JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Comparative Evaluation of Three Lignin Isolation Protocols for Various Wood Species

ANDERSON GUERRA, ILARI FILPPONEN, LUCIAN A. LUCIA, AND DIMITRIS S. ARGYROPOULOS*

Organic Chemistry of Wood Components Laboratory, Department of Forest Biomaterials Science & Engineering, North Carolina State University, Raleigh, North Carolina 27695-8005

Milled wood lignin (MWL), cellulolytic enzyme lignin (CEL), and enzymatic mild acidolysis lignin (EMAL) were isolated from different wood species and characterized by various techniques. The EMAL protocol offered gravimetric lignin yields 2−5 times greater than those of the corresponding MWL and CEL. The purities of the EMALs were 3.75−10.6% higher than those of their corresponding CELs, depending upon the wood species from which they were isolated. Molecular weight analyses showed that the EMAL protocol isolates lignin fractions that are not accessed by the other procedures evaluated, while ^{31}P NMR spectroscopy revealed that MWL is more condensed and bears more phenolic hydroxyl groups than EMAL and CEL. The yields and purities of EMAL, MWL, and CEL from hardwood were greater than those obtained for the examined softwoods. Structural details obtained by DFRC (derivatization followed by reductive cleavage)/^{31}P NMR revealed different contents of condensed and uncondensed β-*O*-aryl ether structures, dibenzodioxocins, and condensed and uncondensed phenolic hydroxyl and carboxylic acid groups within lignins isolated from different wood species.

KEYWORDS: EMAL; MWL; CEL; DFRC; ^{31}P NMR; ball milling; lignin; southern pine; Douglas fir; white fir; redwood; *Eucalyptus globulus*; compression wood

INTRODUCTION

Lignin is a complex natural polymer resulting from oxidative coupling primarily of (4-hydroxyphenyl)propanoids (*1*). The currently accepted theory is that the lignin polymer is formed by combinatorial-like phenolic coupling reactions, via a radical generated by peroxidase−H_2O_2, where monolignols react endwise with the growing polymer (*2*). Such "random" dehydrogenative reactions produce a heterogeneous and highly cross-linked macromolecule, built up of different interunit linkages such as β-*O*-4, $\beta-\beta$, $\beta-5$, $\beta-1$, 5−5, 4-*O*-5, etc. (*1*). Furthermore, lignin is covalently linked to carbohydrates (*3*, *4*), forming a lignin−carbohydrate network made up of benzyl−ether (*3*, *5*), benzyl−ester (*3*, *6*, *7*), and phenyl−glycoside (*8*−*10*) bonds.

Although lignin has been studied for more than 100 years, its structural details continue to emerge (*11*). One of the most important problems in elucidating the lignin structure has been the isolation of the total lignin from wood in a chemically unaltered form (*11*−*14*). Early lignin preparation techniques used strong mineral acids to reach high lignin yields (*15*). Such drastic conditions, however, were found to cause irreversible reactions that severely alter the structure of the isolated material. Currently, the most used techniques aimed at isolating lignin from wood in a chemically unaltered form are based on the extraction of ball-milled wood by neutral solvents (*12*, *16*). While milled wood lignin (MWL) is extracted from finely milled wood without any previous treatment (*16*, *17*), cellulolytic enzyme lignin (CEL) utilizes cellulolytic enzymes to remove most of the carbohydrate fractions prior to aqueous dioxane extraction of ball-milled wood meal (*12*, *14*). Recent comparison of the chemical structures of MWL and CEL using wet chemistry and modern NMR spectroscopy has revealed that MWL is slightly more condensed than CEL, suggesting that MWL may contain a higher proportion of lignin from the middle lamellae (*14*, *18*). Albeit being extensively used to isolate lignin from different sources, such lignin preparations offer moderate yields, which depend on the severity of the wood pulverization. In general, the more severe the milling conditions, the higher the yields achieved by such isolation processes. However, a steady decrease in β-*O*-4 linkages with increasing ball-milling intensity has been observed (*19*−*21*), showing that substantial lignin depolymerization *via* the cleavage of uncondensed β-aryl ether linkages may take place under severe mechanical action (*21*). In this light, intensive milling protocols offered by vibratory or orbital milling devices should be considered with caution since they provide higher lignin yields within relatively short milling intervals at the expense of the integrity of the lignin macromolecule and associated condensation and oxidation reactions (*19*−*21*).

Recent progress toward isolating lignin from wood has shown that a novel procedure using the combination of enzymatic and

* To whom correspondence should be addressed. Phone: (919) 515-7708. Fax: (919) 515-6302. E-mail: dsargyro@ncsu.edu.

10.1021/jf062433c CCC: $33.50
Published on Web 12/20/2006

mild acidolysis (EMAL, enzymatic mild acidolysis lignin) isolates lignin that may be more representative of the total lignin present in milled wood (*11*, *21*). Because a mild acid hydrolysis can liberate lignin from lignin−carbohydrate complexes, known to preclude lignin isolation in high yields, it can be combined with low severity of milling, facilitating the isolation of less modified lignin in high yields from milled wood (*21*). We have recently shown that the yield of EMAL from Norway spruce is about 4 times greater than that of the corresponding MWL and about 2 times greater compared to that of CEL isolated from the same batch of milled wood (*21*). Comparison of the chemical structures of EMAL, MWL, and CEL revealed only subtle differences, showing that EMAL is released by cleaving lignin−carbohydrate bonds rather than other linkages within the lignin macromolecule. Molecular weight distribution analyses also pointed out that the EMAL protocol allows the isolation of lignin fractions from spruce that are not accessed by any other lignin isolation procedures (*21*). In this study we have further explored the extent to which EMAL may be more representative of the total lignin in wood than CEL and MWL. To do this, EMAL, MWL, and CEL were isolated from four different species of softwoods and one hardwood and characterized by wet chemistry, quantitative ^{31}P NMR spectroscopy, and derivatization followed by reductive cleavage (DFRC) coupled with ^{31}P NMR. Moreover, we used the virtues of such lignin isolation protocols to better understand how the wood species affects the lignin yield, purity, structure, and molecular weight distribution when isolated with the same method.

MATERIALS AND METHODS

Isolation of EMALs, MWLs, and CELs. EMAL, CEL, and MWL were isolated form Douglas fir (*Pseudotsuga menziessi*), white fir (*Abies concolor*), redwood (*Sequoia sempervirens*), eucalyptus (*Eucalyptus globulus*), and normal and compression wood from southern pine (*Pinus palustris*). The wood chips from each different wood species were ground to pass a 20-mesh screen in a Wiley mill and Soxhlet extracted with acetone for 48 h. The resulting Wiley-milled wood powder was air-dried and stored in a desiccator under vacuum. The *E. globulus* wood powder was submitted to an alkaline extraction with (0.075 mol/L) NaOH for 1 h (liquid-to-wood ratio 50:1) to remove tannins before use (*22*). Rotary ball milling was performed in a 5.5 L porcelain jar in the presence of 474 porcelain balls (9.4 mm diameter), which occupied 18% of the active jar volume. A 100 g portion of extractive-free wood powder was loaded into the jar, creating a porcelain ball/wood weight ratio of 16.6. The milling process was conducted at room temperature for up to 28 days with a rotation speed of 60 rpm (*21*). EMALs were isolated from ball-milled wood as previously reported (*21*). MWL was isolated from the extractive-free wood according to the method described by Björkman (*16*, *17*). CEL was isolated from the insoluble material obtained after isolation of MWL according to the method of Chang et al. (*12*) modified by Ikeda et al. (*14*) and Holtman et al. (*18*). Both preparations were purified as described elsewhere (*16*).

Determination of Lignin Purity. The purities of EMAL and CEL were calculated by summing the acid-insoluble (Klason lignin) and acid-soluble lignin contents, measured according to the method reported by Yeh et al. (*23*).

Acetobromination Derivatization Procedure. Acetobromination was used as the derivatization method of choice for all samples prior to size exclusion measurements (*21*). Approximately 2.5 mL of a mixture composed of 8 parts of acetyl bromide and 92 parts (v/v) of glacial acetic acid was added to about 10 mg of a lignin sample (EMAL, MWL, or CEL) in a 15 mL round-bottom flask. The flask was sealed and placed in a water bath set at 50 °C for 2 h with continuous magnetic stirring. The solvent was rapidly evaporated at 25−28 °C in a rotary evaporator connected to a high-vacuum pump and a cold trap. The residue was immediately dissolved in THF (5 mL) and subjected to size exclusion analyses.

Size Exclusion Chromatography (SEC). SEC of EMAL, MWL, and CEL samples was performed on a size exclusion chromatographic system (Waters system) equipped with a UV detector set at 280 nm. The analyses were carried out at 40 °C using THF as the eluent at a flow rate of 0.44 mL/min. A 120 μL volume of the sample dissolved in THF (2 mg/mL) was injected into HR5E and HR 1 columns (Waters) connected in series. The HR5E column specifications allow for molecular weights up to 4×10^6 g/mol to be reliably detected. The SEC system was calibrated with polystyrene standards in the molecular weight range of $(890-1.86) \times 10^6$ g/mol, and Millenium 32 GPC software (Waters) was used for data processing.

Quantitative ^{31}P Nuclear Magnetic Resonance. Quantitative ^{31}P NMR spectra of all lignin preparations were obtained using published procedures (*21*, *24*, *25*). To improve resolution, a delay time of 5 s was used and a total of 256 scans were acquired.

DFRC/^{31}P NMR. DFRC was performed as described by Lu and Ralph (*26*). The precise amounts of the lignin and precautions due to the ensuing ^{31}P NMR steps were nearly identical to those reported elsewhere (*21*).

RESULTS AND DISCUSSION

Our continuing efforts to better understand the lignin isolation process from wood have prompted us to examine various salient features of lignin isolation variables. In this study, therefore, we evaluated the yield, purity, molecular weight, and structure of lignin samples isolated from different wood species using different isolation protocols. To ensure that the effects of wood pulverization on the lignin structure would not lead to a misinterpretation of our data, MWL, CEL, and EMAL were isolated from the same batch of ball-milled wood (rotary ball milling). Douglas fir, white fir, and redwood were used in this work to evaluate the aforementioned effects on lignin samples from different species of softwood, while eucalyptus was chosen as a source of lignin from hardwood. To better understand the structural differences between lignin from regular and reaction wood, the three lignin preparations were also isolated from normal and compression wood of southern pine.

Lignin Yield and Purity. The yields of MWL, CEL, and EMAL isolated from the different wood species are shown in **Figure 1**. As previously observed, the yields of EMAL were found to be greater than those of the corresponding MWL and CEL, regardless of the wood species from which they were isolated. The yields of MWL (w/w, based on the amount of Klason lignin of the starting wood and the isolated lignin) varied from 1.34% to 34%, with eucalyptus giving a higher yield as compared to the yields of MWL from softwoods. Overall, the yields of MWL were found to be lower than 16% for all evaluated softwoods. Such low yields are not totally surprising, however, when viewed in light of the recent conclusions of Hu et al. (*20*) and Fujimoto et al. (*19*), where the extent of extractable MWL was shown to be dependent upon milling severity. To isolate MWL in higher yields, more extensive milling is required (*14*). Intensive milling protocols offered by vibratory- or orbital-milling devices should be, however, considered with caution since they provide higher lignin yields within relatively short milling intervals at the expense of the integrity of the lignin macromolecule and associated condensation and oxidation reactions (*14*, *19*−*21*).

To improve yields while minimizing the extent of mechanical action, the insoluble material from aqueous dioxane extraction of MWL can be treated with cellulolytic enzymes (*14*, *18*). This enzymatic treatment removes the majority of the carbohydrates, and a subsequent aqueous dioxane extraction solubilizes another lignin portion (CEL) that is considered to be lignin associated with carbohydrates (*12*, *18*). In this way, Ikeda et al. (*14*) have suggested the combination of these two preparations (MWL and

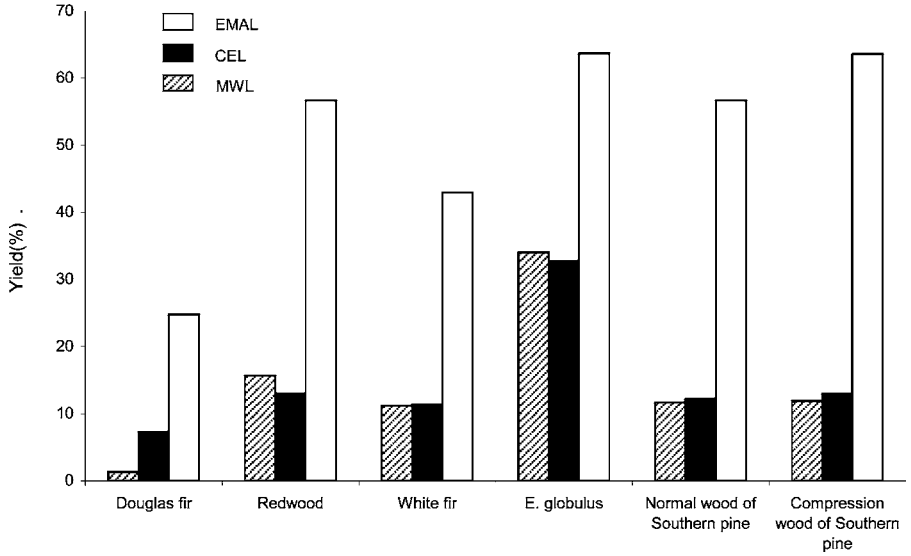

Figure 1. Gravimetric yields of EMAL (open bars), CEL (black filled bars), and MWL (dashed bars) isolated from the same batch of milled Douglas fir, redwood, white fir, *E. globulus*, and normal and compression wood of southern pine.

CEL) to increase the overall yield of pure lignin. **Figure 1** shows that such enzymatic hydrolysis permits isolating more lignin from wood. The yields of CEL were found to be similar to those of the respective MWL, except for Douglas fir, where the yield of CEL was 7 times greater than that of MWL. Albeit being extracted from the residual wood meal using the same solvent system as MWL, our data are not supportive of the combination of MWL and CEL to further increase the yields of isolated lignin as described by Ikeda et al. (*14*). Moreover, a recent solution-state nuclear magnetic resonance study has also revealed that some structural differences exist between these two lignin preparations (*18*).

We have recently shown that the combination of enzymatic and mild acidolysis permits isolating lignin that may be more representative of the total lignin present in milled wood (*11*, *21*). Because a mild acid hydrolysis can liberate lignin from lignin−carbohydrate complexes, known to preclude lignin isolation in high yields, it offers the possibility of isolating less modified lignin in high yields from milled wood (*11*, *21*). The data of **Figure 1** show that the concerted effect of enzymatic and mild acid hydrolysis offered significant yield improvements over the traditional procedures for isolating lignin. It is noteworthy that the amount of lignin isolated by using the EMAL protocol was higher for all different species of softwoods analyzed so far as well as for hardwood and even for isolating lignin from compression wood. Ongoing work in our laboratory has indicated that the yield of EMAL is also higher for wheat straw (data not shown). It is also significant that the liberation of lignin from lignin−carbohydrate complexes provided by the mild acid hydrolysis step offers the possibility of obtaining high yields without applying more severe mechanical action onto the material. The data of **Figure 1** show that the yields of EMAL were from 1.9 to 5.3 times greater than those of the corresponding MWL and CEL isolated from the same batch of milled wood. A closer inspection of such data points out that for all softwood species evaluated so far the yields of EMAL were greater than the combined yields of MWL and CEL. This finding indicates that EMAL includes not only the lignin fraction normally isolated as MWL and CEL, but also macromolecules that are not accessed by any other available lignin isolation protocol.

Other virtues of EMAL appear during the comparison of its isolation procedure to the MWL and CEL protocols. Both procedures require a two-step purification stage that is extremely time-consuming. Such purification steps are not needed in the EMAL procedure since most of the non-lignin contaminants (carbohydrates) may readily be removed by the mild acidolysis stage (*11*). Moreover, the aqueous dioxane extraction step used to isolate MWL and CEL (two times, each 24 h) is much longer than the 2 h of mild acidolysis required to isolate EMAL. Even though faster and simpler than the MWL and CEL protocols, the EMAL isolation procedure must be carried out carefully, since slightly higher concentrations of HCl may seriously compromise the structure of the isolated lignin (*11*).

Another aspect of our data shows that different wood species offer different yields when isolated with the same isolation procedure; i.e., the yields of EMAL, MWL, and CEL from *E. globulus* were found to be greater than those obtained for the examined softwoods (**Figure 1**). Moreover, a comparison of the lignin yields from southern pine, redwood, Douglas fir, and white fir shows that different species of softwood display different behaviors when submitted to the same isolation procedure. More specifically, the yields of isolated lignin from Douglas and white fir were found to be somewhat lower than those obtained from redwood and pine. The data of **Figure 1** show that even different species of the same genus (Douglas and white fir) offer different yields when submitted to the same isolation procedure. The yields of MWL and CEL from normal and compression wood of pine, however, were found to be quite similar, while the yield of EMAL from compression wood was 6.9% higher than the corresponding yield from normal wood.

The abnormal low yields of lignin isolated from Douglas fir, however, demand further attention. Such low yields might be related to the high amounts of polysaccharides present in this species of softwood. Willför et al. (*27*) have analyzed the content and composition of carbohydrates comprising polysaccharides in 12 different species of softwoods and found that Douglas fir contained the largest contents of galactoglucomannans, mannans for short, and cellulose. Besides the enumerated limitations of MWL, such a significant amount of mannans may require an enzymatic solution with high mannanase activity during the isolation of CEL and EMAL. As such future efforts with such species may take into account this suggestion. Furthermore, limitations imposed to the milling process for this wood species due to the formation of wood aggregates arising from its high resin content cannot be ruled out.

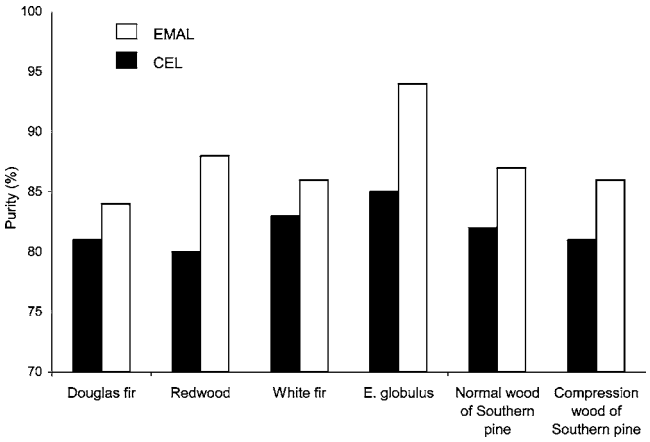

Figure 2. Purities of EMAL (open bars) and CEL (black filled bars) isolated from the same batch of milled Douglas fir, redwood, white fir, *E. globulus*, and normal and compression wood of southern pine.

Lignin samples isolated from wood still contain associated carbohydrates and other non-lignin contaminants, regardless of the isolation and purification procedures applied (*11*, *12*, *14*). Lignin−carbohydrate linkages exist in wood and are known to be of benzyl−ester, benzyl−ether, and phenyl−glucoside types (*1*). Such interactions between lignin and carbohydrates preclude the isolation of lignin in high yields and purities; i.e., the purities of CEL isolated from the different wood species evaluated in this study and purified according to Björkman (*16*) varied from 80% to 85% (**Figure 2**). All of these lignin−carbohydrate bonds are, however, susceptible to acid hydrolysis. In the conditions applied to isolate EMAL, the complete cleavage of the phenyl−glucoside bonds has been shown by model compound studies to be accomplished, whereas the non-phenolic benzyl−ether ones were found to be more stable under such conditions (*28*, *29*). The benefits of the mild acid hydrolysis of lignin−carbohydrate linkages were, however, apparent as far as the purities of the EMALs are concerned. From the data shown in **Figure 2** it is obvious that all EMALs evaluated so far have purities from 3.75% to 10.6% higher than those of their corresponding CELs, depending upon the wood species they are isolated from. The purities of MWL samples were not determined in this study. Nevertheless, previous work has pointed out that the purities of EMALs from both poplar and spruce are higher than those of the corresponding MWLs (*11*). The purities reported in **Figure 2** are based on the sum of Klason and UV-soluble lignin contents, which represents the total amount of lignin contained in CEL and EMAL after removal of non-lignin contaminants through a severe hydrolysis with 72% (w/w) H_2SO_4.

The EMALs isolated from the different wood species were found to have different purities (**Figure 2**). More specifically, the EMAL isolated from *E. globulus* appears to be less contaminated by non-lignin materials than the EMALs from softwoods, while the EMAL from Douglas fir was found to have the highest content of such contaminants. Albeit the prevailing consensus that lignin is cross-linked to different polysaccharides in the cell wall and that such cross-linking might be one of the reasons for the low MWL yields, the exact frequency of lignin−carbohydrate bonds in different wood species is still a matter of discussion (*1*, *3*, *4*, *6*−*9*, *21*, *28*, *29*). Lawoko et al. (*28*) have recently reported, for example, that lignin without covalent bonds to carbohydrates does not exist in spruce wood. The differences in the yields and purities of EMALs reported in **Figures 1** and **2**, respectively, are supportive of the existence of a different microstructure and the presence of a variety and variable abundances of lignin−carbohydrate bonds among the different wood species.

Molecular Weight Distribution. It was recently reported that acetobromination represents a facile and rapid alternative to the complete solubilization of sparingly soluble lignin samples, while still allowing for an accurate analysis (*21*). By dissolving a lignin sample in neat acetyl bromide diluted with glacial acetic acid (8:92, v/v), the primary alcoholic and the phenolic hydroxyl groups are acetylated, while the benzylic α-hydroxyls are displaced by bromide (*26*). Similarly, benzyl aryl ethers are quantitatively cleaved to yield aryl acetates and acetylated α-bromo products (*26*). The concerted effect of acetylation when coupled with the polarity induced by the selective α-bromination caused every lignin sample examined so far to become highly soluble in THF, allowing rapid SEC analyses. Comparison between acetobromination and acetylation with acetic anhydride/pyridine has shown minor differences in the UV responses and elution profiles, which is supportive of the viability of using acetobromination as a derivatization technique to sparingly soluble lignin (*21*).

The molecular weight distributions of the acetobrominated MWL, CEL, and EMAL were therefore compared by SEC using THF as the mobile phase and UV detection at 280 nm. **Figure 3A** shows a typical set of SEC chromatograms of such lignin preparations, where a distinctly higher molecular weight distribution was found for EMAL than for CEL and MWL. The chromatograms reveal that EMAL is richer in high molecular weight fragments (higher than 100×10^3 g/mol), which appeared in lower abundance in CEL and were completely absent in MWL. This result is consistent with our recent work in which we observed that the absence of the high molecular weight fractions made the apparent weight-average molecular weights of both MWL and CEL isolated from spruce significantly lower than that of the corresponding EMAL (*21*). These data are also supportive of a previous finding stating that the concerted effect of cellulolytic action and mild acidolysis allows for the isolation of lignin fragments that are not accessible by either of the alternative lignin isolation procedures (*21*). A question that emerges at present is whether such material causing the formation of the aforementioned high molecular weight fractions consists of covalently bound lignin or lignin−lignin association. Ongoing work in our laboratory is being carried out in an endeavor to address this very important fundamental issue.

For the purposes of the present investigation, lignin association phenomena were not taken into account to calculate the apparent molecular weight averages reported in **Table 1**. However, to ensure that such association phenomena would not lead to a misinterpretation of the molecular weight distributions, all analyses were carried out on freshly prepared lignin solutions analyzed immediately after derivatization, ensuring that the comparisons made from sample to sample were valid.

The data of **Figure 3B** and **Table 1** point out that EMALs isolated from different wood species display different elution profiles and, consequently, different apparent molecular weight averages (M_w and M_n) when the wood is pulverized under the same milling conditions and the lignin extracted by using the same enzymatic and mild acidolysis sequence. As anticipated, a highly polydisperse behavior is apparent in the SEC chromatograms of the EMAL samples as far as their molecular weight distributions are concerned (**Figure 3B,C**). The elution profiles, however, were found to be different among the EMALs isolated from different wood species. Specifically, while the chromatograms of EMAL isolated from southern pine (**Figure**

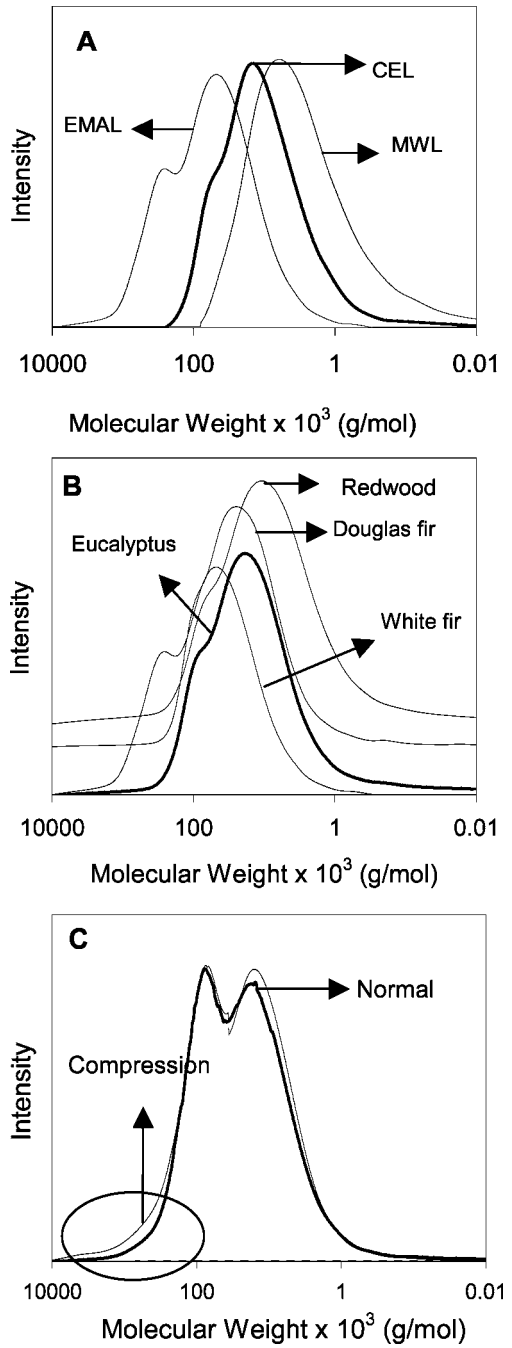

Figure 3. Typical SEC chromatograms of lignin samples isolated from the same batch of milled white fir (**A**). SEC chromatograms of EMAL isolated from milled Douglas fir, redwood, white fir, and *E. globulus* (**B**). SEC chromatograms of EMAL isolated from normal and compression wood of southern pine.

3C) and white fir (**Figure 3B**) displayed a bimodal behavior, the chromatograms of EMAL from Douglas fir, redwood, and *E. globulus* showed only a small shoulder extending over 100 $\times$ 10^3 g/mol. Moreover, a high molecular weigh fraction (albeit of low abundance), extending over 500 $\times$ 10^3 g/mol, was apparent in the chromatograms of EMAL from pine and white fir. Such a fraction, however, was absent in the lignins from the other evaluated wood species.

The bimodality and presence of the aforementioned high molecular weight fraction made the apparent weight-average molecular weight of EMAL from pine greater than those from the other evaluated wood species (**Table 1**). Such an M_w was found to be over 57 $\times$ 10^3 g/mol, while the M_w of EMAL

Table 1. Weight-Average Molecular Weight (M_w), Number-Average Molecular Weight (M_n), and Polydispersity (D) of EMAL, MWL, and CEL Isolated from Different Wood Species

lignin[a]	M_w (g/mol)	M_n (g/mol)	D
	Douglas Fir		
EMAL	38000	7600	5.0
MWL	7400	2500	3.0
CEL	21800	5500	4.0
	White Fir		
EMAL	52000	6300	8.2
MWL	8300	2800	3.0
CEL	21700	4700	4.6
	Redwood		
EMAL	30100	4700	6.4
MWL	5900	2400	2.5
CEL	23000	5400	4.2
	E. globulus		
EMAL	32000	8700	3.7
MWL	6700	2600	2.6
CEL	17200	5500	3.1
	Normal Wood of Southern Pine		
EMAL	57600	9700	5.9
MWL	14900	4700	3.2
CEL	29600	7500	3.9
	Compression Wood of Southern Pine		
EMAL	63500	9300	6.8
MWL	16100	5200	3.1
CEL	27500	7200	3.8

[a] Isolated after 28 days of ball milling. EMAL, CEL, and MWL were isolated from the same batch of milled wood.

isolated from white fir, for which the chromatogram also displayed the high molecular weight fractions but in low abundance, was 52 $\times$ 10^3 g/mol. The molecular weights of EMALs isolated from Douglas fir, redwood, and *E. globulus*, in which the chromatograms did not display bimodality, were 38 $\times$ 10^3, 30 $\times$ 10^3, and 32 $\times$ 10^3 g/mol, respectively. It must be emphasized, however, that the possibility of such differences in the apparent weight-average molecular weight being due to lignin association cannot be ruled out. In this scenario, nevertheless, one should note that lignin from different wood species can display different propensities to associate.

The size exclusion chromatograms of the acetobrominated EMAL isolated from normal and compression wood of southern pine are shown in **Figure 3C**. Comparison of such lignin reveals similar molecular weight distributions, with EMAL from compression wood displaying slightly higher amounts of high molecular weight fragments, which make the M_w of lignin from compression wood slightly greater than that of the corresponding lignin from normal wood (**Table 1**).

Determination of Units Bearing Free Phenolic Hydroxyl Groups. ^{31}P NMR spectroscopy is a reliable method to determine the amounts of various hydroxyl groups within the lignin macromolecule (*24*, *25*). Such hydroxyl groups are revealed and quantified after phosphitylation of lignin with 2-chloro-1,3,2-dioxaphospholane or 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (*24*, *25*). The β-aryl ether content (**Figure 4**) was determined after phosphitylation of the Cα hydroxyl groups in these moieties with 2-chloro-1,3,2-dioxaphospholane (*25*, *30*). The condensed and uncondensed phenolic hydroxyls as well as the carboxylic acids (**Figure 5A**−**C**) were determined by phosphitylation of the lignins with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (*24*). Quantification was then carried out via peak integration using *N*-hydroxynaphthalimide as an internal standard. Details of

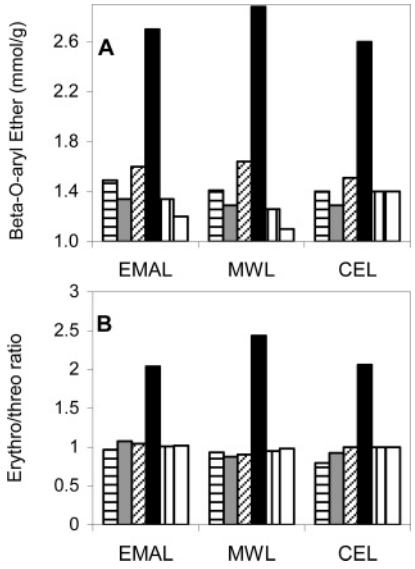

Figure 4. β-*O*-Aryl ether functional group content (**A**) and *erythro*/*threo* ratio (**B**) of EMALs, MWLs, and CELs isolated from the same batch of milled white fir (horizontal dashed bars), redwood (gray filled bars), Douglas fir (diagonal dashed bars), *E. globulus* (black filled bars), and normal (vertical dashed bars) and compression (open bars) wood of southern pine.

signal acquisition, assignment, and integration can be found elsewhere (*24*, *25*).

The data of **Figures 4** and **5** show that some differences exist in the functional group content of EMAL, MWL, and CEL. Such differences, however, were found to be dependent upon the wood species from which the lignin was isolated. As shown in **Figure 4A**, MWL isolated from *E. globulus* has slightly higher amounts of β-*O*-aryl ether linkages than its respective EMAL and CEL. Previously, we have also found that MWL isolated from spruce has higher contents of such linkages than CEL and EMAL (*21*). However, comparison among the three lignin preparations isolated from pine, Douglas fir, redwood, and white fir showed no significant difference in the total β-*O*-aryl ether contents. It is also significant to note that the nearly identical amounts of total β-*O*-aryl ether functional groups of EMAL, MWL, and CEL indicate no evidence of β-aryl ether bond cleavage within the lignin during the mild acid hydrolysis step of the EMAL protocol.

Figure 5 shows the amounts of phenolic hydroxyls as well as carboxylic acid groups determined by quantitative ^{31}P NMR. In general, MWL was found to contain higher amounts of phenolic hydroxyl groups than EMAL and CEL (**Figure 5C**). This finding is not surprising since it is well-known that phenolic-rich lignin structures are more easily and preferentially isolated in the extraction procedure utilized for MWL (*14*). This selective fractionation might explain the much lower contents of such phenolic hydroxyl groups observed for the CELs, since in this work CEL was isolated from the residue of MWL isolation. As shown in **Figure 5C** the total contents of phenolic hydroxyl within EMAL were found to be lower than within MWL, indicating that the aforementioned selective fractionation is minimized by the EMAL protocol.

As expected, MWL was found to contain higher amounts of condensed phenolic hydroxyl than CEL and EMAL (**Figure 5A**). This result is consistent with the recent work of Holtman et al. (*18*), where the degree of condensation of MWL was found to be slightly higher than that of CEL, both isolated from loblolly pine. Such a difference in the degree of condensation might result, at least in part, from different morphological origins. As recently reported by Hu et al. (*20*), lignin samples isolated from milled wood in low yields may be more contaminated by lignin from the middle lamellae (CML), which is known to be more condensed than lignin from the secondary wall (SW). The effect of such contamination, however, diminishes when more lignin from the SW is isolated.

The uncondensed phenolic hydroxyl contents of EMAL were found to be somewhat higher than those of CEL (**Figure 5B**). The possibility that the higher contents of phenolic hydroxyl come from liberation of such groups from β-*O*-aryl ether linkages can be ruled out, because there is no evidence of lignin degradation during the isolation of EMAL (**Figure 4A** and **Table 1**). The higher contents of uncondensed phenolic hydroxyl within EMAL could be rationalized, at least in part, on the basis of the hydrolysis of phenyl−glucoside bonds within lignin−carbohydrate complexes. Complete cleavage of such bonds can be accomplished under the conditions used to isolate EMAL (*28*), liberating new phenolic hydroxyl groups and increasing the purities of EMAL, as shown in **Figure 2**. The contents of carboxylic acids groups were also very similar among EMAL, MWL, and CEL (**Figure 5D**). These similarities provide further support for the effectiveness of the EMAL protocol providing nonoxidized lignin.

In summary, our data are not supportive of the protocol in which MWL and CEL are combined to further increase the yields of isolated lignin (*14*). As was observed in **Figure 5**, the condensed, uncondensed, and total phenolic hydroxyl contents are quite different between such lignin preparations. Moreover, the molecular weight distribution data in **Table 1** also show that MWL and CEL protocols afford isolated lignin fragments with different molecular weights. More significantly, however, the combination of enzymatic and mild acid hydrolysis offers the possibility to isolate lignin samples that are more representative of the total lignin in milled wood. As shown in **Figure 1**, the yields of EMAL were in most cases higher than the combined yields of CEL and MWL with no evidence of structural alteration due to the mild acidolysis involved in the EMAL protocol. This indicates that the cleavage of the lignin−carbohydrate bonds afforded during the mild acidolysis step of the EMAL protocol allows the isolation of lignin fractions that are not accessed by any other isolation procedures. Furthermore, the liberation of lignin from lignin−carbohydrate complexes offers the possibility of obtaining high yields using low-intensity milling, which is desirable to avoid lignin degradation during the wood pulverization as alluded to earlier.

In an effort to better understand how the wood species affects the lignin structure when isolated with the same method, we compared the ^{31}P NMR data obtained for the different EMALs, since such lignin preparation offers the aforementioned benefits over MWL and CEL. As expected, *E. globulus* was found to contain more β-aryl ether structures than all the softwoods evaluated so far (**Figure 4A** and **Table 2**). The value of 2780 μmol of β-aryl ether/g of lignin obtained for *E. globulus* by ^{31}P NMR corresponds to 58.7% β-aryl ether structures within such a lignin, considering that the average molecular weight of one phenylpropane unit (C9) in such a lignin is 211 g/mol, derived from the elemental composition of *E. globulus* dioxane lignin (*22*). Such a value correlates very well with the 60% β-aryl ether structures reported for hardwoods by Adler (*15*). Among softwoods, Douglas fir was found to contain slightly higher contents of such linkages (1600 μmol/g), while southern pine and redwood were observed to contain the lowest values (1340 μmol/g). Lignins in compression and normal wood were found

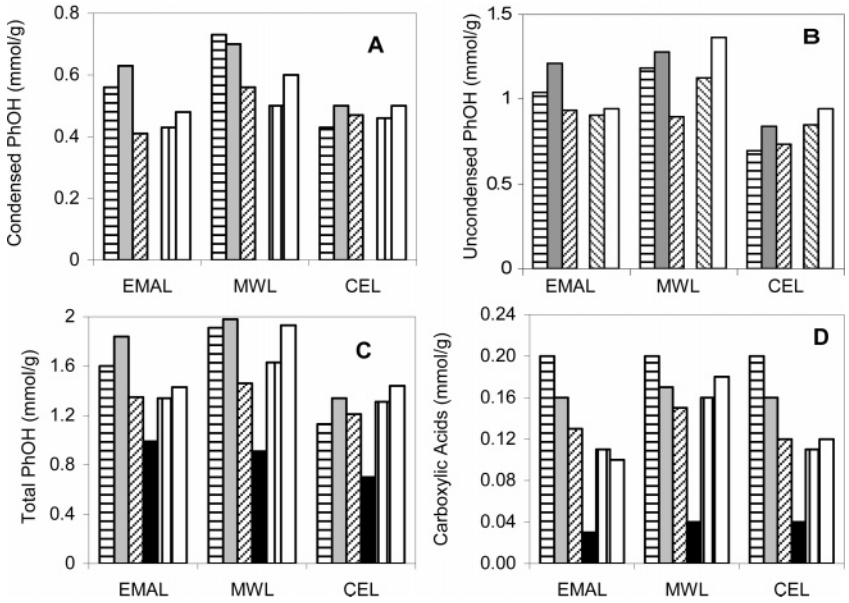

Figure 5. Condensed phenolic hydroxyl (**A**), uncondensed phenolic hydroxyl (**B**), total phenolic hydroxyl (**C**), and carboxylic acid (**D**) group contents of EMALs, MWLs, and CELs isolated from the same batch of milled white fir (horizontal dashed bars), redwood (gray filled bars), Douglas fir (diagonal dashed), *E. globulus* (black filled bars), and normal (vertical dashed) and compression (open bars) wood of southern pine. Due to overlapping, uncondensed and condensed phenolic hydroxyl units have been integrated together to give the total amount of phenolic units reported for *E. globulus*.

Table 2. Functional Group Contents, Yields, and Weight-Average Molecular Weights (M_w) for EMAL Isolated from Different Wood Species[a]

functional group[b]	Douglas fir	white fir	redwood	normal wood of southern pine	compression wood of southern pine	*E. globulus*
total β-aryl ether	1600	1490	1340	1340	1200	2780
syringyl OH	0.00	0.00	0.00	0.00	0.00	620
guaiacyl OH	840	930	1060	790	570	350
p-hydroxyl OH	100	110	160	120	380	20
uncondensed PhOH	940	1040	1220	910	950	overlapped
condensed PhOH	410	560	630	430	480	overlapped
total PhOH	1350	1600	1850	1340	1430	990
carboxylic groups	130	190	160	110	100	150
yield[c] (%)	24.8	42.9	56.7	56.3	65.1	63.7
M_w[d] (g/mol)	38000	52000	30100	57600	63500	32000

[a] Isolated after 28 days of ball milling. [b] Determined by ^{31}P NMR. Values in μmol/g. Error ±20. [c] Based on Klason lignin contents of extracted ground wood meal. [d] Determined by size exclusion chromatography.

to contain 1340 and 1200 μmol of β-*O*-aryl ether functional groups/g of lignin, respectively (**Table 2**). These results indicate a 10.5% decrease in arylglycerol−β-aryl ether linkages in compression wood of southern pine. Such a decrease corroborates recent findings where an approximate 15% decrease in arylglycerol−β-aryl ether linkages in compression wood of loblolly pine has been reported (*31*).

Both *erythro*- and *threo*-stereoisomeric forms of β-*O*-4 structures can also be determined using ^{31}P NMR after derivatization of lignin with 2-chloro-1,3,2-dioxaphospholane, by integrating the regions from 135 to 134.2 ppm and from 134 to 133.4 ppm, which have been attributed to Cα−OH in *erythro*- and *threo*-forms of β-*O*-4 structures, respectively (*25*). As expected, **Figure 4B** shows that the *erythro*/*threo* ratios were similar in all species of gymnosperms, while the predominance of the *erythro*-form was obvious in *E. globulus*. These data corroborate previous findings reported by Akiyama et al. (*32*), where the proportion and amount of *erythro*- and *threo*-forms were described as very similar in softwoods, while, in contrast, for hardwood species the *erythro*-form of β-*O*-4 structures was found to predominate, the extent being dependent upon the wood species. Such selective behavior has been rationalized on the basis of the widely accepted theory for the formation of β-*O*-4 structures in lignin (*15*). According to this theory, the first step in the formation of β-*O*-4 structures is the 4-*O*-coupling of an oligolignol phenoxy radical to a monolignol radical at its side-chain β-position to form a quinone methide intermediate. The next step is water addition to one of the two faces of the quinone methide, leading to the formation of the *erythro*- or *threo*-form. Such water addition, however, depends on the aromatic ring type, solvent, and pH (*33*). Model experimental simulations have shown that the water addition leading to the *erythro*-form is preferred when syringyl-type aromatic rings form the quinone methide (*33*).

The major differences in the hydroxyl functional groups among the EMALs from different wood species are also listed in **Table 2**. The total amount of phenolic hydroxyl groups was slightly higher (6.7%) within lignin from compression wood than normal wood of southern pine. The total H unit content (*p*-hydroxyphenyl moieties) was found to be significantly higher in compression wood, while more G unit content (guaiacyl moieties) was detected within lignin from normal wood. As anticipated, a slightly higher degree of condensation was also observed in lignin from compression wood. Such findings are consistent with the accepted theory that the biosynthesis of normal softwood lignins occurs *via* radical polymerization of

Table 3. Values of Hydroxyl Moiety Contents Determined by DFRC/^{31}P NMR (μmol/g) for EMAL Isolated from Different Wood Species[a]

	phenolic OH content					
EMAL source	S units involved only in uncondensed β-*O*-aryl bonds	G units involved only in uncondensed β-*O*-aryl bonds	H units involved only in uncondensed β-*O*-aryl bonds	G + H units involved only in condensed β-*O*-aryl bonds	dibenzodioxocins	total uncondensed β-*O*-aryl bonds
Douglas fir	not detected	880	60	510	230	940
white fir	not detected	860	40	490	240	900
redwood	not detected	810	41	470	220	851
normal pine[b]	not detected	990	25	240	170	1015
comp pine[b]	not detected	745	74	284	210	819
E. globulus	1430	260	40	overlapped[c]	overlapped[c]	1730

[a] Isolated after 28 days of ball milling. [b] EMAL from normal and compression wood of southern pine. [c] Inadequate resolution for quantification.

coniferyl alcohol, while in compression wood a significant amount of *p*-coumaryl alcohol units are also incorporated (*1*). Due to the absence of an aromatic methoxyl, the possibilities for coupling in the radical polymerization are more complex (*1*). As a result, compression wood lignin has been found to be more condensed (*31*, *34*) and with a larger number of high molecular weight fragments than lignin from normal wood (**Figure 3C**).

The data of **Figure 5A** and **Table 2** also show that *E. globulus* has a lower total phenolic hydroxyl content than all the softwood species. Specifically, while *E. globulus* was found to contain less than 1000 μmol of phenolic hydroxyl/g of lignin, such a content in lignin from softwood ranged from 1340 to 1850 μmol/g. This finding is not surprising, since the total contents of phenolic hydroxyl groups in softwoods have been reported to be somewhat higher than in hardwoods (*35*). Moreover, earlier observations of MWL have indicated that the syringyl units present in hardwood lignins are primarily of the non-phenolic type (*15*, *35*). Such a low content of free phenolic units in hardwood lignins has long been used to explain their relatively poor responses to sulfite treatments used in the preparation of chemimechanical pulps (*36*). The amount of condensed and uncondensed phenolic hydroxyls within EMAL from *E. globulus* was not calculated since the condensed and uncondensed syringyl-type phenolic hydroxyl signals overlap in the ^{31}P NMR. Due to such overlapping, both units have been integrated together to give the total amount of phenolic units reported in **Figure 5C**.

A considerable variation was found among the softwood species in the phenolic hydroxyl group content, which decreases in the order redwood (1850 μmol/g), white fir (1600 μmol/g), Douglas fir (1350 μmol/g), and pine (1340 μmol/g). Redwood and white fir seem to be more condensed than pine and Douglas fir as shown by the total amount of condensed phenolic hydroxyl groups in such lignins (**Figure 5A** and **Table 2**).

Determination of Units Bearing Etherified Phenolic Hydroxyl Groups in β-*O*-Aryl Ether Linkages. Although quantitative ^{31}P NMR has contributed significantly to our understanding of the hydroxyl-bearing functional groups, it cannot offer any information about the etherified or carbon−carbon-linked bonding pattern of lignin (*37*). To overcome this limitation, Tohmura and Argyropoulos (*37*) have recently proposed the combination of DFRC with ^{31}P NMR. In this way, when the aryl ether linkages are selectively cleaved by DFRC (*26*), the corresponding phenolic hydroxyls released can be quantified by ^{31}P NMR. Because ^{31}P NMR can distinguish condensed from uncondensed phenolic hydroxyls, the ^{31}P NMR spectra "after DFRC" offer detailed information about condensed and uncondensed units connected through β-aryl ether linkages as well as dibenzodioxocins (*21*, *37*). The total amounts of uncondensed β-*O*-aryl structures determined by DFRC/^{31}P NMR and thioacidolysis have been shown to be quite similar when both techniques are applied to the same sample of isolated lignin (*21*).

The quantification of the hydroxyl groups released from β-aryl ether structures by DFRC is given in **Table 3**. The total amount of uncondensed β-aryl ether structures within *E. globulus* was significantly higher than that within lignin from softwoods. As well-known, lignins from hardwoods have more uncondensed β-aryl ether structures than lignin from softwood (*2*). The value of 1730 μmol of uncondensed β-aryl ether structures/g of lignin obtained for *E. globulus* corresponds to 36.5% uncondensed β-aryl ether structures within lignin, considering that the average molecular weight of one phenyl propane unit (C9 unit) in such a lignin is 211 g/mol (*22*). The data of **Table 3**, coupled with the data of **Table 2**, show that 62.2% of the total amount of β-*O*-aryl ether structures in *E. globulus* are uncondensed (1730 μmol/2780 μmol), which is in good agreement with the value obtained by thioacidolysis for the same wood species (*22*). The S/G ratio obtained "after DFRC" (83/15) **(Table 3)** is somewhat different from that observed "before DFRC" (63/35) (**Table 2**), indicating that the syringyl units present in *E. globulus* are primarily of the non-phenolic type (*15*, *22*).

The data of **Table 3** show that while the total contents of dibenzodioxocins and condensed β-*O*-aryl ether units were similar among Douglas fir, white fir, and redwood, the total contents of uncondensed β-*O*-aryl ether units were found to be slightly different from species to species. Condensed β-aryl ether bonds refer to structures that are characterized by the covalent attachment of two macromolecules or oligomers that themselves are interlinked via structures other than β-aryl ethers. The total amount of uncondensed β-aryl ether structures was found to decrease in the order Douglas fir (940 μmol/g), white fir (900 μmol/g), and redwood (850 μmol/g). The total amount of uncondensed β-aryl ether linkages in such softwoods was nearly double that of condensed moieties, indicating that about two-thirds of the etherified phenolic moieties in β-aryl ether structures present in EMAL from these species are uncondensed units connected to another phenylpropane unit bearing β-*O*-4, $\beta-5$, and $\beta-\beta$ linkages. One-third of the etherified phenolic moieties in the β-aryl ether structures contained a subsistent group *ortho* to the phenolic hydroxyl, with the majority being dibenzodioxocins. These findings are similar to those reported by Tohmura and Argyropoulos for MWL lignin from black spruce (*37*).

The EMAL from normal wood of southern pine, however, was found to be quite different from that of the other softwoods evaluated so far. The total amounts of condensed β-aryl ether linkages and dibenzodioxocins were somewhat lower while the amount of uncondensed β-aryl ether structures was higher in southern pine. The total uncondensed H unit content (uncon-

densed *p*-hydroxyphenyl moieties), however, was higher in compression wood than in normal wood, while the amount of uncondensed G units was found to be higher in normal wood (**Table 3**). More specifically, 9.0% of the total amount of uncondensed β-aryl ether structures present in compression wood contain at least one *p*-hydroxyphenyl moiety (H unit), while in normal wood only 2.5% of this unit is involved in uncondensed β-aryl ether structures. These results are similar to a recent report by Yeh et al. (*23*), who proposed that the major difference in H units between normal and compression wood is from nonconjugated *p*-hydroxyphenyl moieties. As anticipated, lignin from compression wood was found to contain less uncondensed β-aryl ether structures than lignin from normal wood. The data of **Table 3** show a 19.3% decrease in such uncondensed structures in compression wood lignin. On the other hand, the amount of condensed β-aryl ether structures seems to be at least 18.3% higher in compression wood, with the majority difference being due to a 23.5% increase in dibenzodioxocins in lignin from compression wood when compared with lignin from normal wood.

Conclusions. Overall, MWL, CEL, and EMAL when isolated from four different species of softwood and one hardwood and thoroughly characterized were found to offer different yields, purities, lignin structures, and molecular weights when isolated with the same method. Most significantly, the EMAL protocol was found to offer much higher gravimetric lignin yields and purities than those of the corresponding MWL and CEL isolated from the same batch of milled wood. A more detailed comparison of the lignin yields and purities showed that different species of softwood display different behaviors when submitted to the same isolation procedure.

ACKNOWLEDGMENT

We thank Professor Ronald Sederoff for providing the normal and compression wood of Southern pine. The contributions of Ana Xavier are also acknowledged with respect to the Klason and UV analyses.

LITERATURE CITED

(1) Fengel, D., Wegener, G., Eds. *Wood Chemistry, Ultrastructure and Reactions*; Walter de Gruyter: Berlin, 1989; 613 pp.

(2) Ralph, J.; Lundquist, K.; Brunow, G.; Lu, F.; Kim, H.; Schatz, P.; Marita, J.; Hatfield, R.; Ralph, S.; Christensen, J.; Boerjan, W. Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids. *Phytochemistry* **2004**, *3*, 29−60.

(3) Yaku, F.; Yamada, Y.; Koshijima, T. Lignin-carbohydrate complex. Part IV. Lignin as a side chain of the carbohydrate in Björkman LCC. *Holzforschung* **1981**, *35*, 177−181.

(4) Lawoko, M.; Henriksson, G.; Gellerstedt, G. New method for the quantitative preparation of lignin-carbohydrate complex from unbleached softwood kraft pulp: lignin-polysaccharide networks I. *Holzforschung* **2003**, *57*, 69−74.

(5) Eriksson, Ö.; Goring, D.; Lindgren, B. O. Structural studies on the chemical bonds between lignin and carbohydrates in spruce. *Wood Sci. Technol.* **1980**, *14*, 267−279.

(6) Watanabe, T.; Koshijima, T. Evidence for an ester linkage between lignin and glucuronic acid in lignin-carbohydrate complexes by DDQ-oxidation. *Agric. Biol. Chem.* **1988**, *52*, 2953−2955.

(7) Lundquist, K.; Simonson, R.; Tingsvik, K. Lignin-carbohydrate linkages in milled-wood lignin preparations from spruce wood. *Sven. Papperstidn.* **1983**, *86*, 44−47.

(8) Yaku, F.; Yamada, Y.; Koshijima, T. Lignin-carbohydrate complex Part II. Enzymatic degradation of acid polysaccharides in Björkman LCC. *Holzforschung* **1976,** *30*, 148−156.

(9) Kondo, R.; Sako, T.; Limori, T.; Imamura, H. Formation of glycosidic lignin-carbohydrate complex in the dehydrogenative polymerization of coniferyl alcohol. *Mokuzai Gakkaishi* **1990**, *36*, 332−338.

(10) Xie, Y.; Yasuda, S.; Wu, H.; Liu, H. Analysis of the structure of lignin-carbohydrate complexes by specific ^{13}C tracer method. *J. Wood Sci.* **2000**, *46*, 130−136.

(11) Wu, S.; Argyropoulos, D. S. An improved method for isolating lignin in high yield and purity. *J. Pulp Pap. Sci.* **2003**, *29*, 235−240.

(12) Chang, H.; Cowling, E.; Brown, W. Comparative studies on cellulolytic enzyme lignin and milled lignin of sweetgum and spruce. *Holzforschung* **1975**, *29*, 153−159.

(13) Lee, Z.; Meshitsuka, G.; Cho, N.; Nakano, J. Characteristics of milled wood lignins isolated with different milling times. *Mokuzai Gakkaishi* **1981**, *27*, 671−677.

(14) Ikeda, T.; Holtman, K.; Kadla, J.; Chang, H.; Jameel, H. Studies on the effect of ball milling on lignin structure using a modified DFRC method. *J. Agric. Food Chem.* **2002**, *50*, 129−135.

(15) Adler, E. Lignin chemistry- past, present and future. *Wood Sci. Technol.* **1977**, *11*, 169−218.

(16) Björkman, A. Studies on finely divided wood I. Extraction of lignin with neutral solvents. *Sven. Papperstidn.* **1956**, *59*, 477−485.

(17) Björkman, A. Lignin and lignin-carbohydrate complexes—extraction from wood meal with neutral solvents. *Ind. Eng. Chem.* **1957**, *49*, 1395−1398.

(18) Holtman, K.; Chang, H; Kadla, J. Solution-state nuclear magnetic resonance study of the similarities between milled wood lignin and cellulolytic enzyme lignin. *J. Agric. Food Chem.* **2004**, *52*, 720−726.

(19) Fugimoto, A.; Matsumoto, Y.; Chang, H.; Meshitsuka, G. Quantitative evaluation of milling effects on lignin structure during the isolation process of milled wood lignin. *J. Wood Sci.* **2005**, *51*, 89−91.

(20) Hu, Z.; Yeh, T.; Chang, H.; Matsumoto, Y.; Kadla, J. Elucidation of the structure of cellulolytic enzyme lignin. *Holzforschung* **2006**, *60*, 389−397.

(21) Guerra, A.; Filpponen, I.; Lucia, L.; Saquing, C.; Baumberger, S.; Argyropoulos, D. S. Toward a better understanding of the lignin isolation process from wood. *J. Agric. Food Chem.* **2006**, *54*, 5939−5947.

(22) Evtuguin, D.; Neto, C.; Silva, A.; Domingues, P.; Amado, F.; Robert, D.; Faix, O. Comprehensive study on the chemical structure of dioxane lignin from plantation *Eucalyptus globulus* wood. *J. Agric. Food Chem.* **2001**, *49*, 4252−4261.

(23) Yeh, T.; Tatsuhiko, Y.; Capanema, E.; Chang, H; Chiang, V.; Kadla, J. Rapid screening of wood chemical component variations using transmittance near-infrared spectroscopy. *J. Agric. Food Chem.* **2005**, *53*, 3328−3332.

(24) Granata, A.; Argyropoulos, D. S. 2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholate, a reagent for the accurate determination of the uncondensed and condensed phenolic moieties in lignins. *J. Agric. Food Chem.* **1995**, *43*, 1538−1544.

(25) Argyropoulos, D. S. Quantitative phosphorus-31 NMR analysis of lignin: a new tool for the lignin chemist. *J. Wood Chem. Technol.* **1994**, *14*, 45−63.

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

(27) Willför, S.; Sundberg, A.; Hemming, J.; Holmbom, B. Polysaccharides in some industrial important softwood species. *Wood Sci. Technol.* **2005**, *39*, 245−258.

(28) Lawoko, M.; Henriksson, G.; Gellerstedt, G. Characterization of lignin-carbohydrate complexes from spruce sulfite pulps. *Holzforschung* **2006**, *60*, 162−165.

(29) Kosikova, B.; Joniak, D.; Kosakova, L. The properties of benzyl ether bonds in the lignin-saccharidic complex isolated from spruce. *Holzforschung* **1979**, *33*, 11−14.

(30) Akim, L.; Argyropoulos, D.; Jouanin, L.; Leplé, J.-L.; Pilate, G.; Pollet, B.; Lapierre, C. Quantitative ^{31}P NMR spectroscopy of lignins from transgenic poplars. *Holzforshung* **2001**, *55*, 386−390.

(31) Yeh, T.-F.; Goldfarb, B.; Chang, H.-M.; Peszlen, I.; Braun, J.; Kadla, J. Comparison of morphological and chemical properties between juvenile wood and compression wood of loblolly pine. *Holzforshung* **2005**, *59*, 669−664.

(32) Akiyama, T.; Goto, H.; Nawawi, D.; Syafii, W.; Matsumoto, Y.; Meshitsuka, G. *Erythro/threo* ratio of β-O-4 structures as an important structural characteristic of lignin. Part 4: variation in the *erythro/threo* ratio in softwood and hardwood lignins and its relation to syringyl/guaiacyl ratio. *Holzforshung* **2005**, *59*, 276−281.

(33) Brunow, G.; Karlsson, O.; Lundquist, K.; Sipilä, J. On the distribution of the diastereomers of the structural elements in lignin: the steric course of reactions mimicking lignin biosynthesis. *Wood Sci. Technol.* **1993**, *27*, 281−286.

(34) Önnerud, H.; Gellerstedt, G. Inhomogeneities in the chemical structure of spruce lignin. *Holzforschung* **2003**, *57*, 165−170.

(35) Lai, Y.-Z.; Guo, X.-P. Variation of the phenolic hydroxyl group content in wood lignins. *Wood Sci. Technol.* **1991**, *25*, 467−472.

(36) Sinkey, J. D. Sulphonation treatments for chemimechanical pulping of softwood. *Appita* **1983**, *36*, 301−307.

(37) Tohmura, S.; Argyropoulos, D. S. Determination of arylglycerol-β-aryl ethers and other linkages in lignin using DFRC/^{31}P-NMR. *J. Agric. Food Chem.* **2001**, *49*, 536−542.

Received for review August 23, 2006. Revised manuscript received October 17, 2006. Accepted October 23, 2006. This work was made possible by United States Department of Energy Grant Number DE-FC36-04G014308.

JF062433C