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Solution-State Nuclear Magnetic Resonance Study of the Similarities between Milled Wood Lignin and Cellulolytic Enzyme Lignin

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The structures of milled wood lignin (MWL) and cellulolytic enzyme lignin (CEL) have been analyzed using traditional chemical methods and solution-state NMR techniques. Comparisons of the results obtained reveal that subtle differences exist between the two lignin preparations. Thioacidolysis produced higher monomer yields from CEL than MWL, suggesting MWL has a more condensed structure. Quantitative ^{13}C NMR determined the degree of condensation in MWL to be 0.43 unit per aromatic moiety as compared to 0.36 in CEL. The MWL also contained a lower amount of β-O-4′ substructures per aromatic ring than CEL, 0.41 versus 0.47, respectively. Carbohydrate analysis revealed that the MWL may contain a higher proportion of middle lamella material as compared to the CEL. Because the middle lamella is considered to have a more condensed lignin structure, on the basis of the bulk polymerization theory, these results could explain the differences in β-O-4′ and degree of condensation.

KEYWORDS: Lignin; lignin isolation; milled wood lignin (MWL); cellulolytic enzyme lignin (CEL); HMQC NMR spectroscopy; quantitative ^{13}C NMR spectroscopy; thioacidolysis; GPC

INTRODUCTION

Lignin is arguably the second most abundant biopolymer on earth (*1*) and, as a result, extensive effort has been made to determine the structure of lignin in its native state. However, it is impossible to selectively isolate native lignin from woody material due to its close association with carbohydrates within the cell wall. For this reason, wood samples are subjected to mechanical and/or chemical treatments. The resulting wood meal is then extracted with organic solvent, typically aqueous dioxane (*2*), to obtain a carbohydrate-free lignin. The soluble material is purified and referred to as milled wood lignin (MWL). Although the resulting lignin is of high purity, it typically consists of only ∼25−50% of the theoretical lignin yield (*3*−*6*).

Structurally, MWL is generally considered to be representative of native lignin, even though it is prepared with yields far from quantitative (*7*). Furthermore, lignin yield is dependent upon milling time (*6*), and therefore depolymerization and association with carbohydrates greatly influence the amount and structure of extractable lignin after milling. Although a yield of up to 50% can be achieved by increased milling time (*6*), a MWL more representative of native lignin is not obtained (*8*−*11*).

To improve yields while minimizing the extent of mechanical action, the insoluble material from the aqueous dioxane extraction can be treated with an industrial cellulase (*9*). This enzymatic treatment removes the majority of the carbohydrates. Subsequent aqueous dioxane extraction solubilizes another portion that is referred to as cellulolytic enzyme lignin (CEL), yielding ∼10% of the lignin in wood. CEL is considered to be lignin associated with carbohydrates and is most likely derived from the secondary wall. As CEL is extracted from the residual wood meal using the same solvent system as MWL, CEL is thought to be structurally similar to MWL. If these two preparations are indeed similar, they could be combined to increase the overall yield of pure lignin material.

Previously we reported that DFRC analysis of MWL and CEL produced similar results (*12*), suggesting that the lignin directly associated with carbohydrates and that not associated with carbohydrates are structurally similar. However, DFRC suffers from the fact that it can yield monomers from only β-O-4′ end groups and those linked by β-O-4′ linkages to the lignin macromolecule through both the phenolic hydroxyl and the β-position. Recently, we have shown that DFRC does not completely cleave all available β-O-4′ linkages (*13*). On the basis of this finding, a thorough investigation into the structural similarity between MWL and CEL is warranted. Here we report the results of traditional chemical methods such as nitrobenzene oxidation (*14*) and thioacidolysis (*15*, *16*) as well as modern NMR spectroscopic techniques including quantitative ^{13}C NMR and two-dimensional HMQC NMR to evaluate the similarities and differences between MWL and CEL.

MATERIALS AND METHODS

Materials. DMSO-d_6 was purchased from Cambridge Isotope Laboratories and used as received. 1,4-Dioxane was purchased from Fisher Scientific and distilled over $NaBH_4$ prior to being used. All other

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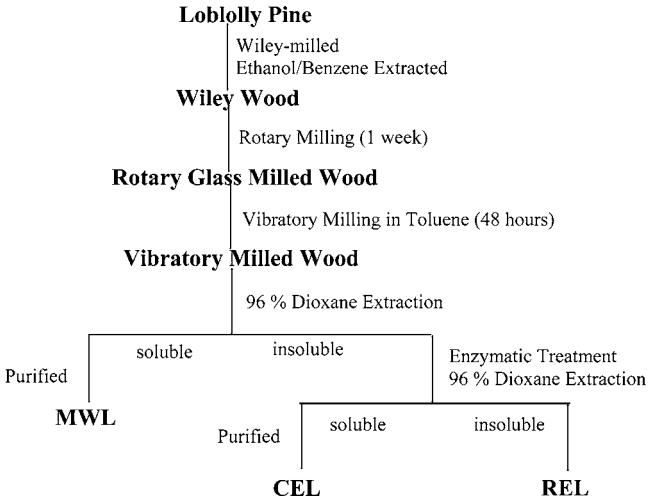

Figure 1. MWL and CEL isolation procedure.

chemicals were purchased from either Fisher Scientific or Aldrich Chemicals and used as received.

MWL and CEL 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 1 week in a 1 gal porcelain jar using glass balls under a nitrogen atmosphere. The rotary-milled wood was then ground for 48 h in a vibratory ball mill (Siebtechnik GmbH, Mulheim, Germany) with steel balls in toluene and blanketed with N_2. MWL was then isolated according to the method of Bjorkman (*3*) and the CEL according to the method of Chang et al. (*9*). The MWL and CEL isolation procedure is summarized in **Figure 1**.

Elemental Analysis. Elemental analyses (C, H, and N) were performed by Complete Analysis Laboratories, Inc., Parsippany, NJ. Oxygen content was determined by difference, 100 − (C + H + N), assuming no other elements were present.

Methoxyl Analysis. Methoxyl analysis was performed according to the modified Zeisel method (*17*).

Carbohydrate Content. Filtrates were analyzed by high-performance anion-exchange chromatography (HPAEC) on an ion-exchange CarboPac PA-1 column using a Dionex DX-500 system equipped with a pulsed amperometric detector with a gold electrode (Dionex, Sunnyvale, CA) and a Spectra AS 3500 autoinjector (Spectra-Physics, Mountain View, CA). Prior to injection, samples were filtered through 0.45 μm HV filters (Millipore, Bedford, MA), and a volume of 20 μL was loaded. Samples were eluted with water at a flow rate of 1 mL/min, with a postcolumn addition of 0.5 mL/min of 250 mM NaOH prior to detection (*18*).

^{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 DMSO-d_6; the DMSO-d_6 cross-peak at δ_C/δ_H 39.5/2.5 was used as an internal reference. Forty milligrams of dry lignin was accurately weighed and dissolved in 0.75 mL of DMSO-d_6. The system was controlled by the SGI INDY host workstation, and the data were 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 precooling and temperature stabilization. All measurements were carried out with a 5 mm i.d. ^{1}H/BB (^{109}Ag−^{31}P) triple-axis gradient probe (ID500-5EB, Nalorac Cryogenic Corp.). The operational frequency for the ^{1}H nucleus was 500.128 MHz, and conditions for analysis included a 90° pulse width of 10 μs and a 1.5 μs pulse delay (d_1).

1D Quantitative ^{13}C NMR Spectroscopy. Quantitative ^{13}C spectroscopy was performed on the same instrument as described above and for the ^{13}C nucleus was 125.032 MHz using a ^{13}C GE probe. The operational frequency for the ^{1}H nucleus was 500.128 MHz. Samples were accurately weighed to 70 mg, dissolved in 0.25 mL of $CDCl_3$, and carefully pipetted into a 5 mm Shigemi tube. To decrease acquisition time 10 μL of a 0.1 mg/μL chromium acetoacetonate solution, a commonly used relaxant, was added to the dissolved sample. Conditions for analysis included a 90° pulse width of 10 μs with an acquisition delay of 1.4 μs and a 1.7 μs pulse delay (d_1).

Carbohydrate overlap in the side-chain region of the ^{13}C NMR spectra can compromise the integration and subsequent quantification of the lignin substructures. A final cellulase treatment was performed using conditions identical to those reported by Chang et al. (*9*). The resulting lignins were freeze-dried and dried over P_2O_5 prior to NMR analysis.

Thioacidolysis Procedure. Thioacidolysis was performed as described by Lapierre et al. (*19*).

Gel Permeation Chromatography (GPC). GPC analyses were performed on a Waters HPLC system at ambient conditions using two μ-Styragel columns (HR-4 and 5E) connected in series. THF was the eluent (0.5 mL/min), and fractions were monitored using refractive index (Waters refractometer model 410) and UV absorbance at 280 nm (Waters UV spectrometer model 484). The acetylated lignins were dissolved in THF at a concentration of 1 mg/mL, and 250 μL of this solution was injected onto the HPLC. Molecular weight determinations were made using polystyrene as a calibration standard.

Table 1. Elemental and Functional Group Analysis of MWL and CEL

	elemental composition[a] (%)				functional group/C_6			
						phenolic	aliphatic	
	C	H	O	N	OCH_3	OH	OH	C=O
MWL	63.2	5.4	31.2	<0.2	0.96	0.17	0.98	0.12
CEL	62.8	5.8	31.1	<0.2	0.94	0.19	1.02	0.08

[a] Elemental analysis based on Klason lignin content.

RESULTS AND DISCUSSION

Table 1 contains information regarding the elemental and chemical compositions of the MWL and CEL. As can be seen the Klason lignin content is much lower for the CEL as compared to the MWL (85 vs 91%). The elemental analyses are corrected to represent only the contributions from lignin and result in chemical compositions of $C_9H_{9.23}O_{3.33}$ for MWL and $C_9H_{9.97}O_{3.34}$ for CEL. It should be noted that a contaminant in the CEL was detected by two-dimensional NMR (vide supra) and may alter the chemical composition somewhat. With the oxygen contents being equivalent, increased hydrogen content for CEL may be indicative of a lower degree of condensation or lower carbonyl content. It is noteworthy that the nitrogen content of the CEL is similar to that of MWL (below detection limit), showing that contamination by enzyme should not be a problem in this analysis.

Laboratory data indicate that CEL and MWL have similar methoxyl contents, 0.94 and 0.96 per aromatic ring, respectively (**Table 1**). This is expected as both MWL and CEL are derived primarily from the secondary wall, and in softwood the primary precursor is guaiacyl-based. As a result, the expected methoxyl content would be close to 1, in agreement with reported literature values (*20*).

The phenolic and aliphatic hydroxyl and carbonyl contents were estimated using quantitative ^{13}C NMR. The hydroxyl contents were similar, with those for CEL being slightly higher for both phenolic, 0.19 versus 0.17, and aliphatic, 1.02 versus 0.98, per aromatic ring. The carbonyl content is slightly higher in the MWL than in CEL, 0.12 for the MWL and 0.08 for the CEL (**Table 1**). The presence of a larger amount of oxidized moieties in the MWL somewhat explains the higher hydrogen content in the CEL. This suggests that either the milling leads

Table 2. Monomer Yields from Chemical Degradation Methods

	DFRC (mol %)	thioacidolysis (mol %)	nitrobenzene oxidation (mol %)
MWL	14.5 ± 0.2	18.1 ± 0.7	31.8 ± 0.9
CEL	14.5 ± 0.4	20.3 ± 1.0	32.3 ± 1.0

to more side-chain oxidation in the MWL or that more end group material is isolated in the MWL portion.

Chemical Degradation Analysis of Lignins. As reported previously, DFRC degradation product yields were similar for both MWL and CEL, as were vanillin yields from nitrobenzene oxidation, leading to the conclusion that MWL and CEL may be similar in structure (*12*). In quantifying the results from the modified DFRC method using the modified thioacidolysis technique, we discovered a discrepancy. Specifically, thioacidolysis has been shown to completely cleave β-O-4′ bonds and result in consistently higher total molar yields than DFRC even though both methods cleave aryl ether bonds (*13*). Although DFRC and nitrobenzene do not reveal a difference between the two lignins, total molar yields for thioacidolysis are 2.2% higher for CEL than for MWL, 20.3 versus 18.1%, respectively (**Table 2**). The higher monomeric yield from thioacidolysis may indicate that the CEL has a larger amount of β-O-4′ substructures than MWL and may have a lower degree of condensation. On the basis of these results, it was determined that the two lignin preparations should be analyzed more closely.

Structural Analysis of Lignins via NMR Techniques. ^{13}C NMR spectroscopy is a reliable method to investigate the structure of the carbon skeleton in lignin. Unlike chemical methods, NMR provides a more comprehensive view of the entire lignin macromolecule. ^{13}C NMR can be used to estimate the abundance of each interunit linkage and can be considered to be quantitative provided adequate experimental conditions are utilized. Specifically, the pulse delay between acquisitions must be sufficiently long to ensure that all carbons have returned to their initial Boltzmann distribution. The pulse delay required to achieve these conditions is generally considered to be 5 times the longest rate of relaxation (*21*). Despite this, ^{13}C NMR suffers the drawback that signals are often overlapped, making the estimation of linkages difficult. As a result, a two-dimensional NMR technique such as HMQC can be considered useful for the elucidation of overlapping signals. Whereas HMQC is almost entirely qualitative, its advantage involves a ^{1}H−^{13}C correlation that stretches the signals onto an $x-y$ axis. Detailed assessment of the HMQC spectrum reveals which signals overlap at what point in the carbon spectrum, enabling more accurate quantification using ^{13}C NMR.

The quantitative ^{13}C NMR spectra for MWL and the oxygenated aliphatic region for the acetylated MWL are shown in **Figure 2**. **Table 3** lists the corresponding peak assignments identified for the lignin preparations (*21*, *22*). The ^{13}C NMR spectra can be broken into structural unit regions and integrated to obtain structural information. **Table 4** lists the various spectral regions identified in the quantitative ^{13}C spectra with results reported as the number of moieties per aromatic ring. Integration was performed by setting a value of 6.12 for the aromatic region (160−100 ppm) representing all aromatic carbons plus a contribution of 0.12 per 100 aromatic units from the side-chain carbons of coniferyl alcohol and coniferaldehydes (*21*).

Integration of the MWL methoxyl peak at 57−54 ppm yields a methoxyl group content of 0.96 methoxyl group per aromatic ring (**Table 4**). This result is identical to the result obtained via chemical analysis (**Table 1**). Surprisingly, NMR analysis of the CEL spectra determined the methoxyl content to be 1.17 methoxyl groups per aromatic ring. This is clearly higher than the 0.94 obtained via chemical analysis (**Table 1**). However, the HMQC spectrum reveals the presence of an overlapping impurity revealed by a correlation at δ_C/δ_H 55.0/5.8 in the CEL spectrum (**Figure 3B**)

Analysis of the carbonyl region (200−190 ppm) of the ^{13}C NMR spectra reveals differences between the two lignin

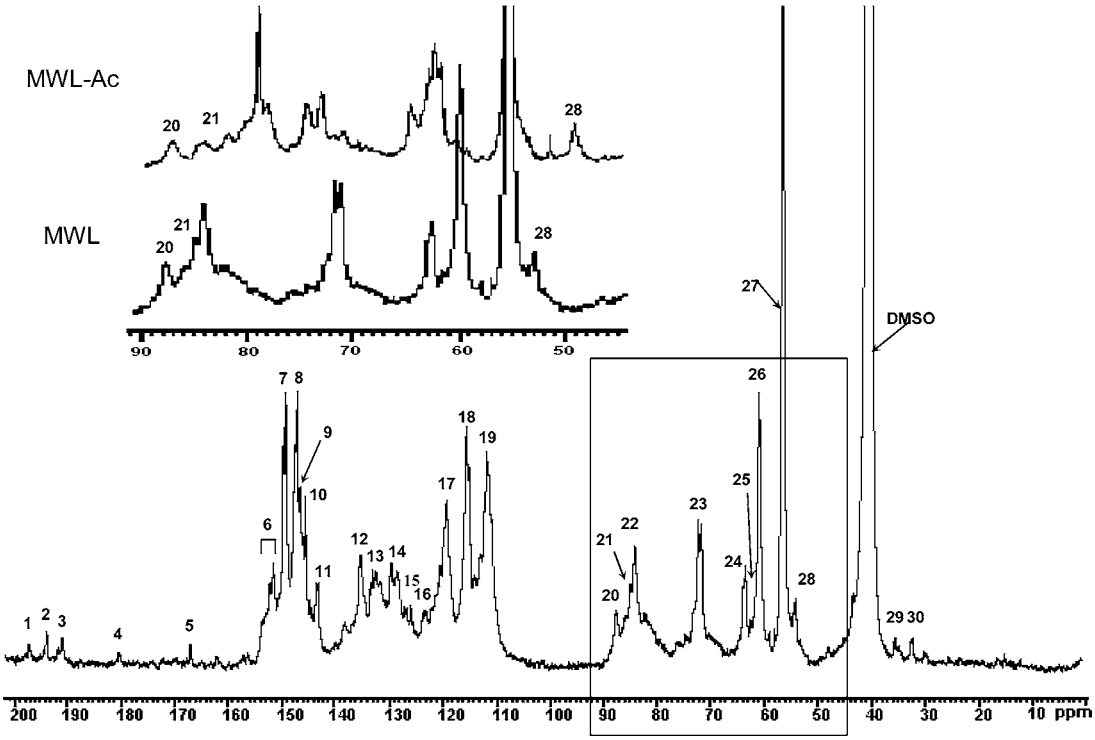

Figure 2. Quantitative ^{13}C NMR spectra of MWL. Included is an expansion of the oxygenated aliphatic region of the MWL and acetylated MWL (MWL-Ac).

Table 3. Assignment of the Quantitative ^{13}C NMR Spectra

peak	δ	assignment	peak	δ	assignment
1	198	C═O in **V**	16	124.5–122	C_1 and C_6 in α-C═O units
2	194	C═O in **III**	17	122–117	C_6 in G units
3	191	C═O in **I**	18	117–113	C_5 in G units
4	181	C═O in **XIV**	19	113–108	C_2 in G units
5	168	C═O in –OAc	20	87	C_α in **V**
6	155–151	$C_3/C_{3'}$ in etherified **V**	21	86.5–85	C_α in **VIII**
		C_α in **III**			C_α and C_β in **IX**
		C_5 in ring B of **XI**			
7	151–148.5	C_3 in etherified G units	22	85–82	C_β (*e*,*t*) in **X**
8	148.5–146.8	C_4 in etherified G units	23	74–71	C_α (*e*,*t*) in **X**
		C_3 in nonetherified **X**			C_γ in **VIII**
9	146.2	C_4 in nonetherified G units	24	64–62	C_γ in **III**, **V**, and **XII**
		C_4 in ring B of **V**			
10	145.2	$C_4/C_{4'}$ in etherified **VI**	25	61.5	C_γ in **VII**
11	143.5	C_3 in ring B of **V**	26	61–59	C_γ in **IX** and **X**
		$C_4/C_{4'}$ in nonetherified **VI**			
12	137–134	C_1 in etherified G units	27	56	$-OCH_3$
13	134–130	C_1 in nonetherified G units	28	54–52	C_β in **V**, **VIII**, and **XII**
		$C_5/C_{5'}$ in etherified **VI**			
14	130–127	C_β in **II**	29	34.5	C_α in **XIII**
		C_α and C_β in **VII**			
15	127–124.5	$C_5/C_{5'}$ in nonetherified **VI**	30	31.5	C_β in **XIII**

Table 4. Quantification of the Spectral Regions of the ^{13}C NMR Spectra

spectral region	chemical shift range (ppm)	no. of moieties per aromatic ring	
		MWL	CEL
methoxyl content	57–54	0.96	1.17[a]
aromatic methine carbons	125–103	2.57	2.64
aromatic carbon–carbon structures	141–125	1.53	1.42
oxygenated aromatic carbons	160–141	2.02	2.06
carbon from carbonyl type structures	195–190	0.12	0.08
degree of condensation	125–103	0.43	0.36

[a] Overestimated due to overlap with unknown contaminant.

preparations. Integration indicates totals of 0.12 and 0.08 carbonyl (−C═O) groups per aromatic ring for MWL and CEL, respectively (**Table 4**). In particular, the peak at 191 ppm, representing vanillin (**I**), is higher in the MWL (0.04 per aromatic ring) than in the CEL (0.02 per aromatic ring), whereas the integrations of the peak at 194 ppm representing coniferaldehyde (**II**) and α-carbonyl type β-O-4′ substructures (**III**) are identical in value (0.04 per aromatic ring). Additionally, the peak at 198 ppm, reported to be an $Ar-CO-CH_2-CH_2$-OH group (**IV**) (*23*), contributes 0.03 unit per aromatic ring in the MWL and 0.01 unit in CEL.

The presence of α-carbonyl type structures indicates oxidation probably occurs during the milling process. The presence of benzaldehyde structures such as vanillin further suggests that the milling process leads not only to side-chain oxidation but also to degradation (*24*). Although the content of these moieties in the lignin is relatively low, there does appear to be some structural modification occurring.

Analysis of the aromatic region of the ^{13}C NMR spectra also reveals differences between the MWL and CEL. The aromatic region can be divided into oxygenated aromatic carbons (160−141 ppm), condensed aromatic carbons (141−125 ppm), and protonated aromatic carbons (125−103 ppm). The latter two regions can provide an estimation of the degree of condensation, for example, C−C linkages in the lignin macromolecule. The condensed aromatic region (141−125 ppm) contains the C_1 side-chain position and the C_5 position from condensed aromatic ring linkages such as phenylcoumaran (β-5′), **V**, or biphenyl (5-5′), **VI**, substructures. However, calculation of the degree of condensation by integration of this region results in some ambiguity as this region is overlapped by the vinylic carbons in coniferyl alcohol (**VII**) and the β-carbon in coniferaldehyde (**I**). Therefore, it may be more precise to use the protonated aromatic region (125−103 ppm) to determine the degree of condensation.

The protonated aromatic region (125−103 ppm) consists of the methine carbons C_2, C_6, and any protonated C_5 carbons and is not expected to contain any overlapping signals. Softwood lignin consists of guaiacyl-based phenylpropanoid units that contain phenolic and methoxyl groups at C_3 and C_4, respectively. Therefore, a completely uncondensed softwood lignin will contain three protonated aromatic carbons per aromatic ring. Three minus the integration of this region (125−103 ppm) yields the degree of condensation. Integration values obtained for the MWL and CEL indicate degrees of condensation of 0.43 and 0.36, respectively, per aromatic ring (**Table 4**).

The oxygenated aliphatic region can also provide more detailed structural information about the quantities of interunit linkages in the lignin preparations. The HMQC NMR spectra are used to determine the unobstructed chemical shifts for important interunit linkages in lignin, for example, β-O-4′, β-β′, and β-5′. **Figure 3** shows the oxygenated aliphatic region (δ_C/δ_H 95−45/6.5−2.5) for MWL and CEL in the two-dimensional HMQC spectra. Qualitatively, the apparent intensity for each signal representing a lignin structural feature in this region appears to be lower in the CEL spectrum than in the MWL, consistent with the 6% higher carbohydrate content in the CEL than in the MWL.

The α-carbon of the β-5′ substructure exhibits a correlation at δ_C/δ_H 88.1/5.5 in the HMQC spectrum of acetylated MWL (**Figure 3C**). The signal in the two-dimensional spectrum is unobstructed in the carbon spectrum, allowing for integration to determine the quantity of these moieties (*25*). As a result, quantitation of the β-5′ substructures leads to the estimations of other substructures, as seen below. The ^{13}C spectra yield values for β-5′of 0.09 per aromatic ring for MWL and 0.07 for CEL (**Table 5**). These values are in agreement with literature values previously reported for MWL (*26*).

In the nonacetylated ^{13}C spectra, the β-carbons of the β-5′ and the β-β′ (**VIII**) substructures overlap at 54−52 ppm and

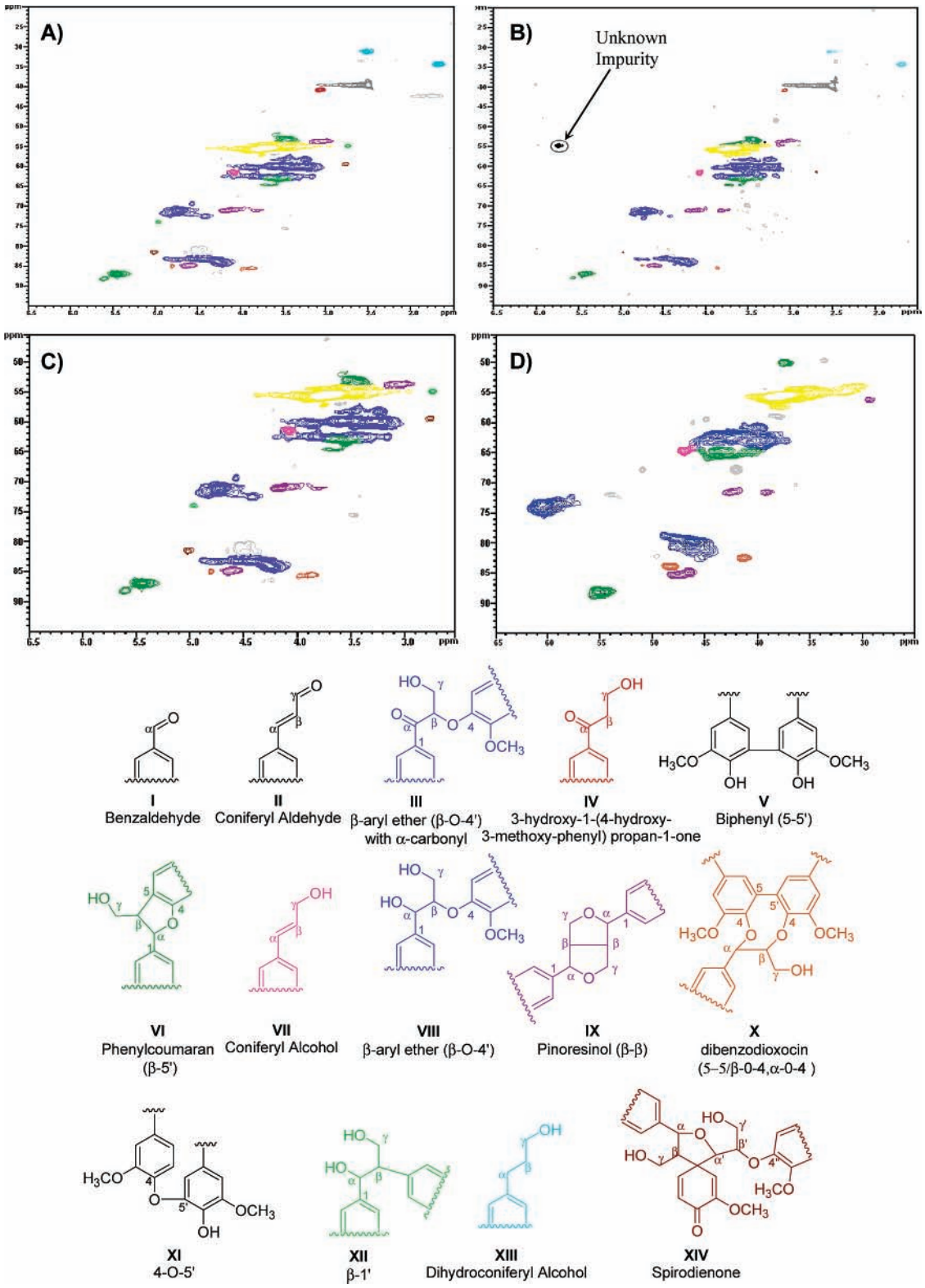

Figure 3. ^{1}H–^{13}C HMQC spectra of the oxygenated aliphatic region of MWL (**A**) and CEL (**B**). Included are the magnified regions of MWL (**C**) and acetylated MWL (**D**).

the amount of *β*-*β′* substructures per aromatic ring is 0.02 for MWL and 0.04 for CEL (**Table 5**). The value for MWL is in agreement with literature data, but the value is somewhat high for CEL (*26*, *27*).

From the acetylated HMQC spectra, the signal for the α-carbon of dibenzodioxocin (**IX**) at δ_C/δ_H 84.5/4.84 is overlapped by only the α-carbon in the *β*-*β′* substructure in the acetylated ^{13}C spectrum (*28*). Therefore, integration of the region from 86 to 83 ppm yields values of 0.08 dibenzodioxocin substructure per aromatic ring in MWL but only 0.07 in CEL (*25*). The values for dibenzodioxocin are slightly higher than those previously reported in the literature (*29*) (**Table 5**).

From the HMQC spectra, the *γ*-carbon for the *β*-O-4′ (**X**) side chain is centered at δ_C/δ_H 59.8/3.0−3.85, is relatively unobstructed, and can be integrated from ∼61.5−57.5 in the ^{13}C spectra. This region is overlapped by the *γ*-carbon from

Table 5. Estimation of Interunit Linkages in MWL and CEL via Quantitative ^{13}C NMR

spectral region	chemical shift range (ppm)	no. of moieties per aromatic ring	
		MWL	CEL
Ar–CHO (**I**)	191	0.04	0.02
Ar–CH=CH–CHO (**II**) + α-C=O in β-O-4 (**III**)	194	0.04	0.04
Ar–CO–CH_2–CH_2OH (**IV**)	198	0.03	0.01
β-5′ (**V**)	Ac(90–86)[a]	0.09	0.07
5-5′ (**VI**)	145–141[b]	0.16	0.12
Ar–CH=CH–CH_2OH (**VII**)	62	0.04	0.05
β-O-4′ (**VIII**)	61.5–57.5[c]	0.41	0.47
β-β′ (**IX**)	54.3–52.0[d]	0.02	0.04
dibenzodioxocin (**X**)	Ac(86–83)[e]	0.08	0.07
β-1′ (**XII**)	Ac(51–48)[f]	<0.01	0.01
Ar–CH_2–CH_2–CH_2OH (**XIII**)	31	0.03	0.02
spirodienone (**XIV**)	181	0.01	0.01

[a] β-5′ (**V**) was directly integrated from 90 to 86 ppm in the acetylated ^{13}C spectrum (inset of **Figure 2**). [b] 5-5′ was determined by integration of 145–141 ppm of the nonacetylated spectrum minus the value of **V**. [c] β-O-4′ (**VIII**) calculated by region of 61.5–57.5 in the nonacetylated spectrum minus the values for **III**, **IV**, **X**, **XII**, and **XIII**. [d] β-β′ (**IX**) calculated by integration of 54.3–52 in the nonacetylated spectrum minus the value of **V**. [e] Dibenzodioxocin was calculated by integration of 86–83 ppm in the acetylated ^{13}C spectrum minus the value of **IX**. [f] β-1′ (**XII**) was calculated by integration of 51–48 ppm of the acetylated ^{13}C spectrum minus the value of **V**.

the dibenzodioxocin substructure, which contains a β-O-4′ linkage, as well as the γ-carbons from substructures **III**, **IV**, **XII**, and **XIII**. Therefore, the amount of β-O-4′ linkages is 0.41 for MWL and 0.47 CEL, both within agreement of literature values (*7*, *26*, *30*). The difference in β-O-4′ content between the two lignin preparations is consistent with previously reported data on the amount of free phenolic content versus etherified β-O-4′ content as obtained by the modified DFRC method (*12*). It can be surmised that CEL is released by degradation of the associated carbohydrate material rather than the degradation of ether bonds within the lignin structure.

The spectral region of 145.5−141 ppm represents etherified and nonetherified C_4 carbons of biphenyl (5-5′) groups and C_3 carbons of phenylcoumaran (β-5′) substructures. Integration of this region results in values of 0.40 and 0.30, respectively, for MWL and CEL. The degree of condensation as determined by integration of the aromatic methine region (125−103 ppm) was previously determined to be 0.43 and 0.36 for MWL and CEL, respectively (**Table 4**). On the basis of these values, either the total amounts of 5-5′ and β-5′ subunits are either slightly underestimated or other structural moieties such as 4-O-5′ (**XI**) are contributing to condensation in the lignin polymer. From the above integrations, MWL and CEL contain 0.16 and 0.12 5-5′ substructure per aromatic ring, respectively. These values agree with those reported in the literature (*27*, *31*, *32*). The apparent increase in condensed linkages in the MWL versus CEL can be attributed to either condensation reactions occurring during milling or morphological origin. Widespread condensation probably does not occur due to the rigidity of the solid lignin matrix; however, condensation may still occur in structures of close proximity. It is likely that at least some of the difference in condensed lignin content between the MWL and CEL results from morphological origin. It has been reported that the initial release of lignin during milling occurs primarily from regions of high lignin content, presumably the cell corners and middle lamella (*6*). These regions are known to contain a higher degree of condensation than the lignin contained in the secondary wall. Because CEL is produced by degradation of carbohydrates, it should be derived primarily from the secondary wall.

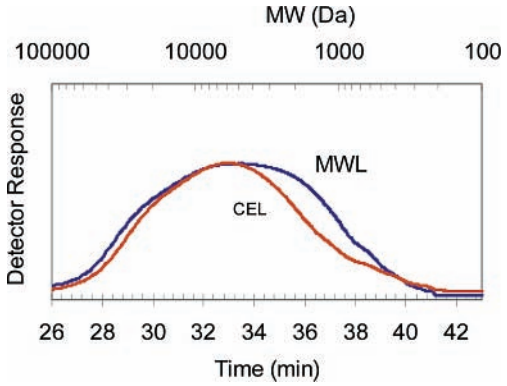

Figure 4. GPC chromatograms of MWL and CEL.

Table 6. Monosaccharide Composition of MWL and CEL

	carbohydrate composition (% monosaccharide composition)				
	xylose	arabinose	glucose	mannose	galactose
MWL	18.8	7.2	41.5	18.8	13.1
CEL	18.5	6.5	42.5	22.6	6.5

Correlations for other minor structural units identified by HMQC include coniferyl alcohol, β-1 (**XII**), dihydroconiferyl alcohol (**XIII**), and spirodienone (**XIV**). As discussed earlier, the γ-carbon of coniferyl alcohol exhibits a cross-peak at δ_C/δ_H 62/4.1, and integration of the peak in the ^{13}C spectra reveals maxima of 0.04 and 0.05 unit of coniferyl alcohol per aromatic ring. This value may be inflated, however, due to overlap with the peak for the γ-carbon of the β-O-4′ substructure.

The correlation for the β-carbon on the side chain of the β-1′ substructure exhibits a cross-peak at δ_C/δ_H 49.9/3.4 in the acetylated HMQC spectra. In the ^{13}C spectra, the peak at 50 ppm is overlapped by that of the β-carbon in the phenylcoumaran (β-5′) substructure. Integration of this region and subsequent subtraction of the contribution from the β-5′ carbon yields values of <0.01 β-1 moiety per aromatic ring in MWL and as much as 0.02 in CEL.

The α- and β-carbons of dihydroconiferyl alcohol have chemical shifts of 35 and 31 ppm. Although there is a slight overlap of the α-carbon, the β-carbon can be integrated to yield a value indicating that MWL contains a maximum of 0.03 dihydroconiferyl alcohol moiety per aromatic ring, whereas CEL contains a maximum of 0.02.

Spirodienone is a newly discovered structural unit in lignin and has a characteristic chemical shift of 181 ppm in the ^{13}C spectrum representing the β-carbon (*33*). Integration indicates that these structures are present at values of 0.01 and <0.01 in MWL and CEL, respectively.

Molecular Weight Distributions. The gel permeation chromatograms of the acetylated MWL and CEL preparations are shown in **Figure 4**. Comparison of the two lignin preparations reveals similar molecular weight distributions, with CEL having a slightly higher M_p (molecular weight at maximum peak height). This is expected on the basis of β-O-4′ content; however, similar molecular weight ranges are expected because the isolation of these portions is more dependent upon the solvent system than the material being extracted.

Carbohydrate Analysis. Monosaccharide compositions of MWL and CEL are listed in **Table 6**. The MWL and CEL have similar amounts of D-xylose, D-glucose, and L-arabinose, but differ in the amounts of D-galactose and D-mannose. The MWL

has a substantially higher amount of D-galactose than CEL, whereas the CEL is richer in D-mannose. This indicates that the CEL contains a higher percentage of glucomannan, which is found primarily in the secondary walls. In addition, it has been reported that polysaccharides rich in L-arabinose and D-galactose are deposited in the middle lamella during the early stages of biosynthesis (*34*). Although the majority of the lignin in CEL and MWL originates from the secondary wall, this may indicate that there is more lignin derived from the middle lamella in MWL than in CEL. Finally, the abundance of glucose, ~42% of the monosaccharides determined, indicates that some cellulose remains in both preparations.

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