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NMR Characterization of Lignins Isolated from Fruit and Vegetable Insoluble Dietary Fiber

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Compositional information for lignins in food is rare and concentrated on cereal grains and brans. As lignins are suspected to have important health roles in the dietary fiber complex, the confusing current information derived from nonspecific lignin determination methods needs to be augmented by diagnostic structural studies. For this study, lignin fractions were isolated from kiwi, pear, rhubarb, and, for comparison, wheat bran insoluble dietary fiber. Clean pear and kiwi lignin isolates allowed for substantive structural profiling, but it is suggested that the significance of lignin in wheat has been overestimated by reliance on nonspecific analytical methods. Volume integration of NMR contours in two-dimensional $^{13}C-^{1}H$ correlation spectra shows that pear and wheat lignins have comparable guaiacyl and syringyl contributions and that kiwi lignins are particularly guaiacyl-rich (~94% guaiacyl) and suggest that rhubarb lignins, which could not be isolated from contaminating materials, are as syringyl-rich (~96% syringyl) as lignins from any known natural or transgenic fiber source. Typical lignin structures, including those newly NMR-validated (glycerols, spirodienones, and dibenzodioxocins), and resinols implicated as possible mammalian lignan precursors in the gut are demonstrated via their NMR correlation spectra in the fruit and vegetable samples. A novel putative benzodioxane structure appears to be associated with the kiwi lignin. It is concluded that the fruits and vegetables examined contain authentic lignins and that the detailed structural analysis exposes limitations of currently accepted analytical methods.

KEYWORDS: Dietary fiber; plant cell wall; dioxane−water milled lignin; ball-milling; acidolysis lignin; pear; kiwi; rhubarb; wheat; NMR; HSQC; HMBC; gut fermentation; lignans

INTRODUCTION

According to a definition adopted in 2001 by the American Association of Cereal Chemists (*1*), dietary fiber is "the edible part 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. Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation." Lignins, most simply described as natural aromatic polymers of 4-hydroxyphenylpropanoids, with units connected by ether and carbon−carbon linkages, are regarded as part of the dietary fiber complex according to this definition. Using the enzymatic−gravimetric determination of dietary fiber, lignin is part of the insoluble dietary fiber fraction (*2*). Within some chemical methodologies, for example, in the Southgate (*3*) and Uppsala (*4*) methods, lignin is determined as Klason lignin, whereas the U.K. method of Englyst and Cummings (*5*) does not include an estimation of lignin. However, the determination of Klason lignin does not measure real lignin contents in food; components including cutin, tannin−protein complexes, structural proteins and, in heated foods, Maillard reaction products may also partially analyze as Klason lignin. As a consequence, the information about lignin contents in food is quite confusing (*6*). However, lignins as part of the dietary fiber complex might be more important than previously considered. They have been shown to be efficient antioxidants (*7*−*9*), possibly scavenging superoxide and hydroxyl radicals, inhibiting the activity of xanthine oxidase and glucose-6-phosphate dehydrogenase, and inhibiting nonenzymatic and enzymatic lipid peroxidation (*8*). From studies of the antioxidant activity of different kinds of lignins and lignin model compounds, structure−activity relationships were proposed (*9*, *10*). As part of the cell wall complex, lignins are

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efficient adsorbers of hydrophobic heterocyclic aromatic amines (*11*−*13*). Lignin concentration and composition both influence the adsorption capacities of the cell wall complex (*11*). Animal experiments using chemically induced intestinal carcinogenesis showed that the incidence and multiplicity of small intestinal adenocarcinomas and the number of colon adenocarcinomas were lower in animals fed a lignin-supplemented diet compared to animals fed a normal diet (*14*). Although lignin was usually considered to be nondegradable by gut microorganisms, a seminal study recently showed that lignins are converted into mammalian lignans in rats. Resinol structures within the lignins were proposed to be metabolized to enterolactone by the gut microflora (*15*).

Average daily lignin intake has been calculated to be 1.6−2.0 g/day. However, these values are probably overestimates as they derive from food compositional data in which lignin levels are determined mostly with the Klason method (*6*). Compositional information about lignins in food is rare and concentrated on cereal grains and brans (*15*−*18*) that are believed to be highly lignified. Recently, we analyzed a range of fruit and vegetable insoluble dietary fibers for their lignin contents and compositions using the DFRC methodology (*19*). These studies showed that pear, kiwi, and rhubarb insoluble dietary fibers contained comparatively high amounts of lignin. Moreover, compositions varied from being rich in guaiacyl units (kiwi) to being strikingly rich in syringyl units (rhubarb). The aim of this study was to structurally characterize lignin fractions from pear, kiwi, and rhubarb in more detail by NMR to provide a basis for understanding various health effects. The lignins from these plants were compared to those obtained from wheat bran, traditionally regarded as being a source rich in lignin.

MATERIALS AND METHODS

General. Heat-stable α-amylase Termamyl 120 L (EC 3.2.1.1, from *Bacillus licheniformis*, 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 kindly donated by Novo Nordisk, Bagsvaerd, Denmark. The carbohydrase mixture "Driselase" (from Basidiomycetes, 0.98 U/mg) was from Sigma, St. Louis, MO.

Material. Kiwi fruit (Chinese gooseberries) (*Actinidia chinensis* Planch. 'Hayward', harvested in 2005 in Italy), pears (*Pyrus communis* L. 'Conference', harvested in 2005 in Belgium), and rhubarb (*Rheum rhabarbarum* L. 'Esta', harvested in 2005 in the Rheinland region, Germany) were obtained from a local German supplier, and wheat bran (*Triticum aestivum* L.) was from a local mill, obtained from standardized grain mixtures harvested in northern Germany.

Preparation of Insoluble Dietary Fibers. Kiwi fruit, pears, and rhubarb were prepared for consumption as typically performed in the household kitchen: The kiwi fruits were pared, cores from the washed but unpared pears were removed, and rhubarb leaves were cut and the petioles washed and thinly pared. Fruits and vegetables were crushed, frozen, and lyophilized. The dried material was ground using a mortar and pestle to a particle size smaller than 0.5 mm. Wheat bran was milled to a particle size smaller than 0.5 mm using a fly cutter mill. Isolation of insoluble dietary fibers followed a preparative procedure described by Bunzel et al. (*19*). In brief, starch and proteins of the dried plant materials were degraded in a phosphate buffer using a sequence of a heat-stable α-amylase (pH 6.0, 100 °C, 20 min), a protease (pH 7.5, 60 °C, 30 min), and an amyloglucosidase (pH 4.5, 60 °C, 30 min). Following centrifugation, the residue was washed three times with hot (70 °C) water and once with 95% (v/v) ethanol and acetone and finally dried at 40 °C overnight in a vacuum oven. The dried residues were further extracted with ethanol (8 h), dichloromethane (8 h), and *n*-hexane (8 h) using a Soxhlet apparatus. Finally, the extracted insoluble dietary fibers were dried at 40 °C overnight in a vacuum oven.

Lignin Isolation. Two lignin fractions were isolated. A cellulolytic enzyme lignin, here simply called a "milled (plant) lignin" or ML but similar to the cellulolytic enzyme lignins (CEL) described previously (*20*, *21*), utilizes crude cellulases to treat ball-milled plant material and improve the yield of 96:4 dioxane−water-extractable lignin; this solvent is used directly on the plant material in the traditional Björkman method (*22*). Liberating further lignin from the residue after extraction was via mild acid hydrolysis according to methods described previously (*23*−*27*) to give what are designated here (mild) acidolysis lignins (AL). The advantage of this sequential method is that the highest yield of solvent-soluble lignin is obtained without the incipient acid degradation caused by even mild acidolysis.

Milled Lignins (ML). The extracted dietary fibers (about 10 g) were ball-milled for 2.5 h in 30-min-on 30-min-off cycles, to avoid excessive sample heating, using a custom-made ball mill using an offset $^1/_4$-hp Dayton motor running at 1725 revolutions/min with rotating (0.2 Hz) stainless steel vessels (12.2 cm diameter, 11.4 cm high) containing about 3.7 kg of 5 mm stainless steel ball bearings. Polysaccharides from ball-milled fibers (dry weights: kiwi, 10.9 g; pear, 10.5 g; rhubarb, 10.6; wheat, 20.0 g) were partially enzymatically digested using a carbohydrase mixture (40 mg of Driselase and 45 mL of water per gram of fiber) for 48 h at 37 °C. Residues were centrifuged and washed four times with water (50 mL) and lyophilized. The residues made up between 44 and 62% of the insoluble fiber fractions (kiwi, 58%; pear, 62%; rhubarb, 44%; wheat, 53%). The major part of the dried residues (kiwi, 6.2 g; pear, 6.3 g; rhubarb, 4.6 g; wheat, 8.9 g) were suspended in a dioxane−water (96:4, v/v) solution (*28*) (kiwi, pear, and wheat, 15 mL/g; rhubarb, 25 mL/g) and stirred for 48 h at room temperature protected from light. The suspension was centrifuged, the supernatant was collected, and the residues were resuspended in dioxane−water (96:4, v/v) and stirred for another 48 h. Following centrifugation, the residues were washed with dioxane−water (96:4, v/v), and the combined dioxane−water supernatants were filtered through glass filter crucibles (porosity 3, pore size = 16−40 μm) and evaporated under reduced pressure. Dried filtrates were designated milled lignins (ML) and acetylated as described below. Traditional purification methods (*29*) further fractionate the sample (reducing the amount for analysis) and were not attempted. Purity is not required for 2D NMR analysis of the lignin component; in fact, substantive lignin structural analysis can even be performed on unfractionated ball-milled whole cell walls (*30*, *31*).

Acidolysis Lignins (AL). The centrifugation residues following ML extraction were dried under the fume hood and were used for the isolation of mild acidolysis lignins, similar to the "enzyme mild acidolysis lignins" described by Wu and Argyropoulos (*26*) but not on the whole plant cell wall. Dried residues were transferred into a two-neck flask and suspended in a mixture of dioxane−0.01 M HCl (85:15, v/v). Nitrogen was bubbled through the solution for 10 min before the pipet was pulled out, and the suspension was heated for 2 h under reflux and under a constant flow of nitrogen. Following filtration through glass filter crucibles (porosity 3, pore size = 16−40 μm) and washing of the residues with dioxane−water (85:15, v/v) and dioxane, filtrates were combined and neutralized by adding sodium bicarbonate. Neutralized filtrates were reduced in volume to about 10 mL and added dropwise into 1.1 L of acidified water (pH 2). Before centrifugation, precipitation was completed by allowing the flask to stand for 1 h in the refrigerator. Following centrifugation, residues were washed three times with water, lyophilized, and designated (mild) acidolysis lignin (AL). Acidolysis lignins were acetylated as described below.

Lignin Acetylation. Lignins were solubilized in pyridine (3 mL) and acetic anhydride (3 mL) and acetylated overnight. Following evaporation, the residues were coevaporated with ethanol (four times) to remove residual solvents and finally dried under high vacuum. The procedure resulted in the following amounts of acetylated milled lignin (Ac-ML): kiwi, 282 mg; pear, 301 mg; rhubarb, 159 mg; wheat, 164 mg. Whereas the acetylated milled lignins from kiwi, pear, and rhubarb were readily soluble in acetone, that from wheat was not fully soluble. The following amounts of acetylated mild acidolysis lignins (Ac-AL) were obtained: kiwi, 264 mg; pear, 268 mg; rhubarb, 74 mg; wheat, 8 mg. Again, the acetylated acidolysis lignin from wheat was only partially soluble in acetone. Similarly, a large fraction of the acetylated acidolysis lignin from rhubarb was insoluble in acetone as well as in chloroform.

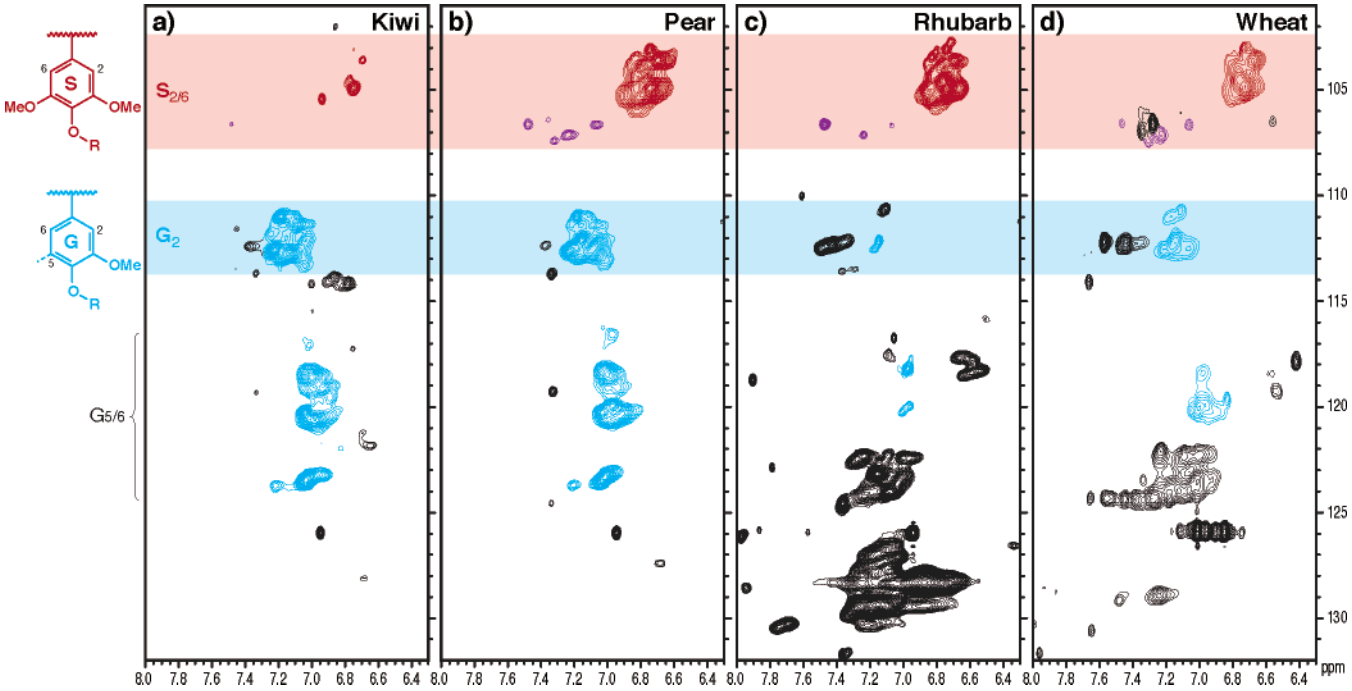

Figure 1. Partial short-range ^{13}C–^{1}H (HSQC) correlation spectra (aromatic regions only) of acetylated milled lignins (Ac-ML) isolated from insoluble fiber fractions from (**a**) kiwi, (**b**) pear, (**c**) rhubarb, and (**d**) wheat. Pear and wheat are syringyl/guaiacyl lignins, whereas kiwi is guaiacyl-rich and rhubarb is particularly syringyl-rich. Semiquantitative syringyl/guaiacyl distributions are given in **Table 1**. Correlations from α-keto syringyl units are colored purple.

NMR Spectroscopy. The NMR spectra were acquired on a Bruker Biospin (Rheinstetten, Germany) DMX-500 instrument fitted with a sensitive cryogenically cooled 5-mm TXI $^{1}H/^{13}C/^{15}N$ gradient probe with inverse geometry (proton coils closest to the sample). Acetylated lignin preparations (60−80 mg) were dissolved in 0.5 mL of acetone-d_6; the central acetone solvent peak was used as internal reference (δ_C 29.8, δ_H 2.04). We used the standard Bruker implementations of 1D (^{1}H) and 2D (gradient-selected, ^{1}H-detected HSQC and HMBC) NMR experiments for structural elucidation and assignment authentication. NMR data for model compounds can be found in the "NMR Database of lignin and cell wall model compounds" (*32*). Normal HSQC experiments, **Figures 1**−**3**, were acquired with the following parameters: acquired from 8.4 to 2.6 ppm in F_2 (^{1}H) using 1120 datapoints (acquisition time = 194 ms), 142−46 ppm in F_1 (^{13}C) using 512 increments (F_1 "acquisition time" = 21.2 ms) of 16 scans with a 1 s interscan delay, total acquisition time of 2 h and 54 min; the d_{24} delay was set to 1.72 ms (∼1/4J). Processing used typical matched Gaussian apodization in F_2 and squared sine-bell in F_1. HMBC experiments, **Figure 4**, had the following parameters: acquired from 11 to 0 ppm in F_2 (^{1}H) using 3K datapoints (acquisition time = 280 ms), 210−0 ppm in F_1 (^{13}C) using 480 increments (F_1 "acquisition time" = 9 ms) of up to 80 scans with a 1 s interscan delay, 80 ms long-range coupling delay, total acquisition time of up to 14 h and 45 min. Processing to a final matrix of 2K by 1K datapoints used typical Gaussian apodization in F_2 (LB −30, GB 0.5) and squared sine-bell in F_1. One level of linear prediction in F_1 (32 coefficients) gave improved F_1 resolution but was not required.

Volume integration of contours in HSQC plots was accomplished using Bruker's TopSpin 1.3 software as described recently (*33*). For quantification of syringyl/guaiacyl distributions, only the carbon-2 correlations from guaiacyl units and the carbon-2/6 correlations from syringyl units were used, and the guaiacyl integrals were logically doubled. No correction factors were necessary. For quantification of the various interunit linkage types, the following well-resolved contours (**Figures 2** and **3**) were integrated: **A**α, **B**α, **C**α, **D**α, **F**α (and **F**β if **F**α was not fully resolved), **S**α, **X1**γ, and **X7**β. Integral correction factors determined previously (*33*) were used: **A**α 1.00, **B**α 0.71, **C**α 1.06, **D**α 0.87, and **X7**β 0.77; **S**α, **F**α, and **X1**γ were either not or not reliably determined and are assumed as 1.00; the response factor for converting **F**β integrals to **F**α integrals was determined to be 2.00 from samples in which both correlations were resolved. These values were used to correct the volume integrals to provide the semiquantitative estimates of unit ratios in **Table 1**.

RESULTS AND DISCUSSION

Isolation Procedure. Defining lignins is not trivial because they show considerable diversity (*34*). Their isolation is generally incomplete, especially when nondegradative or mild methodologies are used. The known isolation procedures are not specific for lignins, coextracting non-lignin material that it is not possible to completely remove by further cleanup steps. Detailed structural studies can be carried out using NMR on solubilized finely divided whole cell wall material without requiring any fractionation (*30*, *31*), but isolation was deemed to be necessary here due to the comparatively low lignin levels in some samples and our concern that polyphenolic material might comprise more than authentic lignins. The methodology that is regarded as the least degradative is the isolation of so-called Björkman-lignin or milled (plant) lignin (*28*), although the ball-milling step breaks bonds, complicating any assessment of the native lignins' molecular weight distribution. We chose to increase the yields of lignins soluble in the 96:4 dioxane−water by first enzyme-digesting away a major fraction of the polysaccharides, as described in the cellulolytic enzyme lignin methodologies (*20*, *21*) and as we have used in prior lignin NMR studies (*35*−*37*). The acetylated milled lignins (Ac-ML), with the exception of wheat, isolated in this study were readily soluble in acetone; acetone- and chloroform-insoluble material that most likely is not composed of lignin or coextracted carbohydrates was removed. The same solubility issues were noted using rye insoluble dietary fibers as starting material for the extraction (unpublished results). A further fraction, a mild acidolysis lignin (AL) (*24*, *26*, *27*), was isolated from the residual fiber material following the dioxane−water extraction, using 0.01 M HCl in dioxane to putatively cleave lignin carbohydrate bonds. This mild acidolysis procedure worked well for kiwi and pear, resulting in additional amounts of quite pure lignins suitable for NMR structural characterization. Acidolysis lignin from

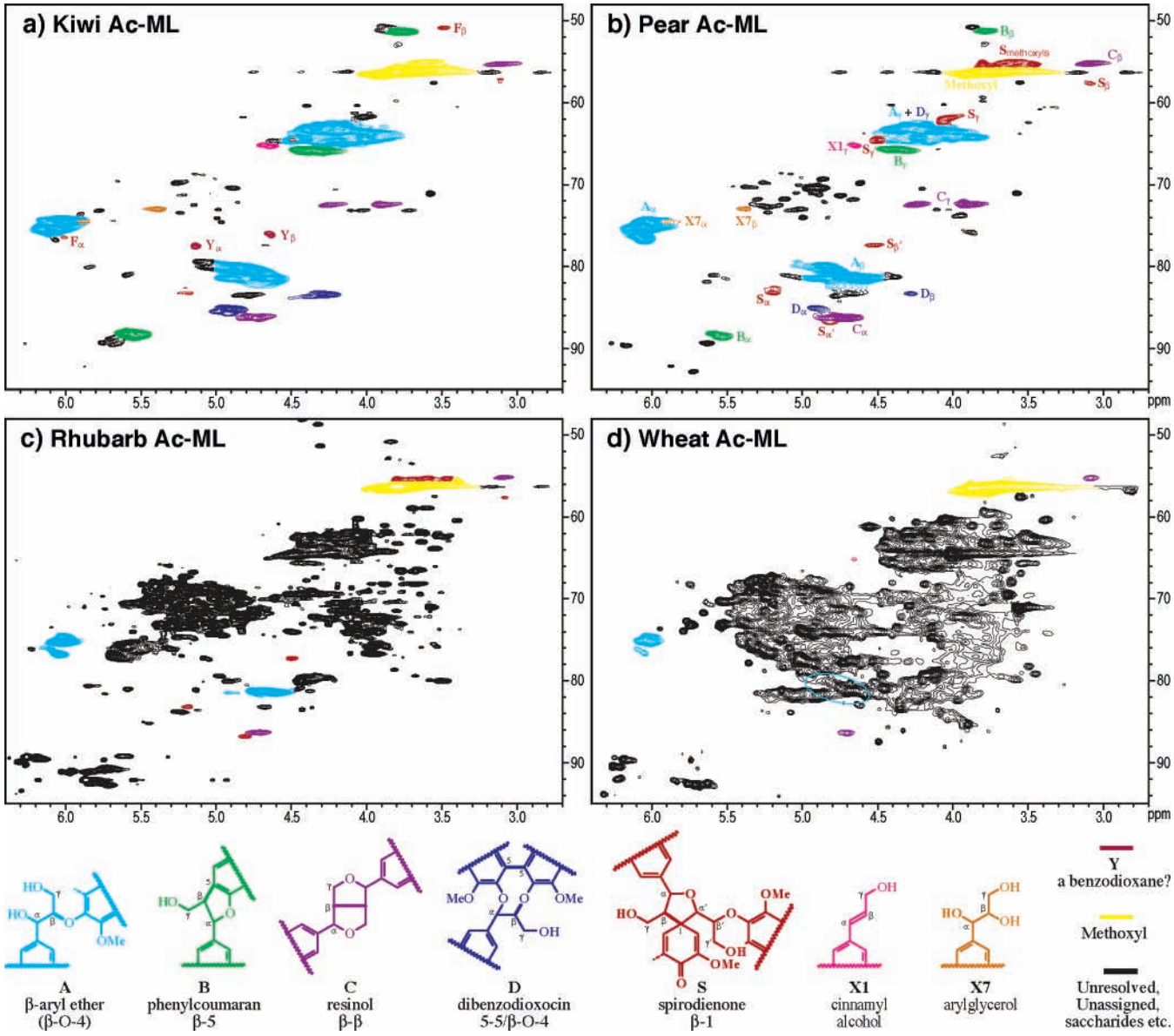

Figure 2. Partial short-range ^{13}C–^{1}H (HSQC) spectra (side-chain regions) of acetylated milled lignins (Ac-ML) isolated from insoluble fiber fractions from (**a**) kiwi, (**b**) pear, (**c**) rhubarb, and (**d**) wheat. Interunit type designations **A**–**D**, **F**, **S**, **X1** and **X7** follow conventions established previously (*33*–*35*, *52*). The β-1-unit **F** is shown in **Figure 3**. New putative benzodioxanes in the kiwi lignin (**a**) are designated **Y**. Semiquantitative distributions of the various unit types are given in **Table 1**.

rhubarb was a nearly black powder (compared to the slightly brown-greenish powders from the other sources) that was not completely soluble in acetone, or chloroform, following acetylation; only the acetone-soluble fraction was used for NMR studies. From wheat, comparably tiny amounts of acidolysis lignin were achieved (yield = 0.4 mg of Ac-AL/g of total insoluble dietary fiber) that again were not completely soluble in acetone following acetylation. Assuming that acidolysis will allow the extraction of higher molecular mass lignins compared to the Björkman methodology, the lignin-like material in wheat seems to be comparatively low molecular weight in nature. With the NMR results from the wheat milled lignin discussed below, it would appear to be inadvisable to further consider wheat insoluble fibers as lignin-rich. Acidolysis lignins were, as previously reported, less contaminated by carbohydrates (*24*), as demonstrated by the HSQC spectra in **Figures 2** and **3**. However, unlike the acidolysis lignins, milled lignins were not precipitated into water to remove low molecular weight saccharides. Saccharide contamination does not significantly interfere with lignin signals in the 2D NMR experiments. The nature of contaminating polysaccharide, aromatic, and other components was not further addressed in this study, which sought out the lignin components.

NMR Characterization: Syringyl−Guaiacyl Nature. Determination of the syringyl/guaiacyl distribution is important because it has been shown to influence the adsorption of heterocyclic aromatic amines (*11*). In addition to the presence of non-etherified phenolic groups, ortho-methoxyl groups positively contribute to the radical scavenging activity of lignins (*9*). The lignins isolated from kiwi and pear in particular were largely typical of lignins from other sources, as seen in **Figure 2**, suggesting that the insoluble dietary fiber from these fruits does indeed contain authentic lignin components. **Figure 1** shows the aromatic regions of HSQC ($^{13}C-^{1}H$ correlation) spectra of the acetylated milled lignins from which the syringyl/guaiacyl distribution is delineated by volume integration of the syringyl-2/6 and guaiacyl-2 contours, as described previously (*33*). From **Figure 1** and from the integral data in **Table 1**, the

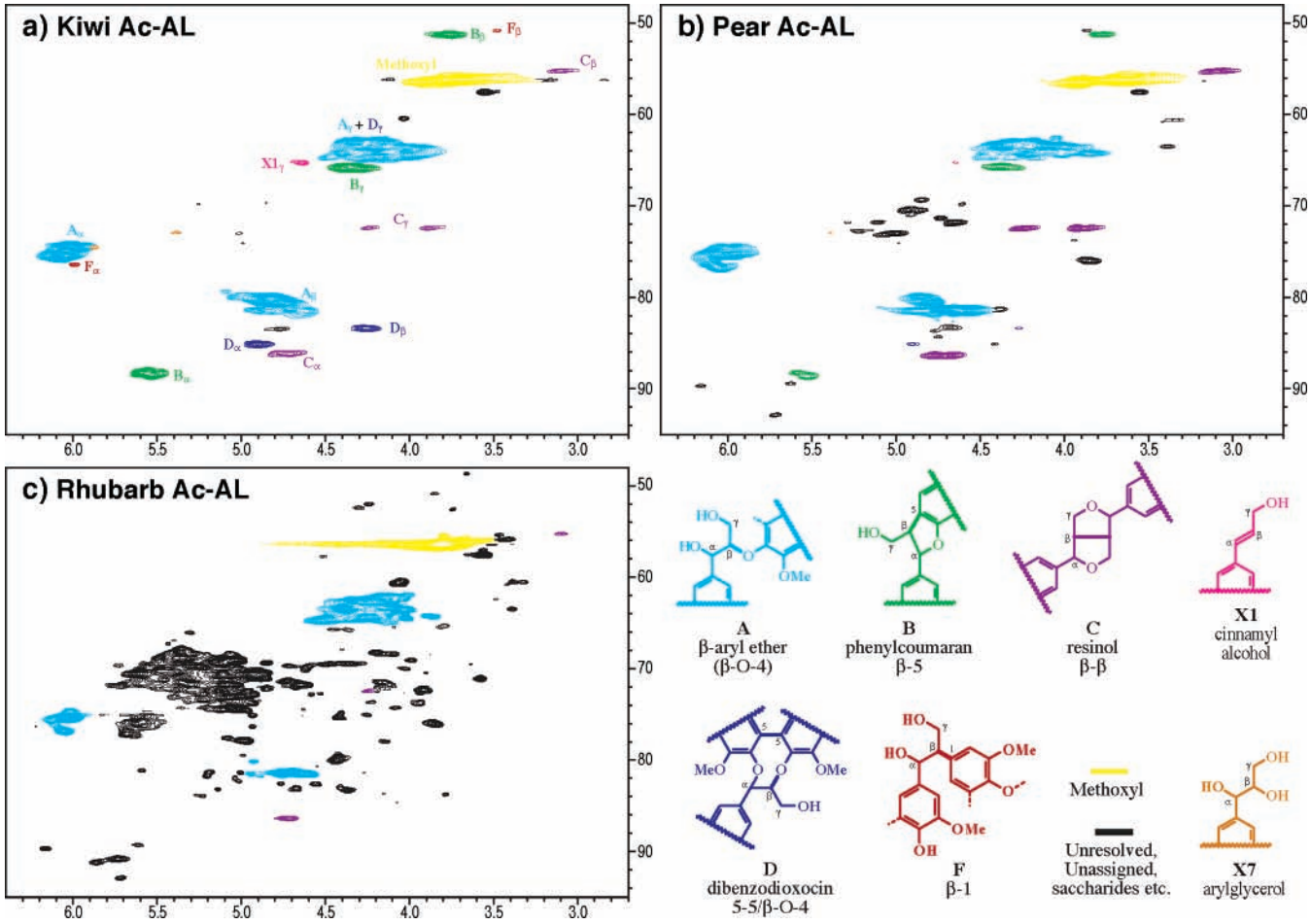

Figure 3. Partial short-range $^{13}C–^{1}H$ (HSQC) spectra (side-chain regions) of acetylated acidolysis lignins (Ac-AL) isolated from the dioxane–water-insoluble residues of insoluble fiber fractions from (**a**) kiwi, (**b**) pear, and (**c**) rhubarb. Interunit type designations follow those used in **Figure 2**, except that **F** is the β-1-structure derived from spirodienones **S** under acidic conditions. Semiquantitative distributions of the various unit types are given in **Table 1**.

pear and wheat milled lignins have roughly equivalent syringyl and guaiacyl levels (55% syringyl and 45% guaiacyl in each), whereas the kiwi lignin is particularly guaiacyl-rich (~94% guaiacyl). Grass (stem) lignins may contain *p*-hydroxyphenyl units derived from *p*-coumaryl alcohol, but these are not evident in the wheat grain lignin, although small amounts of *p*-coumarate appear to be indicated. The rhubarb lignin, although significantly contaminated by unknown components, is strikingly syringyl-rich (~96% syringyl). Such syringyl levels are perhaps higher than the highest reported in nature (*38*), with kenaf bast fiber lignin being among the highest at 85−94% (*39*, *40*). Even transgenics in which syringyl levels have been extraordinarily enhanced by up-regulation of the crucial hydroxylase ferulate 5-hydroxylase, now often more accurately denoted coniferaldehyde 5-hydroxylase, approach only similar levels: about 92% in F5H-up-regulated *Arabidopsis* (*41*) and as high as 93% in poplar or aspen (*42*, *43*). It should be noted that these levels were all measured by different means and so may not be directly comparable.

Measurements from the acetylated mild acidolysis lignins, obtained from the residual material after extraction of simple milled lignins, reveal similar levels, but are even more extreme in some cases (**Table 1**). For example, the rhubarb acidolysis lignin may be almost 98% syringyl. Clearly, this material warrants further examination, along with an elucidation of the other aromatic components that characterize this fraction (**Figure 1c**). Because only small amounts of partially acetone-soluble mild acidolysis lignin were obtained from wheat, characterization of this fraction by NMR was not fruitful.

The syringyl/guaiacyl distributions are comparable to those determined on insoluble fiber by using the DFRC method (**Table 1**) (*16*, *19*). The syringyl/guaiacyl ratio from rhubarb using the DFRC methodology was lower than determined here, probably due to an impurity under the coniferyl diacetate peak in the GC chromatogram, as stated in ref *19*. These quantification methods are not fully comparable, because the DFRC method determines only lignin units connected via β-aryl ethers.

NMR spectra reveal the presence of aldehyde and keto groups also typically identified in isolated lignins. Syringyl-2/6 $^{13}C–^{1}H$ correlations in units with oxidized syringyl α-positions, colored purple in **Figure 1**, show up at higher proton and carbon chemical shifts than their alcohol counterparts. DFRC products from insoluble fiber that had not been subjected to ball-milling also contained evidence of coniferaldehyde and sinapaldehyde endgroups (*19*). In pear, kiwi, and rhubarb, tentative DFRC evidence for both syringyl and guaiacyl α-ketones was also noted. Such observations suggest the presence of oxidized side chains in the native lignin, not just as possible artifacts of the isolation. Studies on model compounds indicated that α-carbonyls in the propanoid chain may decrease radical scavenging efficiency (*9*).

NMR Characterization: Interunit Linkages. Structural details are more evident in the side-chain regions of lignin HSQC spectra (**Figures 2** and **3** and **Tables 1** and **2**). The kiwi

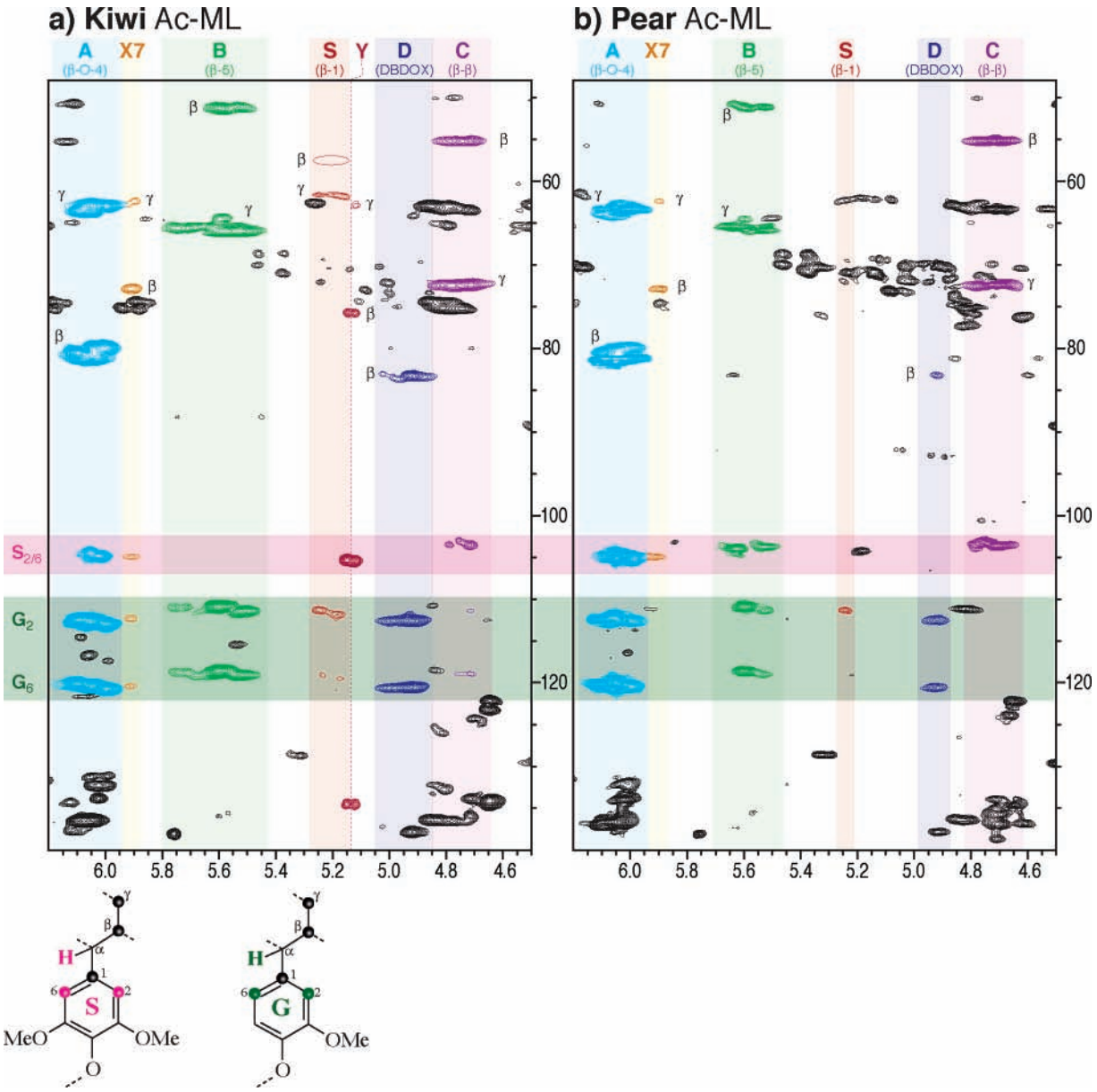

Figure 4. Partial HMBC spectra of acetylated milled lignins (Ac-ML) isolated from the dioxane–water-insoluble residues of insoluble fiber fractions from (**a**) kiwi and (**b**) pear. The correlations highlighted, and colored consistently with correlations and structures in **Figure 2**, are from α-protons to the carbons within three bonds, most diagnostically those to the 2- and 6-positions on the aromatic rings of guaiacyl (G, green row highlighting) and syringyl (S, pink row highlighting) units highlighted in horizontal bands.

and pear milled lignins (**Figure 2a**,**b**), are quite clean, allowing substantive structural analysis. All of the common structures from the various interunit linkage types are readily identified by comparison with various lignin spectra (*33*−*37*). *β*-Aryl ether (*β*-*O*-4, **A**) units predominate, as in all lignins. Phenylcoumarans (*β*-5, **B**), resinols (*β*-*β*, **C**), and dibenzodioxocins (5-5/*β*-*O*-4, **D**) are all revealed, along with the newly identified (*44*, *45*) spirodienones (*β*-1, **S**). So-called "traditional *β*-1-units" **F** (**Figure 3**) are also evident in the kiwi milled lignin (**Figure 2a**). It was formerly a mystery why such units could not be observed in lignins by NMR (*46*) until the discovery that *β*-1-coupled units exist in planta as the intermediate spirodienone **S** products (*34*, *44*, *45*). The reason for their appearance here is presumably because the pH values in the fruit are sufficient to partially cleave the spirodienones either in planta or during lignin isolation. Both cinnamyl alcohol (**X1**) and arylglycerol (**X7**) endgroups are also evident. Identified units are quantified relatively (**Table 1**) via their HSQC volume integrals and calculated as described previously (*33*).

The kiwi milled lignin, being extremely rich in guaiacyl units and therefore more akin to a softwood lignin, has high levels of **B** and **D** units of ∼12 and 8%, respectively (**Table 1**) that require guaiacyl units for their synthesis. With its comparable syringyl and guaiacyl levels, the pear milled lignin is relatively depleted in these units (∼4.5 and 2.1%, respectively) and in **X1** endgroups. However, it is richer (∼10.6 vs 3.9%) in **C** units which, as will be seen below, are largely syringyl units derived from sinapyl alcohol dimerization. The spirodienone units **S** are most clearly seen in the pear and rhubarb lignins (**Figure 2b**,**c**); they were noted previously as being particularly striking in kenaf lignins (*44*). A novel unit in the kiwi lignin defies exact identification at this time. The two maroon contours labeled **Y**α and **Y**β (**Figure 2a**) are particularly reminiscent of the benzodioxanes that are formed when plants utilize 5-hydroxy-

Table 1. NMR- and DFRC-Derived Guaiacyl/Syringyl (G/S) Data and Interunit Linkage Data for Lignins from Kiwi, Pear, Rhubarb, and Wheat Insoluble Fiber[a]

sample[b]	yield[c] (mg/g)	%G	%S	S/G[d]	%**A**	%**B**	%**C**	%**D**	%**S**+**F**	%**X1**	%**X7**
kiwi Ac-ML	26	93.6	6.4	0.07	68.3	12.0	3.9	8.2	2.6	3.7	1.2
kiwi Ac-AL	24	98.4	1.6	0.02	73.0	12.8	3.4	7.0	1.0	2.3	0.4
kiwi DFRC[e]		94.3	5.7	0.06							
pear Ac-ML	29	45.1	54.9	1.2	77.9	4.5	10.6	2.1	2.3	1.7	0.9
pear Ac-AL	26	41.8	58.2	1.4	82.1	4.3	9.7	1.5	1.2	0.7	0.5
pear DFRC[e]		38.8	61.2	1.6							
rhubarb Ac-ML	15	3.6	96.4	27	93.0		5.8		1.3		
rhubarb Ac-AL	7	2.2	97.8	45	95.5		4.5				
rhubarb DFRC[e,f]		13.9	86.1	6.2							
wheat Ac-ML	8	44.6	55.4	1.2	90.5		9.5				
wheat DFRC[e,g]		47.6	52.4	1.1							

[a] G, guaiacyl; S, syringyl; **A**, β-O-4 (β-aryl ether); **B**, β-5 (phenylcoumaran); **C**, β-β (resinol); **D**, dibenzodioxocin; **F**, β-1 ("traditional" form, see **Figure 3**); **S**, β-1 (spirodienone); **X1**, cinnamyl alcohol endgroup; **X7**, arylglycerol endgroup. [b] Fractions: ML, milled lignin; AL, mild acidolysis lignin (from the ML residue); Ac- indicates acetylated samples. [c] Yield is on a total insoluble fiber basis. [d] S/G values become "unstable" at very high guaiacyl or high syringyl values—the %S and %G values are a better indicator in these cases. [e] DFRC S/G data are from whole insoluble dietary fiber following digestion with crude carbohydrolases, not on the isolated lignin fractions. [f] Rhubarb DFRC S/G ratio is a low estimate due to coelution of an impurity with the coniferyl acetate peak. [g] Determined on alkali-extracted insoluble dietary fiber (to remove complication of ferulate-coniferyl alcohol cross-coupling products in grasses).

Table 2. Two-Dimensional Correlation Chemical Shifts for Acetylated Lignins in Acetone-d_6 To Be Used as a Guide for Assigning HSQC Spectra[a]

unit	α	β	γ
A	6.05/75.2 (br)	4.80/80.5 (br)	4.2/63.5 (br)
B	5.56/88.3	3.78/51.3	4.39/65.8
C	4.74/86.3	3.11/55.2	3.89,4.26/72.4
D	4.91/85.1	4.27/83.4	nr
F	6.01/76.4	3.49/50.8	nr
S	5.19/83.2	3.10/57.7	4.05/61.9
S′	4.82/86.8	4.52/77.4	4.49/64.5
X1	6.63/134.2	6.23/122.2	4.65/65.3
X7	5.91/74.5	5.39/73.0	nr
Y	5.13/77.5	4.65/75.9	nr

[a] Chemical shifts are approximate only, especially when correlations are broad (br); the centers of the major peaks only are reported from the kiwi and pear spectra. Most of the γ-correlations are not resolved (nr).

coniferyl alcohol as a surrogate lignin monomer in COMT-deficient plants (*34*, *47*). However, the ^{1}H and ^{13}C chemical shifts do not exactly match. In all likelihood, the component involves sinapyl alcohol addition (see below) to a catechol structure of some kind. Lignans with such structural features are abundant in the literature. For now, the component **Y** is simply tentatively identified as an unknown benzodioxane.

The impure rhubarb milled lignin, being essentially devoid of guaiacyl units, logically has no detectable **B** or **D** units (**Figure 2c** and **Table 1**). β-Ether units **A**, resinols **C**, and spirodienones **S** are the only structures that are readily identified. Nevertheless, such structures in such a syringyl-rich polymer are sufficient to suggest that the rhubarb polymer at least contains authentic lignin domains. Normally, the level of resinol units **C** is higher in high-syringyl lignins, but this was not observed here (<6% in rhubarb vs ~10.6% in pear). Resinol levels might be relevant to the formation of mammalian lignans in the gut; conversion to enterolactone in a rat model has been proposed (*15*). The even less pure wheat isolate (**Figure 2d**) also displays weak traditional **A** and **C** structures. It appears that lignin contents derived from various crude lignin methods have overemphasized the contribution of this component. It is likely that wheat insoluble dietary fiber contains material that can at best be described as "lignin-like." Indications of minimal authentic lignin levels are the low DFRC monomer liberation yields (*16*), the impure milled lignin isolates containing "real lignin" as only a minor component, and the low extraction yield by acidolysis. Lignin-like structures are also considered to be part of the (poly)phenolic domain of suberins, a polymer class differing considerably from lignins (*48*, *49*). However, dicarboxylic acids and long-chain hydroxylated acids (>C_{20}) reported as diagnostic for suberins were found at only trace levels in recently published work characterizing the structure of the external layers of wheat grains (*18*).

Rather clean acidolysis lignins derived from the kiwi and pear insoluble dietary fiber residue (from the milled lignin isolation) support the structural analyses made above. The spectra (**Figure 3**) show essentially the same structures at similar levels (**Table 1**). Spirodienones **S** do not survive acidolysis, cleaving to give "traditional" β-1-structures **F** (**Figure 3a**) (*33*); these units can also be found in the pear acidolysis lignin at lower contour levels (not shown, but quantified in **Table 1**). The novel benzodioxanes in the kiwi milled lignin do not appear in the acidolysis lignin fraction, either because they do not survive the acidolysis conditions or because they are in a lower molecular mass fraction that was completely extracted into the milled lignin fraction with dioxane−water. There remains the possibility that, although quite clearly a cross-coupling product involving a monolignol (sinapyl alcohol, see below), the benzodioxane component may not be an authentic lignin component. Acidolysis lignin from rhubarb was not fully soluble in acetone or chloroform. The acetone-soluble part of this fraction was used to acquire the spectrum in **Figure 3c**, again being rather impure but comprising largely **A** and **C** units, as typical for a high-syringyl lignin.

NMR Characterization: Monomers Involved in the Various Linkage Types. Long-range correlations (via carbons and protons linked via two or three intervening bonds) from HMBC spectra are valuable in providing information on the types of units (guaiacyl or syringyl) involved in each linkage type (*33*, *35*, *42*, *50*, *51*). This is because the equivalent syringyl-2/6 carbons resonate at ~105 ppm, whereas the guaiacyl-2 and -6 carbons, resonating at ~112 and ~120 ppm, are well separated from each other and from the syringyl correlation. Correlations in HMBC spectra are not quantitatively relevant with respect to other correlations in the same spectrum and so cannot be used for determination of S:G ratios, for example. However, they have enormous qualitative value and can be used in

comparisons of the same correlations across samples. In the pear milled lignin (**Figure 4b**) with its comparable levels of syringyl and guaiacyl units, β-ethers **A** and phenylcoumarans **B** both result from coupling of either a coniferyl or sinapyl alcohol monomer to the growing end of the polymer (as seen by the guaiacyl and syringyl nature of these units). In contrast, and as usual in hardwoods and dicots, resinols **C** are almost entirely derived from sinapyl alcohol dimerization and are therefore almost entirely syringyl. New information here is that the spirodienones **S** appear to have resulted primarily or exclusively from coniferyl alcohol addition, as have the dibenzodioxocins **D**. Although structures **D** require guaiacyl units to couple to form the 5-5 moiety, coniferyl or sinapyl alcohol can cross-couple with such moieties to form either guaiacyl or syringyl dibenzodioxocins, but only coniferyl alcohol coupling is evidenced here. Examination of the aromatic regions of the pear lignin HSQC spectra (not shown) indicates that the β-ether unit to which β-1-coupling had occurred had either syringyl or guaiacyl end-units. Correlations were observed at exactly those frequencies noted recently (*44*): guaiacyl-2′ at 6.23/110.9 and 6.29/112.0 ppm, guaiacyl-5′ at 6.15/130.0 and 6.35/131.0 ppm, and syringyl-2′/6′ at 6.36/113.9/6.33/111.4 ppm; the guaiacyl-6′ correlation was not within the acquired region. Again, as noted recently (*33*), the glycerol endgroups **X7** are almost entirely syringyl.

The kiwi lignin (**Figure 4a**), being severely depleted in syringyl units, offers unique insight into the cross-coupling that occurs under such circumstances, due either to chemical cross-coupling propensities or to the availability of monomer units during lignification. Thus, sinapyl alcohol is still quite prevalent in β-ether units **A**, but the phenylcoumarans **B** are now almost exclusively derived from coniferyl alcohol coupling to the phenolic end-unit, as are the spirodienones **S** and dibenzodioxocins **D**. Long-range correlations, in HMBC spectra from either the milled lignin or the acidolysis lignin, from the **F**β proton [50.8 to carbon-β (ex HSQC), 75.5 (α), 64.8 (γ), 137.1 (*1*), 114.2 (*2*), and 121.8 (*6*)], agree closely with guaiacyl β-1-model data, for example, compound 3007 in our NMR database (*32*). As described above for the pear, examination of the aromatic regions in HSQC spectra show that the spirodienones **S** in the milled lignin are all guaiacyl. The resinols **C** that are usually highly derived from sinapyl alcohol in syringyl/guaiacyl lignins are still mainly sinapyl alcohol-derived in this syringyl-depleted lignin, but some coniferyl alcohol product is noted. Whether dimers are all homodimers (i.e., from two sinapyl alcohol monomers or from two coniferyl alcohol monomers) or may result from heterodimerization (one sinapyl and one coniferyl alcohol) cannot be determined from these data. The dominance of resinol structures deriving from sinapyl alcohol would require additional demethylation and dehydroxylation steps for enterolactone/enterodiol formation in gut fermentation. The known precursors secoisolariciresinol and matairesinol and the proposed lignin precursors from pinoresinol and the analogous structures in the lignins contain only guaiacyl units (*15*). The novel putative benzodioxane **Y** becomes even more mysterious from this analysis; it clearly derives from exclusively sinapyl alcohol coupling (with a catechol). Such an event in a syringyl-depleted polymer will eventually need explanation, but again it is likely to result either from the chemical compatibility of sinapyl alcohol for coupling with the catechol or from the production of the unit in a time and space when only sinapyl alcohol monomers are available. There is a minor possibility that the benzodioxane **Y** is a lignan of the type seen in various plants, but lignans are not typically seen contaminating lignin preparations that include exhaustive solvent pre-extraction steps.

In conclusion, the occurrence of authentic lignins has been demonstrated in kiwi, pear, and rhubarb. It is suggested that the significance of lignin in wheat grain has, however, been overestimated by reliance on nonspecific analytical methods, highlighting the need for diagnostic structural studies. The NMR approach used here provides the most readily available detailed structural profile. Syringyl/guaiacyl measures from volume integration of NMR contours support conclusions from DFRC analysis and suggest that rhubarb lignins are among the most syringyl-rich known. Typical lignin structures, including those newly NMR-validated (glycerols, spirodienones, and dibenzodioxocins), are demonstrated via their NMR correlation spectra in the fruit and vegetable samples. Syringyl/guaiacyl levels and unit distributions do not appreciably differ between different lignin fractions, suggesting that such isolates are representative of the native lignin. Resinol structures were largely syringyl even in the highly guaiacyl kiwi lignin. Whether such syringyl analogues of suspected guaiacyl mammalian lignan precursors can be liberated and transformed by gut microflora needs examination. The role of lignin as part of the dietary fiber needs to be more completely evaluated than just measured by, for example, the Klason method. The recent finding of the physiological relevance of lignins in food demonstrates the need for more precise and diagnostic methods for analyzing and characterizing lignins in plant-derived foods, in addition to elucidating their role as mammalian lignan precursors, in adsorbing mutagenic agents, and in influencing the fermentation of cell wall polysaccharides.

ABBREVIATIONS USED

Ac-AL, acetylated mild acidolysis lignin; Ac-ML, acetylated milled lignin; AL, mild acidolysis lignin; ML, milled lignin (a dioxane−water extract of ball-milled insoluble fiber following crude cellulases digestion, akin to a "cellulolytic enzyme lignin").

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