JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Lignins and Ferulate−Coniferyl Alcohol Cross-Coupling Products in Cereal Grains

MIRKO BUNZEL,*,† JOHN RALPH,‡,§ FACHUANG LU,‡,§ RONALD D. HATFIELD,‡ AND HANS STEINHART†

Institute of Biochemistry and Food Chemistry, Department of Food Chemistry, University of Hamburg, Grindelallee 117, 20146 Hamburg, Germany; U.S. Dairy Forage Research Center, Agricultural Research Service, U.S. Department of Agriculture, Madison, Wisconsin 53706; and Department of Forestry, University of Wisconsin−Madison, Madison, Wisconsin 53706

Plant cell walls containing suberin or lignin in the human diet are conjectured to protect against colon cancer. To confirm the existence of authentic lignin in cereal grain dietary fibers, the DFRC (derivatization followed by reductive cleavage) method was applied to different cereal grain dietary fibers. By cleavage of diagnostic arylglycerol-β-aryl (β-*O*-4) ether linkages and identification of the liberated monolignols, it was ascertained that lignins are truly present in cereal grains. From the ratios of the liberated monolignols coniferyl alcohol and sinapyl alcohol, it is suggested that lignin compositions vary among cereals. Furthermore, dimeric cross-coupling products, comprising ferulate and coniferyl alcohol, were identified in most cereal fibers investigated. These ferulate 4-*O*-β- and 8-β-coniferyl alcohol cross-coupled structures indicate radical cross-coupling of polysaccharides to lignin precursors via ferulate.

KEYWORDS: Cereal dietary fiber; lignin; DFRC method; cross-coupling; ferulic acid; coniferyl alcohol

INTRODUCTION

Cereal grains are the most important source of dietary fiber in many industrialized countries. Dietary fiber is a macronutrient defined as "the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine" (*1*). Dietary fiber components are "polysaccharides, oligosaccharides, lignin, and associated plant substances" (*1*). Dietary fiber has marked effects on gut function and may afford protection against colon cancer. Two groups of possible mechanisms for this protection have been proposed. First are mechanisms whereby dietary fiber acts directly by binding carcinogens to undegradable dietary fibers, lowering their effective concentration in the alimentary tract and carrying them out of the body in the feces. The other group of mechanisms is based on the fermentation of some dietary fiber components to short-chain fatty acids, lowering the colon pH; butyrate has been particularly implicated (*2*). Epidemiological studies have shown that plant cell walls containing suberin or lignin may be the most protective, whereas animal carcinogenesis experiments indicate that soluble dietary fiber does not protect against, or may even enhance, carcinogenesis (*3*).

Lignins are polymeric natural products from three primary precursors (*p*-coumaryl, coniferyl, and sinapyl alcohols) arising from an enzyme-catalyzed dehydrogenative polymerization. In the literature, cereal brans are frequently described as highly lignified (*4*, *5*). In most studies lignin was determined as Klason lignin, that is, as an acid-insoluble residue. However, the determination of Klason lignin does not prove that lignin, according to its accepted definition, is present; plant material residues such as structural proteins or waxes may contribute to acid-insoluble residues (*6*, *7*).

In grasses, polysaccharide−lignin cross-linking is mediated by ferulates attached primarily to arabinoxylans (*8*, *9*). Jacquet et al. (*10*) released ferulate−coniferyl alcohol cross-coupling products from grass straw by mild alkaline hydrolysis, affirming that ferulates are at least partially incorporated into lignin by radical-mediated mechanisms (*11*−*15*). The finding also complements the notion that ferulates provide nucleation sites for the process of lignification (*13*, *16*).

The aim of this study was to confirm the presence of authentic lignin in cereal grain dietary fiber and to estimate the monomer composition of cereal grain lignins. Furthermore, we wished to survey commercially important cereal grains for the presence of coniferyl alcohol−(β-*O*-4)- and (β-8)-ferulate cross-products, because only ferulates and dehydrodiferulates were previously identified and quantified in different cereal grains (*17*).

MATERIALS AND METHODS

General. Generally, samples were analyzed in duplicate. Heat-stable α-amylase Termamyl 120 L (EC 3.2.1.1, from *Bacillus licheniformis*,

* Author to whom correspondence should be addressed (telephone +49-40-42838-4379; fax +49-40-42838-4342; e-mail Mirko.Bunzel@uni-hamburg.de).
† University of Hamburg.
‡ U.S. Dairy Forage Research Center.
§ University of Wisconsin−Madison.

10.1021/jf040204p CCC: $27.50
Published on Web 09/18/2004

120 KNU/g), the protease Alcalase 2.4 L (EC 3.4.21.62, from *Bacillus licheniformis*, 2.4 AU/g), and the amyloglucosidase AMG 300 L (EC 3.2.1.3, from *Aspergillus niger*, 300 AGU/g) were from Novo Nordisk, Bagsvaerd, Denmark. Supelclean LC-Si SPE tubes, 3 mL SPE columns, were from Supelco, Bellefonte, PA.

Plant Material. Cereal grains and brans were obtained from local German suppliers. Rye (*Secale cereale* L.) and wheat (*Triticum aestivum* L.) samples (whole grains and brans) were from a local mill and represent standardized mixtures harvested in northern Germany. Maize bran (*Zea mays* L.) was from genuine maize harvested in southern Germany and was kindly donated from Hammermühle Diät GmbH, Kirrweiler, Germany. Whole grains of maize (*Zea mays* L.), barley (*Hordeum vulgare* L.), millet (*Panicum miliaceum* L.), oat (*Avena sativa* L.), rice (*Oryza sativa* L.), spelt (*Triticum spelta* L.), and wild rice (*Zizania aquatica* L.) were purchased from a supermarket (health foods shop) in Hamburg, Germany. Botanical origin was confirmed by the supplier. Grains were visually inspected to be free of contaminating parts of the plant.

Standard Substances. Possible cross-products (compounds **6a**,**b**, **7a**,**b**, and **11a**,**b**, **Figure 1**) were synthesized according to the method of Ralph et al. (*12*) and Helm and Ralph (*18*). Compounds **10a**,**b** (**Figure 1**) were isolated on a preparative scale from plant cell walls (*16*). Briefly, plant cell walls for the isolation process were obtained from corn cell cultures (*14*, *19*). Cell cultures were fed with [$^{13}C_9$]-phenylalanine. Thus, ~40% of ferulates in the cell walls were ^{13}C-labeled. Cell walls were lignified by the addition of coniferyl alcohol. H_2O_2 was generated from glucose and glucose oxidase; wall-bound peroxidase was the sole peroxidase used. Saponification of milled cell walls was carried out by 4 M NaOH at room temperature. The hydrolysate was acidified, and phenolic compounds were extracted into ethyl acetate. Using preparative silica TLC (mobile phase of ethyl acetate/chloroform/acetic acid, 6:3:1) some cross-products were separated from other phenolic compounds. A second preparative silica TLC system was used (mobile phase of ethyl acetate/chloroform/acetic acid, 6:5:1) to isolate a mixture of two stereoisomers of **10** (**a** and **b**). The identification was carried out using the standard array of one- and two-dimensional NMR experiments on a Bruker (Karlsruhe, Germany) DRX 360 MHz spectrometer fitted with an inverse ^1H/broadband gradient probe with three-axis gradients.

Hydroxycinnamyl diacetate standards were prepared by acetylating the appropriate *trans*-hydroxycinnamyl alcohols as previously described by Quideau and Ralph (*20*) and Lu and Ralph (*21*).

Preparation of Insoluble Dietary Fiber (IDF). Samples were milled to a particle size of <0.5 mm. Flours (10 g) were suspended in 0.08 M sodium phosphate buffer, pH 6.0 (300 mL), and 750 μL of α-amylase was added. Beakers were placed in a boiling water bath for 20 min and shaken gently every 5 min. The pH was adjusted to 7.5 with ~60 mL of 0.275 M NaOH, and samples were incubated with 300 μL of protease at 60 °C for 30 min with continuous agitation. After adjustment of the pH to 4.5 with ~60 mL of 0.325 M HCl, 350 μL of amyloglucosidase was added, and the mixture was incubated at 60 °C for 30 min with continuous agitation. Following centrifugation, the residue was washed two times with hot water, 95% (v/v) ethanol, and acetone and finally dried at 60 °C overnight in a vacuum oven. The isolated IDF was corrected for residual protein and ash contents.

Additionally, three different rye bran IDF samples were isolated using varying washing/extraction conditions following the last enzymatic step (incubation with amyloglucosidase): The centrifugation residue was (1) washed once with water; or (2) washed once each with water, 95% (v/v) ethanol, and acetone; or (3) washed twice with water and extracted for 5 h with ethanol and for 5 h with acetone. All samples were dried at 60 °C overnight in a vacuum oven.

Preparation of Alkali-Extracted Dietary Fiber (AEDF). To remove ester-linked monomeric, dimeric, or oligomeric phenolic material that may become confused with polymeric lignin, portions of the isolated IDF were saponified with 2 M NaOH for 18 h under nitrogen. The mixture was acidified (pH 2) with concentrated HCl. Liberated monomeric, dimeric, and oligomeric phenolic compounds were extracted into diethyl ether three times. The ether extract is referred to as EE. Combined ether extracts were evaporated under a stream of filtered air. The aqueous phase that contains most of the cereal fibers was neutralized with NaOH and lyophilized. This alkali-extracted dietary fiber is referred to as AEDF. All calculations are based on the original sample amount of IDF.

Saponification of Ester-Linked Cross-Products from Insoluble Dietary Fiber. IDF (40−90 mg) was weighed into a screw-cap tube, monomethylated 5-5′-diferulic acid (*22*) as internal standard (50 μg, dissolved in dioxane) was added, and saponification with 2 M NaOH (5 mL) was carried out under nitrogen and protected from light for 18 h at room temperature. The pH of the sample was adjusted to <2 with ~0.95 mL of concentrated HCl, and liberated phenolic compounds were extracted into diethyl ether (4 mL, × 3). Extracts were combined and evaporated under a stream of filtered air. Finally, samples were dried under vacuum.

GC-MS Analysis of Cross-Products. Dried extracts were trimethylsilylated by adding 10 μL of pyridine and 40 μL of BSTFA and heating for 30 min at 60 °C in sealed vials. Trimethylsilylated derivatives of cross-products were separated on a model 5980 GC (Hewlett-Packard, Palo Alto, CA) using a 25 m × 0.2 mm i.d., 0.33 μm film, DB-1 capillary column (J&W Scientific, Folsom, CA) and identified by their electron impact (70 eV) mass data collected on a Hewlett-Packard 5970 mass-selective detector. He (0.54 mL/min) was used as carrier gas. GC conditions were as follows: initial column temperature, 220 °C, held for 1 min, ramped at 4 °C/min to 248 °C, ramped at 30 °C/min to 300 °C, and held 40 min; injector temperature, 300 °C; split 1/50. Cross-products were authenticated by comparison of their relative retention times and mass spectra with those of synthesized or isolated standards. Mass spectra were also recorded by using another GC-MS system consisting of a Thermoquest Trace 2000 GC (Austin, TX) and a Thermoquest QSC ion-trap MS using the conditions already described above. Relative retention times against the internal standard (monomethylated 5-5′-diferulic acid) (for structures see **Figure 1**) were as follows: **6a** (0.70), **6b** (0.73), **7a** (0.58), **7b** (0.59), **10a** (0.60), **10b** (0.62), **11a** (0.87), **11b** (0.91). EI mass spectra of the silylated cross-products are given by Grabber et al. (*16*).

Derivatization Followed by Reductive Cleavage (DFRC Method). The DFRC method was performed as described by Lu and Ralph (*23*) with some minor modifications. Briefly, ~50 mg of IDF, or an amount of AEDF or EE resulting from ~50 mg IDF, was weighed into a round-bottom flask. Acetyl bromide reagent (acetyl bromide/acetic acid 20:80 v/v for IDF and AEDF or 8:92 for EE) was added [7.5 mL (IDF), 15 mL (AEDF), 2 mL (EE)]. The mixture was stirred at either 50 °C for 3 h (IDF and AEDF) or room temperature for 4 h (EE). The solvent was removed by rotary evaporation below 50 °C. Acidic reduction solvent (dioxane/acetic acid/water 5:4:1 v/v/v) was added to the evaporation residue [7.5 mL (IDF), 10 mL (AEDF), 2 mL (EE)]. Following the addition of zinc dust (50 mg) the mixture was stirred for 30 min. This mixture, CH_2Cl_2 (10 mL), and saturated NH_4Cl (10 mL) were transferred into a separatory funnel. The internal standard 4,4′-ethylidenebisphenol (0.05 mg in CH_2Cl_2) was added, and the pH of the aqueous phase was adjusted to <3. After vigorous mixing, the organic layer was separated and the extraction was repeated twice with 5 mL of CH_2Cl_2. The combined organic fractions were dried over $MgSO_4$ and evaporated. The dried residues of IDF and AEDF were redissolved in CH_2Cl_2 and transferred into a separatory funnel, and the separation step was repeated. The repeated separation step was not necessary for EE. Following the separation step the residues of IDF, AEDF, and EE were dissolved in 1.5 mL of CH_2Cl_2 and acetylated for 40 min with 0.2 mL of acetic anhydride and 0.2 mL of pyridine. Following the acetylation, the residue was obtained by coevaporation with ethanol under reduced pressure (*24*). A silica SPE column was conditioned with 2 mL of CH_2Cl_2. The acetylation product was redissolved in 200 μL of CH_2Cl_2 and transferred to the column. The products were eluted using 9 mL of $CHCl_3$/ethyl acetate 10:1. After evaporation without heating, the samples were dissolved in CH_2Cl_2 (200 μL) and analyzed by GC-MS and GC-FID.

GC-MS and GC-FID of DFRC Monomers. The DFRC monomers were authenticated by comparison of their relative retention times and mass spectra with those of synthesized standards using a GC-MS system consisting of a Thermoquest Trace 2000 GC and a Thermoquest QSC ion-trap MS. Acetylated monomers were separated using a 25 m × 0.2 mm i.d., 0.33 μm film, DB-1 capillary column (J&W Scientific).

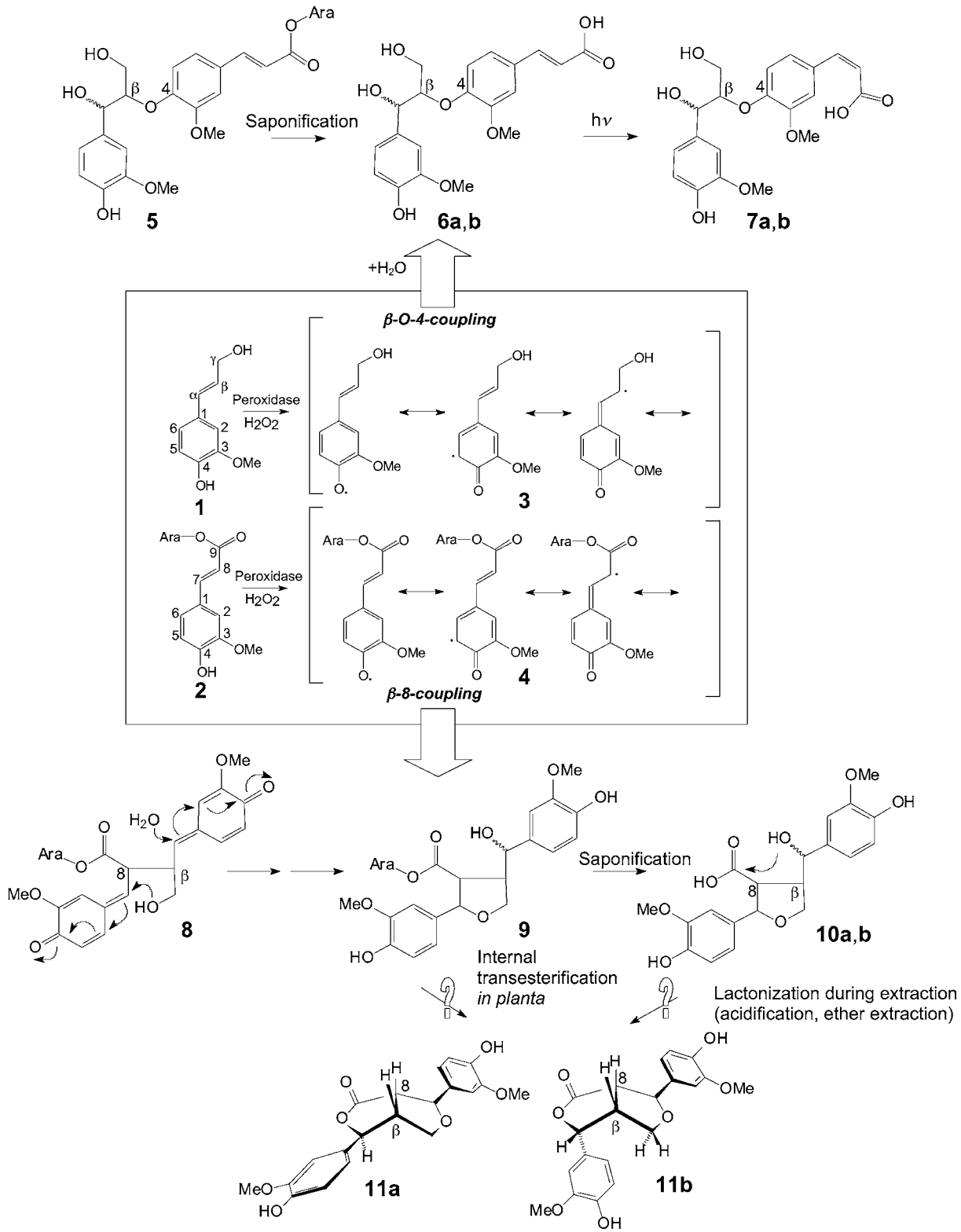

Figure 1. Radical or "active" mechanism for the incorporation of ferulates into lignins; formation of dimeric cross-products. Further structures are theoretically possible (*16*); only detected structures are shown. Radical cross-coupling products **5** and **9** are formed by oxidative coupling of coniferyl alcohol **1** with ferulate **2** and addition of water [Ara represents the polysaccharide (arabinoxylan) chain]. Compounds **6**, **10**, and **11** are saponification products arising during the analytical process. Addition of **a** and **b** indicates possible stereoisomers.

He (0.54 mL/min) was used as carrier gas. GC conditions were as follows: initial column temperature, 140 °C, held for 1 min, ramped at 3 °C/min to 250 °C, ramped at 10 °C/min to 300 °C, and held for 10 min; injector temperature, 220 °C; split, 1/20. Quantification was carried out using a Hewlett-Packard 5980 GC-FID system (Hewlett-Packard, Atlanta, GA): capillary column, 30 m × 0.2 mm i.d. SPB-5 (Supelco, Bellefonte, PA); carrier gas, He (1 mL/min); injector temperature, 220 °C; detector temperature, 300 °C. GC conditions were as follows: initial column temperature, 160 °C, ramped at 10 °C/min to 300 °C, and held for 5 min. Monomer determination was carried

out using response factors [coniferyl diacetate (G), 1.39; sinapyl diacetate (S), 1.44] derived from monomer standards against the internal standard.

Determination of Acetyl Bromide-Soluble Lignin (ABSL). The acetyl bromide lignin assay was performed as detailed by Fukushima and Hatfield (*25*). Briefly, in a screw-cap tube 100 mg of IDF, or an amount of AEDF resulting from 50 mg of IDF, was digested with 4 mL of a 25% acetyl bromide solution at 50 °C for 2 h. The tube was cooled, and 12 mL of acetic acid was added. After centrifugation, 0.5 mL (or 1 mL) of this solution was added to a tube containing 2.5 mL of acetic acid and 1.5 mL of 0.3 M NaOH. After mixing, 0.5 mL of 0.5 M hydroxylamine hydrochloride solution was added, and the volume was made up to 10 mL with acetic acid. The absorbance of the mixture was read at 280 nm. In the same way a sample blank was run. The concentration was determined using the equation

$$c\ [\mathrm{mg\ mL^{-1}}] = \frac{A_{\mathrm{sample}} - A_{\mathrm{blank}}}{\epsilon\ [\mathrm{cm^{-1}\ mg^{-1}\ mL}] \times L\ [\mathrm{cm}]}$$

with c = ABSL concentration in the final solution, L = cell thickness, and ϵ = absorptivity of lignin standard. For these calculations an absorptivity of 17.57 /cm/mg mL was used. This absorptivity is an average value calculated from absorptivities of lignin standards from grasses (corn rind, bromegrass stem, oat straw, and wheat straw) (*25*).

RESULTS AND DISCUSSION

Ferulate−Monolignol Cross-Products. The bifunctional nature of ferulic acid led to early speculation that it may act as a cross-link between polysaccharides and lignin. Later it was demonstrated that ferulates are lignin−polysaccharide cross-linking agents (*8*, *26*, *27*). Two mechanisms are discussed by which polysaccharides can become cross-linked to lignin during plant growth and development (*27*). The first mechanism has been described in the literature as a "passive" or "opportunistic" mechanism in which phenols add nucleophilically to quinone methide intermediates (*28*). Reactive quinone methides are produced during coupling of a monolignol at its β-position with a growing lignin oligomer/polymer at its 4-*O*-position. This mechanism can be demonstrated by model studies (*29*) but requires that the ferulate compete with other phenols and the more prevalent water. More recently it was shown that ferulates are at least partially incorporated into lignin by an "active" radical-coupling mechanism. This is a logical mechanism over which the plant has considerably more control and is simply an extension of the mechanism leading to ferulate dehydrodimers that cross-link polysaccharides (*30*). Moreover, it was shown that ferulates react with monolignols (*12*) and may function as initiation or nucleation sites for the lignification process (*13*). The release of ferulate−coniferyl alcohol cross-coupling products, and smaller amounts of ferulate−sinapyl alcohol and ferulate−*p*-hydroxycinnamyl alcohol cross-products, confirmed this "active" mechanism (*10*). Therefore, as a first step, we screened different insoluble cereal dietary fibers for ferulate−coniferyl alcohol cross-products, which should give the first indications for "real" lignin precursors in cereal grains. These lignin precursors are thought to be sites of growth for the polymeric lignin. If these precursors are detected, lignin should be found, assuming that polymerization will not generally stop at the dimeric stage. Following alkaline hydrolysis and extraction, several ferulate−coniferyl alcohol cross-products were identified. **Table 1** gives an overview of the identified cross-products in the different cereal insoluble dietary fibers (IDF). With the exception of maize (whole grain and bran) the *threo*- and *erythro*-stereoisomers of 4-*O*-β-coupled ferulate−coniferyl alcohol cross-products (**6a**, **6b**) were identified in all investigated cereal fibers. The *cis*-isomer of the *threo*-4-*O*-β-coupled ferulate−coniferyl alcohol cross-product **7a** was solely identified in rye IDF. A *trans/cis*-isomerization may be caused by light, for example, during sample preparation and product isolation. Because an unambiguous quantification was not plausible, "quantitative" aspects are merely based, where possible, on comparisons of peak areas relative to an internal standard. Bearing in mind these limitations, 4-*O*-β-coupled cross-products seem to be most prominent in rye IDF. Inspection of GC-FID chromatograms showed that peak areas of **6a** and **6b** in rye IDF are comparable with peak areas resulting from individual dehydrodiferulic acids. To date, eight dehydrodiferulic acids resulting from five possible coupling modes (8-5′-, 8-*O*-4′-, 8-8′-, 5-5′-, and 4-*O*-5′-coupling) were identified in cereal grains (*17*). The amounts of individual dehydrodimers of ferulic acid that are the major dimers in these cereal dietary fiber fractions and that elute in the same area of the chromatogram as the cross-products range between 0.2 mg/g of rye IDF (an 8-8′-coupled dehydrodiferulic acid) and 1.2 mg/g of rye IDF (an 8-5′-coupled dehydrodiferulic acid) (*17*). The 4-*O*-β-coupled ferulate−coniferyl alcohol cross-products were also identified by Jacquet et al. (*10*) in wheat and oat straws and by Grabber et al. (*16*) in artificially lignified primary maize walls. In contrast to the results of Jacquet et al. (*10*), 4-*O*-β-coupled cross-products of ferulate and sinapyl alcohol or of ferulate and *p*-coumaryl alcohol were not detected here.

Table 1. Identified[a] Ferulate−Coniferyl Alcohol Cross-Products from Different Cereal Grain Insoluble Dietary Fibers (for Structures See **Figure 1**)

	compound						
IDF	**6a**	**6b**	**7a**	**10a**	**10b**	**11a**	**11b**
barley	+	+	−	−	−	−	−
maize	−	−	−	−	−	−	−
millet	+	+	−	−	−	−	−
oat	+	+	−	−	+	−	−
rice	+	+	−	−	−	−	−
rye	+	+	+	+	+	+	+
spelt	+	+	−	−	+	+	−
wheat	+	+	−	−	+	+	−
wild rice	+	+	−	−	−	−	−

[a] +, identified; −, not detectable (compounds may be present but in amounts below the detection limit of the method used).

Long-range $^{13}C-^{1}H$ correlation (HMBC) NMR experiments previously detected an association between ferulate and ryegrass lignin via an 8-β-linkage (*13*) of the monoepoxylignanolide type (**11**). In some cereal IDFs here we identified the cross-products **11a,** and **11b,** which were particularly prevalent in rye IDF. The identification of **11a**,**b** remains surprising because their formation requires an intramolecular transesterification. This transesterification necessitates the ablation of the cross-product from the polysaccharide chain (**Figure 1**). During the preparation procedure of IDF, liberated cross-products should be extracted in the washing steps with ethanol and acetone. To verify that compounds **11a**,**b** are not just extraction residues from the IDF preparation procedure, different preparation procedures were investigated: Isolated rye bran IDF was (1) washed once with water; (2) washed once each with water, ethanol, and acetone; and (3) washed twice with water and extracted for 5 h with ethanol and 5 h with acetone. In the alkaline hydrolysates of all of the differently treated IDFs, **11a** and **11b** were detected in comparable amounts. This shows that it is unlikely that **11a**,**b** are extraction residues. Another possibility is that **11a**,**b** are, at least partially, formed from a precursor under conditions used for the analysis of phenolic compounds (saponification, acidification, extraction, and derivatization). The precursor of **11a**,**b**

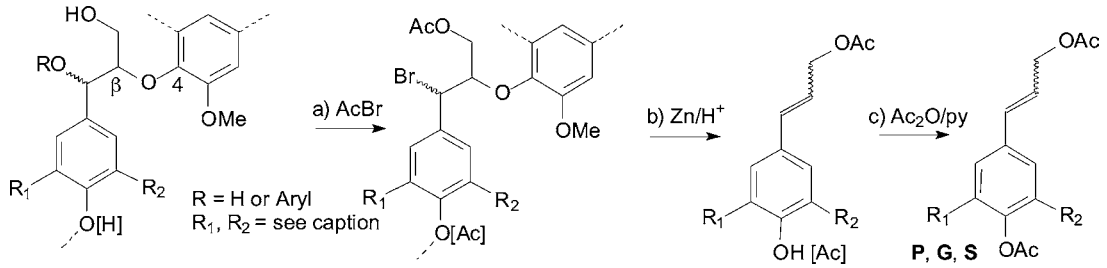

Figure 2. Basic steps of the DFRC method (*23*): (**a**) derivatization with acetyl bromide, accompanied by cleavage of α-ether linkages and cell-wall solubilization; (**b**) reductive cleavage of resulting α-bromo-*β*-ethers using zinc in an acidic medium; (**c**) following acetylation, 4-acetoxycinnamyl acetate monomers **P** ($R_1 = R_2 = H$; cleaved from *p*-coumaryl units originally derived from the monolignol *p*-coumaryl alcohol), **G** ($R_1 = OMe$, $R_2 = H$; derived from guaiacyl units from the monolignol coniferyl alcohol), and **S** ($R_1 = R_2 = OMe$; from syringyl units derived from the monolignol sinapyl alcohol) are identified by GC-MS and quantified by GC-FID.

should be compound **9** (**Figure 1**), which is still associated with the polysaccharide chain and acts as a cross-link between the polysaccharide and the developing lignin. Following the saponification, the acidic solution before and during the ethyl ether extraction is a suitable medium for a lactonization. Such a mechanism would be consistent with the above-mentioned results from the extraction experiments. This theory is fortified by the observation that in all fibers containing **11a**,**b** one or both of the stereoisomers of compound **10** were identified. **10a**,**b** are saponification products of **9** and are proof of a monolignol−ferulate−polysaccharide cross-link containing a coniferyl alcohol *β*-8-ferulate linkage. Recently, Grabber et al. (*16*) also detected compounds **10** and **11** in artificially lignified primary maize cell walls.

A recently published paper (*31*) claims proof that ferulate ethers in grasses are predominantly attached to the α-position of lignins, implicating the "passive" mechanism. The findings here and elsewhere of radical-coupling products, from "active" radical coupling, simply do not support such a contention. We suspect that the methodology used is not as specific for α-ethers as claimed. Our inability to detect ferulate−sinapyl alcohol cross-products may relate to the limited lignification in these tissues; sinapyl alcohol is typically incorporated later in the lignification process in a variety of plant systems (*32*).

Detection of Lignin Structures Using the DFRC Method. Most frequent interunit linkages in lignins are arylglycerol−*β*-aryl ethers, which are diagnostic for polymers formed by endwise coupling of monolignols with the growing polymer. To confirm the presence of actual lignin in cereal grains, a more definitive test for the presence of *β*-*O*-4-linkages connecting hydroxycinnamyl alcohol-derived units is required. Thioacidolysis (*33*−*35*) and the DFRC method (*21*, *23*, *36*) are both diagnostic for such linkages. We used the DFRC method, which partially converts lignins to the monolignols, as their peracetates, from which they are biosynthesized. This conversion is based on a selective cleavage of *β*-*O*-4-ether linkages in lignins. The DFRC and thioacidolysis methods are more explicit for the presence of lignin in the analyzed fibers than other degradation techniques often used to identify lignins. Lignins and other cell wall phenolics (e.g. ferulic acid, *p*-coumaric acid) may be degraded to the same hydroxybenzaldehyde products in the nitrobenzene oxidation, for example (*33*). The reaction basis of the DFRC method is shown in **Figure 2**.

Cereal grain dietary fibers, rye (whole grain and bran), wheat (whole grain), and maize (bran) IDFs, were analyzed using the DFRC method. Liberated acetylated monolignols were identified by GC-MS and quantified by GC-FID, and ratios between liberated lignin monomers were calculated. Lignin contents were estimated from the acetyl bromide-soluble lignin (ABSL) method (*37*, *38*). Due to the relatively high amounts of ferulate−coniferyl alcohol cross-products in rye IDF, this material was chosen for preliminary studies. Maize IDF was considered to be interesting because we did not detect any cross-products in these fibers.

To demonstrate "polymeric" lignin structures using the DFRC method but also for the determination of ABSL, it was essential to produce alkali-extracted dietary fibers (AEDF). Such fibers should be free of, for example, the crossed dimers **5** (**Figure 1**), which will also liberate coniferyl acetate. Although these crossed dimers are sites of growth for polymeric lignin, these compounds should not be considered as part of lignin due to their low molecular weights. To make sure that these dimers are not misleadingly identified as lignin, the IDF was saponified using 2 M NaOH to release them. Simple centrifugation of this preparation would result in a loss of alkali-soluble lignin. Therefore, the solution was acidified, and liberated hydroxycinnamates, which absorb at 280 nm and thus contribute to ABSL, and low molecular weight lignin-like fragments, for example, ferulate−coniferyl alcohol cross-products that would be mistaken for polymeric lignin using the DFRC method, were extracted into diethyl ether (fraction EE). "Polymeric" lignin should not extract into diethyl ether. The aqueous solution was neutralized and lyophilized. The residue bore a high salt content, but increasing reagent volumes proportionately made the implementation of the mentioned procedures possible. Using this procedure we can exclude the possibility that low molecular weight phenolic compounds interfere with the detection of "polymeric" lignin, an interference that should be excluded especially for the coniferyl alcohol lignin structures.

Using the DFRC method, coniferyl and sinapyl acetates were produced from all fibers investigated. This demonstrates that *β*-ether units derived mainly from monolignol−oligolignol coupling reactions are present, establishing the existence of lignin in cereal grains. Coniferyl and sinapyl acetates were identified from rye IDF, rye bran IDF, maize bran IDF, and wheat IDF and from their alkali-extracted fibers (**Figure 3**). Detection of *p*-coumaryl acetate in these low-lignin samples was not possible. Interfering peaks, which possibly result from polysaccharide degradation products not separated by solid-phase extraction, impede the identification and quantification of *p*-coumaryl acetate. Nevertheless, in the corresponding ether extracts (fractions EE) the identification of small amounts of *p*-coumaryl acetate was possible.

The presence of lignin in whole grain and bran rye IDF was expected due to their high levels of ferulate−coniferyl alcohol cross-products. The ratio between released sinapyl alcohol (as diacetate) and coniferyl alcohol (as diacetate) on a molar basis (S/G ratio) was calculated. In whole grain rye IDF and AEDF,

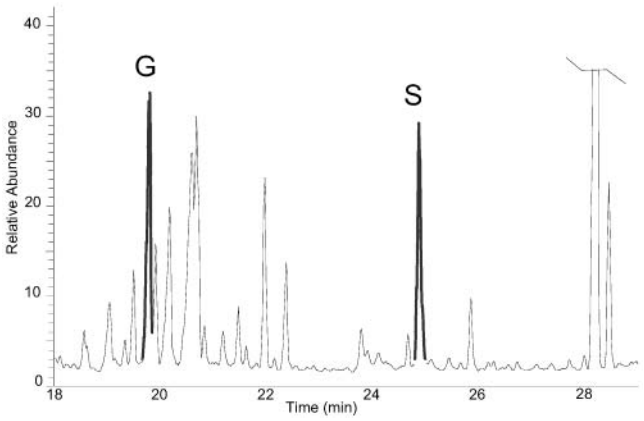

Figure 3. GC-MS chromatogram of DFRC monomers from rye alkali-extracted insoluble dietary fiber. G and S are 4-acetoxycinnamyl acetates, derived from guaiacyl and syringyl units as defined in **Figure 2**.

Table 2. Acetyl Bromide Soluble Lignin (ABSL) Contents of Different Cereal Grain Fractions (IDF, Insoluble Dietary Fiber; AEDF, Alkali-Extracted Dietary Fiber; EE, Ether Extract from Alkaline-Hydrolyzed Dietary Fiber) and S/G and G/S Ratios (S, Sinapyl Diacetate; G, Coniferyl Diacetate, Ratios Approximated to One Decimal Place) As Determined Using the DFRC Method

	ABSL (%)	ratio S/G	ratio G/S
rye (whole grain)			
IDF	8.5	0.3	3.0
AEDF	5.8	0.5	1.9
EE		0.1	13.5
rye (bran)			
IDF	6.7	0.4	2.4
AEDF	4.5	0.5	2.3
EE		0.1	13.3
maize (bran)			
IDF	14.1	1.6	0.6
AEDF	5.7	2.5	0.4
EE		0.1	7.3
wheat (whole grain)			
IDF	14.1	0.7	1.5
AEDF	5.7	1.1	0.9
EE		0.2	4.6

and also in rye bran IDF and AEDF, coniferyl alcohol dominated (**Table 2**). The EE that should contain low molecular weight lignin fragments including the cross-products, some of them 4-*O*-β-linked, showed even lower S/G ratios. This agrees with the fact that we found only ferulate−coniferyl alcohol dimeric cross-products. However, small amounts of sinapyl alcohol and *p*-coumaryl alcohol were also detected from the EE. The contents of ABSL in rye IDF (bran or whole grain) ranged between 6.7 and 8.5% in IDF and between 4.5 and 5.8% in AEDF (**Table 2**).

Lapierre et al. (*39*) recently described evidence for a lignin−heteroxylan association in maize bran heteroxylan samples. Therefore, it was surprising that we did not identify ferulate−monolignol cross-products in our maize samples. However, using the DFRC method, the existence of lignin structures was verified. Maize IDF and AEDF released sinapyl alcohol monomers in excess of coniferyl alcohol monomers. Although no dimeric cross-products were identified, maize IDF contained low molecular weight lignin-like material as shown by the application of the DFRC method to the maize IDF EE. Contrary to the other maize fractions, and in agreement with the rye and wheat IDF EE, coniferyl alcohol dominated over sinapyl alcohol in the EE.

The influence of ester-bound hydroxycinnamates on the determination of ABSL becomes especially clear when the ABSL contents of maize IDF, rich in ferulates and diferulates (*17*) (14.1% ABSL), are compared with maize AEDF (5.7% ABSL). ABSL contents for AEDF are still not absolutely reliable due to UV-active carbohydrate degradation products that can inflate the values (*38*).

Whole grain wheat IDF and AEDF showed S/G ratios of 0.7 and 1.1, respectively (**Table 2**). As expected in the EE, coniferyl alcohol clearly dominated. Contents of ABSL were determined at 8.9% (IDF) and 6.1% (AEDF).

The yields of the liberated monomers based on a dietary fiber and especially on an ABSL basis are surprisingly low. The molar sum of the monomers coniferyl alcohol and sinapyl alcohol ranged between about 5 and 15 μmol/g of dietary fiber and between 50 and 250 μmol/g of ABSL. In grass stems, for example, values of ~350 μmol/g of lignin would be expected, whereas in woods it is 800 μmol/g or more. There are several possible explanations for the very low yield on an ABSL basis. The easiest explanation would be an overestimation of the ABSL contents. Although we tried to minimize the influence of phenolic compounds other than lignin, ABSL determinations may still be inflated by other compounds detectable at 280 nm. Another possible explanation is that the matrix of the cereal fibers investigated prevents a complete solubilization in the reaction medium. A penetration of the active reagents to the inner reaches of the cell wall is not possible, and a complete conversion from lignin to monolignols is not feasible. A further possibility is based on a potentially different structure of cereal grain lignins compared to straw, wood, and other lignins that show higher yields using the DFRC method. This would signify that, in cereal grain lignins, monomers are bound to a lesser degree via ether linkages that are cleavable using the DFRC method. That is, they are more "condensed", a term used to denote lignin units coupled by other than cleavable ether units, for example, 5-5, β-5, β-β, 4-*O*-5, and β-1.

In conclusion, the results of this study demonstrate rather unambiguously that cereal grains are indeed lignified. Using the DFRC method on rye, maize, and wheat dietary fibers, diagnostic monomeric hydroxycinnamyl acetates from β-ether-linked units were liberated. Furthermore, we found monolignol β-*O*-4- and β-8-ferulate cross-products, implicating radical cross-coupling mechanisms in the wall. In future projects a more detailed structural characterization of cereal grain lignins is needed to explain their possible effects, especially binding of carcinogens in the human gastrointestinal tract.

NOTE ADDED IN PROOF

Since completion of this work, very recent reports have indicated that it is possible to liberate thioethylated monolignols from some cereal grains using the popular alternative to DFRC, analytical thioacidolysis (*34*, *35*). These reports did not, however, exclude the interference of low molecular weight phenolic compounds.

ABBREVIATIONS USED

ABSL, acetyl bromide soluble lignin; AEDF, alkali-extracted dietary fiber; DFRC, derivatization followed by reductive cleavage; EE, ether extract (from alkaline-hydrolyzed dietary fiber); G, coniferyl diacetate; IDF, insoluble dietary fiber; P, 4-acetoxycinnamyl acetate; S, sinapyl diacetate.

ACKNOWLEDGMENT

We are grateful to John Grabber for the suspension-cultured cell walls from which coniferyl alcohol−ferulate cross-products were isolated and to Hoon Kim and Jane Marita for general help and discussions.

LITERATURE CITED

(1) Prosky, L. When is dietary fiber considered a functional food? *Biofactors* **2000**, *12*, 289−297.

(2) Plaami, S. P. Content of dietary fiber in foods and its physiological effects. *Food Rev. Int.* **1997**, *13*, 29−76.

(3) Harris, P. J.; Ferguson, L. R. Dietary fibres may protect or enhance carcinogenesis. *Mutat. Res.* **1999**, *443*, 95−110.

(4) Glitso, L. V.; Jensen, B. B.; Knudsen, K. E. B. In vitro fermentation of rye carbohydrates including arabinoxylans of different structure. *J. Sci. Food Agric.* **2000**, *80*, 1211−1218.

(5) Ryden, P.; Robertson, J. A. The effect of fibre source and fermentation on the apparent binding properties of wheat bran preparations for the mutagen 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx). *Carcinogenesis* **1995**, *16*, 209−216.

(6) Manas, E.; Bravo, L.; Saura-Calixto, F. Sources of error in dietary fibre analysis. *Food Chem.* **1994**, *50*, 331−342.

(7) Jin, Z.; Akiyama, T.; Chung, B. Y.; Matsumoto, Y.; Iiyama, K.; Watanabe, S. Changes in lignin content of leaf litters during mulching. *Phytochemistry* **2003**, *64*, 1023−1031.

(8) Lam, T. B. T.; Iiyama, K.; Stone, B. A. Cinnamic acid bridges between cell-wall polymers in wheat and *Phalaris* internodes. *Phytochemistry* **1992**, *31*, 1179−1183.

(9) Ishii, T. Structure and functions of feruloylated polysaccharides. *Plant Sci.* **1997**, *127*, 111−127.

(10) Jacquet, G.; Pollet, B.; Lapierre, C.; Mhamdi, F.; Rolando, C. New ether-linked ferulic acid-coniferyl alcohol dimers identified in grass straws. *J. Agric. Food Chem.* **1995**, *43*, 2746−2751.

(11) Ralph, J.; Helm, R. F.; Quideau, S.; Hatfield, R. D. Lignin-feruloyl ester cross-links in grasses. Part 1. Incorporation of feruloyl esters into coniferyl alcohol dehydrogenation polymers. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2961−2969.

(12) Ralph, J.; Helm, R. F.; Quideau, S. Lignin-feruloyl ester cross-links in grasses. Part 2. Model compound syntheses. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2971−2980.

(13) Ralph, J.; Grabber, J. H.; Hatfield, R. D. Lignin-ferulate cross-links in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. *Carbohydr. Res.* **1995**, *275*, 167−178.

(14) Grabber, J. H.; Hatfield, R. D.; Ralph, J.; Zon, J.; Amrhein, N. Ferulate cross-linking in cell-walls isolated from maize cell-suspensions. *Phytochemistry* **1995**, *40*, 1077−1082.

(15) Quideau, S.; Ralph, J. Lignin-ferulate cross-links in grasses. Part 4. Incorporation of 5-5-coupled dehydrodiferulate into synthetic lignin. *J. Chem. Soc., Perkin Trans. 1* **1997**, 2351−2358.

(16) Grabber, J. H.; Ralph, J.; Hatfield, R. D. Model studies of ferulate-coniferyl alcohol cross-product formation in primary maize walls: Implications for lignification in grasses. *J. Agric. Food Chem.* **2002**, *50*, 6008−6016.

(17) Bunzel, M.; Ralph, J.; Marita, J. M.; Hatfield, R. D.; Steinhart, H. Diferulates as structural components in soluble and insoluble cereal dietary fibre. *J. Sci. Food Agric.* **2001**, *81*, 653−660.

(18) Helm, R. F.; Ralph, J. Lignin−hydroxycinnamyl model compounds related to forage cell wall structure. 1. Ether-linked structures. *J. Agric. Food Chem.* **1992**, *40*, 2167−2175.

(19) Grabber, J. H.; Ralph, J.; Hatfield, R. D. Modeling lignification in grasses with monolignol dehydropolymerisate-cell wall complexes. In *Lignin and Lignan Biosynthesis*; Lewis, N. G., Sarkanen, S., Eds.; American Chemical Society: Washington, DC, 1998; pp 163−171.

(20) Quideau, S.; Ralph, J. Facile large-scale synthesis of coniferyl, sinapyl, and *p*-coumaryl alcohol. *J. Agric. Food Chem.* **1992**, *40*, 1108−1110.

(21) Lu, F.; Ralph, J. The DFRC method for lignin analysis. Part 1. A new method for β-aryl ether cleavage: Lignin model studies. *J. Agric. Food Chem.* **1997**, *45*, 4655−4660.

(22) Bunzel, M.; Ralph, J.; Marita, J.; Steinhart, H. Identification of 4-O-5′-coupled diferulic acid from insoluble cereal fiber. *J. Agric. Food Chem.* **2000**, *48*, 3166−3169.

(23) Lu, F.; Ralph, J. Derivatization followed by reductive cleavage (DFRC method), a new method for lignin analysis: Protocol for analysis of DFRC monomers. *J. Agric. Food Chem.* **1997**, *45*, 2590−2592.

(24) Brunow, G.; Lundquist, K. On the acid-catalyzed alkylation of lignins. *Holzforschung* **1991**, *45*, 37−40.

(25) Fukushima, R. S.; Hatfield, R. D. Extraction and isolation of lignin for utilization as a standard to determine lignin concentration using the acetyl bromide spectrophotometric method. *J. Agric. Food Chem.* **2001**, *49*, 3133−3139.

(26) Lam, T. B. T.; Iiyama, K.; Stone, B. A. Changes in phenolic-acids from internode walls of wheat and phalaris during maturation. *Phytochemistry* **1992**, *31*, 2655−2658.

(27) Ralph, J.; Hatfield, R. D.; Grabber, J. H.; Jung, H. G.; Quideau, S.; Helm, R. F. Cell wall cross-linking in grasses by ferulates and diferulates. In *Lignin and Lignan Biosynthesis*; Lewis, N. G., Sarkanen, S., Eds.; American Chemical Society: Washington, DC, 1998; pp 209−236.

(28) Ralph, J.; Helm, R. F. Lignin/hydroxycinnamic acid/polysaccharide complexes: Synthetic models for regiochemical characterization. In *Forage Cell Wall Structure and Digestibility, International Symposium*; Jung, H. G., Buxton, D. R., Hatfield, R. D., Ralph, J., Eds.; ASA-CSSA-SSSA: Madison, WI, 1993; pp 201−246.

(29) Scalbert, A.; Monties, B.; Rolando, C.; Sierra-Escudero, A. Formation of ether linkage between phenolic acids and Gramineae lignin: a possible mechanism involving quinone methides. *Holzforschung* **1986**, *40*, 191−195.

(30) Ralph, J.; Quideau, S.; Grabber, J. H.; Hatfield, R. D. Identification and synthesis of new ferulic acid dehydrodimers present in grass cell-walls. *J. Chem. Soc., Perkin Trans. 1* **1994**, 3485−3498.

(31) Lam, T. B. T.; Kadoya, K.; Iiyama, K. Bonding of hydroxycinnamic acids to lignin: ferulic and *p*-coumaric acids are predominantly linked at the benzyl position of lignin, not the β-position, in grass cell walls. *Phytochemistry* **2001**, *57*, 987−992.

(32) Terashima, N.; Fukushima, K.; He, L.-F.; Takabe, K. Comprehensive model of the lignified plant cell wall. In *Forage Cell Wall Structure and Digestibility*; Jung, H. G., Buxton, D. R., Hatfield, R. D., Ralph, J., Eds.; ASA-CSSA-SSSA: Madison, WI, 1993; pp 247−270.

(33) Lapierre, C. Application of new methods for the investigation of lignin structure. In *Forage Cell Wall Structure and Digestibility*; Jung, H. G., Buxton, D. R., Hatfield, R. D., Ralph, J., Eds.; ASA-CSSA-SSSA: Madison, WI, 1993; pp 133−166.

(34) Antoine, C.; Peyron, S.; Mabille, F.; Lapierre, C.; Bouchet, B.; Abecassis, J.; Rouau, X. Individual contribution of grain outer layers and their cell wall structure to the mechanical properties of wheat bran. *J. Agric. Food Chem.* **2003**, *51*, 2026−2033.

(35) Begum, A. N.; Nicolle, C.; Mila, I.; Lapierre, C.; Nagano, K.; Fukushima, K.; Heinonen, S.-M.; Adlercreutz, H.; Remesy, C.; Scalbert, A. Dietary lignins are precursors of mammalian lignans in rats. *J. Nutr.* **2004**, *134*, 120−127.

(36) Lu, F.; Ralph, J. The DFRC method for lignin analysis. Part 2. Monomers from isolated lignins. *J. Agric. Food Chem.* **1998**, *46*, 547−552.

(37) Johnson, D. B.; Moore, W. E.; Zank, L. C. The spectrophotometric determination of lignin in small wood samples. *Tappi* **1961**, *44*, 793−798.

(38) Hatfield, R. D.; Grabber, J. H.; Ralph, J.; Brei, K. Using the acetyl bromide assay to determine lignin concentrations in herbaceous plants: Some cautionary notes. *J. Agric. Food Chem.* **1999**, *47*, 628−632.

(39) Lapierre, C.; Pollet, B.; Ralet, M. C.; Saulnier, L. The phenolic fraction of maize bran: Evidence for lignin−heteroxylan association. *Phytochemistry* **2001**, *57*, 765−772.

Received for review April 23, 2004. Revised manuscript received July 16, 2004. Accepted July 16, 2004. We are grateful to the CSREES-USDA-NRI competitive grants program (Grant 2003-01246) for partial funding.

JF040204P