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Toward a Better Understanding of the Lignin Isolation Process from Wood

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The recently developed protocol for isolating enzymatic mild acidolysis lignins (EMAL) coupled with the novel combination of derivatization followed by reductive cleavage (DFRC) and quantitative ^{31}P NMR spectroscopy were used to better understand the lignin isolation process from wood. The EMAL protocol is shown to offer access at lignin samples that are more representative of the overall lignin present in milled wood. The combination of DFRC/^{31}P NMR provided a detailed picture on the effects of the isolation conditions on the lignin structure. More specifically, we have used vibratory and ball milling as the two methods of wood pulverization and have compared their effects on the lignin structures and molecular weights. Vibratory-milling conditions cause substantial lignin depolymerization. Lignin depolymerization occurs via the cleavage of uncondensed β-aryl ether linkages, while condensed β-aryl ethers and dibenzodioxocins were found to be resistant to such mechanical action. Condensation and side chain oxidations were induced mechanochemically under vibratory-milling conditions as evidenced by the increased amounts of condensed phenolic hydroxyl and carboxylic acid groups. Alternatively, the mild mechanical treatment offered by ball milling was found not to affect the isolated lignin macromolecular structure. However, the overall lignin yields were found to be compromised when the mechanical action was less intense, necessitating longer milling times under ball-milling conditions. As compared to other lignin preparations isolated from the same batch of milled wood, the yield of EMAL was about four times greater than the corresponding milled wood lignin (MWL) and about two times greater as compared to cellulolytic enzyme lignin (CEL). Molecular weight distribution analyses also pointed out that the EMAL protocol allows the isolation of lignin fractions that are not accessed by any other lignin isolation procedures.

KEYWORDS: Enzymatic mild acidolysis lignin; MWL; CEL; DFRC; ^{31}P NMR; spruce; vibratory milling; ball milling; lignin

INTRODUCTION

Lignin is a heterogeneous and highly cross-linked macromolecule that represents the second most abundant natural polymeric material on earth (*1*). Despite extensive investigations, the complex and irregular structure of lignin is not completely understood (*2*). It is known that the bulk of lignin in wood consists of nonphenolic aryl-glycerol-β-O-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 within the lignin macromolecule (*1*, *3*). Furthermore, lignin is covalently linked to carbohydrates (*4*, *5*) forming a lignin−carbohydrate network made up of benzyl-ether (*4*, *6*), benzyl-ester (*4*, *7*, *8*), and phenyl-glycoside (*9*−*11*) bonds.

One of the most important problems in elucidating lignin structure has been the isolation of the total lignin from wood in a chemically unaltered form (*12*−*14*). Despite many efforts, the isolation of a highly representative and totally unaltered native lignin is still a challenge (*14*, *15*). A major advance toward this objective was made when Björkman (*16*, *17*) developed a lignin isolation procedure based on the extraction of extensive ball-milled wood by neutral solvents at room temperature. This procedure has been widely used to isolate lignin from different species (*15*). However, it offers lignin that may not be fully representative of the total lignin present in milled wood (*18*, *19*). The moderate yields usually achieved in this procedure can be increased by up to 50% if one extends the milling time (*20*). However, a milled wood lignin (MWL) less representative of the native lignin is obtained (*14*, *21*).

Another important contribution to the isolation of lignin was introduced by Pew and Weyna (*22*). They treated ball-milled

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wood with cellulolytic enzymes and obtained an insoluble residue containing almost all of the lignin present in spruce and aspen woods. Nevertheless, the residue had as much as 12% of carbohydrates and no further characterization was attempted. Chang et al. (*12*) also treated milled wood with enzymes, but used an enzymatic preparation with greater cellulolytic and hemicellulolytic activities than the enzyme used by Pew. In addition, they extracted the insoluble residue obtained after the enzymatic hydrolysis successively with 96% and 50% aqueous dioxane. The combination of these two fractions offered higher yields than MWL (*12*). However, the lignin fraction soluble in 50% aqueous dioxane contained twice as many carbohydrates as MWL or cellulolytic enzyme lignin (CEL96). Recently, Holtman et al. (*23*) have also reported some subtle structural differences between MWL and CEL.

Wu and Argyropoulos (*15*) have proposed a novel lignin isolation procedure composed of an initial mild enzymatic hydrolysis of milled wood, followed by a mild acid hydrolysis stage. In this procedure, the initial cellulolytic action removes most of the carbohydrates while the mild acidolysis is designed to cleave the remaining lignin−carbohydrate bonds, liberating lignin in high yield and purity. Despite the significant improvements in yield and purity offered by this method, few efforts have been made to further advance the understanding on the effect of each step on the structure of the resulting enzymatic mild acidolysis lignin (EMAL). In a similar procedure aimed at residual kraft lignins, Argyropoulos et al. (*24*) have optimized the enzyme charge to minimize lignin contamination by proteins. Furthermore, Wu and Argyropoulos (*15*) have reported the effects of the acid concentration on the structure of such lignins. Nevertheless, no attempts have been made to unravel and address the effects of the milling conditions on the yield, purity, and structure of EMAL.

In another front, many efforts have been made to elucidate the effects of mechanical action on the structure of lignin. However, the conclusions reached have been limited to the characterization of only 25−30% of the overall lignin actually present within the original wood (*13*, *14*). Furthermore, these studies have been restricted to the characterization of the uncondensed moieties of the lignin. Few efforts have been made to evaluate the effect of milling conditions on the condensed lignin moieties (*25*). Consequently, a study on the effect of milling on the structure of EMALs, which is known to provide lignin in high yield and purity, by using a characterization method designed to quantify condensed and uncondensed structures is warranted.

A novel approach for the quantification of different lignin structures using the combination of derivatization followed by reductive cleavage (DFRC) and quantitative ^{31}P NMR was recently described (*26*). Because quantitative ^{31}P NMR determines the amounts of the various hydroxyl groups, such spectra "before DFRC" provide quantitative information about the aliphatic hydroxyls, carboxylic groups, and condensed and uncondensed units bearing phenolic hydroxyl groups within lignin. Such hydroxyl groups are revealed and quantified by ^{31}P NMR after phosphitylating lignin with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (*26*). Unfortunately, quantitative ^{31}P NMR cannot offer any information about the etherified or carbon−carbon-linked bonding pattern of lignin. However, when the aryl ether linkages are selectively cleaved by DFRC, the corresponding phenolic hydroxyls released can be quantified by ^{31}P NMR. In this way, the ^{31}P NMR spectra "after DFRC" offer detailed information about condensed and uncondensed units connected through β-aryl ether linkages (*26*) as well as dibenzodioxocins.

Overall, therefore, this study applies the recently described procedure to isolate EMAL and then uses the combination of DFRC with quantitative ^{31}P NMR spectroscopy (DFRC/^{31}P NMR) to better understand the lignin isolation process from Norway spruce wood. An assessment on the effects of the isolation procedure on structures never reported before (dibenzodioxocins and β-aryl ether linkages connected to condensed units) is made. The detailed structural analyses of the EMALs obtained are also compared to MWL and CEL isolated and purified from the identical batch of milled wood.

MATERIALS AND METHODS

Isolation of EMALs, MWL and CEL. Unbleached Norway spruce thermomechanical pulp (TMP) was sampled in a Swedish mill. The TMP was of approximately 38% consistency and 85 mL of Canadian Standard Freeness prepared by one-stage refinement and a subsequent reject refinement (about 20%) stage. The pulp was sampled at the press stage after the refined and refined reject pulps had been combined. This pulp currently represents a standard sample, which is the subject of Cost Action E 41 entitled "Analytical Tools with Applications for Wood and Pulping Chemistry" operated by the European Union. The pulp was 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. Rotary ball milling was performed in a 5.5 L porcelain jar in the presence of 474 porcelain balls (9.4 mm in diameter), which occupied 18% of the active jar volume. One hundred grams 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 25 days with a rotation speed of 60 rpm. Vibratory milling was performed in a 0.6 L jar loaded with 10 g of extractive-free wood powder and 2.44 kg of stainless steel balls (6.1 mm in diameter), which occupied 32% of the active jar volume, for periods ranging from 2 to 96 h.

EMALs were isolated from samples (10 g) of vibratory- or rotary ball-milled wood according to the procedure described by Wu and Argyropoulos (*15*). The ground wood meal was treated with cellulase (Iogen, Canada; filter paper activity, 130 FPU mL^{-1}) in a previously optimized (*24*) ratio of 40 FPU/g wood. The enzymatic hydrolyses were carried out at 40 °C for 48 h using 50 mM citrate buffer (pH 4.5) at 5% consistency in an orbital water bath shaker. The insoluble material remained after the enzymatic hydrolysis was collected by centrifugation (2000*g*), washed twice with acidified deionized water (pH 2), and freeze-dried. The crude lignin obtained was further submitted to a mild acid hydrolysis using an azeotrope (bp, 86 °C) of aqueous dioxane (dioxane/water 85:15, v/v, containing 0.01 mol L^{-1} HCl) under an argon atmosphere. The resulting suspension was centrifuged (2000*g*), and the supernatant was carefully withdrawn, neutralized with sodium bicarbonate, and finally added dropwise to 1 L of acidified deionized water (pH 2). The precipitated lignin was allowed to equilibrate with the aqueous phase overnight, and it was then recovered by centrifugation, washed (2×) with deionized water, and freeze-dried.

Caution. The acidolysis residue after centrifugation should be carefully decanted and discarded. Efforts to wash it, so as to increase the lignin yields, may cause serious carbohydrate contamination in the final product.

MWLs and CELs were isolated from the extractive-free wood and milled in the vibratory mill for 48 h, according to the methods described by Björkman (*16*, *17*) and Chang et al. (*12*), respectively. Both preparations were purified as described elsewhere (*14*, *16*, *17*).

Determination of Lignin Content. Klason lignin (acid insoluble) and acid soluble lignin contents of wood meal, EMALs, MWLs, and CELs were measured according to the method reported by Yeh et al. (*27*).

DFRC Procedure. DFRC was performed as described by Lu and Ralph (*28*). The precise amounts of the lignin and precautions due to the ensuing NMR steps were nearly identical to those reported by

Tohmura and Argyropoulos (*26*). More specifically, 25 mL of acetyl bromide in acetic acid (8:92, v/v) was added to about 100 mg of a lignin sample (EMAL, MWL, or CEL) in a 50 mL round-bottom flask. The flask was sealed and placed in a water bath set at 50 °C for 2 h with magnetic stirring. The solvent was rapidly evaporated in a rotary evaporator connected to a high vacuum pump and a cold trap. The residue was dissolved in an acidic solvent (dioxane/acetic acid/water, 5:4:1, v/v), zinc dust (500 mg) was added, and the mixture was stirred at room temperature for 30 min. The zinc dust was filtered off, and the filtrate was quantitatively transferred into another 50 mL round-bottom flask. The solvent was evaporated, and the DFRC products were stored at −10 °C for subsequent quantitative ^{31}P NMR.

Thioacidolysis. Thioacidolysis was performed on 5 mg of isolated lignins in 10 mL of reagent according to a published method (*29*). The reagent was prepared by introducing 2.5 mL of BF_3 etherate (Aldrich) and 10 mL of ethane thiol EtSH (Aldrich) into a 100 mL flask and adjusting the final volume to 100 mL with dioxane (pestipur grade). The reagent and 1 mL of a solution of gas chromatography (GC) internal standard (nonadecane C19, 0.50 mg mL^{-1} in CH_2Cl_2) were added to the lignin sample in a glass tube closed with a Teflon-lined screw cap. Thioacidolysis was performed at 100 °C (oil bath) for 4 h. The cooled reaction mixture was diluted with 30 mL of water, and the pH was adjusted to between 3 and 4.0 (aqueous 0.4 M $NaHCO_3$) before extraction with 3 × 30 mL CH_2Cl_2. The combined organic extracts were then dried over Na_2SO_4 and then evaporated under reduced pressure at 40 °C. The final residue was redissolved in approximately 1 mL of CH_2Cl_2 before silylation and GC-MS analyses according to the method of Lapierre et al. (*30*).

Acetobromination Derivatization Procedure. Acetobromination was used as the derivatization method of choice for all samples prior to size exclusion measurements. 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 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 analysis as described below. One precaution, however, that should be mentioned is the fact that the installed bromine on the lignin is a good leaving group. As such, the acetobrominated samples need to be rapidly evaporated from excess solvent using a rotary evaporator in good working order (preferably equipped with a vacuum pump connected to a cold trap). In addition, once the sample is derivatized, it should be immediately diluted with the required amount of THF and never be allowed to dry. THF solutions of acetobrominated lignins should be stored in a refrigerator until further use.

SEC. SEC of EMAL, MWL, and CEL samples were performed on a size exclusion chromatographic system (Waters system) equipped with UV set at 280 nm and refractive index detectors. The analyses were carried out at 40 °C using THF as an eluent at a flow rate of 0.44 mL min^{-1}. One hundred and twenty microliters of the sample dissolved in THF (2 mg mL^{-1}) was injected into a HR5E and HR1 (Waters) system of columns connected in series. The HR5E column's specification allow for molecular weights up to 4 × 10^6 g mol^{-1} to be reliably detected. The SEC system was calibrated with polystyrene standards in the molecular weight range of 890−1.86 × 10^6 g mol^{-1}, and Millenium 32 SEC software (Waters) was used for data processing.

Quantitative ^{31}P NMR. Quantitative ^{31}P NMR spectra of all lignin preparations were obtained using published procedures (*31*−*33*). Approximately 40 mg of dry lignin was transferred into a sample vial, dissolved in 400 μL of pyridine and deuterated chloroform (1.6:1, v/v), and left at room temperature overnight with continuous stirring. *N*-Hydroxynaphthalimide (200 μL, 11.4 mg mL^{-1}) and chromium(III) acetylacetonate (50 μL, 11.4 mg mL^{-1}) were used as an internal standard and relaxation reagent, respectively. Finally, 100 μL of phosphitylating reagent I (2-chloro-1,3,2-dioxaphospholane) or reagent II (2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane) was added and the mixture was transferred into a 5 mm o.d. NMR tube. The spectra were acquired using a Bruker 300 MHz spectrometer equipped with a Quad probe dedicated to ^{31}P, ^{13}C, ^{19}F, and ^{1}H acquisition. Dried material after DFRC was dissolved in 800 μL of pyridine and deuterated chloroform (1.6:1, v/v), and an aliquot of 400 μL was transferred to a vial. Internal standards and relaxation reagents were added, and the mixture was analyzed as described before.

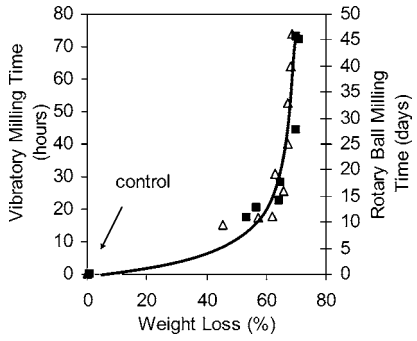

Figure 1. Weight loss during the enzymatic hydrolysis step as a function of vibratory (■) and ball milling (△).

RESULTS AND DISCUSSION

The demonstrated association between lignin and carbohydrates greatly influences the amount and structure of CELs and MWLs that can be extracted from wood (*13*, *15*, *16*, *23*). In general, the longer the milling times are, the higher the yield, but a MWL less representative of the native lignin has been obtained (*13*, *20*). To improve yields while minimizing damage on the lignin structure, the extent of mechanical action during milling must be reduced (*21*). Because a mild acid hydrolysis can liberate lignin from lignin−carbohydrate complexes (known to preclude lignin isolation in high yields), it may facilitate the isolation of less-modified lignin from milled wood (*15*). Consequently, the recently developed procedure aimed at isolating lignin with the combination of enzymatic and mild acidolysis presents a real opportunity in this respect since it can be combined with low-severity milling. In this paper, therefore, the effects of different milling severities on the various steps involved in the isolation of EMAL were evaluated. Vibratory and rotary ball milling were used in order to better understand the effects of severe and mild milling on the yield, purity, and structure of the obtained EMALs.

Effect of Milling on the Efficiency of Enzymatic Digestion. It was initially determined that the efficiency of enzymatic digestion for vibratory- and ball-milled wood varied similarly for both methods of pulverization and was a function of milling time (**Figure 1**). No weight loss was observed when Wiley-milled wood was directly treated with cellulases. Powdered wood before milling consists mostly of large particles with the basic morphological features of the wood unchanged (*34*). Such preparations are known to be impenetrable to cellulolytic enzymes (*35*). After milling, the accessibility of milled wood to cellulase increased, as shown by the wood weight loss observed during the enzymatic treatment (**Figure 1**). The longer the milling times are, the higher the wood weight loss up to 48 h of vibratory or 10 days of ball milling, when the weight loss reached a maximum of 70%. Longer milling times did not lead to greater weight loss (**Figure 1**). Progressive mechanical treatments are known to lead to the formation of increasing amounts of disordered cell wall material, which is rapidly digested by cellulases (*34*). In this way, the more the characteristic structure of wood breaks down, the more effective the enzyme becomes in attacking the disordered cell wall material. Besides cellulose and hemicelluloses, minor amounts of lignin

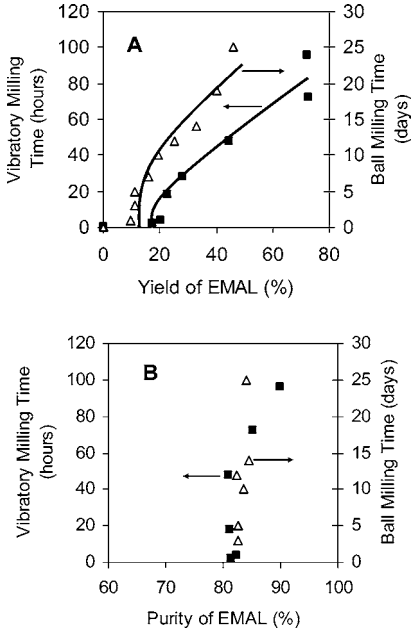

Figure 2. Yields (**A**) and purities (**B**) of EMALs as a function of the vibratory (■) and ball milling (△) times.

also became soluble during the enzymatic treatment. However, no further attempts were made to recover such soluble lignin fractions.

Effect of Milling on Lignin Yield and Purity. The effect of different milling severities on the yield and purity of the resulting EMALs was extensively examined, and the main data are shown in **Figure 2**. As anticipated, progressive mechanical treatments are seen to facilitate the disruption of the wood cell wall structure allowing for more lignin to be extracted. As a result, the longer the milling time is, the higher the EMAL yield, regardless of the type of milling used (**Figure 2A**). However, the combination of enzymatic hydrolysis and mild acid hydrolysis permitted the extraction of high yields of lignin even under the considerably milder rotary ball-milling conditions. More specifically, after 1 day of ball milling, the EMAL yield (w/w, based on the amount of Klason lignin of the starting wood and the isolated lignin) was 10% and it increased 5-fold, reaching about 50% after 25 days of milling. Prolonged ball milling beyond this time was found to offer more lignin, but the material liberated was not extensively examined. As a point of comparison, it is worth mentioning here that only 18.1% of MWL was obtained after 6 weeks of porcelain ball milling (*14*). Moreover, the data show (**Figure 2A**) that the more severe mechanical action applied onto the material by vibratory milling provided significantly higher yields within shorter periods of time (75% after 72 h). Vibratory milling for longer than 72 h had negligible effects on the yield indicating that the maximum EMAL yield obtained from Norway spruce is about 75%. As compared to the MWL and CEL isolated from the same batch of Norway spruce, milled for 48 h (vibratory milling), the yield of EMAL was 3.9 and 1.9 times greater than the corresponding MWL (11.6%) and CEL (23.4%), respectively. These data corroborate previous findings stating that the concerted effect of cellulolytic action and mild acid hydrolysis offers significant yield improvements over the traditional procedures for isolating lignin from wood (*15*, *36*).

Lignin samples isolated from wood still contain associated carbohydrates and other nonlignin contaminants, regardless of the isolation and purification procedures applied (*12*, *14*, *15*). Although always present, the amounts of such nonlignin materials may be affected by different factors, i.e, isolation procedure and wood species. As such, the purity of a lignin preparation, which is based on the sum of Klason and UV soluble lignin contents, represents the total amount of lignin after removal of such contaminants through a severe hydrolysis with 72% (w/w) H_2SO_4. During our work, progressive vibratory or ball milling was found not to have a significant effect on the purity of the lignin isolated as evidenced by the data of **Figure 2B**. The purity of these samples was nearly constant up to 48 h of vibratory milling or 25 days of ball milling. However, a sample isolated after 96 h of vibratory milling reached 90% purity. This increase may suggest that during extended vibratory milling some lignin−carbohydrate linkages may be cleaved. For example, Staccioli et al. (*37*) reported that the amount of ester groups formed by polyoses and lignin decreases due to extensive mechanical treatments performed. Besides esters groups, it is speculated that other types of lignin−carbohydrate interactions may also be cleaved, assisting the isolation of more pure EMAL. The purities of MWL (85.8%) and CEL (81%) isolated from the same batch of milled Norway spruce and purified according to Björkman (*17*) were similar to the values reported for MWL and CEL from different wood species (*12*, *14*, *15*).

Effect of Milling on Molecular Weight of the Lignin. Native lignin samples are usually sparingly soluble in solvents commonly used for SEC, with the EMAL samples studied here being of no exception, regardless of whether they were acetylated in pyridine or phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane. Under these circumstances, the following derivatization scheme for lignin that involved its reactive solubilization in acetyl bromide was established. The system has been extensively examined and offered as an effective alternative to sparingly soluble lignins or lignins requiring mixed polar solvents in the presence of inorganic salts.

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 (*28*). Similarly, benzyl aryl ethers are quantitatively cleaved to yield aryl acetates and acetylated α-bromide products (*28*). The concerted effect of acetylation when coupled with the polarity induced by the selective α-bromination caused every lignin sample examined in this work to become highly soluble in THF, allowing rapid SEC analyses. In an effort to ensure that the aforementioned acetobromination represents a feasible alternative derivatization technique, this procedure was compared with the traditional acetylation derivatization procedure using an organosolv (Aldrich) lignin. This lignin was selected for the comparison since it was completely soluble in THF after acetylation with acetic anhydride/pyridine. The SEC chromatograms displayed in **Figure 3A** show only minor differences in the UV response regarding both absorption and, most importantly, elution profiles. Overall, such data are supportive of the viability of using acetobromination as a derivatization technique since it represents a facile and rapid alternative to the complete solubilization of sparingly soluble lignin samples.

Figure 3B shows a typical set of SEC chromatograms of various lignins isolated from the same batch of Norway spruce after acetobromination. The size exclusion chromatograms of the EMAL samples displayed a highly polydisperse behavior as far as their molecular weight distributions are concerned. A high molecular weight fraction (albeit of low abundance) was apparent. Such polydispersity caused by this higher molecular weight fraction is not obvious neither in the CEL nor in the MWL. This is not totally surprising, however, when viewed in

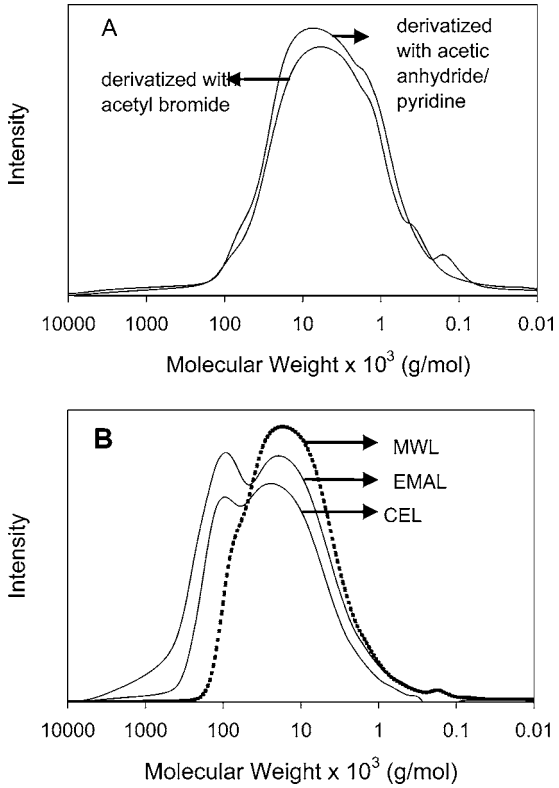

Figure 3. Gel permeation chromatograms of organosolv lignin after acetylation and after acetobromination with acetyl bromide (**A**) and typical SEC chromatograms of lignin samples isolated from the same batch of milled Norway spruce (**B**).

the light of the gelation statistics applied to lignification and delignification (*38*−*40*) as well as the recent conclusions of Lawoko et al. (*41*), where the molecular weight of lignin in spruce is described to be rather large. Furthermore, the often encountered lignin association effects, causing the formation of the aforementioned high molecular weight fraction, cannot be ruled out (*42*).

For the purposes of the present investigation, lignin association phenomena are not taken into account and the apparent molecular weight averages reported in **Tables 1** and **2** are on purpose uncorrected. This is done in order to demonstrate that the EMAL isolation procedure offers significant opportunities for the study of these interactions since the MWL and the CEL procedures do not offer lignin samples with such characteristics (**Figure 3B**). Current work in our laboratory attempts to evaluate the origin and the propensity of various lignin preparations to associate, and this is the subject of a manuscript in preparation.

To ensure that such association phenomena would not lead to a misinterpretation of the effects of milling on 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 are valid. Moreover, our molecular weight calculation strategy was based on integration of the whole SEC chromatogram, without arbitrarily excluding the high and the low molecular mass portions of it, as suggested by Baumberger et al. (*43*).

The data of **Figure 4** and **Table 1** show that for the case of ball-milled wood samples, the apparent molecular weight averages (M_w and M_n) were nearly constant for up to 10 days of ball milling. Longer milling times resulted in significantly greater molecular weight EMAL samples. This is indicative that as the ball milling time is prolonged the accessibility to higher

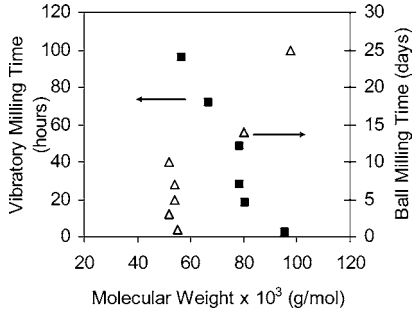

Figure 4. Weight-average molecular weight of EMAL isolated from vibratory (■)- and ball (△)-milled wood.

Table 1. Weight-Average Molecular Weight (M_w), Number-Average Molecular Weight (M_n), and Polydispersity (D) of EMALs Isolated from Ball-Milled Norway Spruce Wood[a]

ball milling time (days)	mol g^{-1} M_w	M_n	D	ball milling time (days)	mol g^{-1} M_w	M_n	D
1	55150	9260	5.9	10	52000	9100	5.7
3	53000	9200	5.8	14	79900	10450	7.6
5	54000	9150	5.9	25	97500	11120	8.8
7	54500	9424	5.7				

[a] Aggregation phenomena are not taken into account for the calculation of these averages.

Table 2. Weight-Average Molecular Weight (M_w), Number-Average Molecular Weight (M_n), and Polydispersity (D) of EMALs, MWL, and CEL Isolated from Vibratory-Milled Norway Spruce Wood[a]

vibratory milling times (h)	mol g^{-1} M_w	M_n	D	vibratory milling times (h)	mol g^{-1} M_w	M_n	D
2	95600	11000	8.7	72	66500	8700	7.6
18	80800	10150	7.9	96	56500	8600	6.6
28	71600	9500	7.5	MWL[b]	23500	6400	3.7
48[b]	78400	8850	8.8	CEL[b]	53850	9450	5.7

[a] Aggregation phenomena are not taken into account for the calculation of these averages. [b] Isolated from the same batch of milled Norway spruce wood.

molecular weight lignin fragments is increased. This becomes more apparent when one couples the data of **Figure 2A** and **Table 1**. It is clear that the concerted effect of mild ball milling followed by the EMAL sequence of enzymatic and mild acidolytic hydrolyses affords the isolation of significant amounts of lignin without any apparent damage on the lignin macromolecular structure. In this light, one should note that efforts to isolate MWL in similar yields necessitate extensive milling (*20*). Despite the fact that a yield of up to 50% can be achieved by increased milling times, a MWL that is less representative of the native lignin is known to be obtained (*12*, *14*, *16*, *21*).

In contrast to ball milling, the M_w and M_n of EMALs isolated from vibratory milled wood monotonically decreased as a function of vibratory milling time (**Table 2** and **Figure 4**). As anticipated, the severe mechanical action exerted on the macromolecule during vibratory milling apparently degrades the lignin macromolecular structure.

For comparative purposes, the size exclusion chromatograms of MWL and CEL isolated from the same batch of milled wood after 48 h of vibratory milling are also included in **Figure 3B**. While the chromatograms of EMAL and CEL display a bimodal behavior, the chromatogram of CEL does not contain the small fraction of the very high molecular weight material. The lack of such high molecular weight components was even more

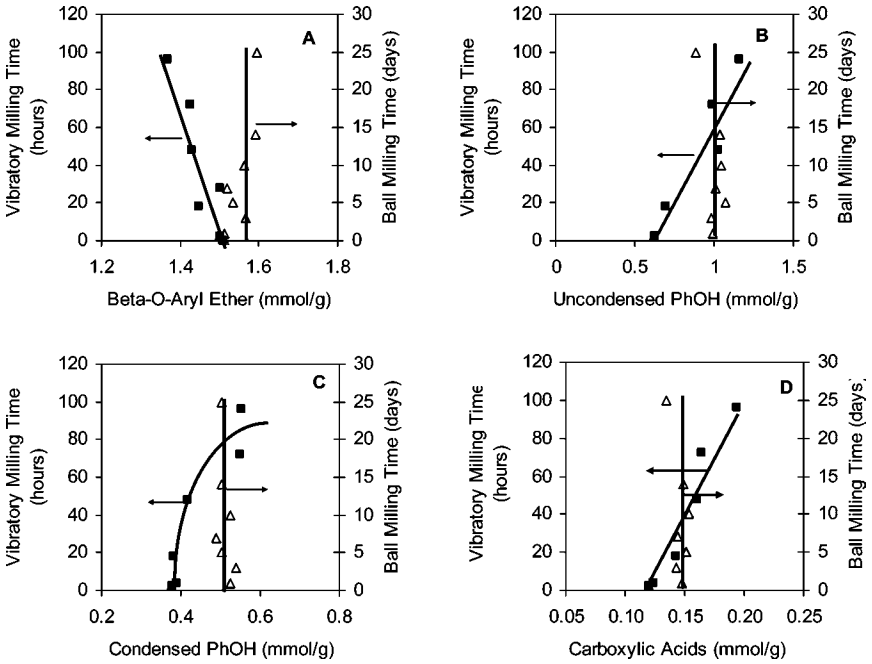

Figure 5. β-O-Aryl ether functional groups (**A**), uncondensed (**B**) and condensed (**C**) phenolic hydroxyl, and carboxylic groups (**D**) of EMALs as a function of the vibratory (■) and rotary (△) ball milling time.

pronounced in the chromatogram of MWL. The absence of the high molecular weight fractions made the weight-average molecular weights of both MWL and CEL significantly lower than the corresponding EMAL. More specifically, the M_w of EMAL isolated after 48 h of vibratory milling was over 78000 g mol^{-1}, while the weight-average molecular weights, determined for MWL and CEL, were 23500 and 53800 g mol^{-1}, respectively (**Table 2**). Overall, these data show that the combination of mild enzymatic and mild acidolyses allows the isolation of lignin fractions that are not accessible by neither of the alternative lignin isolation procedures.

Effect of Milling on Uncondensed β-Aryl Ethers and Other Structures of the Lignin. The various functional groups that define significant aspects on the structure of lignin were then determined using quantitative ^{31}P NMR spectroscopy for all EMAL samples isolated from vibratory- and ball-milled woods. The β-aryl ether structural content (**Figure 5A**) was determined after phosphitylating the Cα hydroxyl groups in these moieties with 2-chloro-1,3,2-dioxaphospholane (*31*, *44*). The condensed and uncondensed phenolic hydroxyls as well as the carboxylic acids (**Figure 5B**−**D**) were determined by phosphitylating the lignins with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (*32*). Quantification was then carried out via peak integration using *N*-hydroxynaphthalimide as an internal standard. Details of signal acquisition, assignment, and integration can be found elsewhere (*31*−*33*).

The data of **Figure 5A** show that while ball milling did not affect the β-O-4 structural content of the resulting EMALs, vibratory milling resulted in extensive cleavage of these lignin moieties. This finding is in accord with the work of Fujimoto et al. (*21*, *45*, *46*) and Ikeda et al. (*14*). A closer inspection of the data of **Figure 5A**, coupled with the data of **Table 2**, shows that when Norway spruce wood was subjected to vibratory milling for a period of 96 h, a moderate decrease (13%) in the β-aryl ether content of the lignin was observed, resulting in extensive lignin depolymerization (from 95600 to 56500 mol g^{-1}). For the same period of vibratory milling, the actual reduction of β-aryl ether content was 0.2 mmol g^{-1}. This reduction was found to be accompanied by an increase in uncondensed phenolic hydroxyls of 0.6 mmol g^{-1} (**Figure 5B**). This overproportional increase in phenolic hydroxyl groups indicates that β-aryl ether cleavage is not the only reaction taking place within lignin during vibratory milling. This is not surprising, since free radicals are known to be formed during mechanical treatment of lignocellulosic material (*47*). Such reactions may be accompanied by a series of autoxidation reactions, which could eventually result in oxidative fragmentation of the lignin macromolecule with the concomitant creation of carboxylic acid groups in the lignin, apparent in vibratory milling EMALs as shown in **Figure 5D**. Under ball-milling conditions, no such oxidation reactions are apparent and the COOH content of the emerging EMALs was found to be remarkably constant throughout the milling period extending into 25 days (**Figure 5D**). Another side reaction that could also take place, once free radicals are formed, is that of radical coupling, causing the creation of condensed carbon−carbon bonds within the lignin. This is evidenced by the increase of condensed phenolic hydroxyls apparent during vibratory milling and totally absent during ball milling (**Figure 5C**).

Effect of Milling on Other Lignin Structures. Most of the recent conclusions regarding the bonding patterns of native lignins have been derived from chemical degradation techniques, such as thioacidolysis, due to its sensitivity and demonstrated lack of artifacts (*48*). In 1997, a new selective β-aryl ether cleavage protocol, termed DFRC, was proposed by Lu and Ralph (*28*, *49*−*51*). The DFRC method uses a mild depolymerizing environment and has the advantage of avoiding the use of malodorous reagents. While DFRC provides a clean and selective protocol for ether scission, the primary DFRC monomers detected and quantified by GC are confined to phenylpropane units connected to β-aryl ether bonds on both sides of the phenyl propanoid units or terminal phenylpropane units connected to the polymer via a β-aryl ether bond. Consequently, by using GC alone for detecting the DFRC monomers, the total amount of β-aryl ether linkages cannot be revealed. For example, if a β-aryl ether connects two macromolecules or oligomers that themselves are interlinked via structures other than β-aryl ethers, the size of the fragments will preclude them from being detected

Table 3. Thioacidolysis and DFRC/^{31}P NMR Data from EMAL Isolated from Norway Spruce Ball-Milled for 25 Days

	μmol/g		
quantified by	G units involved only in uncondensed β-O-aryl bonds	H units involved only in uncondensed β-O-aryl bonds	total uncondensed β-O-aryl bonds
thioacidolysis	721 ± 15 (13.5)[a]	6 ± 1 (0.1)[a]	727 ± 15 (13.6)[a]
DFRC/^{31}P NMR	740 ± 10 (13.8)[a]	70 ± 15 (1.3)[a]	825 ± 15 (15.3)[a]

[a] In mol % (based on C9 unit molecular weight of 187).

by GC as DFRC monomers (*26*, *49*−*52*). The coupling of the DFRC technique with ^{31}P NMR can overcome this limitation and was recently shown to offer new quantitative information about β-aryl ethers linked to condensed and noncondensed aromatic moieties, including dibenzodioxocins (*26*). Such enquiries could thus prove indispensable in further understanding the effects of milling on the structure of isolated EMALs, MWLs, and CELs.

However, to ensure that the conclusions were independently validated, a sample of Norway spruce EMAL, isolated after 25 days of ball milling, was submitted to comparative analyses that included DFRC/^{31}P NMR and thioacidolysis. The comparison between the two analytical protocols is shown in **Table 3**. There is apparently a good agreement between the two methods in the total amount of uncondensed β-O-aryl bonds, demonstrated for the first time on the same sample of isolated lignin.

Because ^{31}P NMR can distinguish condensed from uncondensed phenolic hydroxyls, the combination of DFRC with ^{31}P NMR was used to estimate the effects of milling on the amount of condensed and uncondensed β-aryl ether structures (**Figure 6**). Condensed β-aryl ether bonds refer to structures that connect two macromolecules or oligomers that themselves are interlinked via structures other than β-aryl ethers. By considering the data of **Figure 5A** in conjunction with that of **Figure 6A**, one arrives at the conclusion that the lignin depolymerization that is taking place during vibratory milling is due to the cleavage of uncondensed β-aryl ether linkages. Interestingly, the condensed β-aryl ether structures were found to be resistant to the intense mechanical action caused by vibratory milling and remained unchanged up to 96 h of vibratory milling (**Figure 6B**). **Figure 6** also shows that condensed and uncondensed β-aryl ethers were not affected by ball milling.

Dibenzodioxocins (5-5/β-O-4, α-O-4) can also be determined using the combination of DFRC with quantitative ^{31}PNMR, by integrating the region from 141.2 to 142.4 ppm, which has been attributed to the 5-5′-liberated phenols after these moieties open up during DFRC (*26*). **Figure 6C** shows, for the first time, that the 5-5/β-O-4/α-O-4 ring of dibenzodioxocins is resistant to the mechanical action of vibratory milling.

The functional group content of EMAL, MWL, and CEL determined by quantitative ^{31}P NMR is shown in **Table 4**. The nearly identical amounts of total β-aryl ether functional groups of EMAL and CEL indicate no evidence of β-aryl ether bond degradation within the lignin during the mild acid hydrolysis step of the EMAL protocol. MWL is seen to contain slightly higher amounts of β-aryl ether linkages (~3.7/100 C9) than the EMAL and CEL. However, as shown by its yield and molecular weight, MWL represents the low molecular weight extractable lignin fragments soluble in dioxane rather than the overall lignin from wood.

The aliphatic hydroxyl group data for EMAL, MWL, and CEL were found to be 1.2/C9, 1.0/C9, and 0.9/C9, respectively.

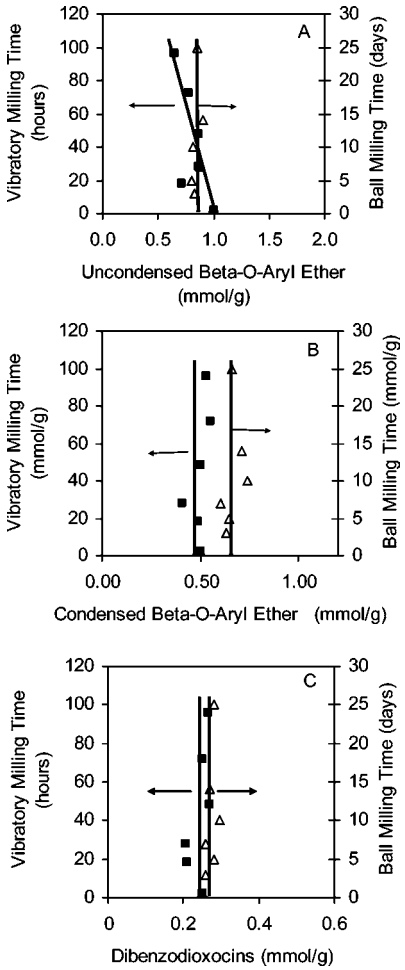

Figure 6. Uncondensed (**A**) and condensed (**B**) phenolic hydroxyl groups (equivalent to the amount of uncondensed and condensed β-O-aryl ether linkages, respectively) and dibenzodioxocins units (**C**) as a function of the vibratory (■) and rotary (△) ball milling time. Condensed β-O-aryl ether linkages are defined as structures that connect two macromolecules or oligomers that themselves are interlinked via structures other than β-O-aryl ethers.

Table 4. Functional Group Contents,[a] Yields, and Weight-Average Molecular Weight (M_w) for EMAL, MWL, and CEL Isolated from the Same Batch of Milled Norway Spruce

functional group	EMAL (per 100 C9)	MWL (per 100 C9)	CEL (per 100 C9)
total β-aryl ether	26.0	29.7	25.7
aliphatic, OH	125	103	92
guaiacyl, OH	16.6	14.4	12
p-hydroxyl, OH	1.4	1.5	1.7
uncondensed PhOH	18.0	15.6	13.4
condensed PhOH	7.6	6.0	7.7
total PhOH	25.6	21.6	21.1
carboxylic groups	2.9	2.3	2.3
yield (%)[b]	44.5	11.4	23.4
M_w (g/mol)	78000	23500	53850

[a] Determined by quantitative ^{31}P NMR (error ± 1) based on a C_9 unit molecular weight of 187 g/mol. [b] On the basis of Klason lignin contents of extracted ground wood meal.

These values compare reasonably well with the literature range (0.8−1.2/C9) (*15*), considering the uncertainties associated with carbohydrates contaminants in this determination.

The contents of carboxylic acid groups (2.3−2.9/100 C9) were also very similar to those reported in the literature for

EMAL and MWL from spruce (*15*). These similarities provide further support for the effectiveness of the EMAL protocol in providing nonoxidized lignin.

The uncondensed phenolic hydroxyl content of EMAL was found to be somewhat higher than that of MWL and CEL. Because there is no evidence of liberation of phenolic hydroxyl groups from β-aryl ether linkages (**Figures 5** and **6** and **Table 4**), the higher content of such functional groups in EMAL is supportive of the notion that this lignin preparation is more representative of the overall lignin present in milled wood. As shown before, one of the effects of vibratory milling is the increase in the amount of uncondensed phenolic hydroxyl groups. Such phenolic groups are seen to increase 3-fold when compared to the reduction of β-aryl ether linkages. Because the yield of EMAL is 3.9 and 1.9 times greater than MWL and CEL, respectively, the higher phenolic contents in such lignins are not surprising when one considers the more representative nature of the EMAL sample.

Conclusions. It can be surmised 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 wood. EMAL is released by cleaving lignin−carbohydrate bonds rather than via the degradation of ether bonds within lignin. 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 provided by the mild acid hydrolysis step offers the possibility of obtaining high yields using low intensity milling. 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.

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