# Structural Analysis of Wheat Straw Lignin by Quantitative $^{31}$P and 2D NMR Spectroscopy. The Occurrence of Ester Bonds and α-O-4 Substructures

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By combining mild alkaline hydrolysis with quantitative $^{31}$P NMR we have been able to arrive at a protocol for determining the various ester linkages and their relative contributions to the overall structure of wheat straw lignin. Additional information on the identity and location of these bonds was sought by the application of GC/MS and two-dimensional $^{13}$C−$^{1}$H heterocorrelation NMR experiments. Milled straw lignin was found to contain about 12 ester units per 100 phenylpropane units. Approximately 77% of the carboxyl fraction of these ester bonds was found to be composed of *p*-coumaric acid while the rest was other aromatic acids bound to lignin via intra- and/or intermolecular ester bonds. In contrast, the hydroxyl fraction of the ester bonds was found to be almost exclusively aliphatic. A small fraction (about 1.6%) of the milled straw lignin units was found to be esterified through the phenolic hydroxyl groups of C-5 condensed phenolic units. The application of $^{13}$C−$^{1}$H correlative NMR experiments revealed that acylation occurs only at the γ-position of the lignin side chain. Detailed studies of two-dimensional HOHAHA and HMQC experiments failed to show evidence for the presence of α-O-4 substructures in milled wheat straw lignin.

**Keywords:** *Wheat straw lignin; 2D NMR; structural analysis*

## INTRODUCTION

The physicochemical properties of straw lignins are known to be different from those of softwoods or hardwoods, with straw lignins possessing a characteristic alkali solubility (Beckman *et al.*, 1923). Alkali treatments have been used to increase the digestibility of various straws and to manufacture paper (Jackson, 1977; Lachenal *et al.*, 1983; Hartler and Ryrberg, 1985). The solubility of straw lignin in alkali has been attributed mainly to the presence of significant amounts of *p*-hydroxyphenyl (H) residues, which are bound to lignin as *p*-coumarate units (Beckmann *et al.*, 1923; Scalbert *et al.*, 1986a). Ferulic and *p*-coumaric acids in grasses are known to be implicated in the cross-linking of cell wall carbohydrates with lignin (Smith, 1955; Higuchi *et al.*, 1967; Scalbert *et al.*, 1985). Obviously such cross-linking can have a dramatic influence on the mechanical properties and biodegradability of straws (Hartley, 1982; Eraso and Hartley, 1990). Furthermore, the nature of lignin−hydroxycinnamic acid−polysaccharide interactions in plant cell walls is fundamental to our understanding of cell wall biosynthesis and biodegradation (Ralph *et al.*, 1992). Ferulic acid is known to be esterified with carbohydrates and etherified with lignin. However, the actual chemistry of its attachment to lignin is not well understood (Ralph *et al.*, 1992; Helm and Ralph, 1992; Jacquet *et al.*, 1995). Similarly, *p*-coumaric acid is known to be extensively esterified with lignin, but the regiochemistry of lignin acylation is still a matter of debate (Helm and Ralph, 1993; Ralph *et al.*, 1994; Shimada *et al.*, 1971). The following description of events represents the state of our knowledge as far as the biosynthetic pathways leading to such species are concerned. The α-position of quinone methides, forming during the dehydrogenative polymerization process (Adler, 1977), apart from being attacked by water, may also be attacked by free acids and alcohols, leading to α-esters and α-ethers. This is the case of feruloyl esters and phenolic glycosides of *p*-coumaric acid, where the free phenol or the free carboxylic acid groups respectively may trap quinone methides by an addition reaction to yield α-ethers and α-esters (Scalbert *et al.*, 1986a,b). Alternatively, feruloyl esters can directly participate in the free radical polymerization process, giving rise to a number of different structures (Fry, 1986). Furthermore, enzymatically preesterified *p*-coumaric acid with *p*-hydroxycinnamyl alcohol monomers may cause the formation of *p*-hydroxycinnamyl *p*-coumarates which could participate in the formation of the lignin macromolecule by conventional oxidative coupling reactions to yield γ-*p*-coumaroylated lignin (Ralph *et al.*, 1994).

While the ether linking of ferulic acid to the α-position of the lignin side chain via "opportunistic" quinone methide trapping is still speculative, it has been reported by Ralph *et al.* (1992) that feruloyl esters, if present in the lignifying matrix, are capable of participating in the free radical lignification process. Moreover, the identification of new ether-linked ferulic acid−coniferyl alcohol dimers in grass straws by Jacquet *et al.* (1995) demonstrates the occurrence of radical coupling reactions between ferulic acid and coniferyl alcohol to yield β-aryl ether structures.

On another front, the occurrence of noncyclic benzyl aryl ether substructures in lignin has been the subject of continuing debate (Ede and Kilpelainen, 1995; Ede and Brunow, 1992; Lundquist, 1981). Despite the fact that such structures have been postulated to be among the most significant labile interunit linkages in lignin, a commonly quoted figure for their abundance is about

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6−9% (Adler, 1977), as obtained by acidolysis, while NMR spectroscopy showed less than 3% in spruce lignin and ≃5% in birch (Lundquist, 1981). Recently the application of homo- and heteronuclear 2D NMR techniques showed that if noncyclic benzyl aryl ethers are present in soluble wood lignin samples they are at a level below the 0.3% detection limit of these experiments (Ede and Kilpelainen, 1995; Ede and Brunow, 1992).

There are many such examples, where magnetic resonance techniques when applied to lignins have proved to be excellent analytical tools for the structural elucidation of these complex biopolymers. Accordingly, the work of our laboratory has been focused on the development of $^{31}$P-based novel magnetic resonance methods aimed to expand the frontiers of application of NMR to lignin analysis (Argyropoulos, 1994, 1995; Granata and Argyropoulos, 1995; Jiang *et al.*, 1995). These NMR techniques are capable of detecting and quantitatively determining all functional groups in lignin possessing labile hydroxyl groups, i.e., aliphatic OH, the various forms of phenolic OH, and carboxylic acids. On the basis of this methodology we undertook to study the nature of ester bonds in wheat straw (*Triticum aestivum*) lignin. By combining mild alkaline hydrolysis with quantitative $^{31}$P NMR we have been able to arrive at a protocol for determining the various ester linkages and their relative contributions to the overall structure of wheat straw lignin. Our technique has been applied on samples of milled wheat straw (ML), dioxane acidolysis wheat straw lignin (AL), and a milled wood lignin from black spruce (BSL), the latter being documented not to contain any appreciable amounts of ester bonds.

Additional information on the identity and location of these bonds was sought by the application of GC/MS and two-dimensional $^{13}$C−$^{1}$H heterocorrelation NMR experiments (Ede and Brunow, 1992; Bax and Subramanian, 1986; Summers *et al.*, 1986), while the occurrence of noncyclic benzyl aryl ethers was evaluated by using homonuclear Hartmann−Hahn (HOHAHA) and heteronuclear multiple quantum coherence (HMQC) experiments (Bax and Subramanian, 1986; Summers *et al.*, 1986; Bax and Davis, 1985).

## MATERIALS AND METHODS

**Preparation of Milled (ML) and Dioxane (AL) Lignins.** Milled lignins (Obst and Kirk, 1988) were prepared from ultraground extractive free powder according to Bjorkman's procedure. Extractive free powders were ultraground for 3 weeks in a rotatory ball mill. The ML fraction was then extracted with 96:4 dioxane/water (v/v). The residue was concentrated under reduced pressure and freeze-dried. Purification ensued by dissolution of the lignin in 90% acetic acid. The solution was then added dropwise to stirred water. The precipitated lignin was centrifuged and freeze-dried. It was then dissolved again in a mixture of 1,2-dichloroethane/ethanol (2:1, v/v) and precipitated in diethyl ether. The resulting product was about 82% lignin as evidenced from UV lignin content measurements.

ML (%): C (54.58), H (5.56), O (31.02), $OCH_3$ (14.45)

The dioxane lignin was prepared by refluxing ground straw in 0.01 M HCl in dioxane under a nitrogen stream for 2 h (Monties, 1988). The reaction mixture was allowed to cool and rapidly filtered, and the filtrate was neutralized with sodium bicarbonate, concentrated under reduced pressure, and precipitated by addition to water. Elemental and methoxy group analyses were carried out by Schwarzkopf Microanalytical, Woodside, NY.

AL (%): C (57.45), H (5.86), O (30.2), $OCH_3$ (12.45)

**Lignin Alkaline Hydrolysis.** Lignin (100 mg) was dissolved in 10 mL of 2 M NaOH and stirred at 25 °C for 48 h. Such reaction periods have been shown to result in complete ester hydrolysis (Scalbert *et al.*, 1985). However, this issue was further examined in this effort, confirming that these are indeed the limiting hydrolysis conditions. After the hydrolysis, the reaction mixture was acidified with 1 M HCl and centrifuged. The solution was extracted twice with ethyl acetate, dried over $Na_2SO_4$, and evaporated under reduced pressure. The residue was finally examined by GC/MS using a DB1 column and an isothermal temperature profile at 70 °C for the first 2 min, followed by a 10 °C/min temperature gradient to 300 °C and finally an isothermal period at 300 °C for 5 min.

The solid residue, after centrifugation, was washed with water, centrifuged, and freeze-dried. The product was then washed with ethanol, 1:1 ethanol/ether, and finally ethyl ether, recentrifuged, and dried in a vacuum oven at 25 °C.

**Lignin Methylation and Acetylation (Crawford and Pometto, 1988).** Lignin (200 mg) was suspended in 5 mL of diethyl ether and then treated with an excess of diazomethane for 24 h at room temperature. The treatment was repeated three times. The mixture was centrifuged, washed with ethyl ether, and centrifuged again. The residue was dried under reduced pressure. Acetylation was carried out with pyridine/acetic anhydride (1:1) at 25 °C for 48 h.

**Permanganate Oxidation (Kirk and Adler, 1970; Tanahashi and Higuchi, 1988).** Lignin (100 mg) was dissolved in 10 mL of 1 M NaOH at 50 °C, and 6.1 mL of dimethyl sulfate and 10 mL of 30% NaOH were added in small portions during a 3 h period. The reaction mixture was then acidified with concentrated HCl, centrifuged, washed with water, centrifuged, suspended in water, and freeze-dried. A 50 mg portion of the methylated lignin was dissolved in 10−20 mL of 1:1 *tert*-butyl alcohol/water. This solution was then added to 75 mL of 1% $Na_2CO_3$ (w/v). The solution was kept at 80 °C with stirring, and 5% $KMnO_4$ was added in small portions during a 3 h period. After that, 5 mL of 95% ethanol was added and the suspension was filtered and washed with hot 1% $Na_2CO_3$. After cooling, the solution was extracted with ethyl ether and hexane. It was then neutralized with 2 M $H_2SO_4$ and concentrated under reduced pressure at 50 °C. To the concentrate was added 30 mL of 1% $Na_2CO_3$, and the mixture was heated at 50 °C with 10 mL of 30% $H_2O_2$. After 10 min the heating bath was removed and 5% $KMnO_4$ was added to obtain a colored solution, followed by an addition of $Na_2S_2O_5$ to obtain a brown color. The pH was finally adjusted to 1−2 with 2 M $H_2SO_4$, and the solution was extracted several times with chloroform dried over $Na_2SO_4$ and evaporated under reduced pressure. The residual solvent was eliminated under reduced pressure, and the residue was analyzed by gas chromatography, using a DB1 column and an isothermal temperature profile at 70 °C for the first 2 min, followed by a 10 °C/min temperature gradient to 300 °C and finally an isothermal period at 300 °C for 5 min.

**Quantitative $^{31}$P NMR (Granata and Argyropoulos, 1995; Jiang *et al.*, 1995; Argyropoulos, 1994).** A solvent mixture composed of pyridine and deuterated chloroform in a 1.6:1 v/v ratio was prepared. The solution was protected from moisture with molecular sieves (3A) and kept in a sealed container under nitrogen. A solution was then prepared by utilizing the above preparation; chromium(III) acetylacetonate (Aldrich, Milwaukee, WI, 5.0 mg/mL) and cyclohexanol (Aldrich, 10.85 mg/mL) served as relaxation reagent and internal standard, respectively. Thirty milligrams of dry lignin was accurately weighed into a 1 mL volumetric flask. The sample was then dissolved in 0.5 mL of the above solvent mixture. The tetramethylphospholane (100 mL) was then added, followed by the internal standard and the relaxation reagent solution (100 mL each). Finally, the solution was made up to the 1 mL mark with more solvent mixture. The volumetric flask was tightly closed and shaken to ensure thorough mixing.

The $^{31}$P NMR spectra were obtained on a Varian XL-300 spectrometer by using methods identical to those described by Granata and Argyropoulos (1995). More specifically, an observation sweep width of 6600 Hz was used, and the spectra were accumulated with a delay time of 25 s between successive pulses. All chemical shifts reported are relative to the reaction

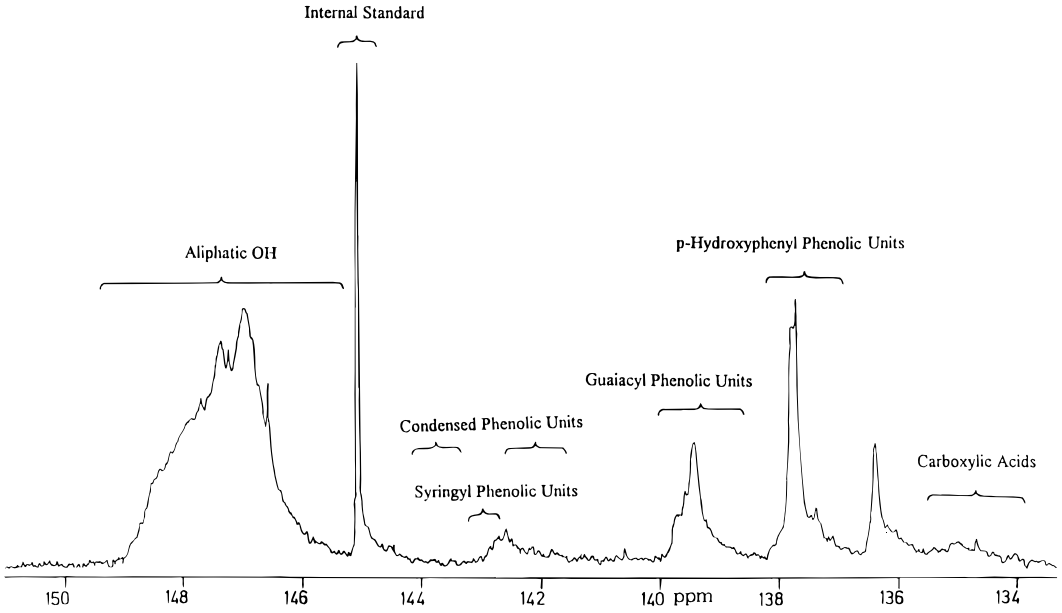


**Figure 1.** $^{31}$P NMR of wheat straw lignin phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane.

**Table 1. Phenolic, Aliphatic, and Carboxylic Hydroxyl Groups Present on the Examined Lignins Obtained by Triplicate Quantitative $^{31}$P NMR Measurements**[a]

| | OH (mmol/g) | | | | | | |
|---|---|---|---|---|---|---|---|
| lignin sample | COOH | H[b] | G[c] | S[d] | condensed phenolic | aliphatic | total phenolic |
| ML[e] | 0.12 | 0.68 | 0.51 | 0.09 | 0.18 | 3.49 | 1.46 |
| ML after alkaline hydrolysis | 0.26 | 0.22 | 0.51 | 0.08 | 0.26 | 3.98 | 1.07 |
| ML after methylation and alkaline hydrolysis | 0.27 | 0.00 | 0.00 | 0.00 | 0.08 | 4.06 | 0.08 |
| AL[f] | 0.18 | 0.50 | 0.51 | 0.10 | 0.13 | 3.80 | 1.24 |
| AL after alkaline hydrolysis | 0.46 | 0.27 | 0.50 | 0.10 | 0.26 | 4.22 | 1.13 |
| AL after methylation and alkaline hydrolysis | 0.47 | 0.00 | 0.00 | 0.00 | 0.12 | 3.96 | 0.12 |
| BSL[g] | 0.10 | 0.06 | 0.76 | 0.08 | 0.30 | 4.48 | 1.20 |
| BSL after alkaline hydrolysis | 0.12 | 0.05 | 0.60 | 0.08 | 0.32 | 4.50 | 1.50 |

[a] Lignin samples phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane. [b] H: *p*-hydroxyphenyl phenolic OH. [c] G: guaiacyl phenolic OH. [d] S: syringyl phenolic OH. [e] ML: milled wheat straw lignin, $C_9H_{9.1}O_{3.3}(OCH_3)_{1.0}$. [f] AL: dioxane acidolysis wheat straw lignin, $C_9$ formula: $C_9H_{9.5}O_{3.0}$ $(OCH_3)_{0.8}$. [g] BSL: black spruce milled wood lignin.

product of water with tetramethyldioxaphospholane, which has been observed to give a sharp signal in pyridine/$CDCl_3$ at 132.2 ppm.

The $^{31}$P NMR data reported in this effort are averages of three experiments. The maximum standard deviation of our results was $2 \times 10^{-2}$ mmol/g,while the maximum standard error was $1 \times 10^{-2}$ mmol/g.

**Two-Dimensional NMR Spectroscopy (HMQC and HOHAHA) (Ede and Kilpelainen, 1995; Ede and Brunow, 1992; Ralph *et al.*, 1994).** Such NMR spectra were acquired at 25 °C using 30 mg of lignin dissolved in 0.6 mL of $CDCl_3$ on a Varian Unity 500 NMR spectrometer using a 5 mm inverse detection probe (DHP). The chemical shifts were referenced to $Me_4Si$. HMQC spectra were acquired over a 9.5 ppm window in $F_2$ ($^1$H) and 140 ppm in $F_1$ ($^{13}$C) with GARP-1 decoupling. BIRD presaturation of $^1$H$-^{12}$C magnetization and a pre-HMQC delay of 400 ms were used. 2K × 256 increments were acquired with simultaneous data acquisition. After $F_1$ zero-filling, Fourier transformation, and squared cosine bell apodization, the transformated data matrix was 1024 ($F_2$) × 512 ($F_1$) real points. HOHAHA spectra were acquired over a 9.5 ppm window in both $F_2$ and $F_1$, with MLEV-17 spin lock length of 80 ms. 2K × 256 increments were acquired. After $F_1$ zero-filling, Fourier transformation, and squared cosine-bell apodization, the transformed data matrix was 1024 ($F_2$) × 256 ($F_1$) real points.

## RESULTS AND DISCUSSION

**The Nature and Amount of the Various Ester Bonds**. Three lignin samples (milled wheat straw (ML)), dioxane acidolysis wheat straw (AL), and black spruce milled wood (BSL) lignins) were phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane in the presence of a known amount of cyclohexanol as internal standard and submitted to quantitative $^{31}$P NMR analyses (Granata and Argyropoulos, 1995). The obtained spectra showed well-resolved signals for the different hydroxyl groups present within the various lignin preparations. Figure 1 shows a typical spectrum together with the detailed signal assignment based on earlier efforts (Granata and Argyropoulos, 1995; Jiang *et al.*, 1995). Two different groups of signals due to aliphatic and aromatic carboxylic acids could also be detected, for samples ML and AL; however, their proximity prohibited their individual contributions from being evaluated. Thus in the following account this distinction will be made only on a qualitative basis. Table 1 displays the quantitative data on the distribution of the various OH groups for all the experiments carried out in this work.

Despite the fact that the detected carboxylic acids are about 2.4 and 3.4 per 100 $C_9$ units in ML and AL, respectively, a comparison with literature accounts could not be made since, to the best of our knowledge, such information has not been reported before. Jung and Himmelsbach (1989) have reported the concentrations of the nitrobenzene oxidation products of various straw lignin preparations. However, the alkaline nature of the nitrobenzene oxidation media they used precludes us from making direct comparisons with our

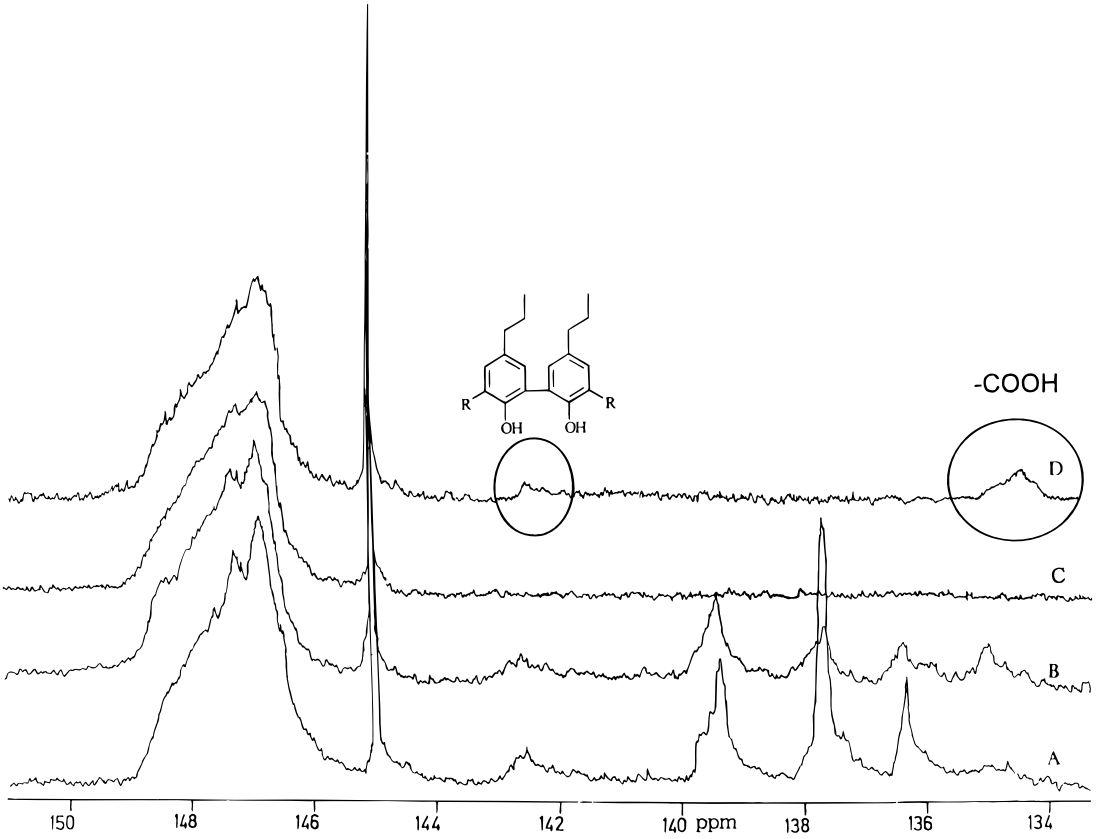


**Figure 2.** Comparison of $^{31}$P NMR spectra of wheat straw lignin phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (A), after alkaline hydrolysis (B), after diazomethane methylation (C), and after diazomethane methylation followed by alkaline hydrolysis (D).

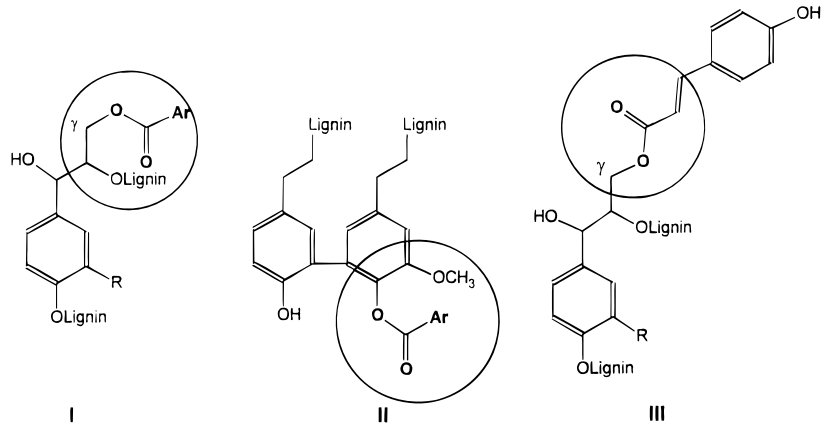


**Figure 3. I**: Intra- and/or intermolecular ester bonds of wheat straw lignin side chain. **II**: Phenolic groups in C-5 condensed structures selectively esterified in wheat straw lignin. **III**: Ester bonds on wheat straw lignin terminal units.

data. For the studied samples of wheat straw lignins, two classes of carboxylic acids could be distinguished in comparable amounts, (i) free benzoic and cinnamic acids and (ii) aliphatic acids (probably due to residual hemicelluloses). After the samples were subjected to alkaline hydrolysis (compare Figure 2A to Figure 2B), the overall amounts of carboxylic acids were found to increase by 0.14 and 0.28 mmol/g in ML and AL, respectively. This increase apparently corresponds to the presence of appreciable amounts of ester bonds (Kim *et al.*, 1995; Imamura *et al.*, 1994; and Takahashi *et al.*, 1988). Qualitatively, these bonds were found to be due mainly to aromatic acid residues. The precise definition of these ester linkages was carried out by further careful experimentation. The relative contributions of ester structures, shown in Figure 3, were evaluated by obtaining quantitative $^{31}$P NMR spectra before and after alkaline hydrolysis and before and after diazomethane methylation followed by alkaline hydrolysis. GC/MS analyses of the organic solvent extractable fraction after alkaline hydrolysis, mainly composed of monomeric or dimeric units, supplied further information on ester units of types **I** and **III** (Figure 3).

The distribution of free phenolic units in the examined straw lignins was found to be significantly different from that of the softwood milled wood lignin. In fact, the phosphitylated samples showed the presence of considerable amounts of *p*-hydroxyphenyl and guaiacyl units, while syringyl units were detected only in low amounts. The occurrence of *p*-hydroxyphenyl units in wheat straw lignins has been widely reported (Nimz *et al.*, 1981; Higuchi and Kawamura, 1966; Erickson *et al.*, 1973; Higuchi *et al.*, 1967), and their quantitative estimation has been made on the basis of permanganate oxidation experiments (Erickson *et al.*, 1973). In fact the *p*-hydroxyphenyl units have been considered to be due

mainly to the presence of esterified *p*-coumaric acid (Higuchi *et al.*, 1967). The amount of *p*-coumaric acid recovered after alkaline hydrolysis has actually been estimated (Scalbert *et al.*, 1986b). A sophisticated multistep strategy to estimate the cinnamic acid links between lignin and polysaccharides has been devised by Lam *et al.* (1992). More specifically, this procedure allows the determination of cinnamic acids bound by esters, ethers, or ester−ether linkages. As such it has been established that *p*-coumaric acid is linked to lignin via ester or ether bonds while ferulic acid constitutes a bridge binding to polysaccharides via ester bonds and to lignin via ether bonds (Helm and Ralph, 1993, 1992; Jacquet *et al.*, 1995).

In this effort we found that the *p*-hydroxyphenyl units are partly esterified in the examined straw lignins. In actual fact after alkaline hydrolysis the amount of *p*-hydroxyphenyl units was reduced from 0.68 to 0.22 mmol/g in ML and from 0.5 to 0.27 mmol/g in AL. An examination of the nature of the monomeric acids released upon alkaline hydrolysis inquired about the presence of ester bonds on terminal units. More specifically this was carried out after alkaline hydrolysis, recovery of the acidic fraction, organic solvent extraction, and GC/MS analyses. For ML only *p*-coumaric acid and no traces of ferulic acid and/or benzoic acids were detected. Therefore, the *p*-hydroxyphenyl units which were found to be eliminated after alkaline hydrolysis can be considered to be due to *p*-coumarate residues as shown in Figure 3, **III**. Recently, Ralph *et al.* (1994) have actually elaborated on the scheme of *p*-coumaric acid incorporation into maize lignin.

As expected, the lignin originating from wood (BSL) showed no variation in any of its hydroxyl and carboxyl groups after alkaline hydrolysis, indicating the absence of ester bonds. Efforts to detect cinnamic or benzoic acid terminal units in BSL showed no such species.

In contrast to *p*-hydroxyphenyl units, the guaiacyl and syringyl units of the ML and AL straw lignins were found to remain unchanged after alkaline hydrolysis, while C-5 substituted phenolic units were increased (Table 1). This indicates that while the guaiacyl and syringyl groups are not involved in ester bonds, C-5 substituted phenolic units are selectively esterified, possibly as shown in Figure 3, **II** by an amount of 0.08 mmol/g in ML and 0.13 mmol/g in AL. Further evidence supporting our findings arises from the work of Ralph *et al.* (1995), where they reported the isolation of ferulate dimers from the saponification extracts of grass cell wall material. The fact that the guaiacyl and the syringyl hydroxyl values remained unchanged after alkaline hydrolysis for both straw lignins and the wood lignin further supports the validity of our technique, pointing to the absence of artifacts that may be caused by the hydrolysis of ester bonds during the alkaline treatment.

The carboxylic acid units were found to be mainly esterified with the lignin side chains. This was clearly illustrated in a series of experiments where the lignin, after diazomethane methylation, was hydrolyzed by alkali. In fact after methylation the only $^{31}$P NMR signal received was due to aliphatic alcohols (Figure 2C), while after alkaline hydrolysis, besides the expected increase of the aliphatic hydroxyls (145−149 ppm) (Table 1, Figure 2D), only a small signal due to C-5 substituted phenolic structures (Jiang *et al.*, 1995) appeared (142.5 ppm).

Table 2 summarizes the actual amounts of ester bonds calculated on the basis of $^{31}$P NMR to be present in the examined milled (ML) and dioxane (DL) wheat lignins. Furthermore, an effort is made to define the relative

**Table 2. Estimation of Ester Bonds in Milled and Dioxane Wheat Lignins Obtained by Quantitative $^{31}$P NMR before and after Alkaline Hydrolysis**

| lignin sample | ML[a] (% of $C_9$ units)[c] | AL[b] (% of $C_9$ units)[c] |
|---|---|---|
| total esters[d] | 11.7 | 10.0 |
| Carboxylic Fraction | | |
| *p*-coumaric acid[e] | 9.0 | 4.5 |
| other aromatic acids (inter-/intramolecular ester bonds) and traces of aliphatic acids[f] | 2.7 | 5.5 |
| Alcoholic Fraction | | |
| aliphatic OH (lignin side chain)[g] | 10.1 | 7.7 |
| phenolic OH groups from condensed units[h] | 1.6 | 2.3 |

[a] ML: milled wheat straw lignin. [b] AL: dioxane acidolysis wheat straw lignin. [c] Calculated on the basis of $C_9$ units as obtained from elemental analysis performed on ML and AL samples. [d] Calculated on the basis of COOH increase and *p*-hydroxyphenyl OH decrease after alkaline hydrolysis (reported in Table 1). [e] Calculated on the basis of *p*-hydroxyphenyl OH decrease after alkaline hydrolysis (reported in Table 1). [f] Calculated on the basis of COOH increase after alkaline hydrolysis (reported in Table 1). [g] Calculated on the basis of aliphatic OH increase after alkaline hydrolysis (reported in Table 1). [h] Calculated on the basis of increase in phenolic OH groups from condensed units after alkaline hydrolysis (reported in Table 1).

**Table 3. Comparison of Guaiacyl/Syringyl (G/S), Guaiacyl/*p*-Hydroxyphenyl (G/H), and *p*-Hydroxyphenyl/Syringyl (H/S) Ratios As Measured by $^{31}$P NMR, Permanganate Oxidation, and $^{31}$P NMR after Alkaline Hydrolysis**

| chemical treatment | G/S | G/H | H/S |
|---|---|---|---|
| ML[a] | | | |
| $^{31}$P NMR | 5.6 | 0.8 | 7.5 |
| $KMnO_4$ oxidation | 6.5 | 2.5 | 2.7 |
| $^{31}$P NMR after alkaline hydrolysis | 6.3 | 2.2 | 2.9 |
| AL[b] | | | |
| $^{31}$P NMR | 5.1 | 1.0 | 5.0 |
| $KMnO_4$ oxidation | 4.7 | 1.7 | 2.8 |
| $^{31}$P NMR after alkaline hydrolysis | 5.0 | 1.8 | 2.7 |

[a] ML: milled wheat straw lignin. [b] AL: dioxane acidolysis wheat straw lignin.

contributions of the various acids and the aliphatic and/or phenolic hydroxyls in these esters. Both lignin samples were determined to contain about 10−12% of ester units expressed on the basis of 100 phenylpropane units. About 77% of the carboxyl fraction of the ester bonds present in ML was found to be composed of *p*-coumaric acid while the rest was other aromatic acids bound to lignin via intra- and/or intermolecular ester bonds. In contrast, the hydroxyl fraction of the ester bonds was found to be almost exclusively (86%) aliphatic. This finding further confirms that esterification occurs mainly with the lignin side chains as previously stated by Ralph *et al.* (1994), for corn lignin. Nevertheless, about 1.6% of the ML lignin units are esterified through the phenolic hydroxyl groups of condensed phenolic units. The lignin sample isolated by mild dioxane acidolysis showed a somewhat lower amount of ester bonds compared to the ML sample. However, the amount of *p*-coumarate esters in DL is about half of the amount detected in ML, while the percentage of inter- and/or intramolecular ester bonds with other aromatic acids is about 5.5%. One may speculate that the acidic isolation conditions of the DL sample caused the hydrolysis of the *p*-coumarate esters. The hydroxyl fraction of the ester bonds was once again found to be mainly aliphatic.

**Guaiacyl/Syringyl/*p*-Hydroxyphenyl (GSH) Ratios Evaluated by Quantitative $^{31}$P NMR and Permanganate Oxidation.** The data obtained by quan-

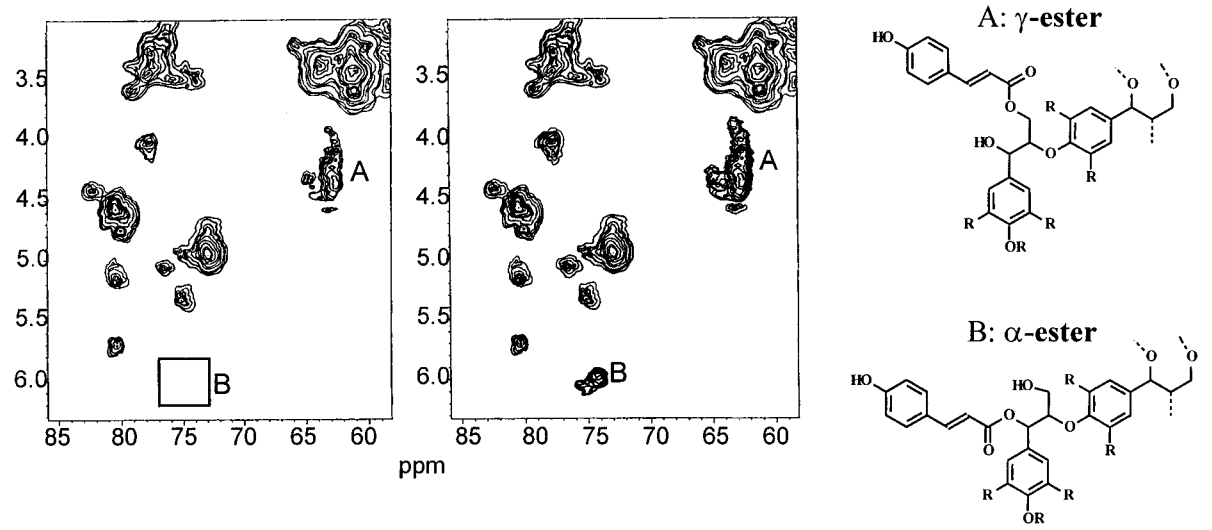


**Figure 4.** HMQC spectra of milled wheat straw lignin (left) and acetylated milled wheat straw lignin (right). Cross peaks labeled A are due to the presence of $\gamma$-ester bonds ($H_\gamma-C_\gamma$), and cross peaks labeled B are due to $\alpha$-esters.

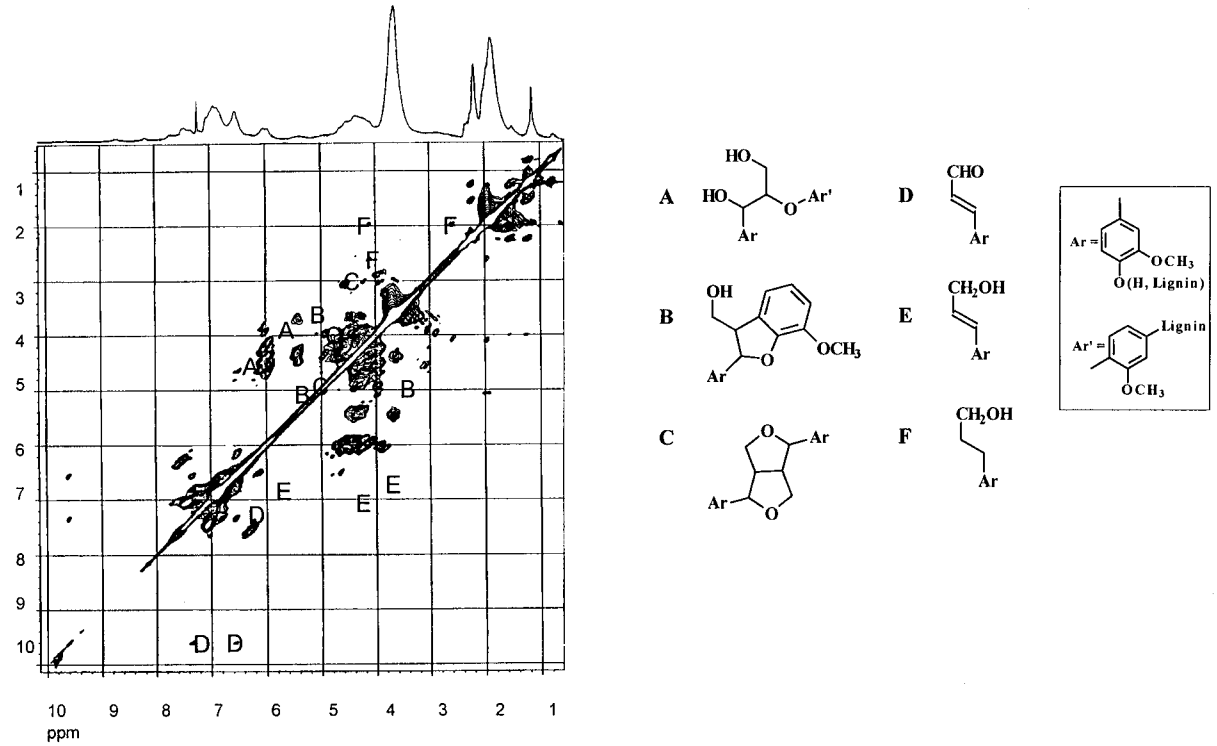


**Figure 5.** Phase sensitive 2D HOHAHA spectrum of acetylated wheat straw lignin, acquired with 80 ms spin lock. The *J* connectivity patterns for structures **A**−**F**, shown in this figure, are mapped out in Table 4.

titative $^{31}P$ NMR of straw lignin was subjected to confirmation by comparison with values obtained by permanganate oxidation. This effort provided another unique opportunity to compare values obtained by quantitative $^{31}P$ NMR with those obtained by permanganate oxidation. Table 3 shows the guaiacyl/syringyl (G/S), guaiacyl/*p*-hydroxyphenyl (G/H), and *p*-hydroxyphenyl/syringyl (H/S) ratios as obtained from permanganate oxidation and $^{31}P$ NMR measurements. At first glance the ratios seem to be remarkably different. However, this was found to be due to the extensive presence of ester bonds in the straw samples. Since the procedure of permanganate oxidation requires an initial methylation of the lignin phenolic moieties, which is carried out under strongly alkaline conditions, it is expected that the esters will hydrolyze, resulting in significant changes in the amounts of *p*-hydroxyphenyls. In fact when the permanganate oxidation ratios were compared with those obtained from quantitative $^{31}P$ NMR for samples previously submitted to alkaline hydrolysis, the close agreement became obvious (Table 3). This effort confirms once again the accuracy and wide applicability of quantitative $^{31}P$ NMR toward lignin structural studies. Moreover, for straw lignins quantitative $^{31}P$ NMR may provide an accurate insight into their structure since it allows the quantitative determination of *p*-hydroxyphenyl groups due to *p*-coumarate and/or to ether-bound units.

**The Topochemistry of Lignin Acylation.** A number of issues in relation to the regiochemistry of ester bonds in lignin require attention. Bamboo lignin has been reported to contain both $\alpha$- and $\gamma$-esters of *p*-coumaric acid (Shimada *et al.*, 1971; Nakamura and Higuchi, 1976), while, on the basis of two-dimensional $^{13}C-^{1}H$ heterocorrelation NMR experiments, maize lignin has been reported to contain *p*-coumarate esters, selectively esterifying the $\gamma$-position of the side chains (Ralph *et al.*, 1994). The occurrence of *p*-coumarate

**Table 4. Assignment of Cross Peaks Obtained from the Phase Sensitive 2D HOHAHA Spectrum of Acetylated Milled Wheat Straw Lignin[a]**

| cross peak[b,c] | $\delta$ (ppm) |
|---|---|
| A | 6.07−4.65 (α−$\beta$) |
| | 6.00−4.00, 4.20, 4.36 (α−$\gamma$) |
| B | 5.46−3.68 (α−$\beta$) |
| | 3.68−4.33, 4.52 ($\beta$−$\gamma$) |
| | 5.46−4.3, 4.4 (α−$\gamma$) |
| C | 4.78−3.07 (α−$\beta$) |
| | 3.07−3.95, 4.26 ($\beta$−$\gamma$) |
| | 4.78−3.95, 4.26 (α−$\gamma$) |
| D | 6.57−7.41 (α−$\beta$) |
| | 7.41−9.65 ($\beta$−$\gamma$) |
| E | 9.65−6.57 (α−$\gamma$) |
| | 6.57−6.15 (α−$\beta$) |
| | 6.15−4.67 ($\beta$−$\gamma$) |
| | 4.67−6.57 (α−$\gamma$) |
| F | 2.61−1.97 (α−$\beta$) |
| | 1.97−4.08 ($\beta$−$\gamma$) |
| | 4.08−2.61 (α−$\gamma$) |

[a] Spin lock of 80 ms used. [b] Cross peaks in Figure 5. [c] Structure assignments shown in Figure 5.

esters at the $\gamma$ position of the lignin side chains cannot be rationalized purely on a chemical basis, but it may arise from a distinct biochemical pathway that requires the enzymatically mediated preesterification of *p*-coumaric acid with *p*-hydroxycinnamyl alcohol units.

In an effort to offer further details on the regiochemistry of acylation in straw lignins, we carried out a two-dimensional NMR experiment involving a one-bond proton−carbon correlation (HMQC), since such inverse detection experiments have been reported to offer enhanced sensitivity over their normal mode counterparts (Summers *et al.*, 1986; Bax and Davis, 1985). The contour plot in Figure 4(left) shows a section of the HMQC experiment on the milled straw lignin (ML) sample. The contour cluster labeled A at $\delta$ 3.8−4.8, 62−66 ppm testifies that an acylation occurs at the $\gamma$-position. This contour cluster, responsible for $\gamma$-esters, may also contain minor contributions from the primary hydroxyls of carbohydrates and carbohydrates with acylated primary alcohol groups. The authenticity of this assignment is based on HMQC experiments of model esters previously reported by Ralph's group, Helm and Ralph (1993) and Ralph *et al.* (1994). The absence of cluster B (due to the presence of α-esters) in the spectrum of the nonacetylated lignin further signifies that, in wheat straw lignin, acylation occurs only at the $\gamma$-position of the side chain. In a reference acetylated sample two contour clusters (Figure 4(right)) labeled A and B at $\delta$ 3.8−4.8, 62−68 ppm and $\delta$ 5.9−6.2, 73−76 ppm respectively due to the presence of $\gamma$- and α-esters were found.

**The Occurrence of Noncyclic Benzyl−Aryl−Ether Bonds.** The occurrence of noncyclic benzyl aryl ether substructures in lignin has been the subject of significant debate. Such structures have been claimed to be present in amounts varying from less than 3% to greater than 8% (Adler, 1977; Ede *et al.*, 1990). For wheat straw lignin, *p*-coumaric and ferulic acids have been invoked to occur as α-aryl ethers on the basis of acid-catalyzed hydrolysis assays (Zhai and Lai, 1995). However, in recent years Ede has reported the application of two-dimensional Hartmann−Hahn (HOHAHA) and HMQC NMR experiments to the structural analysis of milled wood lignin (Ede and Kilpelainen, 1995; Ede and Brunow, 1992). These techniques have been demonstrated to be sensitive, rapid, and unambiguous probes for the α-O-4 structures in soluble lignin samples. This is because within the HOHAHA spectrum there is a distinct correlation due to $H_\alpha-H_\beta$ which occupies a region that does not overlap with any other lignin structures. In addition, the $H_\alpha-C_\alpha$ correlation in the HMQC spectrum of lignin also occupies a unique spectral region. To date all HOHAHA and HMQC spectra of milled wood lignins have shown no correlations that can be assigned to α-O-4 structures. In an effort to examine if such correlations are present in milled straw lignin we performed the same experiments (HOHAHA and HMQC sequences) on acetylated samples. Comparison of the chemical shifts of the cross peaks with data from appropriate models (Lundquist, 1992; Brunow *et al.*, 1989) allowed the assignment of cross peaks A−F (Figure 5) which correspond to structures **A**−**F** (Table 4 and Figure 5). In particular the correlations due to $\beta$-O-4 aryl ether structures were evident (peaks A and structure **A**, Figure 5, Table 4) while the correlations at $\delta$ 5.4−4.7 ppm ($H_\alpha-H_\beta$, in HOHAHA spectra) (Figure 5) were not found. Cross peaks present in the region at $\delta$ 5.4−81 ppm in the HMQC spectra (correlation $H_\alpha-C_\alpha$) (Figure 4) should indicate the occurrence of α-aryl ether bonds. In the HMQC spectra of both acetylated and nonacetylated wheat straw lignins such peaks could not be found. The absence of such correlations shows that, if the α-O-4 structure is present, it must be in levels lower than the detection limits of both HOHAHA and HMQC experiments, which has been estimated to be less than 0.3 unit per 100 $C_9$ units (Ede and Kilpelainen, 1995). However, the possibility of having this acid-labile structure hydrolyzed during the lignin isolation procedure cannot be excluded.

## APPENDIX

The percentage of ester bonds was calculated as follows. The $^{31}P$ NMR spectra of the lignins recorded on weighed samples in the presence of a known amount of internal standard allowed the quantitative determination of the amount (mmol/g) of all functional groups possessing a labile hydroxy group (Argyropoulos, 1994). The ester bond contents were evaluated by comparing the $^{31}P$ NMR spectra before and after alkaline hydrolysis. In fact, the ester bond hydrolysis led to an increase of carboxylic acids and to a decrease of hydroxyphenyl groups, the latter being due to the hydrolysis of *p*-hydroxycoumarates (which are lost during the reaction workup). By adding the amount of increase of carboxylic acids and the decrease of *p*-hydroxyphenyl groups it was possible to obtain the amount of ester bonds (mmol/g). In order to express the amount of ester bonds as percentage of $C_9$ units, these data were compared to the amount of $C_9$ units (mmol/g) calculated on the basis of the elemental and methoxy group analyses.

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