Determination of Arylglycerol−β-Aryl Ether Linkages in Enzymatic Mild Acidolysis Lignins (EMAL): Comparison of DFRC/^{31}P NMR with Thioacidolysis[⊥]

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Enzymatic mild acidolysis lignins (EMAL) isolated from different species of softwood and *Eucalyptus globulus* were submitted to comparative analysis that included thioacidolysis, derivatization followed by reductive cleavage (DFRC), and DFRC followed by quantitative ^{31}P NMR (DFRC/^{31}P NMR). While gas chromatography (GC) was used to determine the monomer yields from both thioacidolysis and DFRC, ^{31}P NMR studies quantified the various phenolic hydroxy groups released by DFRC. The monomer yields from thioacidolysis and DFRC were substantially different, with thioacidolysis resulting in higher yields. In contrast, an excellent agreement was obtained in the total number of β-aryl ether structures determined by thioacidolysis and DFRC/^{31}P NMR, indicating that the combination of DFRC with quantitative ^{31}P NMR overcomes, at least in part, the limitations presented by the DFRC method. Both thioacidolysis and DFRC/^{31}P NMR were further used to better understand the lignin isolation process from wood. The results show that mild rotary ball milling minimizes, but does not prevent, the degradation of β-*O*-4 structures during the early stages of wood pulverization. The extent of such degradation was found to be higher for *E. globulus* than for a variety of softwoods examined. Furthermore, the structures of the EMALs isolated at yields ranging from 20% to 62% were very similar, indicating structural homogeneity in the lignin biopolymer within the secondary wall.

Lignin is a complex and irregular natural polymer built up of different interunit linkages.[1] While the bulk of lignin in wood consists of nonphenolic β-aryl ether units, other units, such as phenylcoumaran (β-5), resinol (β-β), and dibenzodioxocins (5-5/β-*O*-4, α-*O*-4), are also present in lower amounts within the lignin macromolecule.[1,2] According to the current understanding, almost all lignin macromolecules in softwood and softwood pulps are covalently linked to polysaccharides, mainly hemicelluloses.[3,4]

A primary problem in elucidating lignin structure has been the isolation of total lignin from wood in a chemically unaltered form.[5−9] Overall, two approaches have been used to isolate lignin from lignocellulosics: acidolysis methods[10,11] and extraction of lignin after ball milling.[5,6,12] Despite resulting in lignin preparations with high yields and purities, severe acid concentrations (usually 0.2 M) trigger some changes in lignin structure.[10,11] On the other hand, traditional ball-milling-based methods, such as the milled wood lignin (MWL) and cellulolytic enzyme lignin (CEL) protocols, offer less modified lignin than those obtained by severe acid treatments. However, the yields of such lignins are dependent on milling intensity.[7−9,13] Intensive milling protocols offered by vibratory- and orbital-milling devices provide higher lignin yields within relatively short milling intervals, although at the expense of the integrity of the lignin macromolecule and associated condensation and oxidation reactions.[8,9,13,14] Low-intensity milling minimizes structural changes during wood pulverization, but moderate yields are usually achieved under such conditions. As such, the representative nature of the resulting lignin may be questionable.

In a recent series of papers, we have demonstrated a novel procedure, using a combination of enzymatic and mild acidolysis (EMAL), that isolates a lignin fraction that may be more representative of the total lignin present in milled wood.[9,12,15,16] 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 milling severity, facilitating the isolation of less modified lignin in high yields and purities from milled wood.[9,15,16] Comparison of the chemical structure of EMAL, MWL, and CEL has revealed only subtle differences, suggesting that EMAL is released by cleaving lignin−carbohydrate bonds rather than other linkages within lignin macromolecule.[9,12,15] Consequently, the aforementioned protocol presents a real opportunity to improve lignin yield and purity from different softwood and hardwood species.

Another important issue in elucidating lignin structure has been the development and implementation of lignin analysis methods. Most of the conclusions regarding native lignin bonding patterns have been derived from degradative methods, such as thioacidolysis,[17] hydrogenolysis,[18] derivatization followed by reductive cleavage (DFRC),[19] acidolysis,[10,20] oxidation,[2] and ozonation.[22] Despite the development of alternative degradative methods,[23,24] two of the most effective methods for elucidating lignin structure have proven to be thioacidolysis and DFRC. Thioacidolysis is a solvolysis reaction in dioxane−ethanethiol catalyzed by boron trifluoride ethereate, which depolymerizes lignin through β-aryl ether bond cleavage and the consequent formation of trithioethyl monomeric compounds.[17,25] On the other hand, the DFRC method uses considerably milder depolymerization conditions and a more simplified procedure to selectively degrade β-aryl ether structures, forming a simpler mixture of primary monomers.[19] Although DFRC offers a clean and selective protocol for β-aryl ether bond cleavage, this method provides lower monomer yields than thioacidolysis. This difference has been attributed to an inefficient cleavage of β-aryl ether bonds during the DFRC protocol and rationalized on the basis of an undefined complexation of zinc with the ether bond.[25]

Despite such limitation, DFRC is a flexible method due to its three distinctly separated steps, allowing modifications designed to the quantification of different monomeric units[8] and structures.[26] On the basis of this flexibility, Tohmura and Argyropoulos[26] proposed the combination of DFRC with quantitative ^{31}P NMR data. Since quantitative ^{31}P NMR spectroscopy determines the number of the various hydroxy groups, such spectra "before DFRC" provide quantitative information about the aliphatic hydroxy and carboxylic groups as well as the condensed and uncondensed units bearing

[⊥] Dedicated to the memory of Dr. Anderson Guerra, a wonderful and creative colleague who passed away at the beginning of a very promising career.

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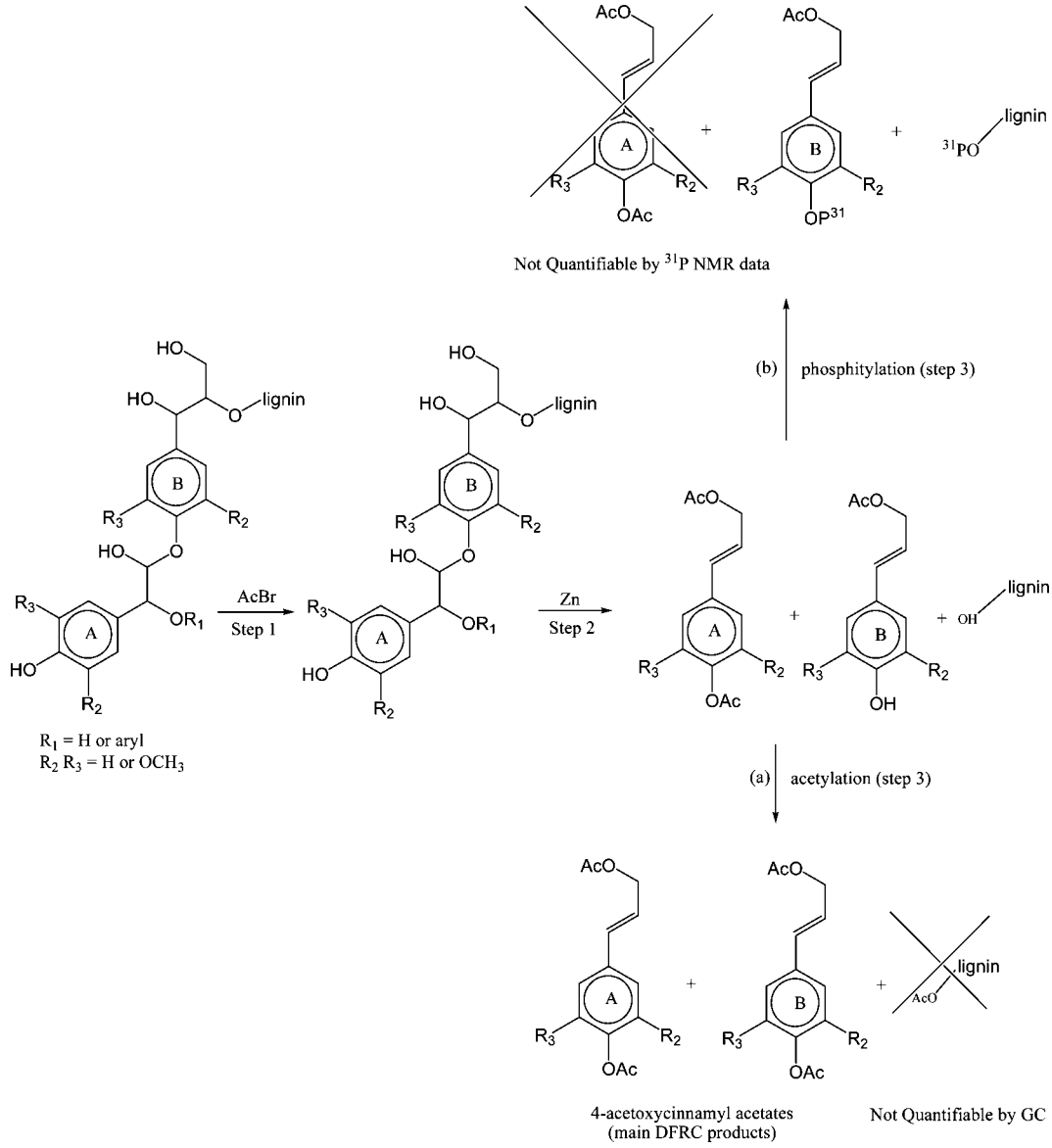

Figure 1. Reaction scheme for DFRC and DFRC/^{31}P NMR analytical methods.

phenolic hydroxy groups within lignin.[26] Unfortunately, quantitative ^{31}P NMR data do not provide information about the lignin's etherified or carbon−carbon-linked bonding pattern. However, when the aryl ether linkages are selectively cleaved by DFRC, the corresponding phenolic hydroxy groups released can be quantified by ^{31}P NMR spectroscopy. While primary DFRC monomers, detected and quantified by GC, are confined to phenylpropane units linked by a *β-O*-4 linkage through both the *β*- and the 4-position, the ^{31}P NMR spectra "after DFRC" have no such limitation and offer detailed information about the total contents of condensed and uncondensed units connected through *β*-aryl ether linkages.[9,26] Therefore, a comparison between the uncondensed phenolic hydroxy groups released by DFRC using quantitative ^{31}P NMR data and the monomer yields from thioacidolysis is warranted.

In this study, enzymatic mild acidolysis lignins (EMALs) were isolated from different wood species and subjected to thioacidolysis and DFRC degradation. While the monomer yields from thioacidolysis were determined by gas chromatography, the resulting product mixture from DFRC was analyzed by quantitative ^{31}P NMR spectroscopy. In addition, both techniques were applied on milled woods and EMALs isolated from different wood species milled for different time periods in an attempt to better understand the lignin isolation process from wood.

Results and Discussion

Comparison of DFRC/^{31}P NMR, Thioacidolysis, and DFRC Treatment Performed on EMALs Isolated from Different Wood Species. Most of the recent conclusions regarding the bonding patterns of native lignins have been derived from chemical degradation techniques, such as thioacidolysis[17] and DFRC.[19] While the degradation of arylglycerol−*β-O*-aryl ether linkages proceeds in a single thioacidolysis step, the DFRC protocol involves three different steps (Figure 1). More specifically, thioacidolysis follows a pathway similar to Kraft pulping, in which the thiol group displaces the α-hydroxy or α-ether group and the *β-O*-aryl ether to form an episulfide-type intermediate, which is further converted into a pair of *erythro* and *threo* trithioethyl phenylpropane compounds (for a detailed mechanism, see ref 17). As anticipated, the DFRC involves three steps: (i) acetyl bromide derivatization; (ii) reductive ether linkage cleavage; and (iii) acetylation (Figure 1, route a). Despite evidence that both techniques selectively degrade the predominant *β-O*-aryl ether structures within the lignin structure, it has been shown that the monomer yields from thioacidolysis and DFRC treatments, determined by GC analyses, are not comparable, thioacidolysis providing higher monomer yields.[25]

Table 1. Thioacidolysis, Conventional DFRC, and DFRC/^{31}P NMR Data from EMAL Isolated from White Fir

quantified by	G units involved only in uncondensed β-*O*-aryl bonds (μmol/g)	H units involved only in uncondensed β-*O*-aryl bonds (μmol/g)	total uncondensed β-*O*-aryl bonds (μmol/g)
DFRC	725	24	749
thioacidolysis	882	30	912
DFRC/^{31}P NMR	860	40	900

Despite the aforementioned limitation, we recently showed that DFRC followed by quantitative ^{31}P NMR (DFRC/^{31}P NMR) and thioacidolysis provide comparable results in terms of uncondensed β-aryl ether contents for lignin isolated from unbleached Norway spruce thermomechanical pulp.[9] These data suggested that the quantification of the hydroxy groups released during DFRC by ^{31}P NMR spectroscopy gives a better estimation of the total amount of uncondensed β-aryl ether bonds than the monomer yields from DFRC quantified by GC. To further validate this hypothesis, a sample of EMAL isolated from white fir wood was submitted to comparative analyses that included thioacidolysis, conventional DFRC, and DFRC/^{31}P NMR (Table 1). As expected, the total amount of uncondensed β-aryl ether linkages estimated by the monomer yields from thioacidolysis was much higher than when estimated by the monomer yields from DFRC. While the monomer yields from DFRC were 749 μmol/g, the total amount of uncondensed β-aryl ether structures estimated by thioacidolysis monomer yields was found to be 912 μmol/g. These data are in good agreement with literature values[25] and confirm that the monomer yields from thioacidolysis are about 20% higher than from the conventional DFRC. This difference has been ascribed to the inefficient cleavage of β-aryl ether bonds during the DFRC protocol and rationalized on the basis of an undefined complexation of zinc with the ether bond.[25]

The data of Table 1 also show that the total amount of uncondensed β-aryl ether linkages determined by DFRC/^{31}P NMR was much higher than the value estimated by the monomer yields released by DFRC and quantified by GC. This difference is due mainly to the detection systems used to quantify the DFRC products. According to the mechanism operating during DFRC,[19] the primary alcoholic and phenolic hydroxy groups are acetylated during the acetyl bromide derivatization step (Figure 1, step 1), while the benzylic α-hydroxy groups are displaced by bromide. Similarly, benzyl aryl ethers are quantitatively cleaved to yield aryl acetates and acetylated α-bromides. During the subsequent reductive cleavage step (Figure 1, step 2), β-*O*-aryl ether linkages are selectively cleaved, quantitatively releasing DFRC monomers and various phenolic hydroxy groups, depending on the linking patterns present in the lignin.[19,26] In the conventional DFRC protocol (Figure 1, route a), the ensuing acetylation step (step 3) is designed to facilitate monomer identification and quantification by GC analysis.[19,26] As anticipated, the primary DFRC monomers detected and quantified by GC are confined to an uncondensed portion of the lignin (i.e., a monomer linked by a β-*O*-4 linkage through both the β- and the 4-position). Consequently, quantification of monomers from DFRC by using GC alone cannot estimate the total amount of β-aryl ether linkages within lignin.[26] On the other hand, when the products from DFRC are analyzed by quantitative ^{31}P NMR spectroscopy (Figure 1, route B) instead of GC (route A), the total amount and identity of free phenolic OH released from β-*O*-aryl ether linkages after DFRC can be determined.[9,15,16,26] Obviously, the acetylation of the free phenolic hydroxy groups present in the starting lignin during the acetyl bromide derivatization step (step 1, Figure 1) preclude them from being detected by ^{31}P NMR spectroscopy since they can no longer be phosphitylated. Consequently, all the "new" phenolic hydroxy groups that can be determined after DFRC originate from an equimolar amount of cleaved β-aryl ether bonds. In this way, the total amount of uncondensed phenolic hydroxy groups determined after DFRC (Table 1) is equivalent to the total amount of uncondensed β-*O*-aryl ether structures present within the lignin macromolecule.[26] By using this approach, the total amount of arylglycerol−β-*O*-aryl ether linkages can be estimated since the quantification of released OH groups are not limited to phenylpropane units connected to β-*O*-aryl ether bonds on both sides of the phenyl propanoid units or terminal phenylpropane units connected to the polymer via a β-*O*-aryl ether bond. Furthermore, the combination of DFRC with ^{31}P NMR spectroscopy also distinguishes condensed from uncondensed β-aryl ether linkages. To avoid misinterpretation, the total number of condensed phenolic units released from β-aryl ether linkages are not included in the data of Table 1 since such structures cannot be determined by DFRC/GC or thioacidolysis.

While thioacidolysis detects monomers released through β-aryl ether cleavage,[17,25] DFRC/^{31}P NMR measures the β-aryl ether linkages themselves by quantifying all hydroxy groups released by reductive cleavage (Figure 1). Even though they are different approaches measuring similar but not completely equal responses, the two methods have shown good agreement in the estimation of the total number of uncondensed β-aryl bonds (Table 1 and ref 9). In an attempt to ensure that these excellent agreements between the two methods are not merely coincidence, six samples of lignin isolated from different wood species were analyzed by DFRC/^{31}P NMR and thioacidolysis (Table 2). Both techniques provided comparable amounts of syringyl (S), guaiacyl (G), and *p*-hydroxyphenyl (H) units involved only in uncondensed β-aryl linkages. Consequently, the total amounts of uncondensed β-aryl ether structures determined by thioacidolysis and DFRC/^{31}P NMR were very similar in all EMAL samples evaluated so far, regardless of the wood species. For example, the thioacidolysis and DFRC/^{31}P NMR data were found to be 1728 versus 1730 μmol/g, respectively, for the EMAL from *E. globules* and 919 versus 940 μmol/g, respectively, for EMAL from Douglas fir, indicating a good agreement for hardwood and softwood lignins. However, when considering the data of Table 2, the absence of degradation products derived from H units in the thioacidolysis products from *E. globules* is noteworthy. This absence is in complete agreement with the work of Evtuguin et al.,[11] and it has been rationalized on the basis of the low yields of H-type lignin structures in the thioacidolysis.[11,27] Furthermore, both techniques also showed remarkable similarities for determining the total number of guaiacyl units involved in uncondensed β-aryl bonds in lignins isolated from normal and compressed woods (Table 2). However, in the particular case of lignin from compression wood, a significant difference was observed in the amount of uncondensed H units determined by thioacidolysis and DFRC/^{31}P NMR: 204 versus 74 μmol/g, respectively. In an effort to ensure that such a difference was not due to a coelution of contaminants with the H-monomer from thioacidolysis during the GC/FID quantification, the thioacidolysis products of EMAL from pine compression wood were analyzed by GC/MS. Accordingly, the presence of any contaminant coeluting with the monomers from H or G units would be promptly recognized by their fragmentation pattern. Nevertheless, under the GC conditions, no such coelution was apparent and the molecular ions and prominent fragments of both monomers from thioacidolysis were identical to those reported by Rolando et al.[27] To date, the observed discrepancy between the thioacidolysis and DFRC/^{31}P NMR data specifically confined to the uncondensed H units in compression wood remains unresolved. The results collated in Tables 1 and 2 show that the combination of DFRC with quantitative ^{31}P NMR

Table 2. Thioacidolysis and DFRC/^{31}P NMR Data from EMAL Isolated from Different Wood Species

quantified by	S units involved only in uncondensed β-*O*-aryl bonds (μmol/g)	G units involved only in uncondensed β-*O*-aryl bonds (μmol/g)	H units involved only in uncondensed β-*O*-aryl bonds (μmol/g)	total uncondensed β-*O*-aryl bonds (μmol/g)
		EMAL from Douglas fir		
thioacidolysis	nd[a]	885 ± 6	34 ± 2	919 ± 9
DFRC/^{31}P NMR	nd	880 ± 16	60 ± 2	940 ± 28
		EMAL from white fir		
thioacidolysis	nd	882 ± 38	30 ± 2	912 ± 37
DFRC/^{31}P NMR	nd	860 ± 26	40 ± 2	900 ± 27
		EMAL from redwood		
thioacidolysis	nd	855 ± 13	24 ± 1	879 ± 14
DFRC/^{31}P NMR	nd	810 ± 16	41 ± 2	851 ± 17
		EMAL from S. pine		
thioacidolysis	nd	1001 ± 4	29 ± 1	1030 ± 6
DFRC/^{31}P NMR	nd	990 ± 20	25 ± 2	1015 ± 20
		EMAL from pine compression wood		
thioacidolysis	nd	735 ± 33	204 ± 8	939 ± 25
DFRC/^{31}P NMR	nd	745 ± 22	74 ± 3	819 ± 24
		EMAL from spruce		
thioacidolysis	nd	741 ± 21	26 ± 2	766 ± 22
DFRC/^{31}P NMR	nd	730 ± 20	40 ± 3	770 ± 20
		EMAL from *E. globulus*		
thioacidolysis	1416 ± 40	312 ± 8	nd	1728 ± 30
DFRC/^{31}P NMR	1430 ± 40	260 ± 5	40 ± 2	1730 ± 35

[a] nd = not detected.

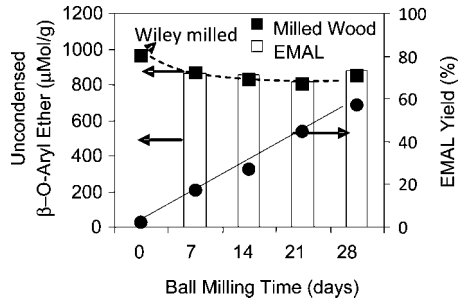

Figure 2. Total yield of monomers from thioacidolysis of EMAL (open bars) and ball-milled wood (black squares) for redwood as a function of the ball-milling time.

data overcomes, at least in part, the aforementioned DFRC limitation, providing results comparable to thioacidolysis.

Effect of Milling on Arylglycerol−*β*-Aryl Ether Structures. Since thioacidolysis and DFRC/^{31}P NMR provide comparable results, both techniques were used to better understand the lignin isolation process from wood. Figure 2 shows the typical effect of different milling severities on the total amount of uncondensed β-aryl ether structures determined by thioacidolysis. An advantage of thioacidolysis is that it allows the evaluation of the milling effects on β-aryl ether structures determined directly on Wiley- and ball-milled wood without requiring lignin isolation. As illustrated in Figure 2 for redwood, the monomer yields from Wiley-milled wood (without ball milling) were greater than from ball-milled woods for all the wood species evaluated, indicating the degradation of β-aryl ether structures during ball milling at room temperature. However, the extension of such degradation was found to be wood specific. While the difference between the monomer yields from thioacidolysis of Wiley-milled and ball-milled softwoods varied from 8% to 9%, the degradation of β-aryl ether structures during ball milling of *E. globulus* was found to be about 12%. These findings are in agreement with the work of Fujimoto et al.[14] and Hu et al.,[13] confirming that β-*O*-4 structures in hardwoods are degraded to a greater extent than in softwoods. For example, at an extractable MWL yield of 25%, 22% of the β-*O*-4 structures in sweetgum were found to be degraded,[14] whereas only about 12% of the β-aryl ether structures in loblolly pine were affected.[13] Furthermore, data in Figure 2 also show that longer milling time under the conditions evaluated in this work does not affect the integrity of lignin's polymeric chains, and the total amount of β-aryl ether linkages remained remarkably constant from 7 to 28 days of milling, irrespective of the wood species.

The uncondensed β-aryl ether contents were also determined using thioacidolysis for all EMAL samples isolated from woods ball-milled for different periods of time (Figure 2). As expected, extending the milling time from 7 to 28 days did not affect the contents of such structures within the resulting EMALs. These data corroborate previous findings, where the total amounts of phenolic hydroxy and carboxylic acid groups, S to G ratio, and total β-aryl ether structures were described as very similar in EMALs isolated from hardwoods[16] and Norway spruce[9] ball-milled for up to 28 days. It is also significant to note in Figure 2 the good agreement between the uncondensed β-aryl ether contents determined for ball-milled woods and isolated lignins, demonstrated for the first time in the same EMAL samples. Such similarities confirm previous estimates[9,12,15] and indicate that 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 woods.

Despite accumulating evidence that lignin is degraded during wood pulverization,[9,13,14] the benefits of ball milling are apparent as far as the yields of EMAL are concerned (Figure 2). When Wiley-milled wood was treated with cellulase enzymes followed by mild acid hydrolysis, relatively low lignin amounts were obtained (1% based on the amount of Klason lignin of the starting wood and isolated lignin). Powdered wood before milling is known to be impervious to cellulolytic enzymes,[28] which precludes EMAL isolation from Wiley-milled wood. Progressive mechanical treatments are known to lead to the formation of increasing amounts of disordered cell wall material, which is rapidly digested by cellulase enzymes, facilitating lignin extraction from wood.[9,31] Consequently, the longer the milling time is, the higher the EMAL yields are (Figure 2), although both yields and milling requirements have been shown to be wood specific.[15,16]

Under the conditions applied in this study, the yields of almost all EMALs isolated from woods milled for 28 days were greater than 53%.[15] On the basis of such yields and the extensive β-aryl ether degradation determined by thioacidolysis (Figure 2), it can be concluded that the combination of enzymatic hydrolysis and mild acidolysis favors the extraction of high lignin yields under the relative low intensity milling provided by ball-milling devices, which minimized undesired mechanical fragmentation reactions within the lignin macromolecule. At this point, it is important to

highlight that the degradation of the β-aryl ether structures has been estimated to be about 25% when milling under different conditions is continued until 40% of lignin in wood becomes extractable.[13,14] However, it is important to note that extractable lignin represents the maximum amount of lignin that might be obtained from milled wood as crude MWL rather than the gravimetric yield of purified MWL. It is known that approximately 30% of crude MWL is usually lost in the first purification step when crude MWL is dissolved in 90% AcOH and precipitated in water.[32] Therefore, about 25% of the β-aryl ether structures are degraded during the extensive milling required to obtain only 28% of purified MWL (40% $\times$ 0.7). In contrast, the combination of low-intensity milling with enzymatic hydrolysis and mild acidolysis allowed the extraction of more than 50% of purified lignin with less than 10% degradation of β-*O*-4 moieties (Figure 2), although the β-aryl ether bond degradation that takes place during the early stages of milling cannot be prevented.

While thioacidolysis provides structural information without requiring lignin isolation, DFRC/^{31}P NMR offers new insights into the lignin structure. Since ^{31}P NMR data can distinguish condensed and uncondensed phenolic hydroxy groups, its combination with DFRC can be used to estimate the total amounts of condensed and uncondensed β-aryl ether structures. Furthermore, by integrating the region from 141.2 to 142.4 ppm in the ^{31}P NMR spectrum after DFRC, the dibenzodioxocin structures can also be quantified.[15,16,26]

Figure 3 shows the quantification of the hydroxyl groups released from β-*O*-4 structures by DFRC performed on EMALs isolated from woods ball-milled for different periods of time. As anticipated, dibenzodioxocin structures (Figure 3c) and condensed (Figure 3b) and total (condensed + uncondensed) β-aryl ether structures (Figure 3a) were remarkably constant from 7 to 28 days of ball milling, regardless of the wood species evaluated. These data are consistent with those reported in Figure 2 and support the contention that the integrity of the lignin's polymeric chains is not seriously affected by low-intensity ball-milling devices.[9,16] On the other hand, intensive milling protocols, such as vibratory- and orbital-milling devices, have been shown to seriously affect the lignin structure through oxidative reactions.[8,9]

The data in Figure 3 also show significant differences among lignins from different wood species. As shown in Figure 3a, the total amount of β-aryl ether structures within *E. globulus* was found to be significantly higher than for the softwoods. The greater content of such structures may explain, at least in part, the relative facility with which pulping and bleaching of *E. globulus* occurs when compared to other wood species.[31] Furthermore, the unusually high contents of uncondensed β-aryl ether structures within *E. globulus* have been used to explain its low propensity to associate in organic solvents.[32] For the softwood lignins, the condensed β-aryl ether structures (Figure 3b) were slightly higher in EMAL from spruce, while no differences were observed among such structures in Douglas fir, white fir, and redwood. Dibenzodioxocin contents (Figure 3c) were found to slightly decrease in the order of spruce (270 μmol/g), w. fir (240 μmol/g), D. fir (230 μmol/g), and redwood (220 μmol/g), confirming previous findings.[9,15] The amount of condensed β-aryl ether structures and dibenzodioxocins within EMAL from *E. globulus* was not calculated since the condensed and uncondensed S-type phenolic hydroxy signals overlap in the ^{31}P NMR spectra. Due to such overlapping, both units have been integrated together to give the total amount of β-aryl ether structures reported in Figure 3a.

Effect of Milling on Other Structures. It is noteworthy that the EMAL isolated at different yields have similar structures. As illustrated in Figure 4 for lignins from redwood and *E. globulus*, the total amounts of the various groups that define significant aspects of the lignin structure were very similar within the EMALs isolated at yields ranging from 20% to 62%. For the purposes of this investigation, the total phenolic OH, condensed phenolic OH, S/G

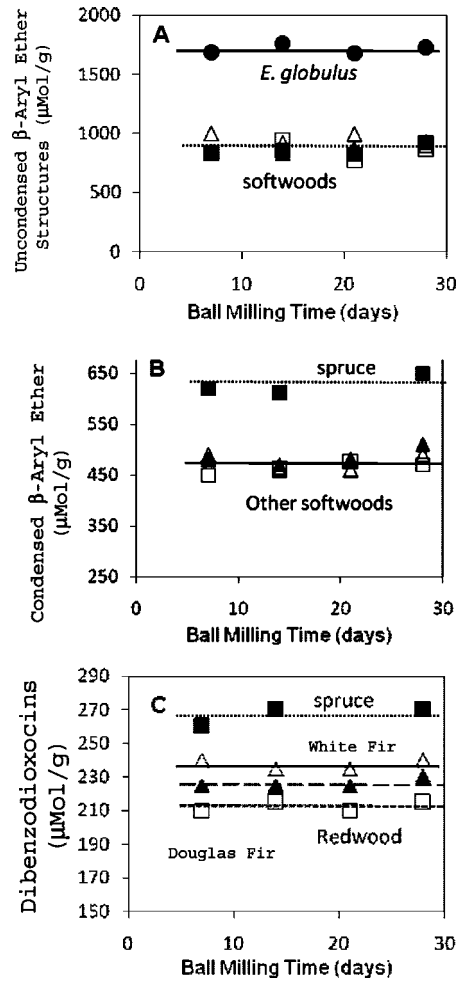

Figure 3. Total (A) and condensed (B) phenolic hydroxy groups (equivalent to the amount of total and condensed β-aryl ether linkages, respectively) and dibenzodioxocin units (C) within the EMAL isolated from *E. globulus* (black circles), white fir (open triangles), Norway spruce (black squares), Douglas fir (black triangles), and redwood (open squares) as a function of the rotary ball-milling time. Condensed β-aryl ether linkages are defined as structures that connect two macromolecules or oligomers that themselves are interlinked via structures other than β-aryl ethers.

ratios with free phenolic OH, carboxylic acids, and β-aryl ether contents of EMALs obtained at different yields were determined by quantitative ^{31}P NMR spectroscopy before DFRC. The total free phenolic hydroxy contents, condensed phenolic hydroxyl contents, and carboxylic groups are quantified by ^{31}P NMR spectroscopy after phosphitylating lignin with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane,[29] and the total β-aryl ether contents were determined indirectly from the α-hydroxyl groups after derivatization with 2-chloro-1,3,2-dioxaphospholane.[9,15]

The structural similarities observed could be rationalized on the basis that the combination of enzymatic and mild acid hydrolysis provides lignin that is little contaminated by lignin from the middle lamella (ML). As recently suggested by Hu et al.,[13] lignin in the secondary wall (S_2) has more linkages with carbohydrates and becomes extractable only after extensive carbohydrate degradation. Therefore, the lignin fraction liberated during the early part of ball milling may originate mainly from lignin in ML, which is known to be of different chemical composition when compared to lignin from S_2.[13,33] The high relative contents of lignin from ML contaminate lignin preparations obtained at low yields, explaining the structural differences observed in MWL released during the early stages of ball milling, when low yields are obtained.[13] However, the effects of such contamination diminish as the yield

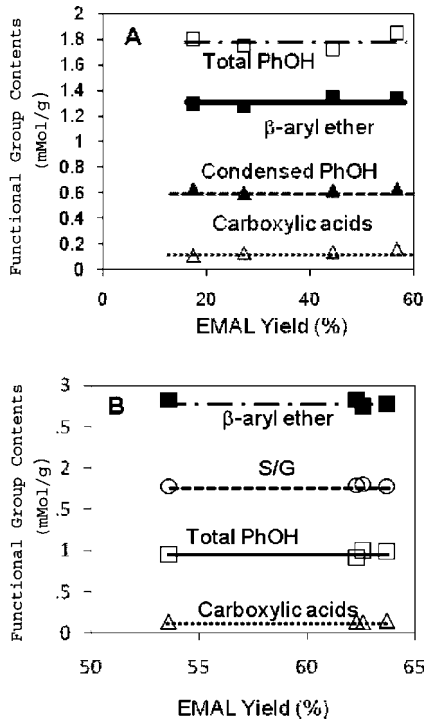

Figure 4. Total β-aryl ether structures (black squares), total phenolic hydroxy (open squares), condensed phenolic hydroxy groups (black triangles), and carboxylic acid groups (open triangles) within the EMAL isolated from redwood (A) and *E. globulus* (B) as a function of the EMAL yield.

of lignin from the S_2 increases. Since the EMAL yields were always higher than 20%, regardless of the ball-milling time evaluated, the contamination by ML lignin is minimized and the EMALs isolated at yields ranging from 20% to 62% have similar structures (Figure 4). Furthermore, these data suggest that lignin in the secondary wall of softwoods and *E. globulus* are uniform in structure. Overall, it is important to note that the S:G ratio *E. globulus* ranges from 1.7 to 1.8 (Figure 4). This is in excellent agreement with most literature accounts and represents confirmation that the analytical methods as applied in this effort are credible and that the EMAL samples are representative of the native lignins.

In conclusion, the present data show that the monomer yields from DFRC determined by gas chromatography are about 20% lower than from thioacidolysis. However, the combination of DFRC with ^{31}P NMR spectroscopy overcomes, at least in part, the DFRC limitation and provides results comparable to thioacidolysis. Furthermore, mild rotary ball milling was found to minimize but did not prevent the degradation of β-*O*-4 structures during the early stages of wood pulverization. The extent of such degradation seems to be higher for *E. globulus* than for a variety of softwoods examined. The similarities in the structures of the EMALs isolated at yields ranging from 20% to 62% indicated structural homogeneity in the lignin biopolymer within the secondary wall.

Experimental Section

Isolation of EMALs, MWLs, and CELs. Enzymatic mild acidolysis lignins (EMALs) were isolated from Douglas fir (*Pseudotsuga menziessi*), white fir (*Abies concolor*), redwood (*Sequoia sempervirens*), eucalyptus (*Eucalyptus globulus*), and normal and compression wood from Southern pine (*Pinus palustris*) according to the previously described procedures.[5,9,15]

Thioacidolysis. Thioacidolysis was performed on 5 mg of isolated lignins or 20 mg of Wiley- or ball-milled wood in 10 mL of reagent according to a published method.[17,31] Each sample was analyzed in triplicate.

DFRC and DFRC/^{31}P NMR. The DFRC was performed as described by Lu and Ralph.[19] The precise amounts of the lignin and precautions due to the ensuing ^{31}P NMR spectroscopy steps were nearly identical to those reported elsewhere.[9,15,26] Each sample was analyzed in triplicate.

Quantitative ^{31}P NMR Data. Quantitative ^{31}P NMR spectra of all lignin preparations were obtained using published procedures.[34,35] To improve resolution, a delay time of 5 s was used and a total of 256 scans per sample were acquired.

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