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Identification and Stereochemical Characterization of Lignans in Flaxseed and Pumpkin Seeds

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Phytoestrogens of the lignan type are widely distributed in plant-derived food items and are believed to protect against hormone-dependent cancer. The richest known dietary source of lignans is flaxseed. Flaxseed has been reported to contain glycosides of secoisolariciresinol as the major lignan, together with small amounts of matairesinol, isolariciresinol, and pinoresinol. Secoisolariciresinol, but none of the other lignans, has so far been identified in pumpkin seeds. In the present study, two different methods for the hydrolysis of lignan glycosides are compared. Artifact formation and loss of lignans under acidic conditions were observed. Lariciresinol was identified by GC−MS analysis in two different types of flaxseed (*Linum usitatissimum* L. and *Linum flavum* L.) and in pumpkin seeds (*Cucurbita pepo* L.) for the first time. Likewise, the novel lignan demethoxy-secoisolariciresinol was tentatively identified in the flaxseed samples. Stereochemical analysis by chiral HPLC of several lignans isolated from flaxseed showed that secoisolariciresinol, matairesinol, and lariciresinol consisted predominantly of one enantiomer.

KEYWORDS: Flaxseed; *Linum usitatissimum* L.; yellow flaxseed; *Linum flavum* L.; pumpkin seeds; *Cucurbita pepo* L.; lignan extraction; chiral HPLC; secoisolariciresinol; matairesinol; lariciresinol; isolariciresinol; pinoresinol

INTRODUCTION

Lignans are phytoestrogens occurring in various plant foods (*1*−*3*). Interest in lignans and other phytoestrogens has grown in recent years because of their putative beneficial health effects. Epidemiological data suggest that phytoestrogens protect against hormone-dependent tumors, e.g., breast and prostate cancer (*4*−*7*). Estrogenic and anti-estrogenic (*5*, *8*−*11*), anti-aromatase (*12*, *13*), and anti-oxidative (*14*−*17*) properties might be responsible for these effects. Flaxseed is known as the richest dietary source of lignans, with glycosides of secoisolariciresinol (**I**, **Figure 1**) and matairesinol (**II**) as the major compounds [370 mg/100 g and 1 mg/100 g, respectively (*18*)]. Significant amounts of secoisolariciresinol (21 mg/100 g of dry weight), but no other lignans, were found in pumpkin seeds (*5*).

After ingestion, the plant lignans secoisolariciresinol and matairesinol are deglycosylated and partly converted to the mammalian lignans enterodiol and enterolactone by colonic bacteria. Enterolactone, enterodiol, secoisolariciresinol, and matairesinol are absorbed, metabolized, and excreted in the urine in humans and animals (*19*−*23*). Recently, pinoresinol (**III**), lariciresinol (**IV**) (see **Figure 1**), 7-hydroxymatairesinol, syringaresinol, and arctigenin were identified as further precursors of mammalian lignans (*24*). Matairesinol, lariciresinol, and isolariciresinol (**V**, **Figure 1**) were also identified in human urine (*25*, *26*).

Different methods have been reported for the extraction of lignan glycosides from food and their subsequent hydrolysis. Mazur et al. (*18*) developed a method for the quantitative determination of phytoestrogens in plant foods using isotope dilution GC−MS analysis. Enzymatic hydrolysis with *Helix pomatia* homogenate, which was suitable for the hydrolysis of isoflavonoid but not lignan glycosides, was followed by an acid hydrolysis step to convert lignans to their aglycones. The instability of lignans during the acid hydrolysis step, resulting in the conversion of secoisolariciresinol to anhydrosecoisolariciresinol (**VI**, **Figure 1**) as an artifact, was described as a limitation of this method. To avoid the formation of artifacts, Obermeyer et al. (*27*) used an enzymatic (β-glucuronidase/sulfatase) hydrolysis step only, followed by solid-phase extraction with a C_{18} column. Secoisolariciresinol, but not matairesinol, was detected by HPLC−UV and HPLC−MS analysis. The same hydrolysis and extraction method was applied by Thompson et al. (*28*).

Johnsson et al. (*29*) reported a method for the isolation of secoisolariciresinol diglucoside from flaxseed. Following extraction of defatted flaxseed flour with 1,4-dioxane/95% ethanol (1:1, v/v), chemical hydrolysis with 0.3 M sodium hydroxide was used to liberate secoisolariciresinol diglucoside from the secoisolariciresinol diglucoside polymer in the plant material, which was later described as a straight-strain oligomer of five

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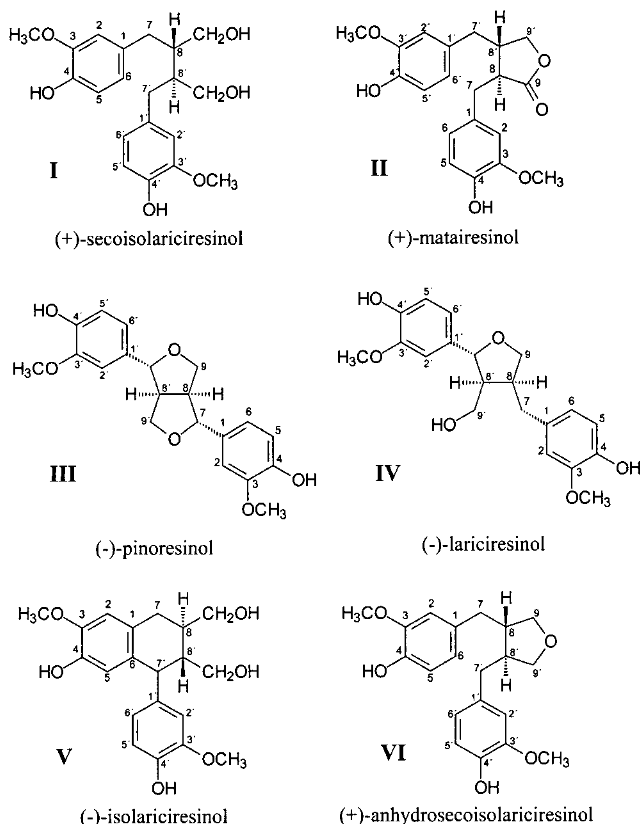

Figure 1. Chemical structures of plant lignans. Nomenclature according to Ayres and Loike (*17*).

secoisolariciresinol diglucoside molecules connected by three 3-hydroxy-3-methyl-glutaric acid molecules (*30*). Extraction with ethanol followed by base-catalyzed hydrolysis of the secoisolariciresinol diglucoside complex is also used in a U.S. patent for extracting lignans from flaxseed (*31*). Meagher et al. (*32*) used methanol/water (80:20, v/v) for the extraction of lignan glycosides, followed by glycoside hydrolysis with hydrochloric acid and extraction of the aglycones with ethyl acetate/*n*-hexane (1:1, v/v). In addition to secoisolariciresinol and matairesinol, isolariciresinol and pinoresinol were identified by GC−MS analysis as minor lignan compounds.

To our knowledge, no lignans other than secoisolariciresinol, matairesinol, isolariciresinol, and pinoresinol have been identified in flaxseed so far. Because pinoresinol and lariciresinol are biosynthetic precursors of secoisolariciresinol in *Forsythia intermedia*, *Linum flavum*, and other plants (*33*, *34*), it is assumed that lariciresinol is likely to be a precursor of secoisolariciresinol in *Linum usitatissimum* as well (*35*).

The first objective of our study was to identify further lignan compounds in two different types of flaxseed (*Linum usitatissimum* L. and *Linum flavum* L.) and in pumpkin seeds (*Cucurbita pepo* L.) by using both an enzymatic and acid hydrolysis method and GC−MS analysis. The second objective was to investigate the occurrence of lignan enantiomers by chiral HPLC separation. The biosynthesis of lignans takes place under regio- and stereochemical control because of certain glycoproteins, so-called "dirigent proteins". They do not have catalytic properties but bind radical monomers to bring them into the correct three-dimensional position for the coupling reaction (*36*−*38*). Bambagiotti-Alberti et al. (*39*) identified two isomers of secoisolariciresinol diglucoside isolated from flaxseed by reverse-phase HPLC on a C_{18} column, likely to be an enantiomeric (or the racemate) and the meso form. Ford et al. (*35*) investigated the biochemical pathway to secoisolariciresinol diglucoside hydroxymethyl glutaryl ester-linked lignan oligomers in flaxseed using radiolabeled lignan precursors. Two diastereomers of secoisolariciresinol diglucoside were separated by HPLC on a C_{18} column after base-catalyzed hydrolysis of an aqueous ethanol extract. After separate enzymatic hydrolysis of each secoisolariciresinol diglucoside isomer and chiral HPLC analysis of the aglycones, the peak resulting from the major secoisolariciresinol diglucoside isomer was identified as (+)-secoisolariciresinol, whereas the minor peak was (−)-secoisolariciresinol. The minor diastereomer of secoisolariciresinol diglucoside presumably originates from (−)-secoisolariciresinol. The ratio of the two secoisolariciresinol diglucoside diastereomers was 99:1.

MATERIALS AND METHODS

Chemicals and Materials. Flaxseed (*Linum usitatissimum* L., Linaceae), marketed by Kluth GmbH & Co. (Norderstedt, Germany); yellow flaxseed (*Linum flavum* L., Linaceae), Optimil Gesundkost GmbH (Köln, Germany); and pumpkin seeds (*Cucurbita pepo* L. *convar citrullina var. styriaca*, Cucurbitaceae), Kluth GmbH & Co. (Norderstedt, Germany), were purchased from a local supermarket. β-Glucosidase from almonds (3.2 U/mg) was purchased from Sigma (Taufkirchen, Germany), β-glucuronidase from *Helix pomatia* (4.8 U/mL) and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Fluka (Taufkirchen, Germany), and deuterated *N*,*O*-bis(trimethylsilyl)acetamide (d9-BSA) from C/D/N Isotopes Inc. (Quebec, Canada). (±)-Secoisolariciresinol and (±)-anhydrosecoisolariciresinol were kindly provided by M. Schöttner (University of Bayreuth, Bayreuth, Germany). (±)-Matairesinol was synthesized in our laboratory as reported previously (*40*). (±)-Lariciresinol was a gift from Dr. L. B. Davin (Washington State University, Pullman, WA). All other chemicals were purchased from Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), or BASF (Ludwigshafen, Germany) and were at least of analytical grade.

Extraction and Hydrolysis. About 10 g of flaxseed or pumpkin seeds was finely ground with a coffee grinder and defatted twice with 100 mL of *n*-hexane under magnetic stirring at 20 °C for 1 h (**Figure 2**). After vacuum filtration and air-drying, 5 g of the defatted material was extracted with 70 mL of ethanol/water (80:20, v/v) for 4 h at 55 °C in a shaking water bath. The ethanolic extract was filtered and concentrated to about 10 mL by rotary evaporation (45 °C, 100 mbar).

Lignan glycosides were hydrolyzed either with enzymes or with acid (**Figure 2**). Five hundred microliters of the concentrated extract was mixed with 10 mL of 0.15 M sodium acetate buffer (pH 5.0) and incubated with 250 μL of β-glucuronidase and 6 mg β-glucosidase for 14 h in a shaking water bath at 37 °C. For acid hydrolysis, 3 mL of the concentrated extract was incubated with 270 μL of concentrated HCl (final concentration 1 M) for 1 h at 95 °C in a shaking water bath, diluted with 4 mL of water, and centrifuged at 3000 rpm for 3 min.

The supernatant of this centrifugation or the incubation mixture of the enzymatic hydrolysis was extracted three times with 10 mL of ethyl acetate/*n*-hexane (1:1, v/v), and the organic phases were collected in a 100-mL round-bottomed flask. After each extraction, the samples were centrifuged at 4000 rpm and 4 °C to accelerate phase separation. The combined organic phases were first concentrated to about 3 mL by rotary evaporation (35 °C, 270 mbar) and then evaporated to dryness in an evaporation centrifuge under reduced pressure at 30 °C. The residues were dissolved in 300 μL of methanol for HPLC analysis.

HPLC Analysis. The hydrolyzed extracts were first analyzed by HPLC on a 250 × 4.6 mm i.d., 5-μm, Prodigy ODS(2) reverse-phase C_{18} column (Phenomenex, Aschaffenburg, Germany). Solvent A was water/methanol (84:16, v/v) adjusted to pH 2.8 with formic acid, and solvent B was 100% methanol. Gradient 1, used after enzymatic hydrolysis, started with isocratic elution at 25% B for 25 min, followed by a convex gradient to 40% B from 25 to 35 min. Gradient 2, used after acid hydrolysis, consisted of a linear gradient from 25% B to 50% B in 55 min. The flow rate was 0.9 mL/min for both gradient programs, and the UV detector was set to 283 nm.

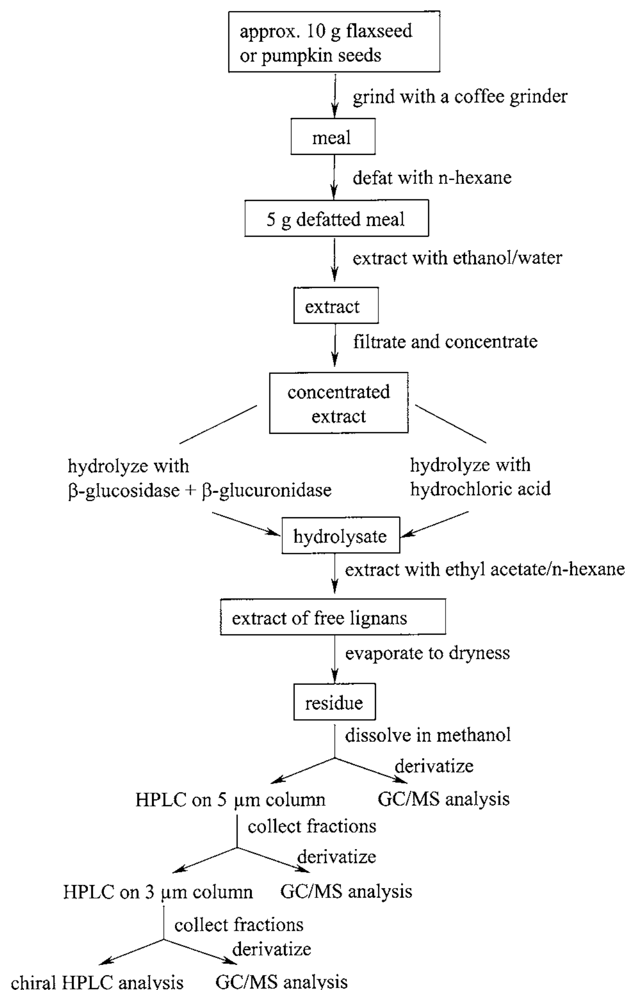

Figure 2. Isolation of lignans from flaxseed and pumpkin seeds.

The HPLC fractions collected on the 5-μm column were further analyzed on a 250 × 4 mm i.d., 3-μm, Prontosil reverse-phase C_{18} column (Bischoff, Leonberg, Germany). The mobile phase consisted of water with 0.05% formic acid (solvent A) and 100% acetonitrile (solvent B). For all lignan fractions except the isolariciresinol fraction, a linear gradient over 50 min from 20% B to 50% B was used with a flow rate of 0.8 mL/min. The isolariciresinol fraction was separated by isocratic elution with a solvent mixture of 80% A and 20% B. A diode array detector was used, and UV spectra were recorded from 200 to 400 nm.

Following HPLC on the 3-μm column, the separation of lignan enantiomers was carried out on a 250 × 4.6 mm i.d., 10-μm, Chiralcel OD chiral HPLC column (Daicel Chiral Technologies, Illkirch, France) with diode array detection. The mobile phase consisted of absolute ethanol (solvent A) and *n*-hexane (solvent B). All separations were achieved by isocratic elution with individual solvent ratios and flow rates for each lignan as follows : secoisolariciresinol and anhydrosecoisolariciresinol, 70% B, 0.5 mL/min; lariciresinol, 65% B, 0.5 mL/min; matairesinol, 85% B, 0.5 mL/min. The enantiomeric excess of enantiomers was calculated according to enantiomeric excess (%) = |% peak area (+)-enantiomer − % peak area (−)-enantiomer|.

GC−MS Analysis. The residues of the collected HPLC fractions were treated with 10 μL of BSTFA or d9-BSA overnight at 20 °C. The injection volume was 2 μL. The residues of the total extracts after hydrolysis were partially treated with 30 μL of BSTFA or d9-BSA, and 1 μL was injected. Analyses were carried out on a Finnigan GCQ ion-trap mass detector under the following conditions: 28.7 m × 0.25 mm i.d., 0.25-μm, MDN-5S fused silica capillary column (Supelco, Taufkirchen, Germany); flow of He, run pressure from 10 to 20 psi; column temperature program, 1 min isothermic at 60 °C, ramp from 60 to 250 °C at 30 °C/min, 10 min isothermic at 250 °C, ramp from 250 to 275 °C at 1 °C/min; injector temperature, ramp from 50 to 275 °C at 8 °C/s; transfer line temperature, 275 °C; ion source temperature, 225 °C; ionization energy, 70 eV. Full scan mass spectra (*m/z* 50−750) were recorded at a rate of two spectra per second. Individual lignans were detected by selecting characteristic ions from the full scan spectra using the Finnigan GCQ 2.31 data analysis software.

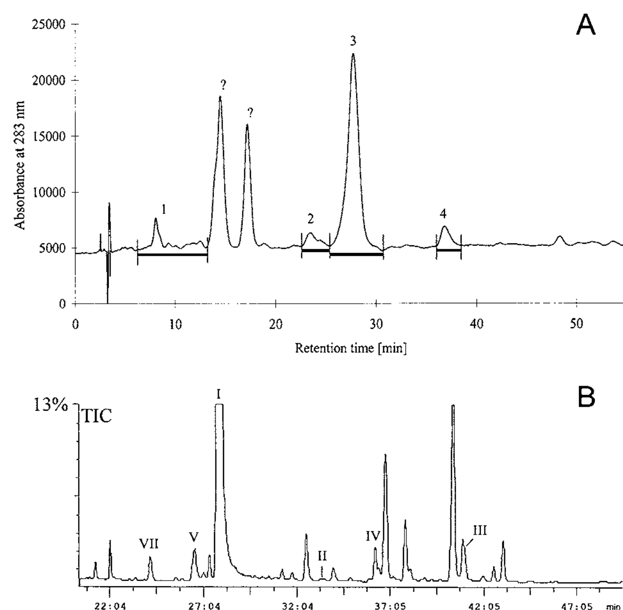

Figure 3. Extract from flaxseed after enzymatic hydrolysis. (A) HPLC analysis: peak 1, isolariciresinol; 2, lariciresinol; 3, secoisolariciresinol; 4, pinoresinol. (B) GC−MS analysis: peak **I**, secoisolariciresinol; **II**, matairesinol; **III**, pinoresinol; **IV**, lariciresinol; **V**, isolariciresinol; **VII**, demethoxy-secoisolariciresinol.

RESULTS AND DISCUSSION

For the isolation and analysis of lignans, the steps listed in **Figure 2** were carried out. Briefly, lignan glycosides were extracted from ground and defatted flaxseed with 80% aqueous ethanol and hydrolyzed either enzymatically with a mixture of β-glucosidase and β-glucuronidase or chemically with hydrochloric acid. The lignan aglycones were either separated by HPLC or directly analyzed by GC−MS after trimethylsilylation. The collected HPLC fractions were further analyzed by chiral HPLC or by GC−MS. Identification of the isolated lignans is based on a comparison of their retention times and mass spectra with those of authentic reference compounds (secoisolariciresinol, matairesinol, lariciresinol, anhydrosecoisolariciresinol) or with mass spectra reported in the literature. Trimethylsilylation with deuterated reagent gave rise to modified mass spectra that confirmed the assigned structures.

Isolation and Identification of Lignans from Flaxseed (*Linum usitatissimum* L.). When the lignan glycosides extracted from flaxseed were subjected to enzymatic hydrolysis and the aglycones were analyzed by HPLC, three major and three minor peaks were observed in the chromatogram (**Figure 3A**). Fractions 1−4 were collected as indicated in **Figure 3A**, trimethylsilylated, and analyzed by GC−MS. The two large HPLC peaks eluted between fractions 1 and 2 were also analyzed by GC−MS, but the mass spectra were not characteristic for lignans.

HPLC fraction 1 contained a lignan tentatively identified as isolariciresinol. No reference compound was available, but the mass spectrum of its TMS derivative (**Figure 4**) exhibited the same characteristic ions at *m/z* 648, 558, 527, 468, 455, 437,

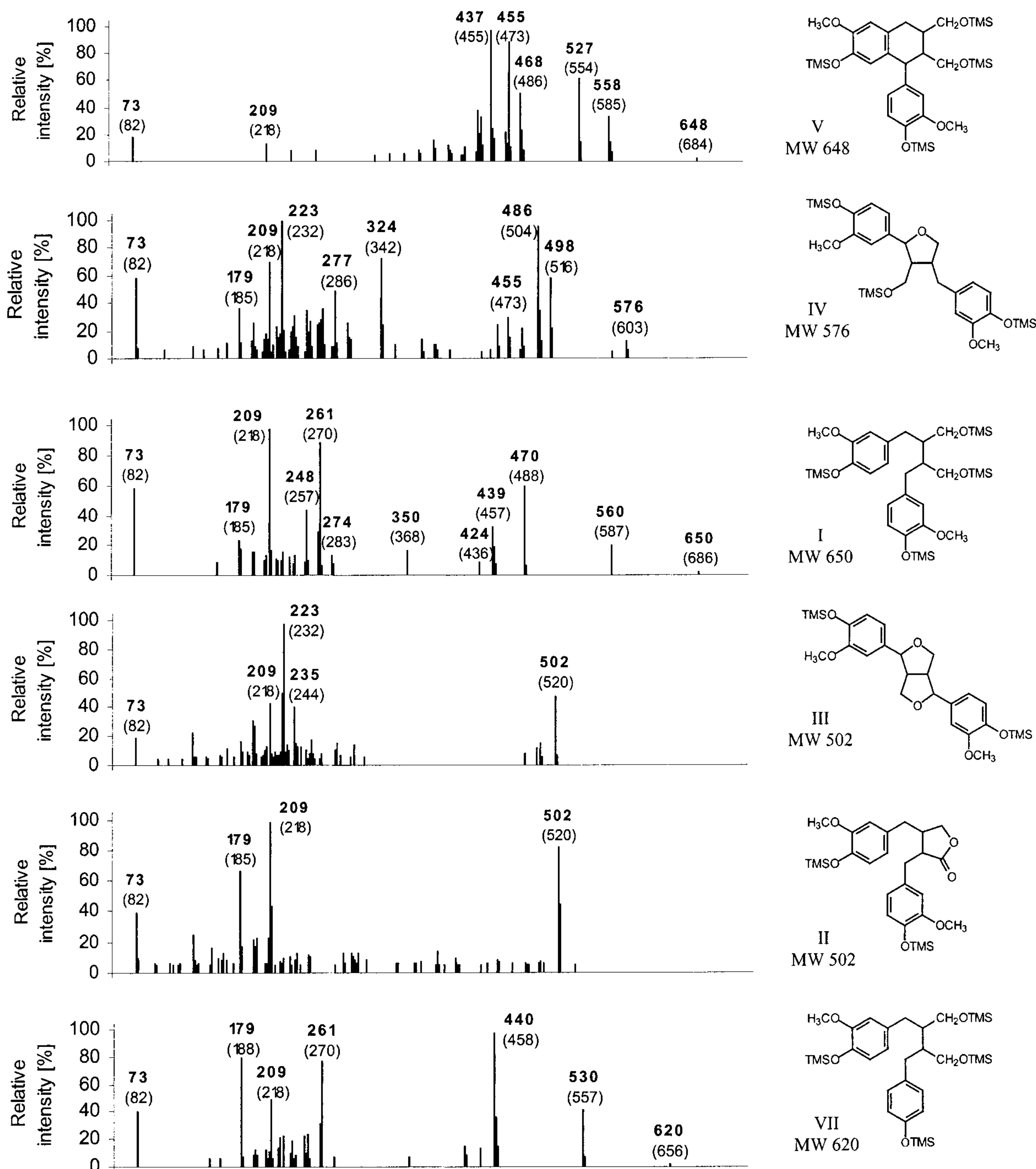

Figure 4. Mass spectra (TMS derivatives) of lignans present in flaxseed and identified by GC–MS after enzymatic hydrolysis. The figures in parentheses represent the corresponding *m/z* values after trimethylsilylation with d9-BSA. **I**, secoisolariciresinol; **II**, matairesinol; **III**, pinoresinol; **IV**, lariciresinol; **V**, isolariciresinol; **VII**, demethoxy-secoisolariciresinol.

424, and 209 as reported in the literature (*26*, *32*, *41*). The shift of the ion masses caused by deuterated TMS groups (**Figure 4**) was consistent with the presence of four hydroxyl groups.

Lariciresinol, a lignan not previously identified in flaxseed, was detected in HPLC fraction 2. Characteristic ions in the mass spectrum of its TMS derivative (**Figure 4**) are at *m/z* 576, 498, 486, 455, 324, 277, 223, 209, and 179. The same fragment ions of lariciresinol were also described by Bannwart et al. (*26*) and Ekman (*41*). Authentic lariciresinol was at hand as a reference compound and had an identical GC retention time and mass spectrum.

HPLC fraction 3 contained the most intense peak, which was identified as secoisolariciresinol by its mass spectrum (**Figure 4**), with ions at *m/z* 650, 560, 470, 439, 424, 350, 274, 261, 248, 209, and 179. The same mass spectrum was also observed for the reference compound.

In HPLC fraction 4, pinoresinol was detected by GC−MS analysis. The mass spectrum (**Figure 4**) exhibited the same ions at *m/z* 502, 235, 223, and 209 as reported by Meagher et al. (*32*) and Ekman (*41*).

The lignans isolariciresinol, lariciresinol, secoisolariciresinol, and pinoresinol identified in HPLC fractions 1−4 were also detected by GC−MS analysis of the complete extract (**Figure 3B**). Secoisolariciresinol represented the largest peak, but isolariciresinol, lariciresinol, and pinoresinol were also clearly detectable in the profile of the total ion current (TIC). Two additional lignans could be identified in the complete extract, viz., matairesinol by its mass spectrum (**Figure 4**) and com-

parison with an authentic reference compound and a novel compound tentatively assigned the structure of secoisolariciresinol lacking one methoxy group (demethoxy-secoisolariciresinol, **VII**) because of its mass spectrum (**Figure 4**). The mass spectra of all lignans containing the butanediol moiety, e.g., isolariciresinol, secoisolariciresinol, and demethoxy-secoisolariciresinol, are characterized by the consecutive loss of two TMSOH moieties (90 mass units each); this fragmentation occurs only once in lariciresinol and not at all in matairesinol or pinoresinol (**Figure 4**). Ions with *m/z* 179 and 209 are fragment ions formed through benzylic cleavage and containing one aromatic ring and one hydroxy group (*m/z* 179) or one hydroxy and one methoxy group (*m/z* 219).

When the collected HPLC fractions were rechromatographed by HPLC using a C_{18} column with 3-μm particle size, it was noted that fraction 2 contained isolariciresinol in addition to lariciresinol. This was confirmed by GC−MS analysis and suggests a conversion of lariciresinol to isolariciresinol. Therefore, it is not clear at this time whether isolariciresinol in fraction 1 is an authentic compound extracted from flaxseed or an artifact due to the instability of lariciresinol. The instability of lariciresinol under acidic conditions was also described by Haworth and Kelly (*42*). Moreover, the conversion of lariciresinol into isolariciresinol during HPLC fractionation was observed in studies on the in vitro metabolism of secoisolariciresinol with liver microsomes, where lariciresinol is one of the major metabolites of secoisolariciresinol (unpublished data).

HPLC fraction 3, containing secoisolariciresinol, could be separated into one major and one minor peak on a C_{18} column with 3-μm particle size. Both peaks were identified as secoisolariciresinol by GC−MS, and are possibly two diastereomers as reported by Bambagiotti et al. (*39*).

In a control experiment, water instead of the flaxseed extract was subjected to the enzymatic hydrolysis and extraction procedures. When the derivatized extract was analyzed by GC−MS, only traces of secoisolariciresinol were detected, which could have originated from the hydrolytic enzymes. Incubation of authentic secoisolariciresinol with the hydrolytic enzymes did not cause the formation of other products. In summary, our studies of the enzymatically hydrolyzed flaxseed extract have confirmed the presence of secoisolariciresinol, matairesinol, and pinoresinol in flaxseed and, for the first time, identified lariciresinol and demethoxy-secoisolariciresinol. Isolariciresinol, which was detected ealier by Meagher et al. (*32*), is generated from lariciresinol as an artifact under acidic HPLC conditions. Its presence in flaxseed could not be clearly determined.

When the acid-hydrolyzed flaxseed extract was analyzed by HPLC, a large amount of polar material was eluted during the first 10 min, followed by four major and several minor peaks (**Figure 5A**). HPLC fractions 1−6 were collected as indicated in **Figure 5A** and analyzed by GC−MS after derivatization. Isolariciresinol was detected mainly in HPLC fraction 1 and only in traces in fraction 2, whereas secoisolariciresinol represented the major lignan and was identified in fraction 3. HPLC fractions 4 and 5 contained matairesinol and pinoresinol, respectively. In fraction 6, a major lignan was tentatively identified as anhydrosecoisolariciresinol (**VI**) on the basis of its mass spectrum (**Figure 6**), which suggested the presence of two aromatic hydroxyl groups but no butanediol structure because of the lack of TMSOH elimination. The identification of anhydrosecoisolariciresinol was corroborated by the mass spectrum of the authentic reference compound. Dehydration of secoisolariciresinol to anhydrosecoisolariciresinol under acidic conditions has been reported previously (*18*, *32*). Furthermore,

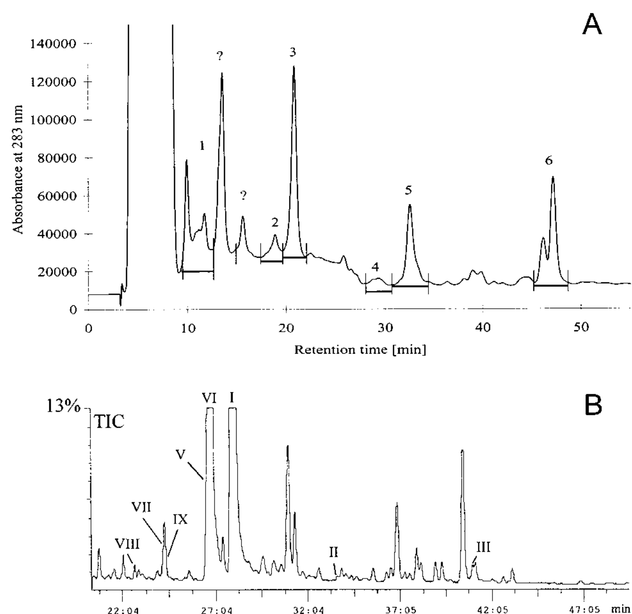

Figure 5. Extract from flaxseed after acid hydrolysis. (A) HPLC analysis: peak 1, isolariciresinol; 2, lariciresinol; 3, secoisolariciresinol; 4, matairesinol; 5, pinoresinol; 6, anhydrosecoisolariciresinol. (B) GC–MS analysis: peak **I**, secoisolariciresinol; **II**, matairesinol; **III**, pinoresinol; **V**, isolariciresinol; **VI**, anhydrosecoisolariciresinol; **VII**, demethoxy-secoisolariciresinol; **VIII**, demethoxy-anhydrosecoisolariciresinol; **IX**, demethoxy-isolariciresinol.

isolariciresinol detected in fraction 1 is likely to be generated from lariciresinol as an artifact during acid hydrolysis. Traces of isolariciresinol detected in HPLC fraction 2 could derive from a small amount of lariciresinol in this fraction that is converted into isolariciresinol during fractionation. As discussed earlier, the authenticity of isolariciresinol in flaxseed is not clear.

When the complete acid-hydrolyzed extract was analyzed by GC−MS, the most intense peaks were identified as secoisolariciresinol and anhydrosecoisolariciresinol, whereas matairesinol and pinoresinol were detectable only as minor peaks (**Figure 5B**). The search for characteristic ions clearly confirmed the presence of matairesinol (*m/z* 502, 209, and 179) and pinoresinol (*m/z* 502, 235, and 223). Likewise, isolariciresinol (*m/z* 558, 527, and 455) and demethoxy-secoisolariciresinol, (*m/z* 620, 530, and 440) were detected, both of which coeluted with other substances of the complete extract. Lariciresinol, which had been identified in the enzyme-hydrolyzed extract, was found neither in the complete acid-hydrolyzed extract nor in the collected HPLC fractions. This might be explained by the above-mentioned instability of lariciresinol, which might lead to a complete conversion of lariciresinol into isolariciresinol under the acid hydrolysis conditions. However, when small amounts of authentic lariciresinol were subjected to acid hydrolysis, neither lariciresinol nor isolariciresinol was detected.

Two other compounds with lignan-type fragmentation patterns were detected in the complete extract by GC−MS analysis. They were tentatively identified as anhydrosecoisolariciresinol and isolariciresinol lacking one methoxy group (demethoxy-anhydrosecoisolariciresinol, **VIII**, and demethoxy-isolariciresinol, **IX**) on the basis of their mass spectra (**Figure 6**). The mass spectra of demethoxy-anhydrosecoisolariciresinol and demethoxy-isolariciresinol are very similar to those of anhydrosecoisolariciresinol and isolariciresinol, respectively (**Figure 4**), except for the decrease of the molecular ions by 30 mass units as a result of the lack of a methoxy group. In the control experiment without flaxseed extract, no lignans were detected by GC−MS analysis after acid hydrolysis.

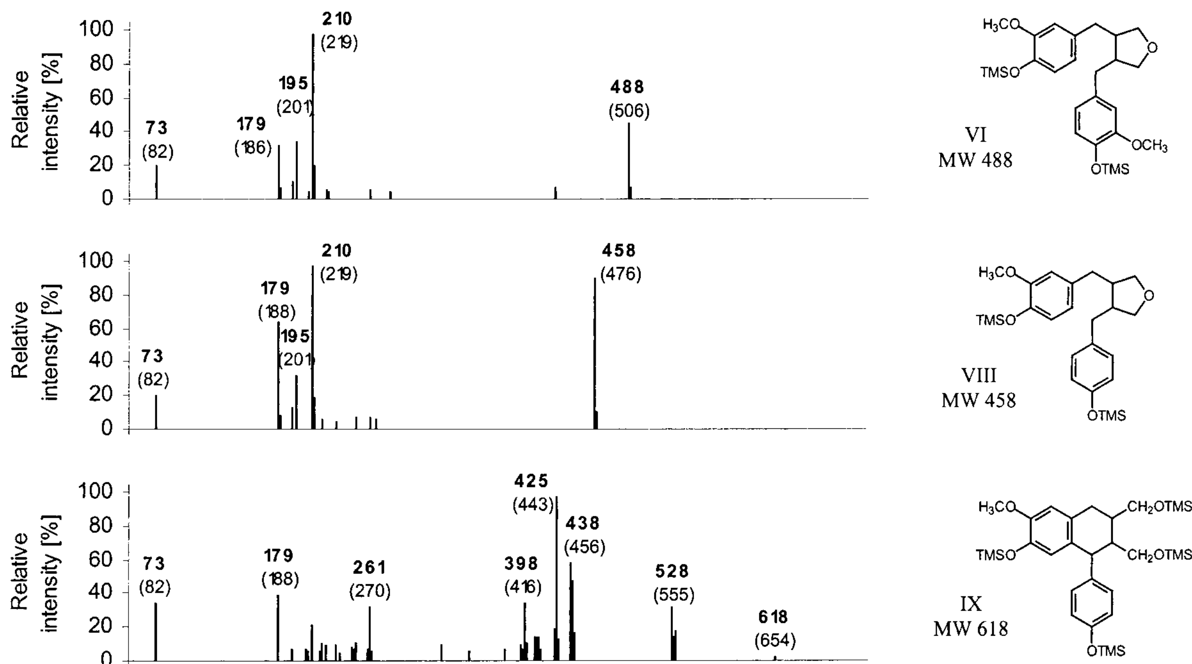

Figure 6. Mass spectra (TMS derivatives) of lignans present in flaxseed and identified by GC–MS after acid hydrolysis. The figures in parentheses represent the corresponding *m/z* values after trimethylsilylation with d9-BSA. **VI**, anhydrosecoisolariciresinol; **VIII**, demethoxy-anhydrosecoisolariciresinol; **IX**, demethoxy-isolariciresinol.

Comparing the results from enzymatic and acid hydrolysis of the flaxseed extract, secoisolariciresinol, matairesinol, pinoresinol, and isolariciresinol, as well as demethoxy-secoisolariciresinol, were found after both types of hydrolysis. Lariciresinol was detectable only after enzymatic hydrolysis, apparently because of its instability under acidic conditions, which might cause the formation of isolariciresinol. Anhydrosecoisolariciresinol and demethoxy-anhydrosecoisolariciresinol are assumed to be artifacts generated from secoisolariciresinol and demethoxy-secoisolariciresinol, respectively, during acid hydrolysis. Indeed, authentic secoisolariciresinol incubated under the conditions of acid hydrolysis gave rise to the formation of anhydrosecoisolariciresinol. Demethoxy-isolariciresinol might be liberated from its glycoside only upon acid hydrolysis, or it might be an artifact formed from another, yet unknown substance. Overall, the enzymatic hydrolysis is preferable because of the lower level of artifact formation.

Isolation and Identification of Lignans from Yellow Flaxseed *(Linum flavum* L.). A study of the lignans present in yellow flaxseed was conducted in the same way as described for flaxseed and gave very similar results. The same major lignan peaks as observed for flaxseed were found in the HPLC analysis after both enzymatic and acid hydrolyses, and the same lignans were identified by GC−MS in the collected HPLC fractions, with the exception of pinoresinol, which was not detected in yellow flaxseed. When the fraction containing secoisolariciresinol was further separated on a 3-μm HPLC column, again one major and one minor secoisolariciresinol peak were detected.

Isolation and Identification of Lignans in Pumpkin Seeds (*Cucurbita pepo* L.). Pumpkin seeds are a known source of secoisolariciresinol (*5*). For the identification of other lignans, the same extraction and hydrolysis methods were used as described for flaxseed. Because no clear secoisolariciresinol peak was detectable by HPLC analysis of the enzyme-hydrolyzed pumpkin seed extract, no fractionation was carried out. GC−MS analysis of the complete extract revealed secoisolariciresinol as the major lignan compound, but traces of lariciresinol were also detected. After acid hydrolysis, anhydrosecoisolariciresinol, but neither secoisolariciresinol nor lariciresinol, was detected by GC−MS analysis of the complete pumpkin seed extract. Obviously, secoisolariciresinol is completely converted to anhydrosecoisolariciresinol under these hydrolysis conditions and at the much lower concentrations compared to flaxseed. Likewise, lariciresinol is not stable under these conditions, as expected from the flaxseed studies. These results again indicate that the enzymatic hydrolysis of lignan glycosides is preferable to acidic hydrolysis, especially for lignans occurring only in trace amounts. For the first time, lariciresinol was identified as a second lignan in pumpkin seeds, but only in trace amounts.

Stereochemistry of Flaxseed Lignans. For an investigation of their stereochemistry, the lignans secoisolariciresinol, anhydrosecoisolariciresinol, matairesinol, and lariciresinol were isolated from flaxseed and purified by HPLC. The purified lignans were separated into their enantiomers by HPLC on a chiral column and compared with racemic reference compounds. Secoisolariciresinol was isolated after both enzymatic and acid hydrolysis, whereas anhydrosecoisolariciresinol and matairesinol were collected after acid hydrolysis and lariciresinol after enzymatic hydrolysis only. From yellow flaxseed, only secoisolariciresinol isolated after enzymatic and acid hydrolysis was analyzed by chiral HPLC.

The (±)-secoisolariciresinol reference compound was separated into the (+)- and (−)-enantiomers, with the first peak relating to (−)-secoisolariciresinol and the second peak to (+)-secoisolariciresinol (**Figure 7A**). The peaks were assigned according to literature data (*43*). Secoisolariciresinol isolated from both flaxseed types was chromatographed on the 3-μm column, and the major secoisolariciresinol peak was collected. This peak was separated by chiral HPLC into one major peak of (+)-secoisolariciresinol and one minor peak of (−)-secoisolariciresinol (**Figure 7B**). GC−MS analysis of the (+)- and (−)-enantiomers from chiral HPLC separation confirmed that both peaks have the mass spectrum of secoisolariciresinol. These results suggest that the biosynthesis of secoisolariciresinol in flaxseed and in yellow flaxseed is not completely stereoselective.

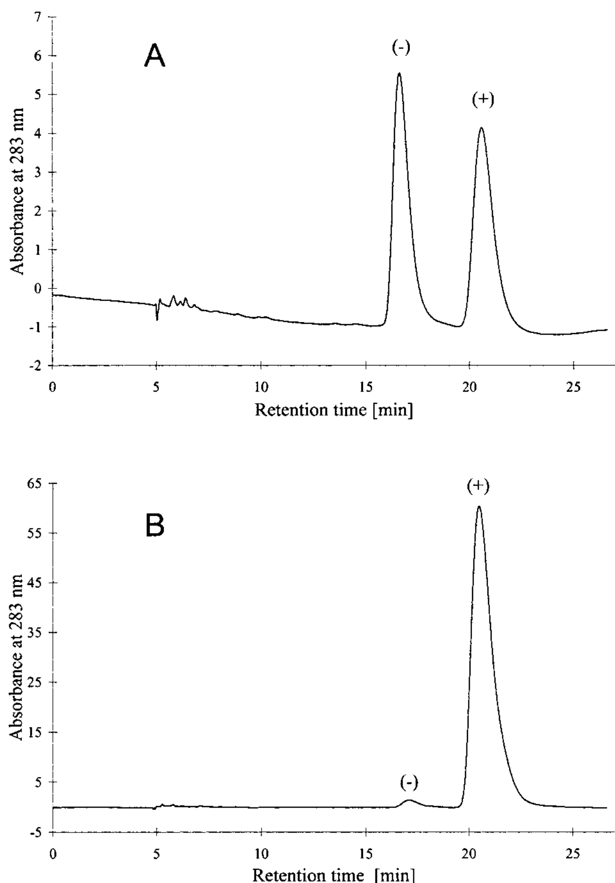

Figure 7. Chiral HPLC analysis of (A) (±)-secoisolariciresinol reference compound and (B) secoisolariciresinol isolated from flaxseed.

The enantiomeric excess of the (+)-enantiomer was determined as approximately 97% for flaxseed and 91% for yellow flaxseed. The results from yellow flaxseed were unexpected, as Xia et al. (*34*) describe the biosynthesis of only the (−)-enantiomer in cell-free extracts from *Linum flavum* roots.

The reference compound (±)-anhydrosecoisolariciresinol could also be separated into its two enantiomers by chiral HPLC. Chiral analysis of anhydrosecoisolariciresinol from the flaxseed extract resulted in one major enantiomer with an enantiomeric excess of approximately 96%. This ratio of enantiomers is about the same as for secoisolariciresinol from flaxseed and consistent with the proposal that anhydrosecoisolariciresinol is formed from secoisolariciresinol as an artifact, although no information is available on the elution order of (+)- and (−)-anhydrosecoisolariciresinol.

From the biosynthetic point of view, it is expected that the major enantiomers of matairesinol and lariciresinol are (+)-matairesinol and (−)-lariciresinol (*33*). The racemic reference compounds could not be completely separated, and the elution order of the enantiomers is unknown. Chiral HPLC analysis of matairesinol from flaxseed revealed only one peak, whereas lariciresinol from flaxseed was separated into one major and one minor peak. However, the minor lariciresinol peak had a retention time different from that of the reference compound and was identified by GC−MS as isolariciresinol, presumably formed from lariciresinol as described above. A small peak with the same retention time that was identified as isolariciresinol by GC−MS was also present in the (±)-lariciresinol reference compound. Therefore, our data suggest that one enantiomer of each matairesinol and lariciresinol occurs in large excess, but its stereochemistry could not be clarified. Moreover, the presence of small amounts of the other enantiomers of matairesinol and lariciresinol in flaxseed cannot be ruled out because of the incomplete chromatographic separation and the very small amounts of these lignans available for chiral analysis.

ABBREVIATIONS

BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; d9-BSA, deuterated *N*,*O*-bis(trimethylsilyl)acetamide; GC−MS, gas chromatography−mass spectrometry; HPLC, high-performance liquid chromatography; ODS, octadecylsilyl; RP, reverse-phase; TIC, total ion current; TMS, trimethylsilyl.

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LITERATURE CITED

(1) Mazur, W. Phytoestrogen content in food. *Baillière's Clin. Endocrin. Metab.* **1998**, *12*, 729−742.
(2) Mazur, W. M.; Wähälä, K.; Rasku, S.; Salakka, A.; Hase, T.; Adlercreutz, H. Lignan and isoflavonoid concentrations in tea and coffee. *Br. J. Nutr*. **1998**, *79*, 37−45.
(3) Mazur, W. M.; Uehara, M.; Wähälä, K.; Adlercreutz, H. Phytoestrogen content of berries, and plasma concentrations and urinary excretion of enterolactone after a single strawberry-meal in human subjects. *Br. J. Nutr*. **2000**, *83*, 381−387.
(4) Sung, M. K.; Lautens, M.; Thompson, L. U. Mammalian lignans inhibit the growth of estrogen-independent human colon tumor cells. *Anticancer Res*. **1996**, *18*, 1405−8.
(5) Adlercreutz, H.; Mazur, W. Phyto-oestrogens and western diseases. *Ann. Med*. **1997**, *29*, 95−120.
(6) Ingram, D.; Sanders, K.; Kolybaba, M.; Lopez, D. Case-control study of phyto-estrogens and breast cancer. *Lancet* **1997**, *350*, 990−994.
(7) Adlercreutz, H.; Mazur, W.; Bartels, P.; Elomaa, V.-V.; Watanabe, S.; Wähälä, K.; Landström, M.; Lundin, E.; Bergh, A.; Damber, J.-E.; Åman, P.; Widmark, A.; Johansson, A.; Zhang, J.-X.; Hallmans, G. Phytoestrogens and prostate disease. *J. Nutr.* **2000**, *130*, 658S−659S.
(8) Welshons, W. V.; Murphy, C. S.; Koch, R.; Calaf, G.; Jordan, V. C. Stimulation of breast cancer cells in vitro by the environmental estrogen enterolactone and the phytoestrogen equol. *Breast Cancer Res. Treat.* **1987**, *10*, 169−175.
(9) Adlercreutz, H.; Mousavi, Y.; Clark, J.; Höckerstedt, K.; Hämäläinen, E.; Wähälä, K.; Mäkelä, T.; Hase, T. Dietary phytoestrogens and cancer: in vitro and in vivo studies. *J. Steroid Biochem. Mol. Biol.* **1992**, *41*, 331−337.
(10) Mousavi, Y.; Adlercreutz, H. Enterolactone and estradiol inhibit each other's proliferative effect on MCF-7 breast cancer cell lines in culture. *J. Steroid Biochem. Mol. Biol.* **1992**, *41*, 615−619.
(11) Rickard, S. E.; Thompson, L. U. Phytoestrogens and lignans: effects on reproduction and chronic disease. In *Antinutrients and Phytochemicals in Food*; Shahidi, F., Ed.; American Chemical Society: Washington, DC, 1997; pp 273−296.
(12) Adlercreutz, H.; Bannwart, C.; Wähälä, K.; Mäkelä, T.; Brunow, G.; Hase, T.; Arosemena, P. J.; Kellis, J., Jr.; Vickery, L. E. Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens. *J. Steroid Biochem. Mol. Biol.* **1993**, *44*, 147−153.
(13) Wang, C.; Mäkelä, T.; Hase, T.; Adlercreutz, H.; Kurzer, M. S. Lignans and flavonoids inhibit aromatase enzyme in human. *J. Steroid Biochem. Mol. Biol*. **1994**, *50*, 205−212.
(14) Kitts, D. D.; Yuan, Y. V.; Wijewickreme, A. N.; Thompson, L. U. Antioxidant activity of the flaxseed lignan secoisolariciresinol diglycoside and its mammalian lignan metabolites enterodiol and enterolactone. *Mol. Cell. Biochem*. **1999**, *202*, 91−100.

(15) Harper, A.; Kerr, D. J.; Gescher, A.; Chipman, K. J. Antioxidant effects of isoflavones and lignans, and protection against DNA oxidation. *Free Radical Res.* **1999**, *131*, 149−160.

(16) Niemeyer, H. B.; Metzler, M. Antioxidant activities of lignans in the FRAP assay (ferric reducing antioxidant power assay). In *Biologically-active Phytochemical in Food*—*Analysis*, *Metabolism*, *Bioavailability and Function*; Pfannhauser, W., Fenwick, G. R., Khokhar, S., Eds.; Royal Society of Chemistry: Cambridge, U.K., 2001; pp 394−395.

(17) Ayres, D. C.; Loike, J. D. Introduction. In *Lignans: Chemical*, *Biological and Clinical Properties*; Phillipson, J. D., Ayres, D. C., Baxter, H., Eds.; Cambridge University Press: Cambridge, U.K., 1990; pp 1−11.

(18) Mazur, W. M.; Fotsis, T.; Wähälä, K.; Ojala, S.; Salakka, A.; Adlercreutz, H. Isotope dilution gas chromatographic−mass spectrometric method for the determination of isoflavonoids, coumestrol and lignans in food samples. *Anal. Biochem*. **1996**, *233*, 169−180.

(19) Axelson, M.; Setchell, K. D. R. Conjugation of lignans in human urine. *FEBS Lett*. **1980**, *122*, 49−53.

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

(21) Stitch, S. R.; Touba, J. K.; Groen, M. B.; Funke, C. W.; Leemhuis, J.; Vink, J.; Woods, G. F. Excretion, isolation and structure of a new phenolic constituent of female urine. *Nature* **1980**, *287*, 738−740.

(22) Axelson, M.; Setchell, K. D. R. The excretion of lignans in rats—Evidence for an intestinal bacterial source for this new group of compounds. *FEBS Lett*. **1981**, *123*, 337−342.

(23) Boriello, S. P.; Setchell, K. D. R.; Axelson, M.; Lawson, A. M. Production and metabolism of lignans by the human fecal flora. *J. Appl. Bacteriol*. **1985**, *58*, 37−43.

(24) Heinonen, S.; Nurmi, T.; Liukkonen, K.; Poutanen, K.; Wähälä, K.; Deyama, T.; Nishibe, S.; Adlercreutz, H. In vitro metabolism of plant lignans: New precursors of mammalian lignans enterolactone and enterodiol. *J. Agric. Food Chem*. **2001**, *49*, 3178−3186.

(25) Bannwart, C.; Adlercreutz, H.; Fotsis, T.; Wähälä, K.; Hase, T.; Brunow, G. Identification of *O*-desmethyl-angolensin, a metabolite of daidzein, and of matairesinol, one likely plant precursor of the animal lignan enterolactone, in human urine. *Finn. Chem. Lett*. **1984**, *4*−*5*, 120−125.

(26) Bannwart, C.; Adlercreutz, H.; Wähälä, K.; Brunow, G.; Hase, T. Detection and identification of the plant lignans lariciresinol, isolariciresinol and secoisolariciresinol in human urine. *Clin. Chim. Acta* **1989**, *180*, 293−302.

(27) Obermeyer, W. R.; Musser, S. M.; Betz, J. M.; Casey, R. E.; Pohland, A. E.; Page, S. W. Chemical studies of phytoestrogens and related compounds in dietary supplements: Flax and chaparral. *Proc. Soc. Exp. Biol. Med*. **1995**, *208*, 6−12.

(28) Thompson, L. U.; Rickard, S. E.; Cheung, F.; Kenaschuk, E. O.; Obermeyer, W. R. Variability in anticancer lignan levels in flaxseed. *Nutr. Cancer* **1997**, *27*, 26−30.

(29) Johnsson, P.; Kamal-Eldin, A.; Lundgren, L. N.; Åman, P. HPLC method for analysis of secoisolariciresinol diglucoside in flaxseeds. *J. Agric. Food Chem*. **2000**, *46*, 5216−5219.

(30) Kamal-Eldin, A.; Peerlkamp, N.; Johnsson, P.; Andersson, R.; Andersson, R. E.; Lundgren L. N.; Åman, P. An oligomer from flaxseed composed of secoisolariciresinoldiglucoside and 3-hydroxy-3-methyl glutaric acid residues. *Phytochemistry* **2001**, *58*, 587−590.

(31) Westcott, N. D.; Muir, A. D. Process for extracting lignans from flaxseed. U.S. Patent 5,705,618, 1995.

(32) Meagher, L. P.; Beecher, G. R.; Flanagan, V. P.; Li, B. W. Isolation and characterization of the lignans, isolariciresinol, and pinoresinol in flaxseed meal. *J. Agric. Food. Chem*. **1999**, *47*, 3173−3180.

(33) Ford, J. D.; Davin, L. B.; Lewis, N. G. Plant lignans and health: Cancer chemoprevention and biotechnological opportunities. In *Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology*; Gross, G. G., Hemingway, R. W., Yoshida, T., Eds.; Kluwer Academic/Plenum Publishers: New York, 1999; pp 675−694.

(34) Xia, Z.-Q.; Costa, M. A.; Pélissier, H. C.; Davin, L. B.; Lewis, N. G. Secoisolariciresinol dehydrogenase purification, cloning and functional expression. *J. Biol. Chem*. **2001**, *276*, 12614−12623.

(35) Ford, J. D.; Huang, K.-S.; Wang, H.-B.; Davin, L. B.; Lewis, N. G. Biosynthetic pathway to the cancer chemopreventive secoisolariciresinol diglucoside−hydroxymethyl glutaryl ester-linked lignan oligomers in flax (*Linum usitatissimum*) seed. *J. Nat. Prod*. **2001**, *64*, 1388−1397.

(36) Davin, L. B.; Wang, H.-B.; Crowell, A. L.; Bedgar, D. L.; Martin, D. M.; Sarkanen, S.; Lewis, N. G. Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. *Science* **1997**, *275*, 362−366.

(37) Davin, L. B.; Lewis, N. G. Dirigent proteins and dirigent sites explain the mystery of specificity of radical precursor coupling in lignan and lignin biosynthesis. *Plant Physiol*. **2000**, *123*, 453−461.

(38) Gang, D. R.; Costa, M. A.; Fujita, M.; Dinkova-Kostova, A. T.; Wang, H.-B.; Burlat, V.; Martin, W.; Sarkanen, S.; Davin, L. B.; Lewis, N. G. Regiochemical control of monolignol radical coupling: A new paradigm for lignan and lignin biosynthesis. *Chem. Biol*. **1999**, *6*, 143−151.

(39) Bambagiotti-Alberti, M.; Coran, S. A.; Ghiara, C. Investigation of mammalian lignan precursors in flax seed: First evidence of secoisolariciresinol diglucoside in two isomeric forms by liquid chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom*. **1994**, *8*, 929−932.

(40) Jacobs, E.; Metzler, M. Oxidative metabolism of the mammalian lignans enterolactone and enterodiol by rat, pig, and human liver microsomes. *J. Agric. Food Chem*. **1999**, *47*, 1071−1077.

(41) Ekman, R. Analysis of lignans in Norway spruce by combined gas chromatography−mass spectrometry. *Holzforschung* **1976**, *30*, 79−85.

(42) Haworth, R. D.; Kelly, W. Constituents of natural phenolic resins. VIII. Lariciresinol, cubebin and some stereochemical relationships. *J. Chem. Soc.* **1937**, 384−391.

(43) Fujita, M.; Gang, D. R.; Davin, L. B.; Lewis, N. G. Recombinant pinoresinol−lariciresinol reductases from western red cedar (*Thuja plicata*) catalyze opposite enantiospecific conversions. *J. Biol. Chem*. **1999**, *274*, 618−627.

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