# Dietary Lignins Are Precursors of Mammalian Lignans in Rats

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ABSTRACT The mammalian lignans enterolactone (ENL) and enterodiol, commonly found in human plasma and urine, are phytoestrogens that may contribute to the prevention of breast cancer and coronary heart disease. They are formed by the conversion of dietary precursors such as secoisolariciresinol and matairesinol lignans by the colonic microflora. The identification of lignins, cell-wall polymers structurally related to lignans, as precursors of mammalian lignans is reported here for the first time. In study 1, rats were fed rye or wheat bran (15% diet) for 5 d. Untreated brans and brans extracted with solvents to remove lignans were compared. ENL was estimated in urine samples collected for 24 h by time-resolved fluoroimmunoassay. ENL urinary excretion was reduced from 18.6 to 5.3 nmol/d ($n = 8$; $P < 0.001$) when lignans were removed from rye bran and from 30.5 to 6.2 nmol/d ($P < 0.001$) when they were removed from wheat bran. These results suggest that lignins, embedded in the cell wall and retained in the bran during solvent extraction, account for 26–32% of the ENL formed from cereal brans. In study 2, rats were fed a deuterated synthetic lignin (0.2% diet) together with wheat bran (15%) for 3 d. The detection of deuterated ENL by LC-tandem MS in urine (20 nmol/d) clearly confirms the conversion of lignin into mammalian lignans. More research is warranted to determine the bioavailability of lignins in the human diet. J. Nutr. 134: 120–127, 2004.

KEY WORDS: • *lignins* • *lignans* • *enterolactone* • *phytoestrogens* • *rats*

Phytoestrogens are plant constituents with estrogenic properties that have been implicated in the prevention of breast and prostate cancers, osteoporosis or cardiovascular diseases (1,2). The most largely studied phytoestrogens are the soy isoflavones. Lignans form another class of phytoestrogens. Two main lignans, enterolactone (ENL)[2] and enterodiol (END) (**Fig. 1**) were first detected in human urine and plasma and were called "mammalian lignans" (3,4). They bind weakly to estrogen receptors (5), have estrogenic effects in cultured cells (6,7) and can modulate the response to endogenous estrogens (8,9).

ENL and END result from the conversion of some dietary precursors by the colonic microflora (10–12). The main precursors identified to date are secoisolariciresinol (SECO) and matairesinol (MAT), which are abundant in flaxseed (Fig. 1) (10,13,14) and present in low amounts in several other food sources (15–17). Other lignan precursors were recently identified by incubating human fecal microflora (18) or feeding rats pure compounds (19,20); these include pinoresinol (PIN), lariciresinol (LAR) and 7-hydroxymatairesinol.

Animal experiments suggest that lignans may play a role in the prevention of cancers. Supplementation of the diet with plant lignans delays the progression of chemically induced mammary tumorigenesis in rats (21–23). Inverse associations between the plasma concentration or urinary excretion of lignans and the risk of breast cancers were also observed (24–28). The effect of mammalian lignans on prostate cancer was also examined. ENL and END inhibit the growth of human prostate cancer cell lines in vitro (29). However the dose used was much higher than that observed in vivo and no association was found between the excretion of ENL and prostate cancer in a case-control study (30).

Lignans may also influence bone metabolism and prevent osteoporosis. Continuous administration of SECO with the diet to nursing rat dams improved bone strength of young female offspring (31). In humans, the levels of ENL urinary excretion were lower in Korean postmenopausal women with osteoporosis compared with healthy women (32). However,

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[2] Abbreviations used: DHP, dehydrogenation polymer; END, enterodiol; ENL, enterolactone; G, guaiacylpropane units; H, *p*-hydroxyphenylpropane units; ISOL, isolariciresinol; LAR, lariciresinol; LC-ESI-MS/MS, LC-electrospray ionization-tandem MS; MAT, matairesinol; MeOH, methanol; PIN, pinoresinol; S, syringylpropane units; SECO, secoisolariciresinol; SYR, syringaresinol; TR-FIA, time-resolved fluoroimmunoassay.


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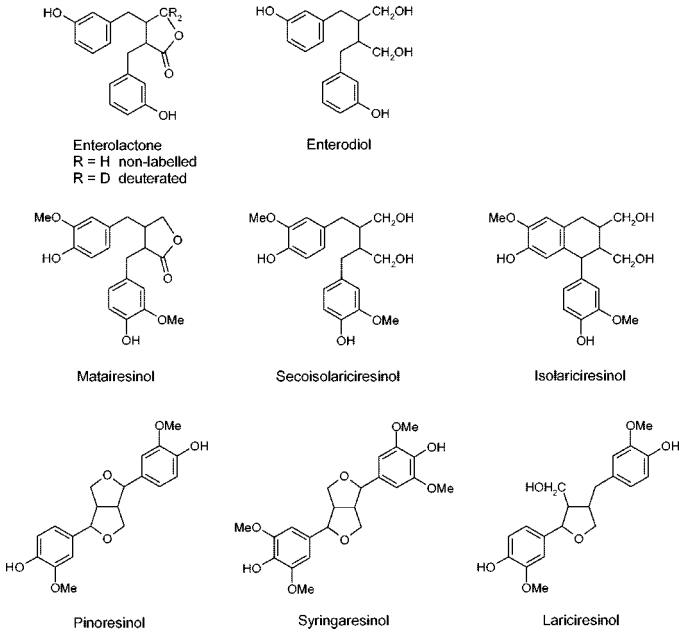


**FIGURE 1** Chemical structures of mammalian lignans and of the plant lignans identified in cereal brans.

the supplementation of postmenopausal women with 40 g of flaxseeds for 3 mo had no effect on biomarkers of bone metabolism (33).

Despite the potential importance of lignans in reducing disease risk, little is known concerning their dietary origin. Flaxseed was recognized as a rich source of lignans and continues to be their best dietary source (34,35). However, the low consumption of flaxseed in most human populations cannot explain the general occurrence of mammalian lignans in human tissues. High ENL and END levels in urine or plasma are generally associated with a high intake of dietary fiber and with the consumption of whole-grain food and fruit and vegetables (16,27,36–40). It was suggested that the lignans contained in these food sources were the precursors of ENL and END. However, two studies in which rats or humans were fed whole-meal cereals or cereal bran showed that the content of SECO and MAT, the two main lignans identified in cereals, was too low by a factor of 5–25 to explain the levels of ENL and END excreted in urine (36,41). The recent identification of a number of lignans in rye (18) and other cereals still does not completely explain the urinary excretion of ENL and END. This suggests the existence of other precursors.

We propose here that lignins are major dietary precursors of mammalian lignans. Lignins are structurally related to lignans but differ from them by their polymeric nature and distribution in plants. They are cell-wall polymers made of phenylpropane units (**Fig. 2**) (42). The most common monomers in lignins are guaiacylpropane units (G); lignins are therefore structurally closely related to SECO and MAT (Fig. 1). Some lignins, like those in cereals, also contain syringylpropane (S) and *p*-hydroxyphenylpropane (H) units. They cannot be easily extracted by solvents unless they are chemically or physically degraded into smaller fragments. Lignins are found in vessels and secondary tissues of all higher plants. They are thus present in a large variety of foods, and are particularly abundant in cereal brans (43). In contrast, lignans are dimers of phenylpropane units, present in vacuoles and extractable by organic solvents (44). They are usually specific to a plant species such as SECO in flaxseeds.

In the present work, we fed rats cereal brans that had and had not had lignans removed by solvent extraction to examine the respective contribution of plant lignans and lignins to the formation of mammalian lignans. We also fed rats a deuterated synthetic lignin and measured the excretion of deuterated ENL in urine. We demonstrate for the first time that lignins are precursors of lignan phytoestrogens.

## MATERIALS AND METHODS

***Preparation of cereal brans.*** Untreated rye and wheat brans were kindly supplied by Celnat (St Germain Laprade, France). Insoluble and soluble dietary fiber were determined on brans by a combination of enzymatic and gravimetric methods (45). To prepare extractive-free brans, 400 g wheat or rye bran were successively extracted in a Soxhlet by ethanol/toluene 2:1 (v/v), ethanol, and water. Extracted brans were then dried at 50°C for 2 d. The weight loss gave the total extractive contents (**Table 1**). To prepare lignin-enriched wheat bran, the extractive-free wheat bran suspended in water (40 g/L) was hydrolyzed by Onozuka cellulase (Yakult & Company, Tokyo, Japan) in 0.05 mol/L acetate buffer, pH 4.7, for 70 h at 37°C. The insoluble residue was recovered by centrifugation (75,000 × *g*, 45 min), washed three times with water and freeze-dried. Lignins were determined by the Klason gravimetric method on the extractive-free and lignin-enriched brans. This gravimetric determination systematically includes a correction for ash-forming compounds present in bran samples (46). The monomeric composition of bran lignins was determined by thioacidolysis with analysis of the lignin-derived monomers by GC-MS as their trimethylsilylated derivatives (47). Thioacidolysis of plant tissues coupled with GC-MS of the lignin-derived products makes it possible to detect small amounts of lignin without interference from other phenolics such as bran ferulic acid esters (47). During this reaction, thioethylated H, G and S monomers are formed from the H, G and S lignin units involved in labile $\beta$-*O*-4 ether bonds, the most frequent interunit linkages of native lignin.

***Determination of lignans in brans.*** The GC-MS method used for analysis of plant lignans MAT and SECO, isolariciresinol (ISOL), LAR, PIN and syringaresinol (SYR) in different brans was optimized from previously published method for the analysis of isoflavonoids and lignans in food matrices (48). Duplicate analyses were carried out for each sample. To clearly differentiate the lignans from the lignins, an additional extraction step was added to the sample pretreatment method. The lignans were extracted from brans or the control purified diet (50 mg) with acetone/water 7:3 (2 mL) at room temperature for 2 min three times. This additional step was necessary because part of

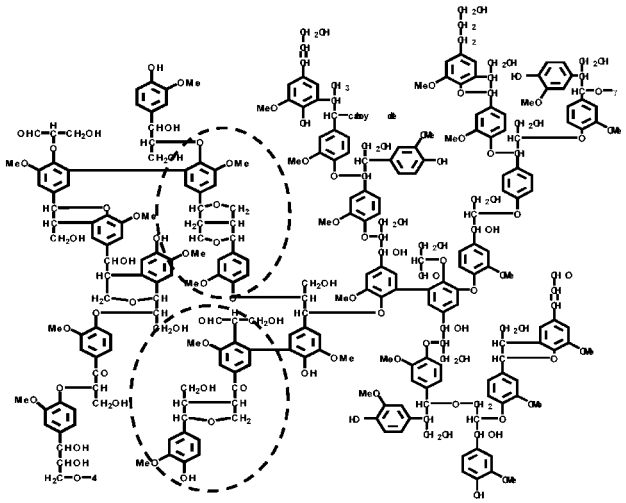

**FIGURE 2** Chemical structure of lignins. Dotted line circles show pinoresinol and lariciresinol structures most likely metabolized into enterolactone (ENL) by the gut microflora.

**TABLE 1**

*Fiber, lignin, total extractives and lignan concentrations of cereal fibers*[1]

| | Fiber | | Total extractives | Lignins | Lignans | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Total | Insoluble | | | SECO | MAT | ISOL | LAR | PIN | SYR |
| | *g/100 g* | | | | *μg/100 g* | | | | | |
| Rye bran | | | | | | | | | | |
| Untreated | 47.7 | 40.6 | 28.9 | 5.4[2,3] | 47 | 91 | 295 | 290 | 321 | 2291 |
| Extractive-free | | | | 7.0[3] | 0.0 | 15.7 | 9.8 | 1.6 | 18.0 | 94 |
| Wheat bran | | | | | | | | | | |
| Untreated | 47.9 | 46.9 | 26.0 | 4.4[2,4] | 142 | 9.4 | 297 | 257 | 106 | 1953 |
| Extractive-free | | | | 5.5[4] | 0.0 | 0.0 | 13.4 | 0.0 | 4.1 | 72 |
| Lignin-enriched | | | | 17.2[4] | 1.6 | 0.0 | 5.3 | 0.0 | 0.0 | 18.8 |

[1] Abbreviations: SECO, secoisolariciresinol; MAT, matairesinol; ISOL, isolariciresinol; LAR, lariciresinol; PIN, pinoresinol; SYR, syringaresinol; H, *p*-hydroxyphenylpropane; G, guaiacylpropane; S, syringylpropane.
[2] Calculated from the lignin content of the extractive-free fraction.
[3] Monomeric composition (molar proportion of thioacidolysis monomers): H units, 11; G units, 57; S units, 32.
[4] Monomeric composition (molar proportion of thioacidolysis monomers): H units, 8; G units, 42; S units, 49.

the insoluble lignins were degraded to lignans when the following sample pretreatment method was applied directly to the brans or to the residue of selected samples, obtained after acetone/water extraction (S. Heinonen, H. Adlercreutz, A. Scalbert, unpublished results). Furthermore, all lignans are extractable by aqueous acetone. The supernatant obtained after centrifugation (5000 × *g*, 10 min) was dried under a nitrogen flow and used for further analyses. Deuterated internal standards ($^2H_6$-MAT, $^2H_6$-SECO and $^2H_8$-anhydrosecoisolariciresinol) and 500 μL of distilled water were added. Enzymatic hydrolysis was carried out with *Helix pomatia* digestive juice in 0.15 mol/L acetate buffer, pH 5. The sample was incubated first overnight at 37°C and then for 1 h at 60°. Hydrolyzed sample was extracted twice with 6 mL of diethyl ether. The combined organic phases were dried, dissolved in 0.5 mL of methanol (MeOH) and stored in the refrigerator. The water phase was subjected to acidic hydrolysis by the addition of 200 μL of 6.0 mol/L HCl followed by incubation of the sample for 2 h at 70°C. The sample was extracted twice with diethyl ether/ethyl acetate, 1:1 (v/v). The organic phases were combined with organic phases obtained after the enzymatic hydrolysis. The sample was completely dried, dissolved in 0.5 mL of MeOH and stored in a refrigerator. The water phase was further hydrolyzed with 1.5 mol/L HCl for 2 h at 100°C, followed by extraction with diethyl ether/ethyl acetate, 1:3 (v/v). The water phase was discarded. The organic phases were combined with the stored organic phases, obtained after enzymatic and mild acid hydrolyses. The sample was dried completely under nitrogen, and dissolved in 200 μL of MeOH.

The sample was applied in 2 × 200 μL of MeOH/$CHCl_3$/$H_2O$, 4:1:1, on a Lipidex 5000 column (0.5 × 5.0 cm in MeOH/$CHCl_3$/$H_2O$, 4:1:1). Lignans were eluted with 4 mL of MeOH/$CHCl_3$/$H_2O$, 4:1:1 (v/v/v). The fraction was dried completely under nitrogen, and dissolved in 0.5 mL MeOH. Further purification of the sample was carried out by chromatography on DEAE Sephadex in hydroxylated form and QAE Sephadex in its acetate form (Pharmacia Fine Chemicals, Uppsala, Sweden) (48). The sample was derivatized with 100 μL of pyridine/hexamethyldisilazane/trimethylchlorosilane (9:3:1) by incubating for 30 min at room temperature; it was transferred to a micro-vial and analyzed by GC-MS. Deuterated internal standards were not available for the new plant lignans; thus, quantification was done using deuterated MAT for analysis of LAR, PIN and SYR, and deuterated SECO and anhydrosecoisolariciresinol for analyses of ISOL and anhydroisolariciresinol, respectively.

***Synthesis of dehydrogenation polymers.*** The labeled dehydrogenation polymer (DHP) was prepared by endwise polymerization of [9,9-$^2H_2$]-coniferyl alcohol (49). Aqueous solutions of coniferyl alcohol (2.0 g in 500 mL distilled water) and 0.05% hydrogen peroxide were simultaneously added dropwise using a micro-pump (flow rate: 20 mL/h) to a solution of horseradish peroxidase (5.0 mg in 500 mL in a 0.07 mol/L phosphate buffer, pH 6.5) for 24 h at room temperature. More peroxidase (5.0 mg) was then added to the reaction mixture and stirred for an additional 24 h. The resulting insoluble polymer was separated by centrifugation (15,000 × *g*, 30 min), dissolved in dichloroethane/ethanol 2:1 (v/v)(50 mL) and added dropwise to excess diethyl ether with vigorous stirring to eliminate the low-molecular-weight products such as monomers, dimers (= lignans) or trimers. The precipitate was recovered by centrifugation (15,000 × *g*, 30 min, 4°C) and washed five times with diethyl ether (5 × 40 mL). The elimination of lignans was confirmed by silica gel TLC with 1,2-dichloroethane/MeOH 20:1 (v/v) as eluant. The insoluble DHP was dried over phosphorus oxide in vacuo (yield, 1.3 g).

The DHP was characterized by thioacidolysis (50). The main monomeric products were 2-methoxy-4-(1,2,3-tris-ethylsulfanylpropyl)-phenol (I), 4-(1,3-bis-ethylsulfanyl-propyl)-2-methoxy-phenol (II) and 4-(3-ethylsulfanylpropenyl)-2-methoxy-phenol (III) with respective yields of 649, 263 and 127 μmol/g (**Fig. 3**). The high yield of (II) and (III) indicates a high content of β-*O*-4–linked coniferyl alcohol end-groups compared with natural lignins (50,51). Intermonomeric linkages were also quantified by thioacidolysis followed by Raney nickel desulfuration (52). The main dimers recovered were of the β-5 type (IV and V) and β-β type (VI) (Fig. 3) (55.4 and 23.4%, respectively, of the total dimers) originating from phenylcoumaran and PIN structures, respectively.

***Rats, diet and sampling procedure.*** Male Wister rats (Iffa-credo, L'Arbresle, France) weighing ~100–120 g were used. Rats were housed two per cage in a room maintained at 22°C with a 12-h light:dark cycle (light from 0800 to 2000 h) with access to food from 1600 to 2000 h. Rats were maintained and handled according to the recommendations of the Institutional Ethics Committee (Institut National de la Recherche Agronomique), according to decree no. 87–848.

In study 1, 48 rats were first adapted for 18 d to a semisynthetic basal diet. The basal diet consisted of casein (150 g/kg diet), wheat starch (755 g/kg diet), mineral mixture (35 g/kg diet), vitamin mixture (10 g/kg diet) and peanut oil (50 g/kg diet). The mineral mixture AIN-93M and vitamin mixture AIN-76A supplemented with choline were used (ICN Biochemicals, Aurora, OH) (53). After adaptation to the basal diet, rats were moved to metabolic cages, randomly assigned to 6 groups and different brans were added to the diets in place of an equivalent weight amount of starch. The six groups of rats were thus fed diets containing 15% rye bran, 10.7% extracted rye bran, 15% wheat bran, 10.2% extracted wheat bran, 3.2% lignin-enriched wheat bran or no bran for 5 d. On d 5, urine samples were collected for 24 h into plastic tubes containing ascorbic acid (Sigma Chemical, St. Louis, MO; 60 mg) and stored at −20°C until further analysis. All rats consumed their feed ad libitum throughout the experiment. Food intakes did not differ during the urine collection period and were (means ± SD) 19.0 ± 2.6, 20.1 ± 3.4, 21.2 ± 2.1, 19.6 ± 2.5, 18.8

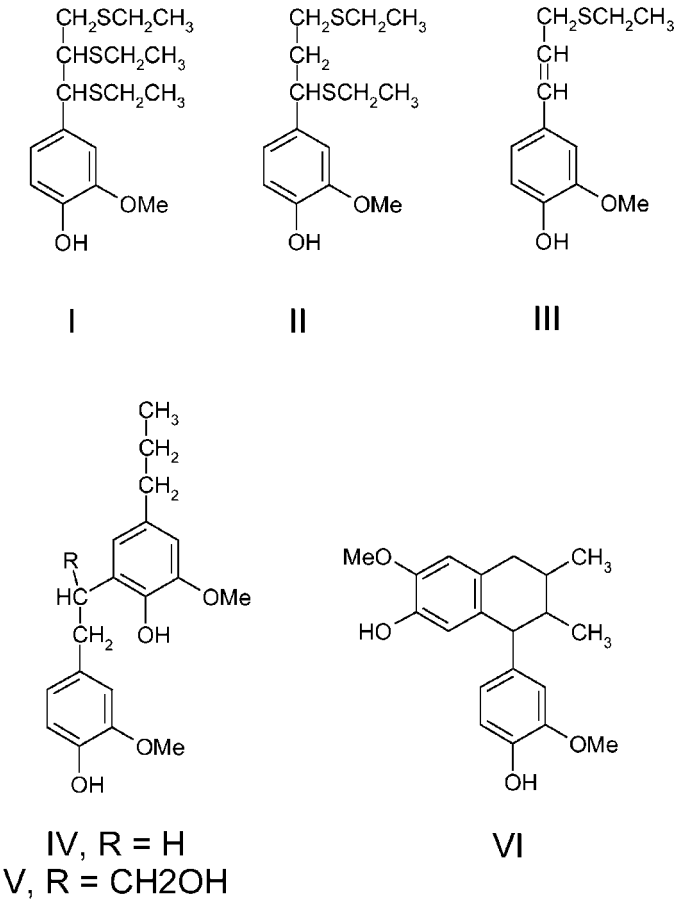


**FIGURE 3** Degradation products formed from lignins by thioacidolysis (I, II and III) and by thioacidolysis followed by Raney nickel desulfuration (IV, V and VI).

± 1.7, 19.8 ± 2.8 g dry matter/d in rats fed rye, extracted rye, wheat, extracted wheat, lignin-enriched wheat brans and controls diets, respectively.

In study 2, 12 rats were first fed the same 15% wheat bran diet as above for 14 d. They were moved to metabolic cages on d 10 and urine samples were collected for 24 h as described above on d 10 and 11. ENL was estimated in these urine samples and the three rats showing the highest level of lignan excretion were selected for feeding the DHP diet. This was done to maximize the chances of detecting labeled ENL when feeding low amounts of labeled DHP to a limited number of rats. On d 15, the three selected rats were fed the same wheat bran diet to which was added 0.2% labeled DHP for 3 d. Three other rats were fed the original 15% wheat bran diet as controls, and urine samples were collected for 24 h on these 3 d. All rats consumed their feed ad libitum throughout the experiment. Food intakes did not differ during the urine collection period and were (means ± SD) 21.8 ± 0.4 and 21.9 ± 2.5 g dry matter/d in rats fed the diet supplemented with DHP and the control diet, respectively.

***Time-resolved fluoroimmunoassay (TR-FIA) for ENL.*** This immunoassay allows the specific estimation of ENL at a low concentration with very low cross-reaction for END (0.28%) and no interference from other compounds that have been investigated (54–56). The assay was carried out as previously described (41). Briefly, urine samples were treated with β-glucuronidase and sulfatase and ENL estimated using the DELFIA kit (Wallac Perkin Elmer, Turku, Finland) and a Victor 1420 multilabel counter.

***Liquid chromatography electrospray ionization tandem MS (LC-ESI-MS/MS) analysis of lignans.*** Urine samples (175 μL) were diluted 10 times with 0.1 mol/L sodium acetate buffer pH 5. Genistein was added as an internal standard (3 μmol/L) and the solution acidified to pH 4.9 with 20 μL of 0.58 mol/L acetic acid and incubated at 37°C for 2 h in the presence of an *Helix pomatia* extract containing 1100 U β-glucuronidase and 42 U sulfatase (G-0876, Sigma Chemical). After acidification to pH 2 with 2 μL of 6 mol/L HCl, the urine samples were extracted twice with ethyl acetate and centrifuged at 2400 × g for 10 min. The supernatant fluid was dried under nitrogen, redissolved in 500 μL of 25% aqueous MeOH and filtered (PTFE membrane, 0.45μm, Millipore, Bedford, MA). A 40-μL aliquot of the filtrate was then injected directly into the LC-ESI-MS/MS system.

LC-ESI-MS/MS analyses were performed on a Hewlett-Packard HPLC system with triple quadrupole MS-MS detection (API 2000, Applied Biosystem, Toronto, Canada). The column was an Hypersil BDS C18 (5 μm, 150 × 2.1 mm, Touzart & Matignon, Les Ulis, France) and the mobile phases consisted of 5% acetonitrile in 0.1% aqueous formic acid (solvent A) and 40% acetonitrile in 0.1% aqueous formic acid (solvent B). The following gradient was applied: 0–3 min, linear gradient from 0% B to 100% B, 3–15 min, 100% B. The flow rate was 0.2 mL/min. Detection was carried out by using electrospray ionization in negative mode at 450°C with a nebulizer pressure of 90 psi, a drying nitrogen gas flow of 11 L/min, a fragmentor voltage of 20 V and a capillary voltage of 4000 V. The MS data were collected in multiple reaction monitoring mode, monitoring the transition of parent and product ions specific to each compound with a dwell time of 500 ms. The selected parent and product ions for ENL, $D_2$-ENL, END, $D_4$-END and genistein internal standard were 297/253, 299/255, 301/253, 305/257 and 269/63, respectively.

***Data analysis.*** All numerical values are expressed as means ± SD Significant differences in ENL excretion levels were determined by one-way ANOVA and Tukey's test in study 1 and by the unpaired two-tailed *t* test in study 2 (Instat, San Diego, CA). ENL excretion values were log-transformed before analysis. Differences with $P < 0.05$ were considered significant.

## RESULTS

***Enterolactone formed from cereal brans.*** Rye and wheat brans were used as dietary sources of lignans and supplemented to the purified diet of rats. Some of these brans were extracted successively with a Soxhlet by ethanol/toluene, ethanol and water to remove lignans. Lignans were analyzed in both untreated brans and extractive-free brans. The total concentrations of lignans were 3335 and 139 μg/100 g for the untreated and extractive-free rye brans, respectively, and 2764 and 89 μg/100 g for the untreated and extractive-free wheat brans, respectively (Table 1). Extractive-free brans then contained virtually no lignans (96–97% removed) but still contained lignins (7.0 and 5.5 g/100 g for rye and wheat brans, respectively; Table 1), which cannot be extracted by solvents.

The bran-supplemented diets were fed to the rats for 5 d and ENL estimated in urine samples collected for 24 h by TR-FIA assay (**Table 2**). The ENL excretion did not differ significantly between rats fed untreated rye and wheat brans. When these brans were extracted with a Soxhlet, the excretion of lignans was significantly reduced to about one fourth the value in rats fed untreated brans but was still 5–6 times higher than that for control rats ($P < 0.001$). This shows that the nonextractable materials in brans still contain ENL precursors different from lignans.

To examine whether these nonextractable ENL precursors could be lignins, we established unequivocally the occurrence of lignins in extractive-free brans using thioacidolysis. The detection of the three thioethylated H, G and S monomers demonstrates that the three lignin units H, G and S are present in the extractive-free bran samples (Table 1). To further establish the involvement of native lignins in the formation of urinary lignans, we treated the extracted wheat bran with a commercial mixture of cellulases and hemicellulases. This treatment removed part of the cell-wall polysaccharides, resulting in a 310% increase in the lignin content in

**TABLE 2**

*Polyphenol intake and enterolactone (ENL) urinary excretion in rats consuming diets supplemented with different cereal brans*

| Diet | Bran in diet | Lignin intake | Lignan intake | | ENL excretion[1] |
|---|---|---|---|---|---|
| | | | Total | ENL precursors[2] | |
| | *g/100 g* | *mg/d* | *nmol/d* | | |
| Rye bran | | | | | |
| Untreated | 15.0 | 159 | 246.6 | 61.3 | 18.6 ± 5.1[a] |
| Extractive-free | 10.7 | 142 | 7.2 | 2.0 | 5.3 ± 3.7[b] |
| Wheat bran | | | | | |
| Untreated | 15.0 | 138 | 217.7 | 45.0 | 30.5 ± 7.0[a] |
| Extractive-free | 10.2 | 108 | 4.3 | 0.2 | 6.2 ± 3.7[b] |
| Extractive-free and treated with cellulases | 3.2 | 99 | 0.4 | 0.0 | 7.7 ± 3.8[b] |
| Control | — | — | 2.4 | 0.2 | 1.0 ± 0.5[c] |

[1] Values are means ± SD, $n = 8$. Means without a common letter differ, $P < 0.001$.
[2] Main enterolactone precursors are secoisolariciresinol + matairesinol + lariciresinol + pinoresinol.

this enzyme-treated bran (Table 1). The amount of this enzyme-treated bran added to the diet was determined to provide the same lignin amount to the rats as was given with the diet supplemented with the extractive-free wheat bran (Table 2). The level of ENL urinary excretion was close to that of rats fed the extractive-free wheat bran diet although the amount of bran ingested by the rats was only 31%. The ENL excretion level thus appears to depend in part on the amount of lignins ingested with the diet.

***Enterolactone formed from lignin dehydropolymers.*** Three rats were first fed a diet supplemented with 15% wheat bran for 14 d. A labeled lignin dehydrogenation polymer (DHP) was then added to this same diet (0.2%) for three more days and urine samples were collected for 24 h over these 3 d. The DHP group was compared with the control group fed the diet supplemented with wheat bran only. The excretion of both unlabeled and labeled-ENL was followed in urine by LC-ESI-MS/MS (**Fig. 4**). Feeding the labeled DHP greatly increased $D_2$-ENL excretion compared with the control rats (19.9 vs. 0.9 nmol/d, $P < 0.001$ at d 3 of the DHP diet) (**Fig. 5**). Excretion levels of unlabeled ENL did not differ between the DHP-fed rats and control rats (the higher means for the DHP-fed rats is explained by the selection of the best lignan producers for feeding the labeled-DHP; see experimental section). The $D_2$-ENL/nonlabeled ENL in the urine of the DHP-fed rats increased after d 1–2 of feeding the labeled DHP, reflecting the time needed for the DHP to be metabolized by the cecal microflora after ingestion. These results show unambiguously that lignins can be degraded into ENL, in a way similar to lignans.

The presence of END was determined in the urine of rats by tandem MS (m/z 301/253). Levels of excretion were low and did not exceed 1.1 nmol/d in rats fed the 15% wheat bran diet. No labeled END (m/z 305/257) could be detected in rats fed the labeled DHP.

## DISCUSSION

Mammalian lignans differ from all other polyphenols detected in human plasma or urine because not all of the dietary precursors that explain their concentrations or levels of excretion have been identified. The most evident dietary sources of mammalian lignans are plant lignans. However, the number of plant foods containing significant amounts of lignans is very

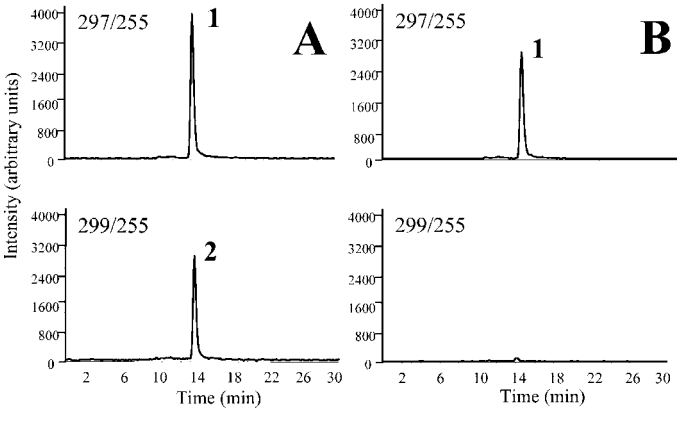


**FIGURE 4** LC-electrospray ionization-MS/MS chromatograms of the urine of rats fed a diet supplemented with labeled lignin dehydropolymer (*A*) or the control diet (*B*).

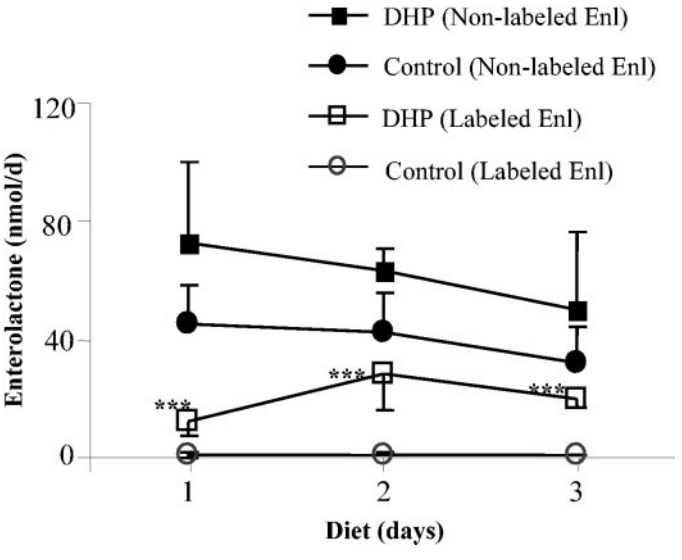


**FIGURE 5** Enterolactone (ENL) excretion in rats consuming a control diet or a diet supplemented with deuterated lignin dehydropolymer (DHP). Values are means ± SD, $n = 8$ (unlabeled DHP) or 3 (labeled DHP). ***Different from respective control, $P < 0.001$.

limited. Flaxseed contains SECO and MAT (14), and sesame seeds contain sesamin and other furanolignans (57). However, both foods are generally consumed in limited amounts and cannot explain the systematic excretion of mammalian lignans in urine. A large variety of grains, fruits, vegetables and beverages such as tea or coffee were also found to contain small amounts of SECO (< 3.5 mg/100 g) and traces of MAT (16,17).

In the present work, if we assume an average recovery yield of 32% in urine (58), SECO and MAT could account for no more than 20 and 14% of the ENL excreted in the urine of rats fed the diets supplemented with rye and wheat brans, respectively, and for 0 and <6%, respectively, when the same brans were previously extracted to remove the major part of the lignans. Other lignans such as PIN and LAR were also shown to be metabolized in high yields into ENL and END when incubated in vitro with a human fecal microflora (18). PIN and LAR were thus also estimated in the bran diets, in addition to SECO and MAT. If we assume the same recovery yield of 32% for the 4 lignans, they would then account for all (111%) and 49% of the ENL excreted after consumption of the rye and wheat bran diets, respectively, but for only 13 and 1%, respectively, when both brans were previously extracted with solvents. This clearly suggests the presence of other precursors.

The contribution of plant lignans to the formation of mammalian lignans could be somewhat underestimated because SYR, the major lignan in rye and wheat bran (3 times more abundant than SECO, MAT, PIN and LAR taken together), can also be converted to ENL and END, although in a yield which does not exceed 7% of those of the other four lignans (18).

Lignins are structurally related to lignans and may also be metabolized into mammalian lignans. Lignins were unequivocally detected by thioacidolysis in rye and wheat brans where they account for ~5% of their weight (Table 1). Native lignins cannot be isolated without substantial degradation due to their insoluble and cross-linked nature (59). To examine whether lignins are effective precursors of mammalian lignans, a lignin-enriched wheat bran and a deuterated synthetic lignin (DHP) were prepared and fed to rats. When the lignin-enriched extractive-free wheat bran (310% lignin-enriched) was compared with the extractive-free wheat bran, ENL yields were similar when calculated on a lignin intake basis (78 and 58 nmol/g, respectively).

It is difficult to obtain pure lignins from complex plant materials and more particularly from materials such as brans, which have a relatively low lignin content compared with other lignocellulosic materials such as wood. For this reason, we prepared a synthetic lignin, free of any other contaminants. These synthetic lignins are prepared by oxidative polymerization of coniferyl alcohol and are called dehydropolymers (DHP). To differentiate ENL originating from the degradation of DHP from that originating from other constituents of the diet, a labeled DHP was prepared from [$\gamma,\gamma-D_2$]-coniferyl alcohol. In the labeled DHP, the distal carbon of the propane side chain of most of the monomeric units carries two deuterium atoms in the place of hydrogen. The ENL that would result from the metabolism of the labeled lignins by the rat microflora would then carry two deuterium atoms.

When the labeled DHP was fed to rats, $D_2$-ENL was specifically detected, showing that lignins can be metabolized into ENL in rats. The yield of $D_2$-ENL was 655 nmol/g DHP (195 $\mu$g/g). This yield is ~10 times higher than that calculated for bran lignins. These differences could be explained either by a limited accessibility to the substrate for bran lignins embedded in the cell wall or to structural differences between native bran lignins and synthetic lignins. To examine the first possibility, extractive-free wheat bran was thinly ground with a Retsch grinder (particle size < 0.5 mm) and fed to rats (not shown). The ENL urinary excretion was not affected by grinding. Therefore, a difference in substrate accessibility cannot be responsible for the difference in ENL yields between native and synthetic lignins.

The most likely explanation is a difference in the structure of the synthetic DHP compared with the bran native lignins. The precursors of mammalian lignans within the lignin are most likely the PIN and LAR structures (see dotted line circles, Fig. 2). The content of such structures in the DHP was determined by thioacidolysis followed by reduction with Raney nickel desulfuration (see experimental section). The PIN structures accounted for 23% of the total dimers recovered after such a chemical degradation. This value is much higher than that commonly observed for native lignins such as those present in cereal bran, and this difference most likely explains the high yield of ENL formation observed when the DHP is fed to the rats (60).

Lignins, because of their polymeric nature and of their embedding in the cell wall, are usually considered inert in the digestive tract. However, the present results show that they are metabolized by the gut microflora to form part of the ENL excreted in urine. Similarly, proanthocyanidins, natural phenolic polymers with antimicrobial properties, were also shown to be degraded into various phenolic acids by the gut microflora (61–63). Another observation not previously noted is the absence of optical activity of ENL and END excreted in urine (64). A lignin origin for mammalian lignans is consistent with the absence of optical activity of lignins due to their biosynthesis through radical polymerization (65). This contrasts with the biosynthesis of plant lignans, which is stereospecific and leads to the formation of optically active lignans (66,67).

The respective contributions of lignins and plant lignans to the genesis of mammalian lignans can be evaluated from the comparison of the ENL excreted in urine after feeding whole brans or extractive-free brans (Table 2). Lignins account for 32 and 26% of the ENL excreted in the rats fed rye and wheat brans, respectively. Lignans present in brans would account for the remaining fraction.

The SECO and MAT intakes in Western populations were calculated using the SECO and MAT content in foods and beverages previously published (68). An intake of 1.5–3 $\mu$mol/d was determined for SECO and MAT in two Dutch and American cohorts (69,70), but this value could be 10 times higher if other lignans such as those estimated here in cereal brans were considered (70). The intake of these lignans explains in part the urinary excretion levels of mammalian lignans (0.5–27 $\mu$mol/d) in various populations (71). However, previously published lignan content values in food may be overestimated. Indeed, the analytical method used differs from the one used here by the mode of extraction of the lignans (48) and also gives positive values for SECO and other lignans when applied to a lignan-free lignin sample (S. Heinonen, H. Adlercreutz and A. Scalbert, unpublished results). Food composition tables for lignins do not exist. More data on lignan and lignin contents in foods will be required to determine their respective contributions to the mammalian lignan formation.

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