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Determination of secoisolariciresinol, lariciresinol and isolariciresinol in plant foods by high performance liquid chromatography coupled with coulometric electrode array detection

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Dedicated to Emer. Univ. Prof. Dr. Gerald Kainz on the occasion of his 85th birthday.

Abstract

The paper describes a method for the determination of selected lignans in plant foods. First, samples were submitted to methanolysis resulting in cleavage of ester bonds between lignan glycosides and organic acids. Glycosidic linkages were then broken by enzymatic hydrolysis using cellulase. The released aglycones were separated isocratically (acetonitrile/10 mM sodium acetate buffer, pH 4.8, 225:775, v:v) by reversed phase high performance liquid chromatography (RP-HPLC) and the compounds were detected coulometrically at four electrodes set on potentials between +260 and +330 mV against palladium reference electrodes. The selectivity and sensitivity of the method allowed quantitation of the lignans secoisolariciresinol, lariciresinol and isolariciresinol in various foodstuffs down to the upper ppb-range with recoveries between 44.7 and 97.0%. Unidentified peaks displaying similar current–voltage curves (CVCs) as the investigated lignans indicated the presence of further possible lignan representatives. In addition, investigation of various foodstuffs involving enzymatic hydrolysis with and without preceding methanolysis showed that the degree of esterification of lignans in plant foods is species dependent.

Keywords: Phytoestrogens; Lignans; Secoisolariciresinol; Lariciresinol; Isolariciresinol; HPLC–CEAD; Food

1. Introduction

Lignans are non-nutrient bioactive secondary metabolites present in a wide variety of plant foods from the ppb- to the percent range [1–4]. Several hundred representatives exist in the plant kingdom with native forms of variable complexity [5]. Lignans may occur glycosidically bound to various sugar residues, esterified or as structural subunits of biooligomers [6–8].

In plants, lignans are supposed to fight phytopathogenic organisms, to protect against stress and to be involved in growth regulation. In mammals, gut metabolites of plant lignans, the so called mammalian lignans, and some of their plant precursors have been reported to exhibit weakly estrogenic, antiestrogenic, anticarcinogenic, antioxidant, antiviral, antifungal and bactericidal effects [9–14].

Due to the structural diversity of the carbohydrate moiety of lignan glycosides and due to the lack of appropriate glycoside standards lignan analysis usually includes release and quantitation of the aglycones. Lignan analysis being a relatively young discipline, only few analysis schemes applicable to quantitation of lignans in trace levels have been published [3,15–20]. The methods by Mazur et al. [15] and Liggins et al. [3], both limited to the determination of secoisolariciresinol (Fig. 1) and matairesinol, include release of lignan aglycones by acid hydrolysis during which both ester linkages and glycosidic bonds are broken. However, secoisolariciresinol is acid labile so that a sum determination of the target lignan and its degradation product anhydrosecoisolariciresinol is required. Although the method developed by Mazur et al. [15] which is a combination of enzymatic and acid hydrolysis has been extended to the analysis of the lignans isolariciresinol, lariciresinol (Fig. 1), pinoresinol and syringaresinol in wine [16] and in cereal brans [18] actual data on the acid stability of these lignans have not been reported in these papers. Penalvo et al. [17] modified the method by

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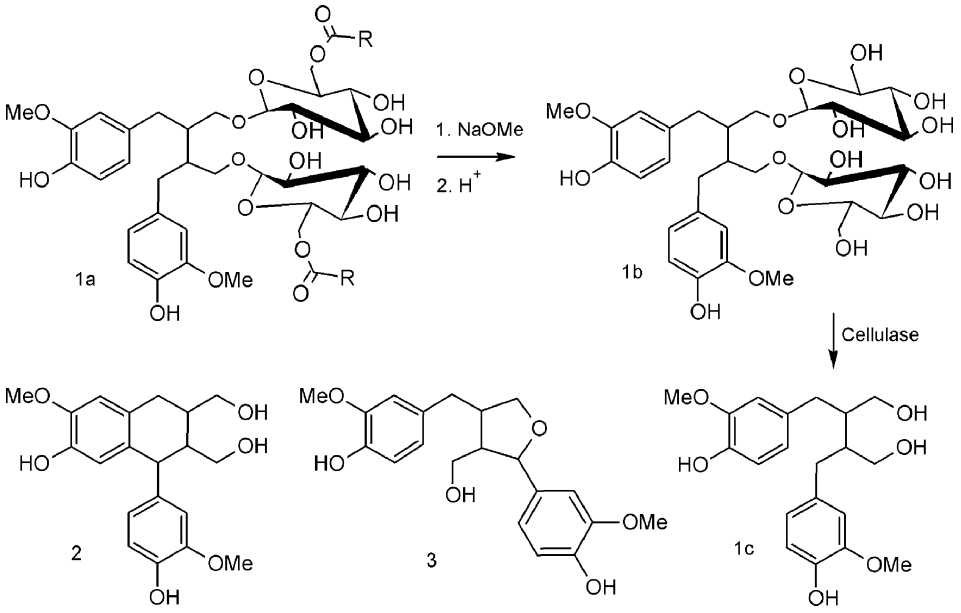

Fig. 1. Stepwise deconjugation of esterified secoisolariciresinol diglucoside (**1a**, R = organic acid) by the two-step method. Methanolysis with sodium methoxide (NaOMe) and subsequent neutralization yields secoisolariciresinol diglucoside (**1b**) which is cleaved into secoisolariciresinol aglucone (**1c**) and two glucose residues by the enzyme cellulase. The structures of isolariciresinol (2) and lariciresinol (3) are given in one line with that of secoisolariciresinol.

Mazur et al. [15] by decreasing the acid concentration from 2.0 to 0.6 M before applying it to the analysis of various lignans in soy based supplements and in sesame seeds [21]. By using enzymatic hydrolysis with β-glucosidase after methanolic aqueous extraction Horn-Ross et al. [19] applied the mildest sample preparation technique of the above cited methods. In order to account also for lignans occurring in more complex native forms which are difficultly accessible to enzymatic hydrolysis Milder et al. [20] combined different deconjugation techniques with the aim of non-destructive liberation of lignan aglycones. In a first step, possible ester bonds were broken by alkaline extraction, subsequently glycosidic bonds were cleaved by enzymatic hydrolysis (Fig. 1).

The commonly used separation techniques in lignan analysis are gas chromatography (GC) [3,8,15,17,18,21–24] and reversed phase high performance liquid chromatography (RP-HPLC) [16,18–23,25–34]. Advantages of GC are higher peak capacities and routine coupling to mass spectrometric detection. However, GC is restricted to volatile compounds which requires derivatization prior to analysis. RP-HPLC is the method of choice for separating polar compounds in their native forms. As UV-detection is neither selective nor sensitive enough for trace analysis of lignans in complex mixtures its application is limited to the detection of the abundant lignans in flaxseed [22,27,29–31]. A versatile alternative to UV-detection proved to be coulometric electrode array detection [16,21,23,25,26,28,32–34] which is 1–2 orders of magnitude more sensitive and selective for electrochemically active compounds like lignans.

The detector of choice in terms of identification and structure elucidation of unknown compounds is the mass spectrometer [7,19,20,22,31]. Milder et al. [20] demonstrated that even coeluting compounds can be reliably quantitated in diluted sample extracts provided that deuterated analoga are used as internal standards. However, the same researchers reported that LC–MS analysis of complex mixtures is prone to signal enhancement or suppression effects resulting in relatively high matrix dependent between- and within-run variations.

The objective of the present work was to develop a simple but nevertheless sensitive and generally applicable non-destructive method for the analysis of some of the most important plant lignans in foodstuffs using HPLC coupled with coulometric electrode array detection. For that, the two-step method of successive methanolysis and enzymatic hydrolysis designed for the analysis of secoisolariciresinol in flaxseed developed by Kraushofer and Sontag [26] should be extended to the analysis of sub- and low ppm levels of the mammalian lignan precursors secoisolariciresinol and lariciresinol as well as of isolariciresinol which is of interest because of its antioxidant activity [13] in various plant foods. In addition, the impact of methanolysis on the liberation of lignans from their complex possibly esterified native forms should be investigated by comparing the concentrations of lignan aglycones released upon enzymatic hydrolysis with and without preceding methanolysis.

2. Experimental

2.1. Chemicals and solutions

Secoisolariciresinol, lariciresinol and isolariciresinol were purchased from Separation Research (Turku, Finland). Cellulase Onozuka R-10 1 U/mg (EC 3.2.1.4), sodium acetate trihydrate, hydrochloric acid, ethanol (absolute ultra pure), anhydrous methanol SeccoSolv®, acetonitrile (Lichrosolve), methanol (Lichrosolve) as well as glacial acetic acid were from Merck (Darmstadt, Germany). *n*-Hexane (HPLC grade) was obtained from Fluka (Buchs, Switzerland). Metallic sodium and

solid carbon dioxide were provided by the Institute of Organic Chemistry (University of Vienna).

Stock solutions containing 190 mg/l isolariciresinol, 206 mg/l secoisolariciresinol and 185 mg/l lariciresinol were prepared in absolute ethanol and stored in tightly sealed 5 ml volumetric flasks under exclusion of light at 4 °C. These solutions were further diluted with absolute ethanol to obtain the spiking solutions for standard addition method or with mobile phase (see below), respectively, for the preparation of calibration standards for HPLC analysis. Sodium methoxide solution was always freshly prepared by dissolving 400 mg of metallic sodium in 100 ml anhydrous methanol. Sodium acetate buffer (0.1 M) used for sample preparation was adjusted to pH 5.0 with glacial acetic acid. Deionized water prepared using a Barnstead EASY pure LF (Dubuque, IO, USA) was used in all experiments.

Mobile phase: 225 ml of acetonitrile were mixed with 775 ml of membrane filtered (PTFE-filters 0.45 μm, Sartorius, Göttingen, Germany) 10 mM sodium acetate buffer (adjusted to pH 4.8 with glacial acetic acid) and degassed by sonication for 15 min.

2.2. HPLC separation and detection

The HPLC system consisted of two ESA HPLC pumps model 420 (ESA, Chelmsford, MA, USA), a six-port injection valve equipped with a 20 μl stainless steel injection loop (Rheodyne, model 7125, Cotati, CA, USA), a Waters Spherisorb ODS2 column (3 μm, 150 mm × 4.6 mm I.D.) equipped with a guard-column of the same stationary phase and an ESA Coulochem Electrode Array System (CEAS) with four cells. The system was controlled by an IMB PC/AT compatible computer installed with ESA chromatographic software (CEAS v4.1 software).

Twenty microliters of standard solutions or sample extracts were injected into the chromatographic system. The compounds were separated isocratically at a flow rate of 0.55 ml/min at 32 °C. The potentials set on the individual channels of the CEAS were +260, +280, +300, +330 mV against palladium reference electrodes. The principle of coulometric electrode array detection has been described in [35].

2.3. External calibration, limits of detection and quantitation, intra- and inter-day repeatability of HPLC-analysis

Five calibration solutions with lignan concentrations of about 3, 75, 200, 500 and 1000 μg/l (prepared by stepwise dilution of a mixed standard solution containing 1030 μg/l secoisolariciresinol, 925 μg/l lariciresinol and 950 μg/l isolariciresinol with mobile phase) were analyzed in the course of each analysis run. Calibration functions were established by linear regression of the peak heights in channel 2 for secoisolariciresinol and isolariciresinol and in channel 3 for lariciresinol on the concentrations.

The limits of detection (quantitation) of the individual analytes in standard solutions were calculated by equating the signal (y) in the respective regression equation $y = k \times x + d$ to the three-fold (10-fold) standard deviation of the noise (determined as standard deviation of 15 measuring points in a 2-min time window free of interferences) and division by the slope (k) of the respective calibration function through the origin ($d = 0$).

The intra-day repeatability of the chromatographic method was determined by five-fold injection of a calibration standard containing 515.0 μg/l secoisolariciresinol, 475.0 μg/l isolariciresinol and 462.5 μg/l lariciresinol and calculation of the relative standard deviations of the peak heights. The inter-day repeatability was assessed on the basis of five injections of the same calibration standard over the period of 5 days. The precision of HPLC-analysis of sample solutions was tested by five-fold injection of two cashew nut extracts obtained via two-step method on 2 days and calculation of the relative standard deviation of the peak heights.

Table 1
Characterization of samples

Common name	Botanical name	Country of origin	Manufacturer
Cashew nut	*Anacardium occidentale*	Sri Lanka	Perlinger
Sesame seed	*Sesamum indicum*	Burkina Faso	Alnatura
Mung bean	*Vigna radiata* var. *radiata*	China	Granovita
Asparagus	*Asparagus officinalis*	Austria	Fresh
Broccoli	*Brassica oleracea* var. *italica*	Italy	Fresh
Carrot	*Daucus carota* ssp. *sativus*	Italy	Fresh
Celeriac	*Apium graveolens* var. *rapaceum*	Spain	Fresh
Courgette	*Cucurbita pepo* ssp. *pepo* var. *giromontiina*	Italy	Fresh
Strawberry	*Fragaria x ananassa*	Spain	Fresh

2.4. Samples

Food samples (250–500 g) were purchased at local supermarkets and drugstores in early 2004. Product data are summarized in Table 1. Prior to analysis ~50 g aliquots were prepared and homogenized in a commercial kitchen blender (Krupps Speedy Pro, Solingen, Germany). An 8–10 g aliquot of ground fatty samples (cashew nuts, sesame seeds) was defatted with 120 ml of *n*-hexane for 2 h by Soxhlet extraction and the solid residue was dried for 30 min at 40 °C under vacuum using a rotary evaporator (BÜCHI Rotavapor E120, Switzerland). Fifty grams aliquots of homogenized asparagus, broccoli, carrots, celeriac, courgette and strawberries were freeze-dried (P. Haack FTS Systems Flexi-Dry TM MP, Vienna, Austria). Ground mung beans, defatted and freeze-dried samples were stored in tightly closed scintillation vials at 4 °C under exclusion of light.

2.5. Enzymatic hydrolysis

0.05–0.1 g of pretreated (ground, defatted or freeze-dried) sample were weighed into a 20 ml glass vial equipped with a screw cap, 3.5 ml of ethanol and 1.5 ml of 0.1 M sodium acetate buffer (pH 5.0) were added and the closed vial was sonicated for 2 h. In order to avoid evaporation of the solvent the water in the

ultrasonic bath was changed every 20–30 min. After separation of the solid residue by centrifugation (Hettich Universal, Tuttlingen, Germany) at 5000 rpm for 15 min the alcohol was evaporated from the supernatant under a constant stream of nitrogen. The aqueous extract was then diluted with 3 ml of 0.1 M sodium acetate buffer (pH 5.0) and incubated with 20 mg of cellulase at room temperature overnight. Subsequently the hydrolysate was applied to a preconditioned (5 ml of methanol, 5 ml of deionized water) solid phase extraction (SPE) cartridge containing 500 mg of C18 sorbent (AccuBond II SPE ODS-C18 cartridge, Agilent Technologies Inc., Palo Alto, CA, USA) and the analytes were eluted with 5 ml of methanol/water (80:20, v:v) after a washing step with 2 ml of methanol/water (20:80, v:v). Depending on the expected lignan concentrations, the SPE-eluate was either diluted with mobile phase or concentrated by evaporation on the rotary evaporator, dissolution of the residue in 200 μl of methanol and dilution with mobile phase to a final volume of 0.5 ml. Prior to HPLC analysis of a 20 μl aliquot 1–2 ml of the obtained solution were filtered through a 13 mm PTFE 0.45 μm syringe filter (Alltech, Deerfield, IL, USA).

2.6. Two-step method

0.05–0.1 g of pretreated (ground, defatted or freeze-dried) sample were weighed into a 20 ml glass vial. Methanolysis was performed by sonication in 5 ml sodium methoxide solution for 3 h during which the water in the ultrasonic bath was changed every 20–30 min. After cooling to room temperature, the solution was neutralized by stepwise addition of small pieces of solid carbon dioxide (~0.5 g). Then 1 ml of 0.1 M sodium acetate buffer (pH 5.0) was added and the pH was adjusted to about five with 70–75 μl of concentrated hydrochloric acid. Subsequently the solution was sonicated for 30 min, the solid residue was separated by centrifugation (15 min, 5000 rpm) and the alcohol was evaporated from the supernatant under a constant stream of nitrogen. The further procedure was equal to that described for enzymatic hydrolysis.

2.7. Qualitative analysis

In sample extracts lignans were identified by comparison of both retention factors and current–voltage curves (CVCs), important tools in peak identification and determination of peak purity, with those of reference compounds in standard solutions measured in the same analysis run. CVCs were obtained by plotting the peak heights of the substances measured in each individual channel against the applied potential.

2.8. Quantitative analysis

In order to obtain an overview of the concentrations of secoisolariciresinol, lariciresinol and isolariciresinol in selected plant foods unspiked samples were analyzed as described above and the lignan concentrations were estimated on the basis of external calibration functions. Quantitation was then performed using standard addition method. For that, four 50 mg aliquots of pretreated, i.e. defatted or freeze-dried samples were spiked with ethanolic standard solutions containing 50, 100, 150 and 200% of the estimated amounts of lignans in the unspiked sample and analyzed together with two unspiked samples according to the analysis schemes of enzymatic hydrolysis and combined methanolysis and enzymatic hydrolysis outlined above. The analyte concentrations in the sample extracts were determined by plotting the peak heights versus the added concentrations of standard compounds, linear regression and division of the y-intersection (d) by the slope (k) of the regression line $y = k \times x + d$ [36].

The recoveries were determined by division of the slope of the regression lines obtained using standard addition method by the slope of external calibration functions recorded in the same analysis run and multiplication by 100 [36].

Limits of quantitation ($S/N = 10$) of target compounds in sample extracts were determined in the same way as those of standard compounds in standard solutions (see above). Consideration of concentration or dilution factors, weighed portion and conversion factors for results on a wet basis yielded the limits of quantitation in the original food sample.

3. Results

Application of the method developed by Kraushofer and Sontag [26] for the determination of secoisolariciresinol in flaxseed to other food matrices revealed that lignan analysis in the sub- and low ppm-range necessitated several changes. The sample preparation protocol had to be modified, the chromatographic conditions adapted and a means of quantitation under consideration of the recoveries for lignans in different matrices had to be found. In addition, experiments had to be performed in such a way as to allow estimation of the extent of esterification of lignans or lignan glycosides, respectively.

3.1. Sample preparation

Enzymatic hydrolysis can be performed either after aqueous ethanolic extraction or subsequent to methanolysis. The former approach allows quantitation of both free and β-glucosidically bound lignans, the latter makes a sum determination of free, β-glucosidically and additionally esterified lignans possible.

3.1.1. Enzymatic hydrolysis

The multi-enzyme complex cellulase was chosen due to its successful application by Kraushofer and Sontag [26] and due to its broad activity range (β-glucosidase, hemicellulase, α-amylase, pectinase) [37]. Enzyme activity tests using carboxymethylcellulose sodium salt as substrate revealed a pH-optimum of about five and only moderate loss of activity in the range from pH 4.5 to 7.0. Specific activities turned out to be highly temperature dependent. During the first 10 min of incubation highest turnover numbers were recorded at 70 °C. However, the specific activity decreased upon prolonged exposure to temperatures higher than 50 °C [38]. Thus, an incubation temperature of 40 °C was chosen as a compromise between

Table 2
Characteristics of the method applied to lignan analysis in standard solutions

Compound	Retention factor ± S.D.[a]	Slope ± S.D. (nA l/mg)[a,b]	Intercept ± S.D. (nA)[a,b]	Standard error	Detector response R.S.D. (%) intra-day[a]	Detector response R.S.D. (%) inter-day[a]
Isolariciresinol	3.55 ± 0.04	243.85 ± 0.93	0.70 ± 0.31	0.81	1.9	5.0
Secoisolariciresinol	5.74 ± 0.05	427.69 ± 1.23	0.86 ± 0.41	1.17	2.0	4.9
Lariciresinol	6.59 ± 0.07	253.94 ± 2.18	−0.17 ± 1.14	1.57	2.5	4.3

[a] $N = 5$.
[b] 95% confidence interval.

long incubation times and gradual deactivation of the enzyme at higher temperatures.

3.1.2. Two-step method

Kraushofer and Sontag [26] and Eliasson et al. [27] achieved most efficient release of secoisolariciresinol or secoisolariciresinol diglucoside, respectively, from flaxseed when methanolysis and, in the case of Kraushofer, also enzymatic hydrolysis were performed in the presence of the solid sample. However, preliminary investigations on mung beans performed according to [26] suffered from a low recovery of secoisolariciresinol (32.8%). To avoid adsorption of lignans to matrix compounds and to improve the solubility of the analytes sodium acetate buffer was added subsequent to methanolysis and addition of solid carbon dioxide. After pH-adjustment and renewed sonication the solid residue was separated by centrifugation and enzymatic hydrolysis was performed as described above. Following this protocol the recovery of secoisolariciresinol could be increased to 79.9%.

3.2. Calibration functions, limits of detection and quantitation, repeatability of HPLC-analysis

Calibration functions for secoisolariciresinol, lariciresinol and isolariciresinol were linear in the investigated concentration range between 3 and 1030 μg/l with correlation coefficients of 0.9999. Retention factors and regression equations are given in Table 2. Limits of detection ($S/N = 3$) in standard solutions were 1.0 μg/l for isolariciresinol and lariciresinol and 0.6 μg/l for secoisolariciresinol. Limits of quantitation ($S/N = 10$) in standard solutions were 3.3 μg/l for isolariciresinol and lariciresinol and 2.0 for secoisolariciresinol. Intra- and inter-day repeatability of detector response in standard solutions are summarized in Table 2. The relative standard deviations of the peak heights in sample (cashew nut) chromatograms recorded on 2 days ($N = 5$) were 1.1% for isolariciresinol, 3.5% for secoisolariciresinol and 3.8% for lariciresinol. In order to compensate for the relatively high inter-day variation of the detector response calibration functions were recorded in the course of each analysis run.

3.3. Analysis of selected foodstuffs

For most sample extracts analytes in chromatograms were sufficiently resolved using a mobile phase containing 22.5% acetonitrile (Fig. 2). Yet, separation of the lignans from matrix compounds in strawberry extracts demanded a reduction of the organic modifier content to 20%. However, as each sample has to be worked-up and injected once before being analyzed by standard addition method (so that the spiking volumes and concentrations of the individual lignans can be calculated), samples requiring reduction of the percentage of acetonitrile could be identified at this stage and analyzed on the same day or on successive days using a mobile phase containing 20% of acetonitrile.

Identification of the analytes was based on comparison of the retention factors and the current–voltage curves in sample extracts with those in standard solutions. CVCs served also as purity check and for the determination of the potentials best suited for quantitation of the individual lignans. In Fig. 3 the CVCs of secoisolariciresinol, lariciresinol and isolariciresinol in a standard solution and in a cashew nut extract are depicted. The potentials for most sensitive detection proved to be +280 mV (channel 2) for secoisolariciresinol and isolariciresinol and +300 mV (channel 3) for lariciresinol.

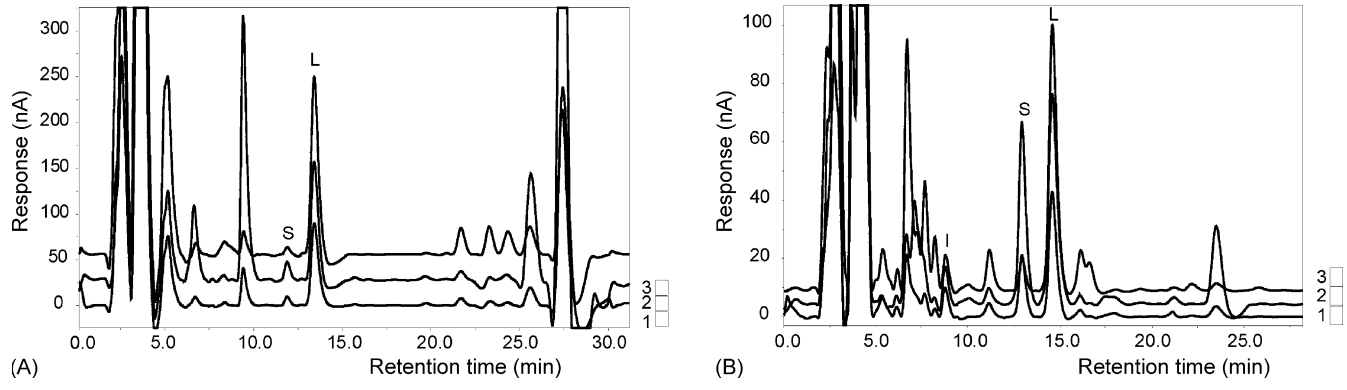

Fig. 2. Electrode array chromatograms of a broccoli extract (A) and a cashew nut extract (B) prepared by the two-step method. S: secoisolariciresinol, L: lariciresinol, I: isolariciresinol.

Table 3
Lignan concentrations and standard deviations determined using standard addition method

Food	Deconjugation method	Content ± S.D.[a] (mg/kg fresh food)		
		Secoisolariciresinol	Lariciresinol	Isolariciresinol
Cashew nut	E	1.63 ± 0.09	4.19 ± 0.33	0.56 ± 0.02
	M + E	1.66 ± 0.08	4.14 ± 0.23	0.81 ± 0.05
Sesame seed	E	<0.48	52.2 ± 2.9	<0.92
	M + E	<0.09	64.4 ± 3.4	<0.18
Mung bean	E	2.60 ± 0.30	1.89 ± 0.12	<0.07
	M + E	3.18 ± 0.15	2.12 ± 0.02	<0.07
Asparagus	E	2.15 ± 0.10	1.05 ± 0.08	<0.17
	M + E	3.19 ± 0.19	1.78 ± 0.10	<0.02
Broccoli	E	0.38 ± 0.03	4.14 ± 0.20	<0.01
	M + E	0.44 ± 0.04	4.81 ± 0.27	<0.02
Carrot	E	0.72 ± 0.05	0.17 ± 0.02	<0.03
	M + E	1.77 ± 0.09	0.45 ± 0.08	<0.10
Celeriac	E	0.40 ± 0.04	0.25 ± 0.02	<0.03
	M + E	1.28 ± 0.08	0.77 ± 0.03	<0.01
Courgette	E	0.30 ± 0.02	0.54 ± 0.04	<0.01
	M + E	0.36 ± 0.01	0.59 ± 0.01	<0.01
Strawberry	E	1.32 ± 0.09	0.43 ± 0.04	<0.02
	M + E	1.33 ± 0.06	0.44 ± 0.03	<0.02

E: enzymatic hydrolysis, M + E: combined methanolysis and enzymatic hydrolysis (two-step method).
[a] ($N = 5$).

The concentrations and standard deviations of secoisolariciresinol, lariciresinol and isolariciresinol in selected foodstuffs determined by single enzymatic hydrolysis and the two-step method are summarized in Table 3. Contrary to the common practice to present results on a dry-weight basis concentrations are given on a fresh weight (as-is) basis which is of greater interest for the calculation of the daily intake. As vegetables and fruits consist of high amounts of water the concentrations in these food groups are considerably lower than the dry-basis values reported in the literature. Due to the high sensitivity of coulometric electrode array detection the limits of quantitation ($S/N = 10$) were generally in the medium ppb-range. They ranged from 0.02 mg/kg in courgette to 0.57 mg/kg in asparagus for isolariciresinol, from 0.03 mg/kg in courgette to 0.36 mg/kg in asparagus for secoisolariciresinol and from 0.04 mg/kg in courgette to 0.54 mg/kg in asparagus for lariciresinol. Higher LOQs (up to 3.07 mg/kg) were observed only in sesame seeds where high dilution was required due to the elevated concentration of lariciresinol. The recoveries differed for the investigated analytes in the same sample matrix as well as for one analyte in different foodstuffs. They ranged from 46.5 to 97.0% for secoisolariciresinol, from 44.7 to 89.5% for lariciresinol and from 71.5 to 80.5% for isolariciresinol (Table 4).

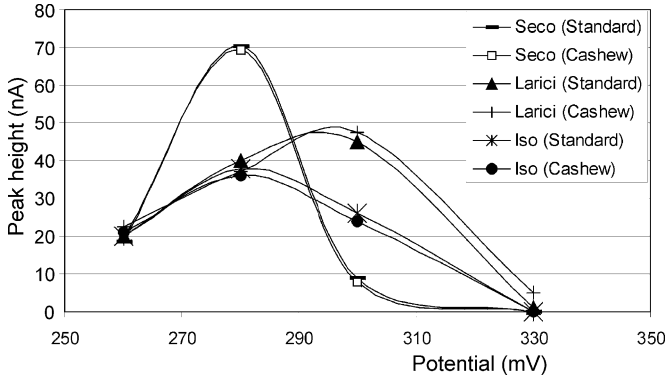

Fig. 3. Current–voltage curves of secoisolariciresinol (Seco), lariciresinol (Larici) and isolariciresinol (Iso) in a standard solution and in a cashew nut (cashew) extract.

Table 4
Recoveries of secoisolariciresinol (seco), lariciresinol (larici) and isolariciresinol (iso) in selected foodstuffs upon analysis including enzymatic hydrolysis with and without preceding methanolysis

Food	Recovery[a] (%)					
	Enzymatic hydrolysis			Two-step method		
	Seco	Larici	Iso	Seco	Larici	Iso
Cashew nuts	82.3	77.7	80.5	78.4	78.6	71.5
Sesame		81.8			83.9	
Mung beans	97.0	85.4		79.9	n.d.	
Asparagus	86.6	83.0		68.8	59.6	
Broccoli	80.6	89.5		56.8	71.2	
Carrot	87.1	n.d.		89.4	n.d.	
Celeriac	81.5	80.0		83.7	75.9	
Courgette	58.8	68.9		46.5	65.6	
Strawberry	60.0	44.7		77.0	77.7	

n.d.: not determined.
[a] ($N = 5$).

4. Discussion

In this paper a non-destructive trace level method for lignan analysis in a variety of foodstuffs using HPLC with coulometric electrode array detection is presented. Contrary to conclusions drawn by other researchers [20], fast and simple sample preparation is well compatible with this detection mode provided that chromatographic conditions and detection potentials are carefully chosen. The high sensitivity and selectivity of coulometric electrode array detection allowed reliable quantitation of the lignans secoisolariciresinol, lariciresinol and isolariciresinol in various plant foods in a wide concentration range with limits of quantitation in the medium ppb-range. Lariciresinol could be detected and quantitated in all investigated foodstuffs and proved to be more abundant than secoisolariciresinol which, together with matairesinol, has been the main target lignan for several years. This finding is in agreement with recently published data on the lignan content of a variety of Dutch plant foods [4].

The analysis method developed in the present work is relatively simple, requiring only solid phase extraction as concentration and purification technique subsequent to methanolysis and enzymatic hydrolysis. Due to the lack of one universal recovery standard for all analytes and sample matrices, quantitation was performed via standard addition method which automatically corrects for losses during sample work-up [36]. The great variability of the recoveries of the individual analytes in the same food matrix and of one analyte in different food matrices underlines the importance of quantitation by standard addition method.

Comparison of the concentrations of secoisolariciresinol, lariciresinol and isolariciresinol obtained via two-step method with those determined using single enzymatic hydrolysis with cellulase shows that enzymatic hydrolysis is not a quantitative deconjugation method for lignans in complex native forms due to poor accessibility of glycosidic bonds between aglycones and esterified sugar moieties. The obtained results confirmed preliminary data published by Milder et al. [4] according to which the degree of esterification of lignan glycosides depends on the plant species. Fig. 4 illustrates the proportion of free and β-glucosidically bound lignans (obtained by enzymatic hydrolysis) to the total, i.e. free, β-glucosidically bound and additionally esterified lignans released using the two-step method.

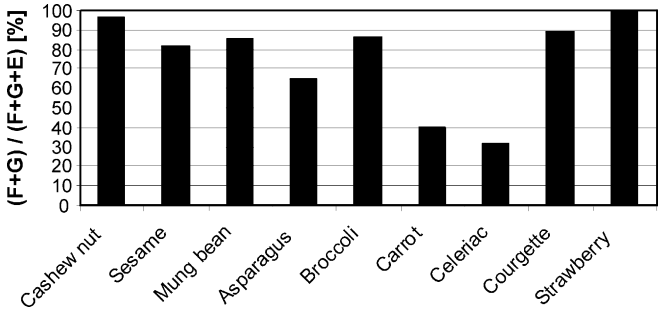

Fig. 4. Percentage of free and β-glycosidically bound lignans (F + G, black bars) to free, β-glycosidically bound and esterified lignans (F + G + E, 100%). The difference between total and free plus glycosidically bound lignans gives the percentage of esterified lignans and esterified lignan glycosides. Lignans denotes the sum of secoisolariciresinol, lariciresinol and isolariciresinol.

Mass spectrometric detection is the technique of choice for identification purposes but susceptible to enhancement or suppression effects [20]. CEAD, on the other hand, is a reliable tool for quantitative analysis but not suited for unequivocal identification of analytes. However, CEAD is selective for electrochemically active compounds and capable of distinguishing between compound classes with different electrochemically active groups, e.g. lignans and isoflavones. As demonstrated in Fig. 3, even the investigated lignans can be differentiated by their CVCs which makes CEAD a useful tool for peak purity checks provided the detection potentials are carefully chosen. In addition, CEAD furnishes complementary information to mass spectrometry on the presence of further compounds with the same or similar electrochemically active groups, thereby giving indications to the possible presence of further lignan representatives.

5. Conclusion

The presented two-step method consisting of successive methanolysis, enzymatic hydrolysis, purification and HPLC-separation coupled with coulometric electrode array detection has been successfully applied to quantitative lignan analysis from the upper ppb- to the ppm-range in various plant foods. Advantages of the method are its simplicity, universal applicability, good sensitivity and compatibility with quantitation using standard addition method. In addition, it provides information on the presence of matrix compounds with similar electrochemically active groups as the investigated lignans which gives a first indication to the possible abundance of further lignans in the sample. Future work will show whether the method can be extended to the analysis of further lignans such as pinoresinol and matairesinol.

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