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Optimization of a Liquid Chromatography−Tandem Mass Spectrometry Method for Quantification of the Plant Lignans Secoisolariciresinol, Matairesinol, Lariciresinol, and Pinoresinol in Foods

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A liquid chromatography−tandem mass spectrometry (LC-MS/MS) method was developed for the quantification of the four major enterolignan precursors [secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol] in foods. The method consists of alkaline methanolic extraction, followed by enzymatic hydrolysis using *Helix pomatia* (*H. pomatia*) β-glucuronidase/sulfatase. *H. pomatia* was selected from several enzymes based on its ability to hydrolyze isolated lignan glucosides. After ether extraction samples were analyzed and quantified against secoisolariciresinol-d_8 and matairesinol-d_6. The method was optimized using model products: broccoli, bread, flaxseed, and tea. The yield of methanolic extraction increased up to 81%, when it was combined with alkaline hydrolysis. Detection limits were 4−10 μg/(100 g dry weight) for solid foods and 0.2−0.4 μg/(100 mL) for beverages. Within- and between-run coefficients of variation were 6−21 and 6−33%, respectively. Recovery of lignans added to model products was satisfactory (73−123%), except for matairesinol added to bread (51−55%).

KEYWORDS: Lignans; HPLC-MS/MS; phytoestrogens; secoisolariciresinol; matairesinol; lariciresinol; pinoresinol

INTRODUCTION

Lignans are polyphenolic compounds in plants derived from the combination of two phenylpropanoid (C6−C3) units (*1*). Several hundred lignans have been discovered in plant species. A small number of these plant lignans are known to be converted by the colonic microflora into the "enterolignans" enterodiol and enterolactone. Enterolignans are present in biological fluids of humans and animals (*2*−*4*). Enterolignans possess several biological activities such as antioxidant, antitumor and (anti-) estrogenic, and inhibit enzymes involved in the metabolism of sex hormones (e.g. SHBG, 5α-reductase, and 17β-hydroxysteroid dehydrogenase (*5*−*10*). Because of these activities, they may affect the development of hormone dependent diseases (*11*, *12*). Increased plasma or serum levels or urinary excretion of enterolactone were associated with reduced breast cancer risk in epidemiological studies (*13*−*15*). Only one of these studies also included enterodiol (*14*), with similar results. However, increased risks were also found (*15*, *16*). There also is some support for a protective effect of lignans against cardiovascular diseases. Negative associations have been found between serum enterolactone and acute coronary events and cardiovascular disease related mortality (*17*, *18*). Besides a negative association between serum enterolactone and F_2-isoprostanes, a marker of in vivo lipid peroxidation, was found in a cross-sectional study in men (*19*).

Until recently, plant lignan research focused on secoisolariciresinol (**1**) and matairesinol (**2**) (**Figure 1**), because they were thought to be the only enterolignan precursors. Secoisolariciresinol and matairesinol are present in grains, seeds, vegetables, and fruits (*20*, *21*). Flaxseed contains by far the highest concentrations of secoisolariciresinol of any food for which data have been published (*20*, *21*). In some human and animal supplementation trials with rye bread or wheat bran the enterolignan excretion was much higher than expected on the basis of the amount of secoisolariciresinol and matairesinol consumed (*22*, *23*). Heinonen and co-workers (*24*) showed that also pinoresinol (**3**) and lariciresinol (**4**) (**Figure 1**) were converted into enterodiol and enterolactone after in vitro fecal incubation (55 and 100% conversion, respectively). In addition, syringaresinol, arctigenin, and 7-hydroxymatairesinol were

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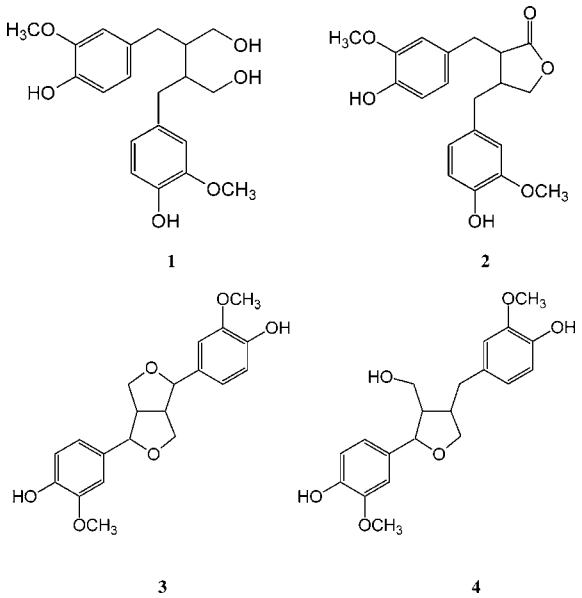

Figure 1. Chemical structures of plant lignans secoisolariciresinol (**1**), matairesinol (**2**), pinoresinol (**3**), and lariciresinol (**4**).

metabolized to enterolignans, but to a smaller extent (4−15% conversion). These newly discovered lignan precursors might explain the difference between lignan intake and excretion in the aforementioned trials, and indeed some of these precursors have been detected in rye bran (*24*). It is important to include these newly discovered enterolignan precursors, especially lariciresinol and pinoresinol in studies that assess the health effects of dietary lignans.

For the analysis of secoisolariciresinol and matairesinol in foods several methods have been published (*25*), but quantitative methods for pinoresinol and lariciresinol are lacking. To enable quantification, lignans have to be extracted from the food matrix. Extraction is usually performed using organic solvents, acid or alkaline hydrolysis, or combinations of these. Alkaline hydrolysis is mainly used to liberate secoisolariciresinol diglucoside from flaxseed (*26*−*28*), since secoisolariciresinol in flaxseed forms oligomers with 3-hydroxy-3-methylglutaric acid via ester bonds, which can be hydrolyzed using alkali. However, we discovered that alkaline hydrolysis also improved lignan yield from other products (this paper). Plant lignans occur bound to one or more sugars as glycosides. Because not all glycosides are known, lignans are usually measured after hydrolysis to aglycones. This can also be achieved with acid hydrolysis, or with enzymatic hydrolysis. Liggins et al. (*29*) showed that optimal acid hydrolysis times vary with the food matrix. In addition, aglycones appeared to be unstable during hydrolysis. Therefore we decided to pursue an enzymatic method. Enzymatic methods include hydrolysis with β-glucosidase (*30*) or *Helix pomatia* β-glucuronidase/sulfatase (*31*, *32*).

Published analytical techniques for the measurement of lignans in foods include reversed-phase high-performance liquid chromatography (RP-HPLC) with ultraviolet (UV) (*26*, *31*) or coulometric electrode array detection (*28*, *33*, *34*), gas chromatography with mass spectrometric detection (GC-MS) (*29*, *32*, *35*), or liquid chromatography with mass spectrometric detection (LC-MS) (*30*, *31*). HPLC-UV is used for quantification of secoisolariciresinol in flaxseed. Because of its limited sensitivity and specificity, it is not suitable for products with low lignan values. Coulometric detection offers adequate sensitivity but proved to be not selective enough for a wide range of foods. A disadvantage of GC-MS is that it requires time-consuming cleanup and derivatization, with consequent risk of losses. Liquid chromatography−tandem mass spectrometry (LC-MS/MS) requires no derivatization and is both specific and sensitive.

Here we present a quantitative method for the determination of the plant lignans secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol in foods. This method consists of combined extraction and alkaline hydrolysis (for solid foods), followed by enzymatic hydrolysis using *Helix pomatia* (*H. pomatia*) β-glucuronidase/sulfatase. After a subsequent ether extraction, lignans are separated and detected using LC-MS/MS and quantified against deuterated standards of secoisolariciresinol and matairesinol.

MATERIALS AND METHODS

Chemicals. Pure standards of secoisolariciresinol (purity ca. 92%) and matairesinol (purity >98%) were obtained from Plantech (Reading, England). Enterolactone was purchased from Fluka Chemie GmbH (Buchs, Switzerland). Lariciresinol isolated from the wood of *Abies sachalinensis* and pinoresinol and pinoresinol diglucoside isolated from *Euconmia ulmoides oliv.* bark were kindly provided by Dr. Ozawa (Rakuno Gakuen University, Japan), Dr. Deyama (Yomeishu Seizo Co., Ltd., Japan) and Dr. Nishibe (University of Hokkaido, Japan). Secoisolariciresinol diglucoside isolated from flaxseed (purity 93%) was purchased from Dr. P. Winterhalter (Technical University of Braunschweig, Germany). Secoisolariciresinol-d_8 and matairesinol-d_6 with isotopic purity >98% (*32*) were synthesized by Dr. K. Wähälä. β-Glucuronidase/sulfatase (*H. pomatia*), β-glucuronidase (bovine liver), and β-glucosidase (almonds) were purchased from Sigma-Aldrich Chemie B. V. (Zwijndrecht, The Netherlands). Rapidase LIQ$^+$ was obtained from DSM Food Specialties (Seclin, France). All other chemicals were of analytical grade, and water was purified with a Milli-Q system.

Sample Pretreatment. *Preparation of Test Products.* All test products used to optimize the method were bought at a local supermarket and prepared the same day. Whole grain wheat bread was cut into pieces, frozen under liquid nitrogen, and freeze-dried the same day. Flaxseed was ground to a powder using a Retsch GP 200 mill (Labotech BV, Ochten, The Netherlands) for 15 s at 8000 rpm and stored at −20 °C until analysis. Broccoli was chopped into pieces under liquid nitrogen, and freeze-dried the same day. After freeze-drying, broccoli and bread were ground to a powder and stored at −20 °C until analysis. Black tea from tea bags was passed through two sieves of 0.8 and 0.355 mm, and the middle fraction was stored at room temperature for use as a test product. For each series of analysis fresh tea was prepared by adding 100 mL of boiling tap water to 1 g of tea. After 5 min it was stirred, and 50 mL was filtered through a 1.2 μm Acrodisc filter (Gelman Sciences, Ann Arbor, MI).

Analytical Scheme. Alkaline extraction of 1.0 g of dry food was performed with 24.0 mL of methanol/water (70% (v/v)) containing 0.3 M sodium hydroxide, in a shaking water bath for 1 h at 150 rpm and 60 °C. After extraction, the pH was adjusted to 5−6 with 750 μL of 100% glacial acetic acid (Supra Pure) and the extract was centrifuged for 10 min at 10 °C and 4500 g. An aliquot of 1 mL was transferred to a preweighed test tube. Methanol was evaporated from this aliquot using a Zymark Turbo vap LV Evaporator (Zymark Corp., Hopkinton, MA) at 60 °C, under a mild nitrogen flow (5−10 psi), until the residual weight of the extract was ≤0.30 g. Then the volume was adjusted to approximately 1.2 mL with sodium acetate buffer (0.05 M, pH 5.0), and the extract was weighed again, to calculate the dilution compared to the original aliquot of 1 mL.

A 1 mL aliquot of this weighed extract or 1 mL of beverage was hydrolyzed by the addition of *H. pomatia* β-glucuronidase/sulfatase (0.83 mg, in 1 mL of 0.05 M sodium acetate buffer, pH 5.0). The samples were incubated overnight at 37 °C. Samples were extracted twice with 2 mL of diethyl ether, and the two organic phases were combined. The diethyl ether was evaporated using a Turbo vap at 30 °C, under a mild nitrogen flow (5−10 psi). The dried samples were redissolved in 0.3 mL of methanol, mixed, and 0.7 mL of water was

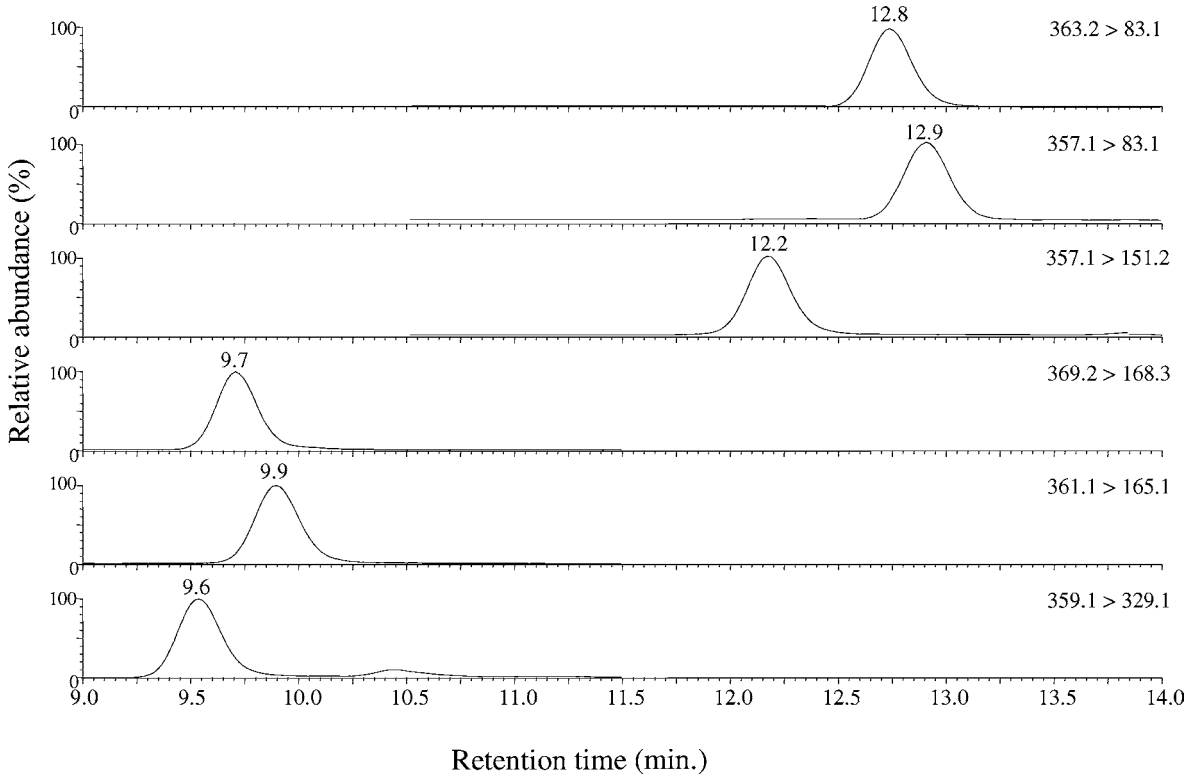

Figure 2. Individual chromatograms of a typical analysis of tea. The chromatograms show the MRM transitions that were used for quantification. From bottom to top, lariciresinol (359.1 > 329.1) and secoisolaricireinol (361.1 > 165.1) with internal standard secoisolariciresinol-d_8 (369.2 > 168.3), pinoresinol (357.1 > 151.2), and matairesinol (357.1 > 83.1) with internal standard matairesinol-d_6 (363.2 > 83.1).

added. A 240 μL aliquot of sample was added to 10 μL of internal standard solution containing 50 ng of secoisolariciresinol-d_8 and 50 ng of matairesinol-d_6 in 30% aqueous methanol. Samples were mixed and transferred to HPLC vials. Samples were analyzed the same day or stored at −80 °C (for a maximum of 1 week) until analysis.

Chromatographic and Detection Conditions. *Chromatographic Conditions.* A Waters (Millford, MA) Alliance chromatography separation Module 2690 was used, which consisted of a chromatographic system, and an autosampler with a cooled sample tray which was set at 10 °C. Separations were performed on a 150 mm × 3.0 mm i.d., 5 μm Symmetry C18 column (Waters), at a flow rate of 0.4 mL/min at 40 °C. The mobile phases A and B consisted of water and methanol, respectively. The gradient was as follows: 0−0.5 min, 30% B; 0.5−12 min, linear gradient from 30 to 50% B; 12−15 min, isocratic at 50% B; 15−15.2 min, linear return to 30% B, 15.2−19 min, isocratic at 30% to equilibrate. The total run time for each sample was 19 min. The sample injection volume was 50 μL. The divert valve was programmed to allow flow into the mass spectrometer from 8 to 19 min of each run.

Detection. Detection was performed with a Micromass Quatro Ultima MS, (Micromass, Manchester, U.K.) equipped with an atmospheric pressure chemical ionization (APCI) source, operated in the negative ion mode. The cone voltage was set at −30 V. Nitrogen at a flow rate of 100 L/h was used as desolvation gas. Source and desolvation temperatures were set at 100 and 500 °C, respectively. Dwell time was set at 0.2 s for all of the compounds. For quantification of lignans, the deprotonated molecules were used as precursor ions and the most abundant fragments after collision-induced association were selected as product ions. Argon was used as collision gas at a pressure of 2.2×10^{-3} mbar The retention times, characteristic precursor/product combinations, and collision energies for each compound were as follows: lariciresinol, 9.6 min, (359.1/329.1), 12 eV; secoisolariciresinol, 9.9 min, (361.1/165.1), 23 eV; pinoresinol, 12.2 min, (357.1/151.2), 12 eV; matairesinol, 12.9 min, (357.1/83.1), 20 eV; secoisolarciresinol-d_8, 9.7 min, (369.2/168.3), 25 eV; and matairesinol-d_6, 12.8 min, (363.2/83.1), 25 eV. A second, less abundant ion was selected for confirmation. However, in samples confirmation was not always possible because of lack of sensitivity. Integration of peak areas was performed using the program Masslynx provided by the MS manufacturer.

Method Validation. *Calibration and Quantification.* Calibration standards with lignan concentrations of 20, 100, 200, 600, and 1000 ng/mL plus 200 ng/mL of the deuterated standards in 30% aqueous methanol were injected three times (at the beginning, middle, and end of each series of analyses). Calibration lines were obtained by plotting the response factor (area lignan/area deuterated standard) against the concentration of the calibration standard. Average calibration lines were constructed from all of the injections. Secoisolariciresinol-d_8 was used to calculate the response factor of secoisolariciresinol and lariciresinol, and matairesinol-d_6 was used for matairesinol and pinoresinol, since these lignans elute close to those deuterated standards (**Figure 2**). All calibration lines were forced through the origin, with correlation coefficients ≥ 0.99.

When MS is used for quantification, signal enhancement or suppression can occur, causing over- or underestimation of the actual concentrations. This is caused by interferences introduced by the food matrix. To adjust for these matrix effects, 200 ng/mL of deuterated standards was added to each sample, and lignan concentrations were calculated on the basis of the response factor (area lignan/area deuterated standard). To establish whether this adjustment was sufficient, calibration lines with 0, 20, 100, 200, 1000, and 2000 ng/mL of all lignan standards were made in 30% aqueous methanol or in food extracts (tea, broccoli, and bread). Each of the calibration standards was injected two times, and the average response factor was used to construct the calibration lines. The slopes of calibration lines with and without food matrix were compared. If the adjustment is sufficient, the slopes of the calibration lines in food extracts and calibration lines in aqueous methanol are equal, even though the intercepts increase when lignans are present in the food extracts. In addition, the effect of dilution of the food extracts was evaluated using bread. Calibration lines with 0−2000 ng/mL of isolated lignans added were constructed in 3.3× concentrated, undiluted, and 2× diluted extract. Each calibration standard was injected three times.

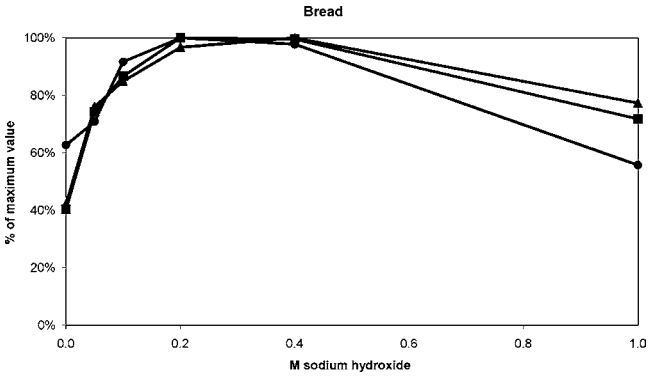

Figure 3. Influence of sodium hydroxide concentration during alkaline extraction on yield of lariciresinol (■), secoisolariciresinol (●), and pinoresinol (▲) from bread, expressed as a percentage of the maximum yield.

Limits of Detection. Limits of detection were determined by triplicate injections of 50 μL of standard mixtures, containing 20 ng/mL of each lignan standard, on 5 different days. The limit of detection was defined as the amount of lignan resulting in an extrapolated signal-to-noise (*S*/*N*) ratio of 3; average *S*/*N* ratios from 5 days were used.

Recovery. Recovery of lignans was determined by spiking known amounts of lignan standards to bread (total method) and tea (method without extraction). For bread the standards were added immediately after the addition of extraction solvent and for tea immediately after filtering the brew and cooling to room temperature. Lignan standards were added at approximately 100 and 200% of the original level in bread and 50 and 100% of the original level in tea. Because matairesinol was not present in well-detectable amounts, it was added at two higher levels.

Precision. To determine within- and between-run variation of the method, duplicate analyses of a broccoli, bread, and tea control sample were carried out on 4−7 separate days.

Stability. The stability of sample extracts was evaluated, to enable storage for up to 1 week at −80 °C before analysis. Seven sample extracts (together containing all four lignans) were analyzed before and after storage at −80 °C for 9 days. The concentrations were compared using Anova for repeated measurements for statistical analysis.

RESULTS

Sample Pretreatment. *Alkaline Extraction.* The combined extraction and alkaline hydrolysis was optimized using broccoli and bread as test products. Broccoli and bread were extracted for 3 h using 0, 0.02, 0.05, and 0.20 M sodium hydroxide, at 20 and 60 °C. For both products the yield of lignans was higher at 60 °C than at 20 °C, irrespective of the sodium hydroxide concentration (results not shown). For broccoli the influence of the sodium hydroxide concentration on the yield of lignans was less than 25% (results not shown), but bread required further optimization. The lignan yield was optimal at 0.3 M sodium hydroxide, when an extended range of 0, 0.05, 0.1, 0.2, 0.4, and 1 M sodium hydroxide was tested (**Figure 3**). Additional influence of the hydrolysis time (0.5, 1, 2, 3, 4 h) at 0.3 M sodium hydroxide was less than 20% (results not shown). Thus 1 h extraction with 0.3 M sodium hydroxide at 60 °C was chosen for the final method.

Subsequently the effects of omitting the alkaline hydrolysis from the method were evaluated. For flaxseed and bread, omitting the alkaline extraction from the analytical method decreased the lignan yield up to 80% (**Table 1**). For broccoli this was also the case, although to a smaller extent. For beverages extraction of lignans from the food matrix is not necessary. Therefore a milder, separate alkaline hydrolysis was used for tea. This hydrolysis was performed at 20 °C and 0.05 M sodium hydroxide. Omitting the alkaline hydrolysis did not affect the yield of secoisolariciresinol, lariciresinol, and pinoresinol, but 4-fold enhanced the yield of matairesinol. Thus analysis of tea was further performed without alkaline hydrolysis.

Table 1. Lignan Yield without Alkaline Hydrolysis Compared to the Method Including Alkaline Hydrolysis

	percentage yield[a]			
	lariciresinol	secoisolariciresinol	pinoresinol	matairesinol
flaxseed	51 ± 4	19 ± 2	46 ± 5	35 ± 1
broccoli	78 ± 10	84 ± 12	98 ± 5	
bread	55 ± 7	39 ± 2	49 ± 3	
tea	95 ± 1	99 ± 16	110 ± 4	420 ± 12

[a] Results are expressed as a percentage of the yield of the method including alkaline hydrolysis (average of duplicate analyses ± SD).

Enzymatic Hydrolysis. *Type of Enzyme.* Four different enzyme preparations, almond β-glucosidase, Rapidase LIQ$^+$, *H. pomatia* β-glucuronidase/sulfatase, and bovine liver glucuronidase were first tested on isolated secoisolariciresinol diglucoside (an alkyl β-glucoside) and pinoresinol diglucoside (an aryl β-glucoside). Rapidase LIQ$^+$ is a commercial enzyme preparation, used in the fruit juice industry. It has a broad range of enzyme activities, such as pectinase and cellulase, but no specified β-glucosidase activity. *H. pomatia* is also not recognized as a β-glucosidase, but it has been used before to liberate lignans (*31*, *32*), and isoflavone aglycones (*36*). The amount and hydrolysis conditions for each enzyme (**Table 2**) were based on enzyme specifications from the manufacturers or previous optimization with test products (results not shown).

Rapidase LIQ$^+$ and *H. pomatia* β-glucuronidase/sulfatase were able to convert both pinoresinol diglucoside and secoisolariciresinol diglucoside to their aglycones (**Table 2**). β-Glucosidase yielded the highest amount of pinoresinol aglycones, but only 13% of secoisolariciresinol aglycones compared to LIQ$^+$. β-Glucuronidase from bovine liver yielded no secoisolariciresinol aglycones and only 11% of pinoresinol aglycones compared to the maximum value obtained with β-glucosidase. Broad unspecified enzyme preparations also have the potential to break down lignan aglycones. Therefore their stability during enzymatic hydrolysis was tested. Recovery was compared to a standard incubated without any enzyme added. There was no lignan breakdown due to the different enzymes (recovery of aglycones 93−117% compared to the standard without enzyme; **Table 2**). The presence of lignans in the enzyme preparations was evaluated by incubation of the enzyme with sodium acetate buffer. None of the enzymes contained lignans except for β-glucosidase (from almonds) and Rapidase LIQ$^+$, which contained traces of lariciresinol.

Thus, both Rapidase LIQ$^+$ and *H. pomatia* were suitable for the hydrolysis of both types of glucosides. *H. pomatia* β-glucuronidase/sulfatase was chosen for further use, since this is standardized, easily available for use in a laboratory environment, and contained no lignans.

Enzymatic Hydrolysis. *Amount of Enzyme.* For the selected enzyme (*H. pomatia)* the amount required to hydrolyze all glycosides was determined for flaxseed, broccoli, and bread (**Figure 4**). If possible, extracts were already diluted before the enzymatic hydrolysis, to avoid signal suppression during MS detection. Flaxseed was diluted 10×, and broccoli was diluted 5×. Bread was analyzed undiluted. For flaxseed, the optimal yield of secoisolariciresinol was achieved at 500 units of

Table 2. Aglycone Yield from Secoisolariciresinol Diglucoside and Pinoresinol Diglucoside and Stability of Lignan Aglycones during Enzymatic Hydrolysis Conditions

		aglycone yield[a] (% of max value)		stability of lignan aglycones[b] (% compared to std without enzyme)			
enzyme	hydrolysis conditions	secoisolariciresinol	pinoresinol	lariciresinol	secoisolariciresinol	pinoresinol	matairesinol
H. pomatia β-glucuronidase/sulfatase	500 units,[c] 16 h, 37 °C	90 ± 5	94 ± 3	97 ± 3	93 ± 5	100 ± 7	101 ± 5
bovine liver β-glucuronidase	500 units,[c] 16 h, 37 °C	0 ± 0	12 ± 2	99 ± 3	97 ± 2	99 ± 1	100 ± 0
β-glucosidase from almonds	50 units,[d] 16 h, 37 °C	13 ± 0	100 ± 10	110 ± 13	105 ± 12	105 ± 1	102 ± 1
Rapidase LIQ+	200 μL, 3 h, 37 °C	100 ± 5	84 ± 8	117 ± 0	113 ± 3	98 ± 2	99 ± 2

[a] Results are expressed as percentages of the maximum value (average of two duplicate analyses ± SD). [b] Results are expressed as a percentage of standards without enzyme incubated for 16 h at 37 °C (averages of duplicate analyses ± SD). [c] Units β-glucuronidase. [d] Units β-glucosidase.

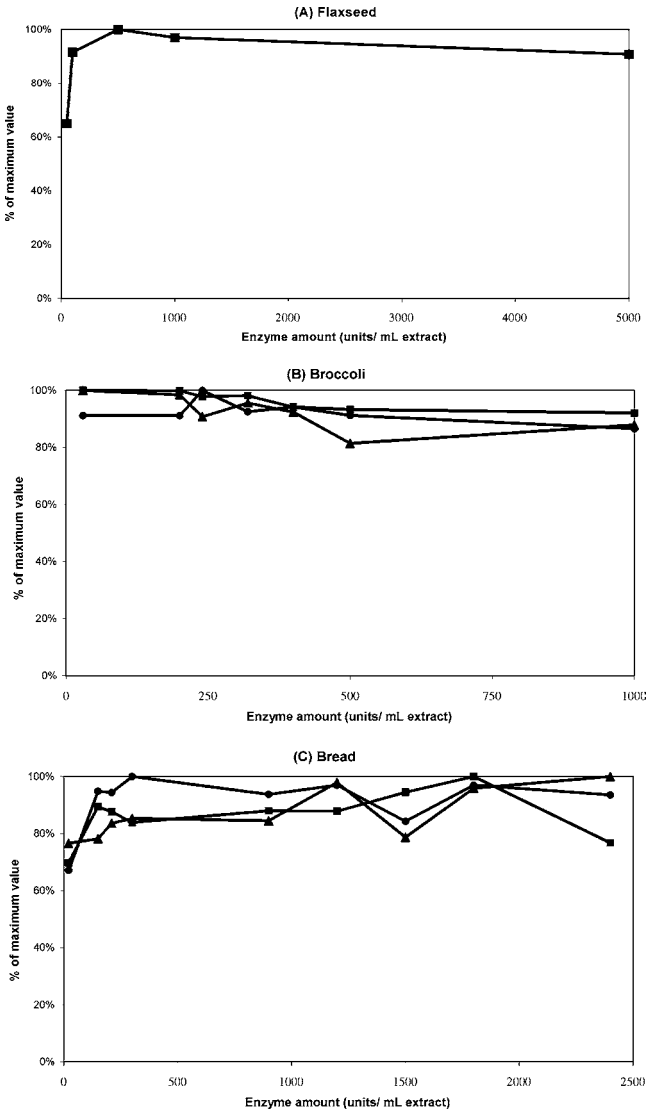

Figure 4. Influence of the enzyme amount (β-glucuronidase units/(mL of extract)) on yield of lariciresinol (■), secoisolariciresinol (●), and pinoresinol (▲) from (**A**) flaxseed, (**B**) broccoli, and (**C**) bread, expressed as a percentage of the maximum yield.

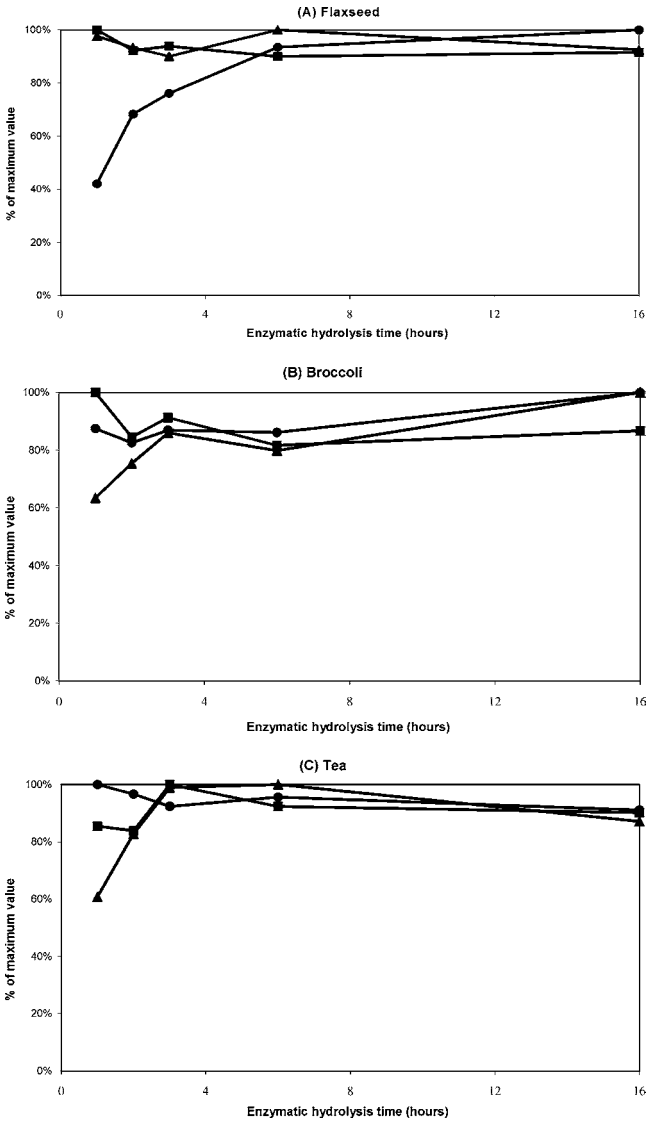

Figure 5. Influence of enzymatic hydrolysis time on yield of lariciresinol (■), secoisolariciresinol (●), and pinoresinol (▲) from (**A**) flaxseed, (**B**) broccoli, and (**C**) tea, expressed as a percentage of the maximum yield.

β-glucuronidase/(mL of extract). Only the results of secoisolariciresinol are shown, because it is present in high amounts compared to the other lignans, which already had a maximum yield at the lowest enzyme amount. Within the tested range, the influence of the enzyme amount was modest for bread and broccoli. No substantial increases in aglycone yield were observed after 500 units/(mL of extract). Thus 500 units of β-glucuronidase activity, corresponding to 0.83 mg of *H. pomatia*/(mL of extract), was chosen for the final method.

Enzymatic Hydrolysis. *Hydrolysis Time.* Subsequently the effect of the hydrolysis time was evaluated (**Figure 5**). For broccoli and tea yield of lariciresinol, secoisolariciresinol, and pinoresinol did not substantially change after 3 h, but for

flaxseed there was still a relatively large increase in secoisolariciresinol between 3 and 6 h. Enzymatic hydrolysis for 16 h was chosen for all products to ensure that optimum lignan yield was reached.

Chromtatographic and Detection Conditions. *Detection.* First we pursued a method with electrochemical detection. This was performed with a coulometric detector (Coularray detector, ESA Chelmsford, MA) set at 300, 350, 650, and 800 mV. However, the specificity proved not to be adequate, resulting in quite a number of interfering peaks. We tried several HPLC columns (with higher resolution than we used for MS detection), combined with several gradients and modifiers and/or simple cleanup procedures, but this did not improve the specificity enough to enable quantification of lignans in our test samples. Our aim was to develop a method with relatively simple sample preparation, and we thus concluded that this was not feasible with electrochemical detection. Therefore MS was chosen for the detection of the lignans. Both electrospray and APCI in the negative and positive mode were tested by infusion of pure enterolactone. Since APCI in the negative mode gave the highest sensitivity for enterolactone, this was also selected for quantification of the plant lignans. Cone voltages and collision energies were optimized using infusion of pure standards of the plant lignans. The most abundant product ions were chosen for multiple reaction monitoring (MRM) transitions. **Figure 2** shows chromatograms of the selected transitions for a typical analysis of tea. Addition of ammonium acetate to the mobile phase largely decreased the sensitivity (especially for pinoresinol), so only water and methanol were used as mobile phases. This did not influence the stability of the retention times. The desolvation gas flow was optimized by injection of standards of 200 ng/mL. Decreasing the desolvation gas flow from 500 to 100 L/h increased the sensitivity, *S*/*N* ratios, and signal stability.

Method Validation. Calibration and Quantification. In most cases, the slope of the calibration lines in the food matrix was larger than the slope of the calibration lines prepared in aqueous methanol, indicating that the control for matrix effects was not optimal. Especially for bread the increases were relatively large (up to 28%). Bread extracts were concentrated 3.3× after the alkaline extraction (methanol was evaporated from 1 mL of extract, until the residual volume was 0.3 mL) to increase the sensitivity of the method. However, concentration also increases the amount of food matrix in the extracts. Indeed the deviations increased when the bread extracts were more concentrated, especially for lariciresinol and pinoresinol. This can be explained by the fact that no deuterated standards for lariciresinol and pinoresinol were available, and corrections are based on secoisolariciresinol-d_8 and matairesinol-d_6. To further investigate this matrix effect, calibration lines were constructed in 3.3× concentrated, undiluted, or 2× diluted bread extracts. Indeed, deviations could be decreased by dilution of the bread extract. For 2× diluted bread extract, the maximum deviation of the slope was 11% (for lariciresinol).

Limits of Detection. The limits of detection ranged from 1.6 (lariciresinol) to 3.6 (pinoresinol) ng/mL (**Table 3**) corresponding to 4−10 μg/(100 g of dry matter) for solid foods and 0.2−0.4 μg/(100 mL) for beverages.

Recovery. The recoveries of lignans added to bread and tea are presented in **Table 4**. The recoveries of pinoresinol and lariciresinol were relatively high (106−123%). The recovery of secoisolariciresinol ranged from 73 to 82%. For matairesinol recovery from bread was low (51−55%), but from tea it was close to 100% (99−102%).

Table 3. Detection Limits[a] for Lignans, as Pure Standards, in Solid Foods and Beverages

compound	pure standards (ng/mL)	solid foods[b] (μg/100 g of DM)	beverages (μg/100 mL)
lariciresinol	1.6 ± 0.2	4 ± 0.5	0.2 ± 0.02
secoisolariciresinol	2.3 ± 0.5	6 ± 1.4	0.2 ± 0.06
pinoresinol	3.6 ± 0.1	10 ± 0.4	0.4 ± 0.01
matairesinol	2.4 ± 0.5	6 ± 1.2	0.3 ± 0.05

[a] Average of five triplicate measurements, ± SD. [b] Dry matter, products were freeze-dried and milled before the analysis.

Table 4. Recovery of Lignans Added to Bread and Tea[a]

	recovery[b] (%)			
	bread (n = 5)		tea (n = 4)	
compound	100% addition	200% addition	50% addition	100% addition
lariciresinol	123 ± 16	115 ± 18	118 ± 28	108 ± 33
secoisolariciresinol	82 ± 21	74 ± 6	73 ± 18	77 ± 28
pinoresinol	110 ± 6	119 ± 9	112 ± 25	106 ± 37
matairesinol[c]	55 ± 5	51 ± 5	99 ± 11	102 ± 16

[a] Lignan standards were added at approximately 100 and 200% of the value in bread and 50 and 100% of the value in tea. [b] Average of duplicate analyses ± SD. [c] Recoveries were calculated by adding two well-detectable amounts.

Precision. The within-run CV was calculated by averaging the CVs of duplicate analyses at 6 (broccoli), 7 (bread), or 4 (tea) separate days. The within-run CV ranged from 6.0% (pinoresinol in tea) to 20.8% (secoisolariciresinol in tea). The between-run variability was calculated after averaging the duplicate values from each day. The between-run CV ranged from 6.2% (lariciresinol in broccoli) to 32.5% (secoisolariciresinol in bread).

Stability. No significant differences were found between sample extracts analyzed directly and extracts analyzed after 9 days storage at −80 °C.

DISCUSSION

In this study we have optimized a quantitative LC-MS method for the four major enterolignan precursors lariciresinol, secoisolariciresinol, pinoresinol, and matairesinol. Previous methods were developed only for the quantification of secoisolariciresinol and matairesinol or for specific lignan-rich foods such as flaxseed.

Combining the methanolic extraction with alkaline hydrolysis proved to be an important step to increase the lignan yield. The largest effect was found for the extraction of secoisolariciresinol from flaxseed. When alkaline hydrolysis was omitted, the lignan yield decreased by 81%. This might be explained by the fact that SDG in flaxseed forms oligomers with 3-hydroxy-3-methylglutaric acid via ester bonds (*37*). These bonds are cleaved during the alkaline hydrolysis, thus releasing lignan glycosides from the oligomer. For bread, omitting the alkaline hydrolysis decreased the yield by 45−61%; for broccoli, the decrease was 2−22%. Possibly, lignan oligomers are also present in other products, and methods with only enzymatic hydrolysis are likely to underestimate the lignan contents of such foods. Alkaline hydrolysis of tea did not influence the yield of lariciresinol, secoisolariciresinol, and pinoresinol and largely decreased the yield of matairesinol. Probably lignans in tea are

already free, so it is not necessary or even detrimental (for matairesinol) to use alkaline hydrolysis.

Enzymatic hydrolysis of isolated lignans showed that β-glucosidase from almonds did not efficiently hydrolyze SDG (aglycone yield only 13% of the maximum value), which might explain the relatively low lignan values for methods that use only β-glucosidase (*30*). *H. pomatia* is not recognized as a β-glucosidase, but has been widely used to hydrolyze lignan (*31*, *32*) and isoflavone glycosides (*36*), and we showed that SDG and PDG were efficiently hydrolyzed. However, β-glucuronidase from bovine liver did not hydrolyze SDG, and only a small amount of PDG. So it is not clear whether β-glucuronidase or other enzyme activities from *H. pomatia* are responsible for the hydrolysis of lignan glucosides.

The sensitivity of the method is adequate for the analysis of a variety of foods with a wide range of lignan levels. The limits of detection of our method, 4−10 μg/(100 g of dry matter) for solid foods and 0.2−0.4 μg/(100 mL) for beverages, compare favorable with other published methods. Only slightly lower detection limits (2−3 μg/(100 g)) have been published for the measurement of secoisolariciresinol and matairesinol in foods using ID-GC-MS-SIM (*32*). Detection limits similar to those we found for fluids have been reported by Nurmi and co-workers (*33*) for coulometric electrode array detection of lignans in wine (0.24−1.2 μg/(100 mL)). For coulometric detection of matairesinol in flaxseed a higher detection limit of approximately 30 μg/(100 g) was found by Kraushofer and Sontag (*28*). Horn-Ross and co-workers (*30*) also reported a higher limit of detection of 25 μg/(100 g of food) for HPLC-APCI-MS.

The relatively high between- and within-run variation is the main limitation of the current method; however, this is not uncommon for quantification relatively close to the detection limits. The variation is largely determined by the matrix of the various products. Broccoli has relatively high lignan contents and could be analyzed after dilution, resulting in within- and between-run CVs that were all less than 15%. For bread, a product with denser matrix and lignan concentrations closer to the detection limits, within-run CVs ranged from 10.1 to 14.7% and between-run CVs from 17.8 to 32.5%. Tea lies more or less between, with within-run CVs ranging from 6.0 to 20.8% and between-run CVs from 9.2 to 18.5%. Horn-Ross et al. (*30*) published the only HPLC-APCI-MS method for the quantification of lignans in plant foods but unfortunately did not document its variability. For the analysis of lignans (including matairesinol) in plasma with HPLC-MS, within-run CVs of 6.1−19.7% were reported by Smeds and Hakala (*38*), for low- to high-concentration levels. Mazur and co-workers (*32*) reported within-run CVs of 4.6 to 6.6% and between-run CVs of 8.2 to 14.6% for GC-MS analysis of secoisolariciresinol and matairesinol in foods.

The slopes of the calibration lines nearly always increased when the matrix was present compared to the calibration lines prepared in aqueous methanol. This was especially the case for lariciresinol and pinoresinol. It can be explained by the fact that no deuterated standards were available for those lignans, and secoisolariciresinol-d_8 and matairesinol-d_6 had to be used for their quantification. To minimize this error, it would be advisable to dilute samples as much as possible, but in practice this is not feasible since dilution also causes loss of sensitivity. Thus all samples were first analyzed undiluted. Only if lignan values in the sample extracts exceeded 1000 ng/mL, samples were reanalyzed with dilution of the alkaline extract. For undiluted sample, the maximum deviation of the calibration line was 26% for lariciresinol in bread, which would lead to an overestimation of the lariciresinol content of 26%. However, by proper dilution overestimation can be reduced to 11%.

The recovery of lariciresinol and pinoresinol was relatively high (102−123%). This can be explained by the above-mentioned matrix effects. For secoisolariciresinol the recovery was approximately 70−80%. For matairesinol added to tea, recovery was approximately 100%, but for bread recovery was low (51−55%). This is probably caused by limited stability of matairesinol aglycones under alkaline conditions. However omitting the alkaline hydrolysis resulted in an even lower yield (**Table 1**). Thus, the yield of matairesinol after alkaline extraction seems to be the result of release from the food matrix on one hand and breakdown of lignan aglycones on the other hand. We decided to retain the alkaline hydrolysis in the analytical method. This will lead to underestimation of matairesinol, but matairesinol is usually present in relatively low amounts in foods, compared to secoisolariciresinol (*20*) and the other enterolignan precursors (unpublished results). If labeled standards for all lignans become available, it would be advisable to add those standards at the beginning of the analytical procedure. This would enable correction of recovery losses and decrease the problem of matrix interferences as well. We added deuterated standards just before the LC-MS analysis, because the stability of lariciresinol and pinoresinol during the analytical procedure differed from that of secoisolariciresinol-d_8 and matairesinol-d_6 (results not shown), and thus earlier addition would cause errors in correction.

In conclusion, combined methanolic extraction and alkaline hydrolysis followed by enzymatic hydrolysis with *H. pomatia* β-glucuronidase/sulfatase is an effective method for the release of lignan aglycones from different kinds of plant foods. Combining the extraction with alkaline hydrolysis was an important step to increase the lignan yield, although it also causes breakdown of matairesinol. The LC-MS/MS method allows very specific and sensitive quantification of all four major enterolignan precursors, secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol. However, the accuracy and precision of the measurement of lariciresinol and pinoresinol could probably be improved if labeled standards for these compounds were also available. This would also allow correction of lignan losses during the analytical procedure. An advantage that this method offers compared to existing techniques is the relatively simple sample preparation. Since it requires no derivatization, as for GC-MS, and specificity is improved compared to coulometric detection, extensive sample cleanup is not necessary.

ABBREVIATIONS USED

APCI, atmospheric pressure chemical ionization; CEAD, coulometric electrode array detection.

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