffer. The serum samples were subjected to the cartridge, washed with 1.0 ml of acetate buffer, and eluted with 1.0 ml of MeOH followed by 1 ml of acetone. The samples were evaporated to dryness with nitrogen at 40 °C in a water bath, redissolved in 300 μl of MeOH/0.1% HAc (20:80, v/v), and stored at −20 °C until analysis. The resulting concentrations of Mat-d_6 and EL-d_6 in the samples were approximately 4600 and 5500 nmol/l, respectively. Thirty microliter of this solution was injected into the HPLC-MS/MS. The calibration standards, QC samples, blank, and zero serum samples were not hydrolysed. To 0.6 ml aliquots of these, 1.0 ml of acetate buffer and internal standards were added as described above and the samples were solid-phase extracted and reconstituted. Internal standards were not added to the blank samples.

2.6. Quantification and quality control

HMR and HEL were quantified as a sum of their two diastereomers. ED and EL were quantified using EL-d_6 as an internal standard and the other lignans using Mat-d_6. For each study sample set, one blank serum, two zero serum samples, two QC samples at each concentration level, and one internal control sample were prepared and analysed. The accuracy was evaluated from the QC samples. The lignan concentrations in the zero samples were subtracted from the concentrations in study samples.

During the enzymatic hydrolysis of the samples, Lar is partly (13%) converted to CLar [4]. Thus the obtained concentrations in study samples were corrected for this conversion.

The statistical differences in serum EL concentrations in study samples collected from men and women were performed using Statistica software for Windows (Stat Soft, Tulsa, OK, USA). The normally distributed data were analysed with one-way analysis of variance (ANOVA) followed by Tukey's least significant difference test. The acceptable level of significance was set at $p < 0.05$.

2.7. Method validation

The method was validated basically according to guidelines of the U.S. Food and Drug Administration (FDA) [42] with slight modifications which are presented below.

2.7.1. Calibration curves and relative extraction recovery

Calibration curves were generated for each analyte by analysing the calibration standards (Section 2.3). The standard curves consisted generally of six standard points, however, for HEL, Mat, and EL, they consisted of five points. The sets of standards for the calibration curve were prepared and analysed in triplicate and each set of standards was analysed twice. 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 and the origin excluded.

For determination of relative extraction recovery, the analytes were spiked in MeOH/acetate buffer solution (20:80, v/v) without blank serum to produce reference standards. The calibration curves for these reference standards were prepared similar to those of the calibration standards. The relative extraction recovery was determined by dividing the slope, i.e. mean k-value ($n = 6$) of the calibration curve of an analyte in the presence and absence of serum.

2.7.2. Precision and accuracy

The inter-assay precision was evaluated using data collected from six HQC, MQC, and LQC samples from three different sample sets extracted on different days. The acceptable intra- and inter-assay variation was an R.S.D. of ≤15%. The intra-assay precision of hydrolysed samples was determined by analysing five parallel internal control samples and the inter-assay precision by analysing one internal control sample each from five different sample sets. The accuracy of the method was evaluated from the same samples. The acceptance criterion for accuracy was that the determined concentration of at least four of the six samples at each concentration level was within ±20% of their theoretical concentration.

2.7.3. Ion suppression

The possible ion suppression effect of the serum matrix on the detector response of the lignans was determined by analysing standard solutions at three concentration levels both dissolved in mobile phase (MeOH–0.1% HAc 20:80, v/v) and spiked into solid-phase extracted (unhydrolysed) blank serum.

3. Results and discussion

3.1. Method validation

In blank serum, traces of EL, Lar, and HEL were detected. The accepted level of analytes in the blank was set at one fourth of that in the LLOQ samples, which is higher than the FDA recommended level of one fifth of the LLOQ concentrations [42]. However, this was considered acceptable, as human serum containing no lignans is practically non-existent. Lignans are natural compounds common in human diet and thus inherently present in almost any human serum sample.

The R.S.D. of the calibration curve slopes in the three replicate samples analysed twice ($n = 6$) varied from 3.5% (Mat and HEL) to 9.4% (ED) in spiked mobile phase and from 6.0% (CLar) to 16.6% (Lar) in spiked and extracted blank serum. The relative extraction recoveries varied from 80% (Mat) to 110% (ED).

The intra-assay R.S.D.s of six LLOQ samples varied from 8.9% (EL) to 18.6% (Seco), and the accuracy of the mean concentration from 81.6% (Seco) to 111.8% (HEL) of the theoretical concentration. The extracted ion MRM chromatograms of the lignans in an LLOQ sample are shown in Fig. 2.

In Table 1, intra- and inter-assay R.S.D.s of six QC samples at three concentration levels are shown. All the compounds had acceptable analysis repeatability (R.S.D. < 15%) at all the concentrations. The accuracy of the determined concentrations was acceptable at all three concentration levels both within- and between-run.

Only ED and EL were present in the internal control samples in amounts sufficient for evaluating the intra- and inter-assay variation. The intra-assay variation of five parallel samples was 10.8% and 4.8% and the inter-assay variation 18.2% and 18.7% for ED and EL, respectively.

The detection limits and linear ranges of the compounds in mobile phase composed of MeOH/0.1% HAc (20/80, v/v) have been reported previously [4]. These detection limits were expected to correspond to the limits in extracted and spiked human blank serum as no significant ion suppression effect of the matrix was detected. The detector response in the presence of matrix compared to the response in mobile phase varied from 87.3% (CLar) to 100.0% (HEL and EL).

3.2. Lignans in study samples

The acceptance criterion for study sample sets was fulfilled, i.e. the determined lignan concentrations of at least one of the two QC samples at the three concentration levels were within ±20% of the theoretical concentrations.

The lignan concentrations in the human serum samples are shown in Table 2 and an example chromatogram of one study sample (woman no. 3) is presented in Fig. 3. EL was detected in all samples. Also ED was detectable in serum both in men and women but in quantifiable concentrations only in two out of five serum samples in women. As EL is formed in the intestine from dietary lignan precursors, such as Mat, Seco [1,2,43], Lar, Pin [2,11], syringaresinol [11], sesamin, and HMR [3], the presence of EL in all measured serum samples of volunteers having their habitual diet suggests the ubiquitous presence of its precursors in the food. EL may be metabolised further to HEL as observed in rat liver microsomes [44] and in male rats administered with EL (unpublished results). Furthermore, HEL may be formed from HMR as indicated by the increased HEL excretion in HMR administered rats [4,7].

The inter-individual variation in EL concentrations was large both in men and in women (CV 97.5% and 77.5%, respectively), which concurs with previous observations in humans [32,45,46]. In this study, the average serum EL concentration in Finnish men, 31.5 nM, was higher than published previously in larger studies (17.3 nM, $n = 1168$ [32] and 16.9 nM, $n = 214$ [33]). Accordingly, the average serum EL concentration in Finnish women obtained in the present study, 36.3 nM, was also higher than reported in larger studies, i.e. 20.5 nM ($n = 1212$) [32] and 24.0 nM ($n = 215$) [34]. However, no significant difference ($p = 0.8$) in average serum EL concentrations between men and women was measured. The differences between EL concentrations in the present and previously published studies may be explained by a small set of serum samples in this study, which is not likely to reflect the serum EL levels in Finnish population in general.

Most of the studies done so far have focused on the analysis of only two plant lignans, i.e. Seco and Mat in human serum. These lignans are known to be present in several dietary sources [1,2,43], and in the present study, Mat was detected in four serum samples out of ten. However, Seco could not be detected in any of the analysed samples, which was somewhat surprising. It is

Table 2
Lignan concentrations (nM) in the serum of five men and five women consuming their habitual Finnish diet

	HMR	Seco	Mat	Lar	CLar	HEL	ED	EL
Women								
1	nd	nd	nd	nd	10.1	nd	nq	55.0
2	nd	nd	nq	87.5	4.16	9.00	6.06	22.5
3	nd	nd	16.2	190	nd	9.95	2.61	76.4
4	nd	nd	nd	48.9	7.23	nq	nd	16.8
5	nd	nd	nd	nq	nd	10.4	nq	110
Men								
1	nd	nd	24.2	nd	nd	6.87	nd	17.2
2	nd	nd	nq	nd	nd	nq	nd	85.8
3	nd	nd	nd	nd	nd	6.87	nd	22.5
4	nd	nd	nq	nd	nd	nd	nq	22.3
5	nd	nd	nd	nd	nd	8.71	nq	9.95

nd, not detected; nq, not quantified, i.e. below LLOQ. Detection limits (nM): Lar 1.21, HMR 3.37, Seco 0.50, Mat 1.10, HEL 0.067, and ED 0.22 (calculated from [4]). LLOQ (nM): Mat 6.98, HEL 5.57, ED 1.99, Lar 8.31.

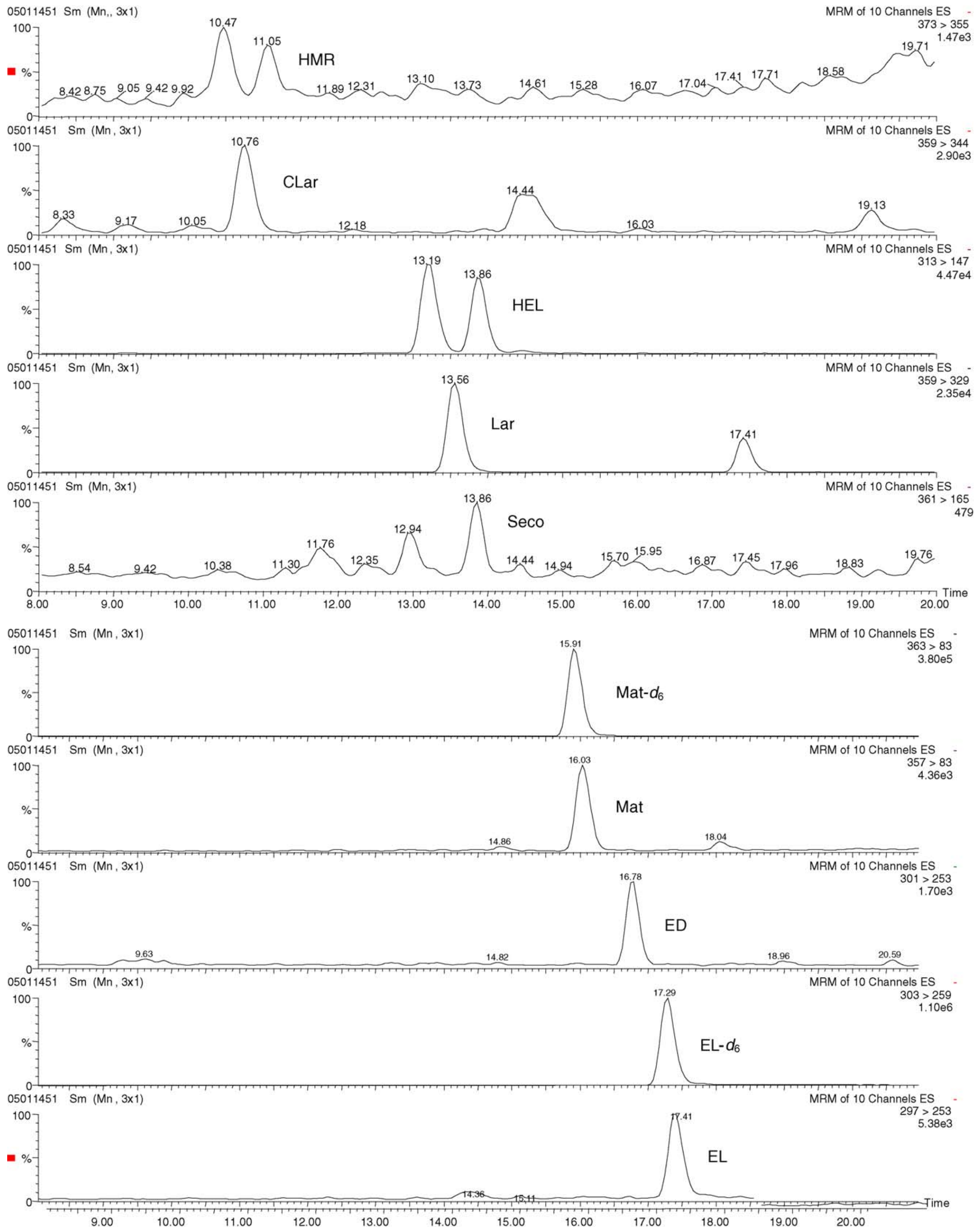

Fig. 2. HPLC-MS/MS-MRM chromatograms of lignans in an LLOQ sample. From above to below the chromatograms represent the following [compound, monitored ions in MS1 and MS2 (*m/z*), and retention time (min)]: HMR, 373 → 355, 10.47 and 11.05 (HMR2 and HMR1, respectively); CLar, 359 → 344, 10.76; HEL, 313 → 147, 13.19 and 13.86 (two stereoisomers); Lar, 359 → 329, 13.56; Seco, 361 → 165, 13.86; Mat-d_6, 363 → 83, 15.91; Mat, 357 → 83, 16.03; ED, 301 → 253, 16.78; EL-d_6, 303 → 259, 17.29; and EL, 297 → 253, 17.41.

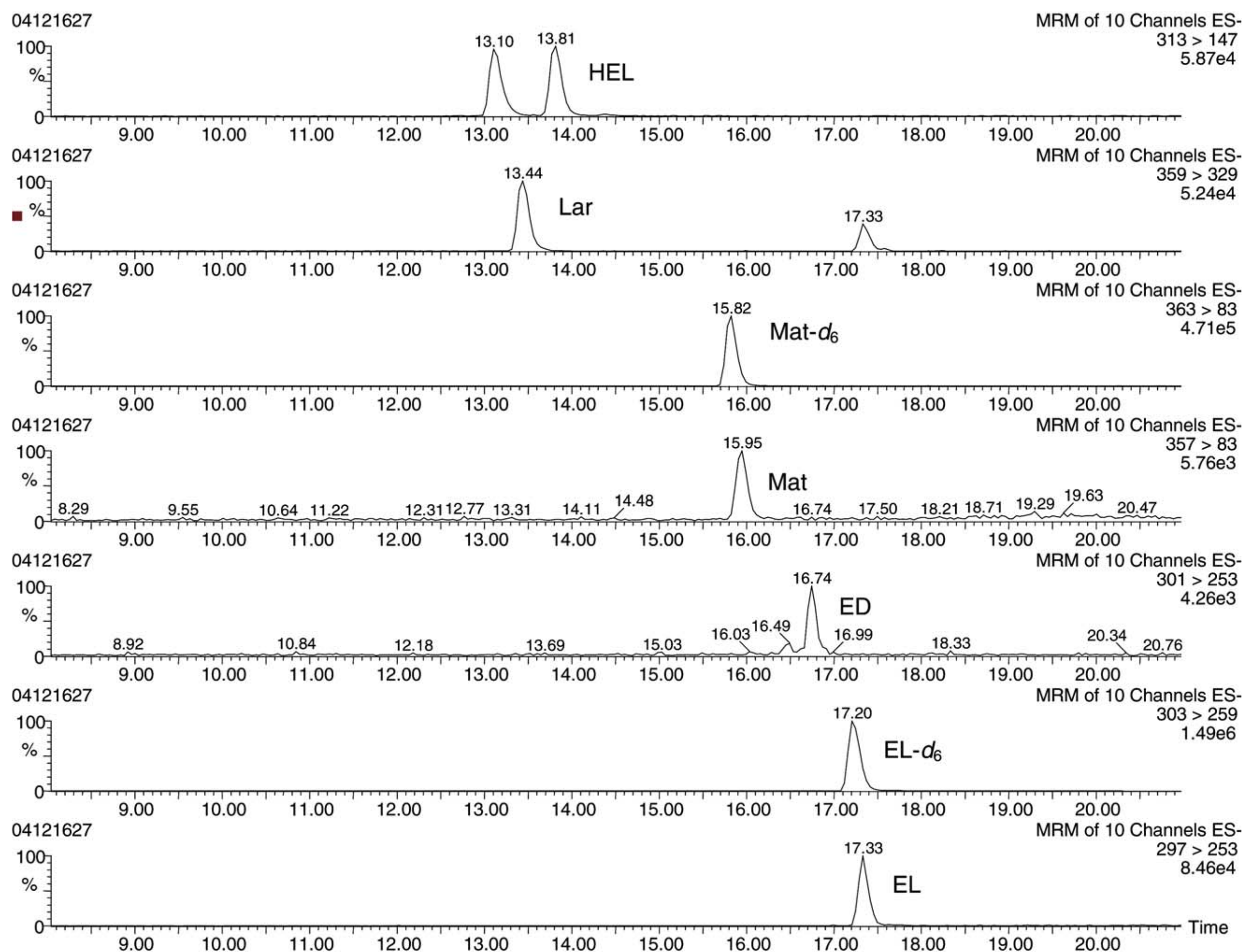

Fig. 3. HPLC-MS/MS-MRM chromatograms of lignans in a study sample. From above to below the chromatograms represent HEL, Lar, Mat-d_6, Mat, ED, EL-d_6, and EL. Monitored ions as in Fig. 2.

possible that plant lignans differ from each other in regard to their absorbability, metabolism, and excretion in humans as we have previously demonstrated in animals [4,6,47,48]. Instead, Lar as well as CLar was quantified in most of the samples collected from women, while they were not detected in any of the male subjects (Table 2). Moreover, in three female samples out of five, Lar was the major serum lignan.

HMR was not detected in any of the serum samples. Previously, HMR has been detected in sesame seeds and in the plasma of human subjects after intake of sesame seeds [3]. Although sesame seeds are rarely consumed in Finland, the compound has been detected in blood bank human plasma in Finland [29]. Thus, the presence of this compound in other dietary sources than sesame seeds is possible.

The recent reports on the presence of Lar and Pin in rye [2,11] and several other foods [2] may explain the presence of Lar in some of the serum samples in this study. Lar as well as CLar were quantified in most of the samples collected from women, while they were not detected in any of the male subjects (Table 2). Moreover, in three female samples out of five, Lar was the major serum lignan.

CLar is known to be formed from Lar under acid conditions [4,49,50]. This suggests that Lar may be at least partly converted to CLar under the acidic stomach conditions and hence explain the presence of CLar in some of the analysed serum samples. Accordingly, CLar detected in food sources [51] may have been produced during the strong acid treatments [4,49,50], although the presence of CLar in dietary sources cannot be excluded.

4. Conclusions

The present HPLC-MS/MS method for human serum allows reliable and sensitive quantification of five plant lignans (HMR, Mat, Seco, Lar, and CLar) and their three major mammalian metabolites (ED, EL, and HEL). The lignans can be quantified at low-nanomolar concentrations and the method is applicable for serum samples with a wide range of lignan concentrations.

In this study we demonstrate for the first time that in men and women consuming their habitual Finnish diet, the serum concentrations of plant lignans may clearly exceed the concentrations of enterolignans. Similar to enterolignans, large inter-individual variation in serum plant lignan profiles were observed. Human serum may as well contain high concentrations of other plant lignans such as Lar or Mat. Thus, depending on the individual diet and metabolic characteristics of the microbiota, the individual profile of lignans in human serum may vary. As the knowledge

of the variety of dietary plant lignans is increasing, their presence in serum and putative importance as biologically active components requires further investigation.

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