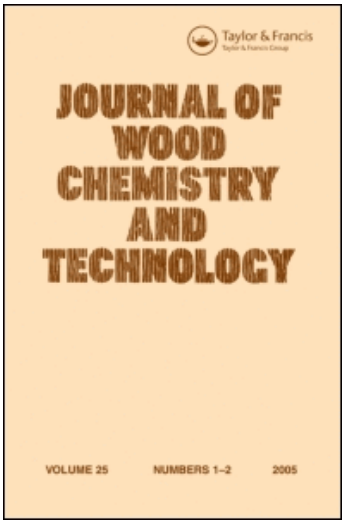

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An NMR Comparison of the Whole Lignin from Milled Wood, MWL, and REL Dissolved by the DMSO/NMI Procedure

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Abstract: Lignins isolated from pine milled wood, milled wood lignin (MWL), and residual enzyme lignin (REL) were compared using modified thioacidolysis, modified DFRC, gel permeation chromatography (GPC), two-dimensional Heteronuclear Multiple Quantum Coherence (HMQC) NMR, and quantitative ^{13}C NMR. Dissolution of the lignin for solution-state NMR was accomplished by utilizing the recently reported DMSO/N-methylimidazole/acetic anhydride solvent system. Contrary to previous reports, comparison of the lignin preparations by thioacidolysis indicated that REL was more structurally similar to the lignin in the milled wood and Wiley wood meal than MWL. Total monomer yields indicated that the MWL was lower in β-aryl ether content than the other preparations, and this was verified by quantitative ^{13}C NMR. NMR analysis indicated that the inter-unit linkages present in all the lignin preparations are consistent with the present knowledge about lignin biosynthesis. The contribution of minor end group structures in the MWL are further decreased in the milled wood, indicating that they are preferentially isolated as low molecular weight material, possibly generated during the milling process. All other structural moieties were similar in all preparations. GPC data indicated that the milled wood and REL both contain a portion of lignin with a molecular weight of 55,000 g/mol. Data

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indicate that the inefficiency of the DFRC method may be related to molecular mobility or accessibility in higher molecular weight portions of the lignin polymer.

Keywords: lignin isolation, milled wood lignin (MWL), residual enzyme lignin (REL), HMQC NMR spectroscopy, quantitative ^{13}C NMR spectroscopy, GPC

INTRODUCTION

Lignin in the cell wall is an amorphous copolymer of phenylpropanoid units linked through both ether and carbon-carbon bonds. It provides mechanical support for the plant, as well as facilitating transport of nutrients and providing defense against attack from microorganisms.[1–5] Therefore, elucidation of the complete structure and macromolecular characteristics of the lignin polymer is of interest from the biological perspective. In addition, lignin must be removed in order to produce high-quality printing and writing paper. As a result, the structure of lignin is of great importance for optimizing the processes and understanding the reactions involved in its removal.

For decades, lignin chemists have been attempting to completely isolate lignin from wood in an unaltered form. However, polymerization of monolignols occurs within the cell wall, embedded in a polysaccharide gel, thereby producing molecular association and possibly covalent bonds.[4,6,7] Consequently, quantitative isolation of the complete lignin polymer has proven impossible.

Milled wood lignin is isolated after milling disrupts the crystallinity of the cellulose in the cell wall and depolymerizes the lignin polymer to some unknown extent.[8–11] Solvent can then penetrate the cell wall and extract some low molecular weight lignin at maximum yields of less than 50% of theoretical.[12] Even with an inability to isolate the whole lignin, MWL has been typically considered to be representative of the structure of native lignin.

There have been many reports on the heterogeneous nature of lignin in wood,[13–15] and therefore, anything less than quantitative isolation of lignin cannot be considered representative. As a result, we have employed a recently documented technique that allows for dissolution of the entire cell wall by acetylation in a DMSO/N-methylimidazole solution in preparation for characterization by solution-state NMR.[16]

It has been reported that extended milling in a rotary ball-mill can reduce the particle size of wood, analogous to vibratory milling, so that MWL can be extracted.[17,18] The advantage of utilizing this technique is that one can avoid the metal contamination imparted by vibratory-milling with steel balls. These metal ions are paramagnetic in nature and will greatly reduce the relaxation time of the carbon and proton nuclei in the NMR solution.[19] The result is a spectrum with extreme line broadening and very poor resolution. In the past, this has not been a problem with MWL isolation because the metal

ions are insoluble in aqueous dioxane, and hence will be retained in the insoluble residual lignin. However, for the analysis of the residue or whole wall material, this is problematic.

In this article, we will employ solution-state NMR techniques to compare rotary-milled wood, the corresponding MWL, and the insoluble residual (REL) material left after the MWL isolation procedure. These materials were subjected to extensive hydrolysis with industrial cellulase to remove the bulk of the carbohydrates [20,21] in order to examine the lignin structure by one-dimensional quantitative ^{13}C NMR and two-dimensional HMQC NMR spectroscopy. The lignins were also analyzed by degradative methods and GPC.

MATERIALS AND METHODS

Materials

Deuterated chloroform ($CDCl_3$) was purchased from Cambridge Isotope Laboratories in sealed ampoules with volumes of 0.25 and 0.75 mL. 1,4-dioxane was purchased from Fisher Scientific and distilled over $NaBH_4$ prior to being used. All other chemicals were purchased from either Fisher Scientific or Aldrich Chemicals and used as received.

All samples were produced from Loblolly pine (*Pinus taeda*). Sapwood was ground to pass a 20-mesh screen in a Wiley mill and Soxhlet-extracted with 1:2 (v/v) ethanol:benzene for 24 h, followed by ethanol for 24 h. Wiley wood meal (100 g) was ground for six weeks in a 1-gallon porcelain jar using porcelain balls under a nitrogen atmosphere. A portion of this material was retained for analysis and is referred to as Porc 6 Wood. (Figure 1).

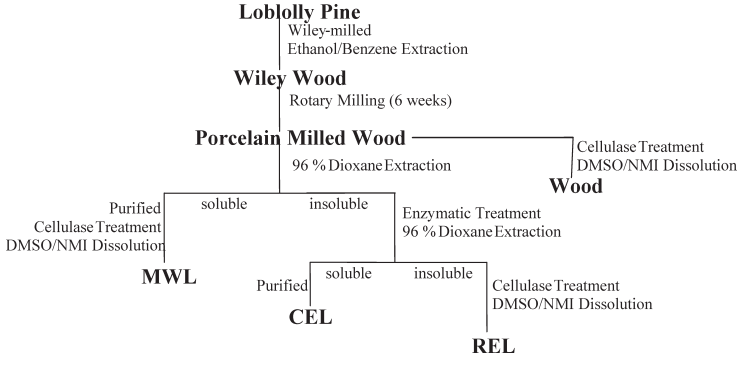

Figure 1. Isolation procedures for various lignin fractions.

Milled wood lignin (MWL) and cellulolytic enzyme lignin (CEL) were then isolated according to Björkman[22] and Chang et al.,[21] respectively These samples were combined and analyzed together, based upon results indicating the similarity between MWL and CEL.[23] This portion will be referred to as Porc 6 MWL. The insoluble residue (REL—residual enzymatic lignin) from the 96% dioxane extraction of the CEL, referred to as Porc 6 REL, was washed with deionized water and freeze-dried.

Sample Preparation

Each sample was subjected to enzymatic hydrolysis treatments to remove carbohydrate material for NMR analysis. The Porc 6 MWL portion was treated only once to assure that carbohydrate overlap would not occur in the quantitative ^{13}C NMR spectrum, as was done in our previous report.[23] Porc 6 Wood and Porc 6 REL have much lower lignin contents, 27% and 42%, respectively, and therefore were treated five times to remove the carbohydrates.

The lignins were freeze-dried after the cellulase treatments and dissolved using a solvent system recently reported by Lu and Ralph.[16] Specifically, 100 mg of dried lignin (over P_2O_5) was carefully weighed into a 20 mL scintillation vial. In the case of the Porc 6 MWL, 2 mL of DMSO and 1 mL of N-methylimidazole were added to the vial and the solution was stirred for 3 h at room temperature. The Porc 6 MWL went almost immediately into solution. The solution was yellowish-orange in color. After 3 h, 0.5 mL of acetic anhydride was added and the mixture allowed to mix for an additional 1.5 h. The color of the solution turned dark brown almost immediately after addition of the acetic anhydride.

The same protocol was used for the Porc 6 Wood and Porc 6 REL. However, because these samples were more difficult to dissolve, longer reaction times were used. The initial DMSO/N-methylimidazole step was extended to 8 h of mixing, and the acetylation step was allowed to proceed overnight. Prior to addition of acetic anhydride, the solution was light brown in color and it appeared that much of the lignin was actually suspended. After acetylation the lignin gradually went into solution. It is interesting to note that the Porc 6 REL was the most difficult sample to dissolve, even more so than the Porc 6 Wood. This fact may be attributable to the higher concentration of high molecular weight lignin material in the Porc 6 REL.

At this point all samples were treated identically. Because the Porc 6 Wood and Porc 6 REL contain considerable amounts of ash from milling with porcelain balls, each sample was filtered through a No. 2 Whatman filter. In each case, only light colored material was left on the filter pad, indicating that the lignin was in solution. The lignin was then precipitated into 2 liters of deionized water and centrifuged at 5,000 rpm for 10 min. The

supernatant was decanted and the precipitated lignin material combined and washed twice with deionized water. All supernatants were collected and filtered through a Whatman 0.45 μm nylon membrane filter and the solid material combined with the rest of the lignin material.

The collected lignin was washed twice with deionized water until the filtrate ran clear, and then dried in a vacuum oven at 40°C and 30″ Hg overnight. The lignin was then dissolved once again in $CHCl_3$ and filtered through a No. 2 Whatman paper filter to remove any remaining ash in the solution. Again, dark material indicative of lignin was not present on the filter. The $CHCl_3$ was removed by a stream of Ar lightly bubbled over the surface the solution. The final lignin material was crushed, ground, and placed in a drying pistol for a minimum of 24 h prior to analysis by NMR. Typical yields were in the range of 110 mg, and loss in yield can be attributed to the excessive handling of the samples.

^{1}H-^{13}C Correlation 2D NMR Spectroscopy

Spectra were recorded on a Bruker AVANCE 500 MHz spectrometer (1996) using an Oxford narrow bore magnet (1989) in $CDCl_3$, the $CDCl_3$ crosspeak at δ_C/δ_H 77.25/7.265 was used as an internal reference. Forty mg of dry lignin were accurately weighed and dissolved in 0.75 mL of $CDCl_3$. The system was controlled by the SGI INDY host workstation and the data was processed with XWIN-NMR. The instrument was equipped with three frequency channels, waveform memory and amplitude shaping, three channel gradient control units (GRASP III), and one variable temperature unit, as well as one unit for pre-cooling and temperature stabilization. All measurements were carried out with a 5 mm ID ^{1}H/BB (^{109}Ag-^{31}P) triple-axis gradient probe (ID500-5EB, Nalorac Cryogenic Corp.). The operational frequency for ^{1}H nucleus was 500.128 MHz and conditions for analysis included a 90° pulse width, a 0.1 s acquisition time and a 1.5 s pulse delay (d_1).

1D Quantitative ^{13}C NMR Spectroscopy

Quantitative ^{13}C spectroscopy was performed on the same instrument as described earlier and for the ^{13}C nucleus was 125.032 MHz using a ^{13}C GE probe. For the Porc 6 MWL, 70 mg was accurately weighed and dissolved in 0.25 mL of $CDCl_3$. For the Porc 6 Wood, 60 mg was dissolved in 0.6 mL of $CDCl_3$. To decrease acquisition time and provide complete relaxation of all nuclei, 10 μL of a 0.25 mg/mL chromium acetoacetonate solution was added to the dissolved samples. Analyses were performed using a 5-mm Shigemi tube, and conditions for analysis included a 90° pulse width, an acquisition time of 1.4 s, and a 1.7 s pulse delay (d_1).

Dipolar Decoupling Solid-State NMR Spectroscopy

A Chemagnetics CMX-200 spectrometer operating at a frequency of 200 MHz for proton and for 50 MHz for carbon was used to obtain all spectra. Samples were placed in a 7.5 mm zirconia magic angle-spinning (MAS) rotor. A standard cross polarization (CP) pulse sequence was utilized with ^{13}C chemical shifts referenced to HMB. Conditions used were as follows: the spinning speed was 5 kHz, the contact time was 2 ms, the pulse width was 6.5 μs, the pulse delay was 3 s, and 10,000 scans were performed per sample at a temperature of 298 K.

Conditions identical to the CP/MAS experiment described above were followed in the dipolar decoupling experiment. In this experiment the high-powered decoupler used to eliminate the carbon-proton interaction is gated off for a period of time prior to acquisition. As a result, spin-lattice relaxation can occur and the magnetization of the carbon is allowed to decompose. This process occurs much more quickly if there is a directly attached proton, only the protonated carbon signals will decay and as result a spectrum containing only quaternary carbons is obtained. In the case of isolated lignins in particular, the overwhelming majority of quaternary carbons are located on the aromatic ring. As a result, the degree of condensation of the lignin polymer can be estimated.

Gel Permeation Chromatography (GPC)

GPC analyses were performed on a Waters HPLC system at ambient conditions using two μ-Styragel columns (HR-1 and 5E) connected in series. DMF was the mobile phase with 0.1 N LiCl added to eliminate lignin association.[24] The fractions were monitored using refractive index (Waters refractometer model 410) and UV absorbance at 280 nm (Waters UV spectrometer model 484). All lignins were dissolved at a concentration of 1 mg/mL. The flow rate was 0.5 mL/min, and the injection volume was 120 μL. Relative average molecular mass determinations were made using polystyrene calibration standards.

Modified Thioacidolysis

Modified thioacidolysis was performed as described previously.[25]

Modified DFRC

The modified DFRC method was performed as described previously.[26]

RESULTS AND DISCUSSION

Comparison of Lignin Preparations by Degradative Techniques

It was previously shown by DFRC that MWL consistently showed a higher total molar yield of degradation monomers than the Wiley Wood from which it was isolated.[26] Additionally, it was shown that the CEL isolated after removal of carbohydrates was structurally similar to the MWL, supporting the data presented in the modified DFRC study.[23] It was suggested based on the DFRC data, however, that the insoluble REL must have a more condensed structure because less DFRC monomers were released. This conclusion was based on the assumption that the DFRC reaction procedure was efficient. However, it was shown that the DFRC method did not completely degrade β-aryl ethers in MWL, resulting in a lower than expected yield of DFRC monomers.[27]

Thioacidolysis on the other hand, completely degraded all β-aryl ethers in MWL and contrary to the DFRC data, the modified thioacidolysis actually showed a higher yield of monomers from the REL (25.5 mol%) than the MWL (18.1 mol%) (Table 1). Both thioacidolysis yield and etherified end groups (Unit A) for the REL were similar to those of the Wiley Wood. This suggests that the REL is actually more similar to the native lignin than MWL. It could also suggest that both the Wiley Wood and REL are largely comprised of secondary wall material, whereas a significant portion of the MWL is from the middle lamella lignin, especially if the yield of MWL is low. These results are in agreement with a recent study on the effect of extensive ball milling on lignin structure.[28] These results suggest that it is necessary to consider the REL portion when attempting to determine the structure of native lignin, or more importantly to study the whole lignin.

The REL portion has been largely ignored because of its relative insolubility in typical solvents. The development of the DMSO/NMI solvent system by Lu and Ralph, however, has afforded the opportunity to study the REL, and the lignin in finely milled wood by solution-state NMR.

Comments on Milling

It is well known that the yield of MWL increases with milling time[12] with structural changes occurring in the isolated lignin to an unknown extent.[8,21,29,30] As a result, it is advisable to minimize the extent of milling when preparing MWL in order to obtain a lignin structure more similar to that of native lignin. Thus, vibratory ball milling is typically limited to obtain MWL yields of $\sim$20% (for softwoods) after purification by the Björkman procedure.[22] Based on results from the modified DFRC method, we showed previously that rotary milling for 6 weeks with porcelain balls would provide a MWL of similar yield, and likely similar chemical content

Table 1. Comparison of the unit composition and total molar yields of lignin preparations analyzed by the modified DFRC and thioacidolysis methods

	Modified DFRC				Modified thioacidolysis		
	Unit composition (mol%)				Unit composition (mol%)		
	Unit A	Unit B	Unit C	Total yield (mol%)	Unit A	Units B + C	Total yield (mol%)
Wiley wood	21.6	6.8	71.6	13.2	25.1	74.9	23.6
Vibratory milled wood	26.7	7.8	65.5	14.8	21.8	78.2	21.8
Vibratory MWL	41.3	7.7	51.0	14.5	33.4	66.6	18.1
Vibratory REL	25.2	5.3	69.5	12.9	23.3	76.7	25.5

to that of the Björkman isolation procedure.[26] In terms of NMR analysis of the entire lignin structure, the rotary-milled wood has the advantage that the milled wood does not contain paramagnetic contamination resulting from the vibratory-milling with steel balls.

Dissolution of Lignin

The Porc 6 Wood and Porc 6 REL were treated five times with an industrial cellulase to remove carbohydrates, then each sample was dissolved using the DMSO/NMI procedure described by Lu and Ralph.[16] The Porc 6 Wood and Porc 6 REL did not dissolve within the prescribed time, therefore as a precaution several hours of mixing and acetylation overnight were performed in order to obtain dissolution. Only the MWL was completely soluble in the DMSO/NMI solution prior to acetylation. This dissolution procedure was not optimized as it was stressed that there was no evidence of structural changes occurring due to the dissolution procedure.[16]

^{1}H-^{13}C Two-Dimensional HMQC NMR

Lu and Ralph recently reported a two-dimensional NMR spectrum of whole acetylated cell wall dissolved using DMSO/NMI.[16] They did not remove the carbohydrates prior to NMR analysis and as a result the lignin exhibits a very low intensity compared to the carbohydrate signals. We removed the bulk of the carbohydrates and our spectra show a higher intensity for the structural moieties in the lignin. In addition, we have been attempting to directly analyze the insoluble material from the lignin isolation process by NMR, and as a result, this new solvent has given us a chance to achieve this goal.

Figure 2 shows the expanded oxygenated aliphatic region for Porc 6 Wood, Porc 6 MWL, and Porc 6 REL. Although the spectra are only qualitative due to varying carbohydrate contents, they are nevertheless informative in the comparison of the lignins. Specifically, all the major structural moieties based on lignin biosynthesis are present; there are no unexplainable signals in the milled wood or REL to indicate that the bonding pattern is dramatically different from the MWL. HMQC indicates that minor substructures, such as β-1′, may be present only in small proportions in the milled wood; however, their signal intensities may be diminished compared to the MWL due to the residual carbohydrate. (Figure 2A) Interestingly, the relatively strong signal at δ_C/δ_H 82.0/5.1–5.2 ppm may correspond to C_α/H_α correlation in the spirodienone (**XIV**) substructure. Zhang and Gellerstedt suggested that these structures may be present in levels as high as 3% in spruce MWL, and confirmed this by quantitative ^{13}C NMR.[31] We have shown previously by quantitative ^{13}C NMR that these are minor structures in pine MWL[23] but, as will be seen later these substructures are not

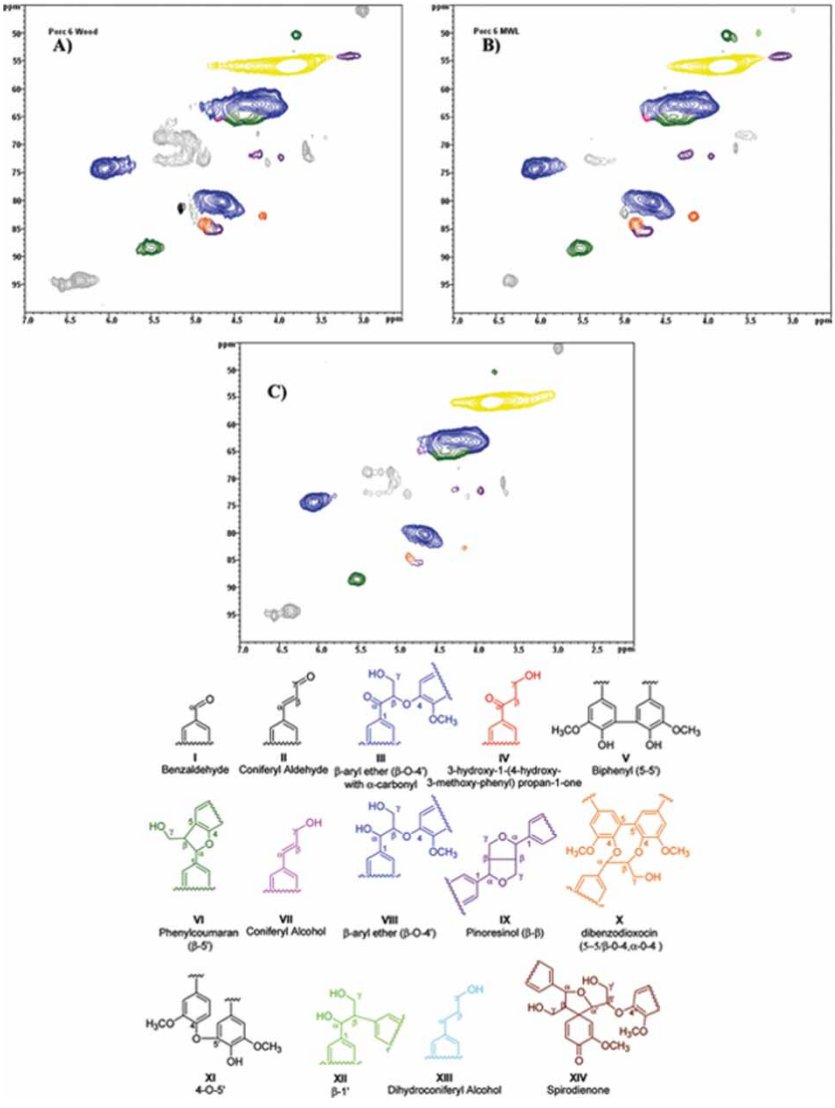

Figure 2. Expansions of the oxygenated aliphatic regions of the HMQC spectra: A) Porc 6 Wood; B) Porc 6 MWL; C) Porc 6 REL.

readily discernible by quantitative ^{13}C NMR of the lignin isolated from milled wood. This suggests that the signal in the 2D NMR may derive from somewhere else.

The strong signal at δ_C/δ_H ~95/6.1–6.6 ppm may be the anomeric carbons from carbohydrates, however, the proton chemical shift (typically 5.2–5.3 ppm) based on literature data is much too far downfield to make

this assignment.[16,32–34] This signal is strong in all preparations, including MWL dissolved using this solvent system, yet it is not present in the MWL spectrum from directly dissolving the acetylated MWL in NMR solvents. As a result, it must be an impurity derived from the dissolution process. DMSO and N-methylimidazole, however, do not have a correlation that would appear in this region of the spectrum.

The signals in the region of δ_C/δ_H 65–75/4.7–5.4 ppm are derived from the C_2 and C_3 carbons of acetylated carbohydrates. Likewise the signals in the region of δ_C/δ_H 69–74/3.5–4.3 ppm result from the remaining carbohydrate carbons and not lignin.[16,32–34]

Quantitative ^{13}C NMR

Recently, we presented a solution-state NMR comparison of Vibratory MWL and Vibratory CEL prepared by the standard methods of Björkman and Chang.[23] In that paper we presented data using both the nonacetylated and acetylated lignins to perform an in-depth comparison of the two lignin preparations. In the present case, we will only be able to make a comparison of the lignins utilizing the acetylated material because the nonacetylated wood and REL are not soluble.

In order to perform quantitative ^{13}C NMR on a lignin sample, a high concentration is required to achieve good signal to noise, and a relaxant is preferable to minimize the experimental time. Chromium acetoacetonate is typically added to decrease the relaxation time of the lignin carbons. To increase the signal to noise ratio, a concentration on the order of 70 mg/0.25 mL is used. Due to difficulty in solubility, the Porc 6 REL was dissolved at concentrations of only 60 mg/0.6 mL. Quantitative carbon for the Porc 6 REL did not yield a spectrum with enough resolution to provide any useful data because it was not depolymerized to a large enough extent. As a result, only comparison between Porc 6 MWL and Porc 6 Wood will be presented.

Figure 3 compares the quantitative ^{13}C NMR spectra for the Porc 6 MWL and Porc 6 Wood. As can be seen, the resolution is higher in the MWL compared to the wood. This is easily explained by the concentrations (i.e., solubility) of each of the preparations in the deuterated solvent. As a result, the signal to noise is much lower for the wood compared to the MWL. In addition, the wood contained 3% ash prior to dissolution, and although the solution was filtered through glass wool, some insoluble porcelain material may be present in the NMR tube, thereby contributing to line broadening. Peak assignments for quantitative ^{13}C NMR spectra are given in Table 2.

Oxidized Carbon Region

Table 3 lists the data for the inter-unit linkages in each of the lignin preparations. The wood contained only vanillin (**I**), ~0.04 carbons per

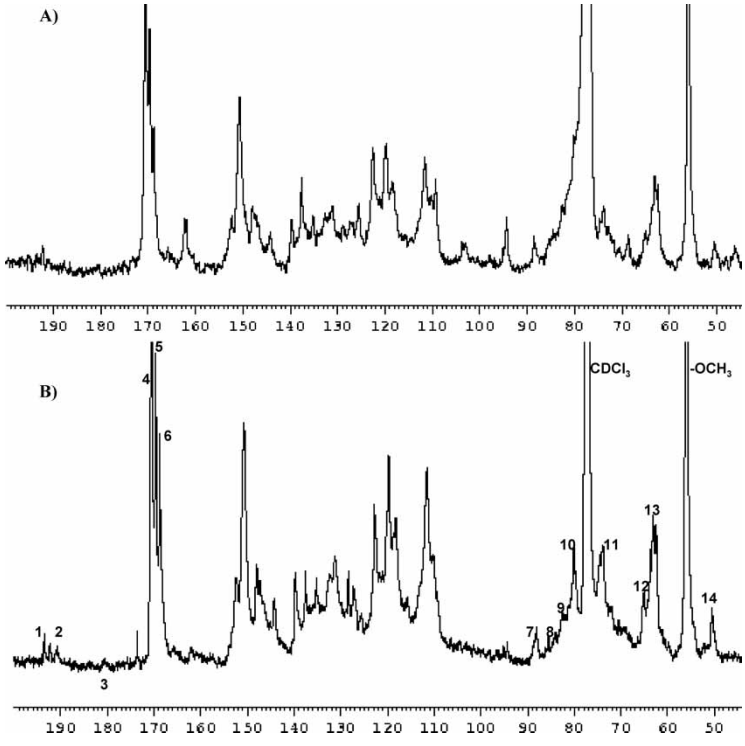

Figure 3. Quantitative ^{13}C NMR spectra for A) Porc 6 Wood and B) Porc 6 MWL, both dissolved and acetylated using the DMSO/NMI dissolution procedure.

aromatic ring, with the other carbonyl moieties either absent or present in small amounts. This is not unexpected as these carbonyl structures are considered end group moieties and therefore will be represented in much smaller amounts in the higher molecular weight lignin obtained from the wood. Although the MWL portion is present in the Porc 6 Wood fraction, it is only about one-fifth of the total lignin and will be expressed to a much lower extent in the wood. Therefore, the carbonyl structures may be present in the lignin isolated from milled wood but are below the detection limit of this technique.

Interestingly, the region from 200–190 ppm exhibited typical values for the MWL, with a total integration of 0.11 carbons per aromatic ring. Absent from this spectrum (Figure 3) is the 3-hydroxy-1-(4-hydroxy-3-methoxy-phenyl) propan-1-one (IV), shown in Figure 2, suggesting that it may be solubilized during the DMSO/NMI dissolution process. Vanillin, with a chemical shift of 191 ppm, is present in levels of 0.04 carbons per aromatic ring. β-O-4′ substructures with α-carbonyl (**II**) and coniferaldehyde (**III**) overlapping at 194 ppm are also present at a combined level

Table 2. Peak assignments for quantitative ^{13}C NMR spectra

Peak number	δ (ppm)	Assignment	Peak number	δ (ppm)	Assignment
1	194	C═O in (**II**) and (**III**)	8	86–85	C_α in (**IX**) and (**X**)
2	191	C═O in (**I**)	9	83	C_β in (**X**)
3	181	C═O in (**XIV**)	10	80	$C_\beta(e,t)$ in (VIII)
4	171	C═O in aliphatic primary -OAc	11	75	C_α (e,t) in (VIII)
5	169.5	C═O in aliphatic secondary -OAc	12	66	C_γ in (**VI**)
6	168.5	C═O in phenolic -OAc	13	64.5–61	C_γ in (**III**), (**IV**), (**VII**), (**VIII**), (**X**), (**XII**), and (**XIII**)
7	90–87	C_α in (**VI**)	14	51-49	C_β in (**VI**) and (**XII**)

of 0.04 carbons per aromatic ring. Spirodienone (**XIV**) at 181 ppm is present at levels about 0.01 per aromatic ring in the MWL but not in appreciable amounts in the Porc 6 Wood. A clearly resolved signal present at 193 ppm accounts for the remainder of the value of the integration for this region.

Table 3. Quantitative determination of interunit linkages for Porc 6 Wood and Porc 6 MWL by ^{13}C NMR.

		Number of moieties per aromatic ring	
Spectral region	Chemical shift range (ppm)	Porc 6 MWL	Porc 6 Wood
Ar-CHO (**I**)	191	0.04	0.04
Ar-CH═CH-CHO (**II**) + α-C═O in β-O-4 (**III**)	194	0.04	Negl.
Ar-CO-CH_2-CH_2OH (**IV**)	198	Negl.	Negl.
β-5′ (**V**)	90–86	0.09	0.10
β-O-4′ (**VIII**)+ Dibenzodioxocin (**X**) + Ar-CH═CH-CH_2OH (**VII**)	64.5–61	0.40	0.47
β-1′ (**XII**)	51–48	0.02	Negl.
Spirodienone (**XIV**)	181	0.01	Negl.

Aliphatic and Phenolic Hydroxyl Content

The hydroxyl contents are represented by the acetates of the primary, secondary, and phenolic hydroxyl groups, respectively, from 171.5–167 ppm. (Figure 3). For the Porc 6 MWL, the aliphatic hydroxyl content, 0.75 carbons per aromatic ring, is much lower than was reported for the MWL prepared by the standard Björkman method (1.02 per aromatic ring).[23] The aliphatic hydroxyl content for the Porc 6 Wood on the other hand is much higher with a value of 1.23 per aromatic ring. The difference in the two MWL preparations could be attributed to structural changes occurring within the isolatable lignin portion during milling. The value for the Porc 6 Wood could be higher because of contribution from carbohydrates. Interestingly the phenolic hydroxyl content of the wood and MWL were quite similar with values of 0.29 and 0.28 per aromatic ring, respectively, which are similar to the value (0.25) reported earlier for spruce MWL.[35] These values, however, were much higher for the Porc 6 MWL (0.28 vs. 0.20 per aromatic ring) compared to another spruce MWL reported previously.[23] The authors attributed the difference to the use of less efficient vibratory ball mill in the latter study.[23] This indicates that the lignin may be depolymerized to a greater extent in the rotary ball milling than in the standard Björkman procedure.

Aliphatic Side Chain Region

Comparison of the oxygenated aliphatic side-chain regions for the two preparations unfortunately will yield less information than a comprehensive comparison utilizing both the acetylated and the nonacetylated spectra. In any case, estimations will be made where possible.

The correlations for C_α/H_α, C_β/H_β, and C_γ/H_γ for the β-O-4′ substructures are centered at δ_C/δ_H 74.0/6.0, 80.0/4.6, and 62–63/4.0–4.4, respectively, as shown in the HMQC spectra. Because the Porc 6 Wood lignin was most easily solubilized in $CDCl_3$, the correlations for the α- and β-carbons are overlapped by the solvent peak. As a result, calculation of the quantity of β-O-4′ substructures (**VIII**) must be calculated based on integration of the region containing the γ-carbons. This region is overlapped by the γ-carbons from other structures, however, most of these can be estimated based on integration of other signals, and as a result, a close approximation can be obtained.

The structures that cannot be completely elucidated based on other signals are the dibenzodioxocin (**X**) and coniferyl alcohol (**VII**). The correlations for the α- and β-carbons in the dibenzodioxocin substructure are present, as seen by the C_α/H_α and C_β/H_β correlations at δ_C/δ_H 84.5/4.8 and 82.6/4.1 in the HMQC spectra. The α-carbon in the quantitative ^{13}C spectrum is overlapped by the α-carbon from the β-β' (**IX**) substructure, whereas the β-carbon is

overlapped by the β-O-4′ β-carbon as well as the solvent peak. Coniferyl alcohol on the other hand will have the α- and β-carbons overlapping the aromatic region at 134 and 123 ppm, respectively, and the γ-carbon at 62–63 ppm.

Dibenzodioxocin is a specific β-etherified substructure that also contains an α-O-4′ linkage to a biphenyl (**VI**) structure, thus forming an 8-membered ring. Coniferyl alcohol is likely incorporated as a monomer during lignin biosynthesis and is present in small amounts in MWL. The amount of coniferyl alcohol was estimated to be a maximum of 0.02 in the by typical analysis of Porc 6 MWL in DMSO,[36] but even this may be high because of overlap of the γ-carbon from β-O-4′. Based on the estimable end group contributions to the Porc 6 Wood, it may be considered that the contribution of coniferyl alcohol to this preparation is only minor.

Integration of the region from 64.5–61 ppm, minus the contributions of (**III**), (**IV**), (**XII**), and (**XIV**), results in 0.47 and 0.40 per aromatic ring for Porc 6 Wood and Porc 6 MWL, respectively. The difference in β-O-4′ content is consistent with the results from the degradative techniques discussed earlier, and confirms that cleavage of the β-aryl ether linkages is far from complete in the DFRC analysis. A discussion of this subject will be presented later.

The β-5′ (**V**) content can be clearly determined based on integration of the area from 90–87.5 ppm, and is 0.09 and 0.10 for Porc 6 MWL and Porc 6 Wood, respectively. These values are in line with those previously reported. It would then follow that integration of the region from 51–49 ppm, comprising the β-carbons of both the β-5′ and β-1′ (**XII**) substructures would yield a maximum value of 0.02 β-1′ moieties for the MWL and essentially zero for the wood. This is consistent with the HMQC spectra, which show a C_β/H_β correlation at δ_C/δ_H 50.0/3.4 ppm in the MWL, and is not present in the wood.

Indications based on comparison of Porc 6 Wood and Porc 6 MWL by one- and two-dimensional NMR are that the β-O-4′ content in the milled wood preparation is higher than that in the MWL. While phenolic hydroxyl content is nearly identical, aliphatic hydroxyl content is unexpectedly low in the Porc 6 MWL. All major inter-unit linkages expected to be present based on the present knowledge of lignin biosynthesis are identifiable by HMQC. In addition, there are no unexplainable signals in the wood or REL. Based on quantitative ^{13}C NMR it is apparent that end group moieties are more prevalent in the MWL as compared to the milled wood. This is not unexpected, as MWL is of lower molecular weight and should contain more end groups.

Dipolar Dephasing Solid-State ^{13}CP/MAS NMR

One of the drawbacks from the comparison of the milled wood and MWL by only the acetylated quantitative ^{13}C NMR spectra is that the degree of

condensation cannot be estimated. Fortunately, this can be easily estimated using the dipolar dephasing solid-state NMR experiment. This is simply a CP/MAS experiment with a short delay period (d_2) where spin-locking is removed to allow spin-lattice relaxation to occur to the extent that the signals from carbons with directly attached protons are decayed. Removal of the protonated carbons leaves a quaternary carbon spectrum from which the degree of condensation can be estimated. Although not quantitative, it is a good tool for comparing the different lignin preparations.

The aromatic region of the nonprotonated spectrum can be divided into the condensed aromatic (141–125 ppm) and the oxygenated aromatic (160–141 ppm) regions. A completely uncondensed guaiacyl lignin will contain two oxygenated aromatic carbons per aromatic ring (C_3 and C_4) and one condensed aromatic carbon (C_1) per aromatic ring. Any condensed linkages in the lignin, predominately β-5′ or 5-5′ substructures, will shift the carbon-carbon linked aromatic carbon to the region of 141–125 ppm. As a result, the degree of condensation can then be estimated by the ratio of Region A (160–141 ppm) to Region B (141–125 ppm) (Figure 4).[37,38]

Results in Table 4 show that the MWL has a degree of condensation as estimated by solid-state NMR of 0.44 per aromatic ring, comparable to that of the Vibratory MWL previously reported.[23] This technique is not quantitative; however, comparisons of similar samples can still be performed. Because the Wiley Wood can be analyzed by solid-state techniques, the degree of condensation can be determined to be 0.30 per aromatic ring, and that of the REL is 0.36 per aromatic ring. These results agree with those from the degradative techniques, indicating that the REL is more similar to the lignin in wood than is the MWL.

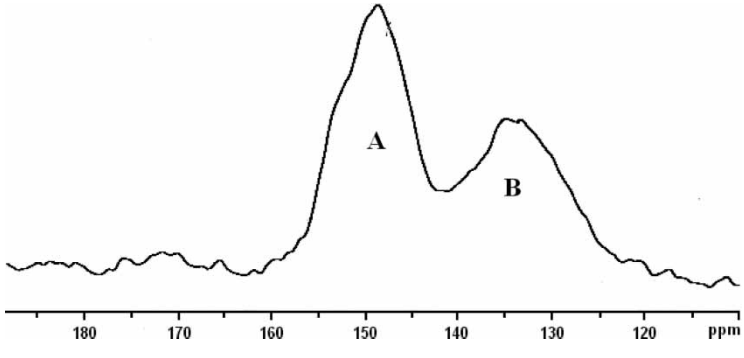

Figure 4. Dipolar dephasing spectrum depicting the two regions of nonprotonated carbons: the oxygenated aromatic carbons (Region A) and the condensed aromatic region (Region B).

Table 4. Estimations of the degree of condensation for Wiley wood, Porc 6 MWL, and Porc 6 REL, as determined by the dipolar dephasing solid-state NMR experiment.

	Estimation of degree of condensation
Wiley wood	30
Porc 6 MWL	44
Porc 6 REL	36

Gel Permeation Chromatography

The average relative molecular mass of the various lignin preparations were determined on acetylated samples in 0.1 N LiCl/DMF (Figure 5). As seen in Figure 5 the Porc 6 Wood sample has a wide average relative molecular mass distribution and as a result, only the M_p (relative molecular mass at the maximum peak height) will be reported. The Porc 6 Wood exhibits four possible peaks, including fairly well resolved peaks at high molecular weight ($\sim$55,000 g/mol) and at very low molecular weight ($\sim$1,200 g/mol). Additionally, two poorly resolved peaks are present as shoulders are also evident, and in the range of the MWL average molecular mass distribution.

Porc 6 MWL is of course isolated from the Porc 6 Wood, and it exhibits the characteristic unimodal distribution expected from a MWL preparation. This is important because it also indicates that aggregation at higher molecular mass is not occurring, thus verifying that the chromatograms of Porc-6 Wood and Porc-6 REL are true representations of their average

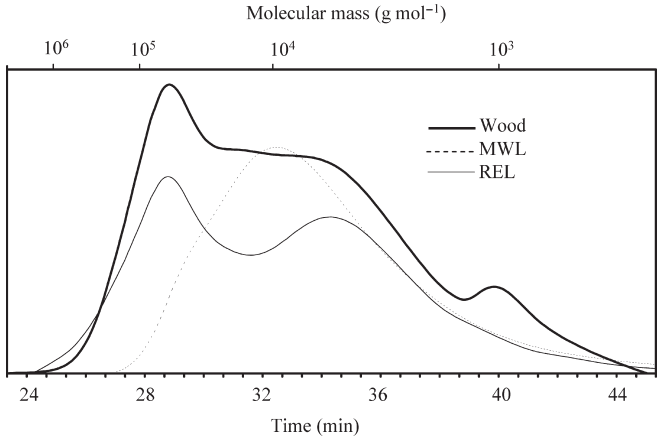

Figure 5. GPC chromatograms of the Porc-6 Wood, Porc-6 MWL, and Porc-6 REL.

relative molecular mass distributions.[24] We have previously reported that the average relative molecular mass of acetylated MWL in THF has an M_w at ~5000 g/mol, whereas in DMF it is closer to 10,000 g/mol.[23,27] The apparent increase in relative molecular mass between THF and DMF is due to the difference in retention times for the polystyrene standards, consistent with previous reports.[39,40] The average relative molecular mass as determined in DMF is closer to that originally reported by Björkman, indicating that possibly the determination of molecular mass relative to polystyrene may be underestimated in THF and that DMF is a more proper solvent for molecular mass determination.[41]

Like the Porc 6 Wood, the Porc 6 REL has a high average relative molecular mass peak at ~55,000 g/mol, as well a lower average relative molecular mass peak at ~6,000 g/mol. It can be seen that the molecular mass portion associated with the MWL, which isolated from the Porc 6 Wood, is clearly absent in the Porc 6 REL. This indicates that the GPC chromatograms are representative of the MWL isolation process. The Porc 6 Wood has a wide lignin average relative molecular mass distribution after milling. From this milled wood the lignin that is free of carbohydrates is readily removed and purified as MWL. The very low average relative molecular mass portion in the wood is not present in either the MWL or the REL, and

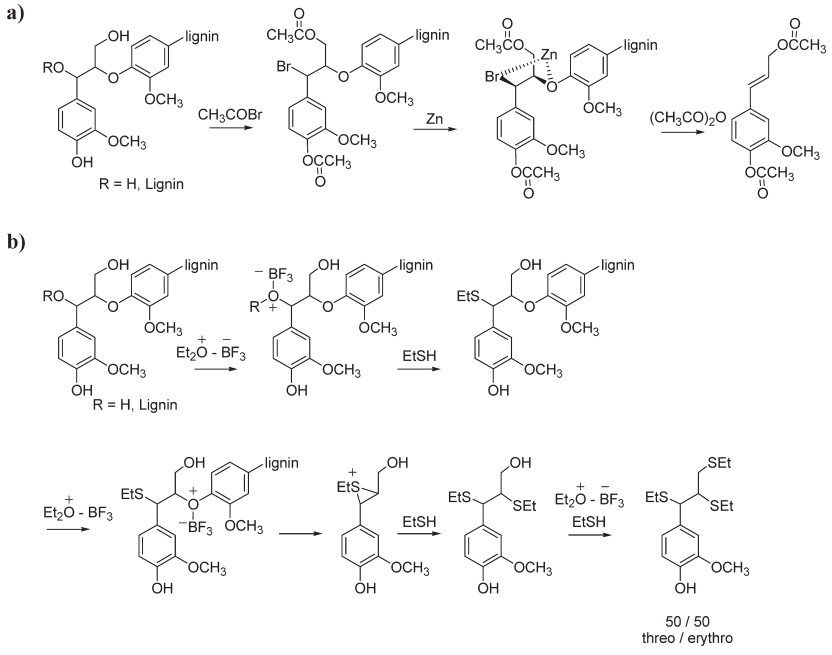

Figure 6. Reaction mechanisms for the a) modified DFRC[42] and b) modified thioacidolysis methods.[43]

is likely removed during the MWL purification process. The presence of a large portion of lower average relative molecular mass material in the REL indicates that association with carbohydrates likely prohibits the solubilization of this lower molecular mass portion of the lignin. After purification and removal of the carbohydrates, the lower molecular mass portion of the REL is likely to solubilize in aqueous dioxane.

Discussion of the Effects of Molecular Mass on the Inefficiency of DFRC

These results indicate that the inefficiencies of the DFRC method likely parallel molecular mass. The MWL exhibits a decrease in total yield of about 20% by the DFRC method, whereas the Wiley Wood, which likely has the highest molecular weight, decreases by almost 45%. The milled wood and REL yields decreased by 32% and 49%, respectively.

The apparent greater inefficiency in β-aryl ether cleavage by DFRC in the high molecular mass materials suggests that accessibility and molecular mobility may be important. Indeed it may be difficult to obtain the orientation necessary to achieve the five-membered ring intermediate in the zinc cleavage step (Figure 6). Thioacidolysis, on other hand, reacts similar to an episulfide intermediate, therefore, molecular mobility is much less important and complete aryl ether cleavage is achieved.

As a result, the data presented in this article indicate that the REL, rather than the MWL, is likely more similar to the structure of native lignin. The REL portion is insoluble due to association with carbohydrates and its high average relative molecular mass, not because it is more highly condensed than the MWL. Furthermore, the REL is probably comprised mostly of secondary wall material, similar to the composition of native lignin, whereas MWL is a lower average relative molecular mass material, and may contain a larger proportion of middle lamella lignin accounting for the higher degree of condensation.

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