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Studies on the Effect of Ball Milling on Lignin Structure Using a Modified DFRC Method

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The structures of milled wood lignin (MWL), cellulolytic enzyme lignin (CEL), and residual lignin (REL) from a loblolly pine were analyzed using a modified derivatization followed by reductive cleavage (DFRC) method developed to allow the quantitative determination of three different structural monomeric products originating in lignin: phenolic β-*O*-4, α-*O*-4, and etherified β-*O*-4 structures. Results show that MWL and CEL are structurally identical, with an increased phenolic β-*O*-4 content compared to that of the original Wiley milled wood. These results indicate that the portion of lignin linked to carbohydrates and that not linked to carbohydrates are structurally the same. Modified DFRC analysis of the effect of ball milling on the structure of lignin in wood, MWL, CEL, and REL indicate that vibratory ball milling does not change the lignin structure provided certain precautions are taken. Specifically, dry vibratory ball milling under a nitrogen atmosphere causes substantial structural changes including condensation, whereas vibratory ball milling in toluene had little effect on the lignin structure. This indicates that the structural differences observed in MWL and CEL arise because of the extraction procedure, which preferentially extracts phenolic lignin structures. MWL and CEL are representative of the total lignin in wood; however, due primarily to the solvent extraction process, higher phenolic hydroxyl contents are observed. Nitrobenzene oxidation showed structural results similar to those from the modified DFRC method.

KEYWORDS: Aryl ether bonds; milled wood lignin (MWL); cellulolytic enzyme lignin (CEL); ball milling; native lignin; structure

INTRODUCTION

Lignin, the second most abundant natural polymeric material on earth, next to cellulose, is extremely complicated, and its structure has not yet been completely elucidated. In situ, lignin has no structural regularity. Unlike most natural polymers, which consist of a single intermonomeric linkage, lignin is a network polymer made up of many carbon-to-carbon and ether linkages. The tight physical binding and chemical linkages between lignin and cell-wall polysaccharides also practically prevents its isolation in an unaltered form. This makes it very difficult to use degradative or nondegradative methods for structural determination. As a result, our understanding of the structure of native lignin has been formed as a sum of the information obtained from different fields of lignin research, namely, studies concerning the elucidation of the mechanisms of lignin biosynthesis (*1*−*3*) and analytical data obtained in studies with isolated lignin specimens (*4*, *5*).

A major problem in native lignin structure elucidation has been in trying to isolate as much of the lignin as possible while minimizing the extent of chemical modification. Traditionally, milled wood lignin (MWL) has been used as a representative source of native lignin. The procedure for isolating milled wood lignin involves the production of wood meal by Wiley milling of the wood, followed by vibratory or rotary ball milling, and subsequent extraction with dioxane−water. The yield of MWL varies depending on the extent of milling, ranging from 25 to 50% (*6*). It is believed that by increasing the extent of milling, and thus MWL yield, a lignin sample more representative of the total lignin in wood is produced. However, severe chemical modification of the lignin occurs. In fact, increases in carbonyl content (*7*, *8*) and phenolic hydroxyl content, as well as decreases in molecular weight (*8*) and cleavage of aryl ether linkages (*9*), have been reported as a result of the MWL isolation procedure. Whiting and Goring (*10*) further showed that MWL is not representative of the whole lignin in wood, but primarily originates in the secondary wall of the cell; with differences in rate of extraction after ball milling due to inherent differences in the chemistry of lignin in the middle lamella and the secondary wall being responsible. However, no systematic study on the extent of structural changes in lignin during milling has been reported.

A number of degradative methods exist which provide information regarding the structure of lignin through the generation

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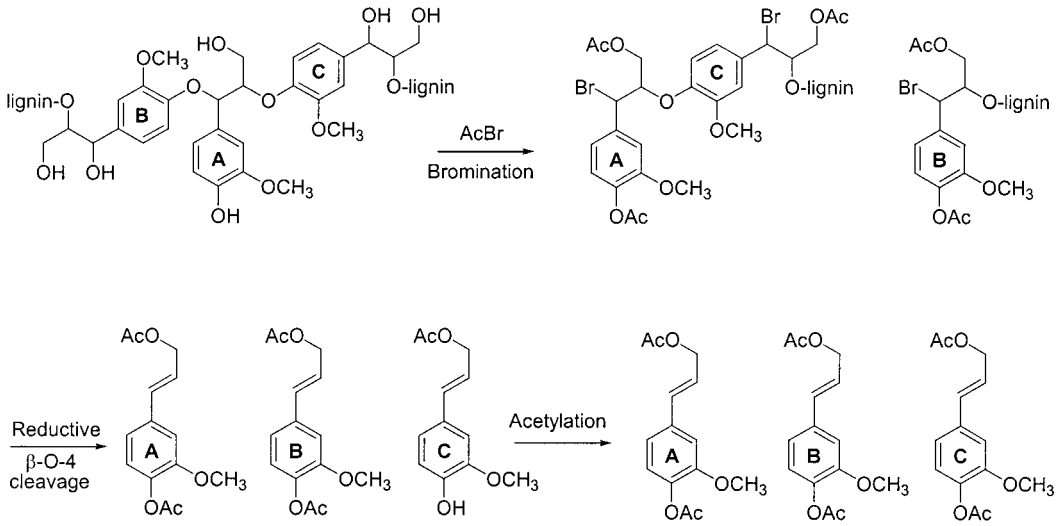

Figure 1. Schematic representation of the original DFRC method: unit A, from phenolic *β-O*-4 units; unit B, from α-*O*-4 units; unit C, from etherified *β-O*-4 units.

of low-molecular-weight compounds (*11*). Through careful analyses of these compounds a detailed picture of the original lignin can emerge. Lignin dimers and trimers, which contain most of the inter-unit linkages, have been extensively reported from hydrogenolysis (*12*), thioacidolysis (*13*−*16*), permanganate oxidation (*17*), and other methods. Unfortunately, these methods are extremely labor intensive, involving extensive sample preparation and reaction times. In addition, quantitative analyses are difficult owing to the low yields of degradation products. Recently, a new method has been developed which appears to eliminate many of the aforementioned problems. This method, derivatization followed by reductive cleavage (DFRC), is based on a partial degradation of lignin using acetyl bromide, resulting in the cleavage of α-aryl ethers and the formation of acetylated α-bromo derivatives, followed by reductive cleavage of the *β*-aryl ether linkages (*18*−*22*). It is reportedly much more quantitative in the yields of low-molecular-weight products, with >95% yield of targeted products in model compound studies. Moreover, DFRC can be used on whole wood samples, eliminating the need for lignin isolation. Most recently, DFRC has been coupled with ^{31}P NMR to enable further characterization of macromolecular lignin, addressing issues concerning degradation product yields (*23*). However, the inability of ^{31}P NMR to be used in samples containing large amounts of carbohydrates limits its utility to isolated lignins (*24*).

The major degradation product from the DFRC method is acetylated coniferyl alcohol, including both cis and trans isomers, originating from uncondensed *β-O*-4 and α-*O*-4 lignin structures (Figure 1). Unfortunately, because of the complete acetylation of all of the generated degradation products in the DFRC process, no determination can be made as to the origin of the various lignin moieties liberated. Therefore, we have modified the DFRC method to enable us to identify and quantify the phenolic *β-O*-4, α-*O*-4, and etherified *β-O*-4 structures (Figure 2). We now describe the modified method and utilize it to study the structure of MWL and the effect of ball milling on lignin structure, comparing our results with those obtained from nitrobenzene oxidation.

MATERIALS AND METHODS

Materials. Loblolly pine (*Pinus taeda* L.) sapwood was ground to pass a 20-mesh screen in a Wiley mill, Soxhlet extracted with ethanol/benzene (1:2, v/v) for 24 h, and followed by ethanol extraction for an additional 24 h (Wiley wood). The resulting Wiley wood was air-dried and stored in a desiccator under vacuum over P_2O_5. Wiley wood (100 g) was ground in a 1-gallon porcelain jar under a nitrogen atmosphere using either glass, porcelain, or Teflon balls. The jar was then placed on a rotary mill for periods of 1 week (rotary glass 1 week wood), 4 weeks (rotary glass 4 week wood, rotary porcelain 4 week wood), and 6 weeks (rotary glass 6 week wood, rotary porcelain 6 week wood, rotary teflon 6 week wood). The rotary glass 1 week wood (10 g) was then ground in a vibratory ball mill (Siebtechnik GMBH, 433 Mulheim 011380, Germany) with stainless steel balls for 48 h under a nitrogen atmosphere in the presence and absence of toluene (vibratory wood, vibratory wood in toluene).

MWL, CEL (cellulytic enzyme lignin), and REL (residual enzyme lignin) were isolated according to Figure 3. Specifically, MWL was isolated according to the method of Björkman (*25*), wherein ball milled wood (25 g) was extracted 2 times with dioxane/water (250 mL; 96:4, v/v) for 24 h under a nitrogen atmosphere. The solution was collected by centrifugation and concentrated (100 mL). The crude MWL, isolated by freeze-drying, was dissolved in acetic acid/water (20 mL; 9:1, v/v) and precipitated into water (400 mL). The precipitated lignin was filtered, dissolved in 1,2-dichloroethane/ethanol (10 mL; 2:1, v/v), and precipitated into ether (200 mL, MWL). CEL was isolated from the 96% dioxane insoluble residue. The residue was washed with water (200 mL) to remove dioxane, and suspended in acetate buffer (250 mL) (4.1 g of sodium acetate and 2 mL of acetic acid in 1 L of water, pH 4.8). Enzyme, (10 mL of Dyadic Super Ace (Dyadic International Inc., Jupiter, FL)), was added, and the reaction slurry was incubated for 24 h at 55 °C. The enzyme treatment was repeated two times. The solution was centrifuged and the enzyme-treated residue was washed with water (200 mL), followed by repeated extraction (2 × 24 h) with dioxane/water (250 mL; 96:4, v/v) under nitrogen atmosphere. The solution was collected by centrifugation and concentrated (100 mL). The crude CEL was freeze-dried and purified as per the MWL. The residue remaining after CEL isolation was freeze-dried to produce the REL sample.

Methylation. All methylations were performed using a diazomethane ether solution produced from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide. For the milled wood (1.0 g) and REL (1.0 g), the samples were suspended in dioxane/methanol (20 mL; 2:1, v/v) followed by the addition of the diazomethane ether solution (5 mL) and stirred for 3 h at room temperature. The ether phase was removed by evaporation, and fresh diazomethane (2 mL) was added. Diazomethane methylation was repeated 6 times. After the final methylation, the solution was removed by evaporation, and the residue was washed with diethyl ether (100 mL) and dried. The completeness of methylation was determined using the $\Delta\epsilon$ method (*11*) and the absence of a bathochromic shift in

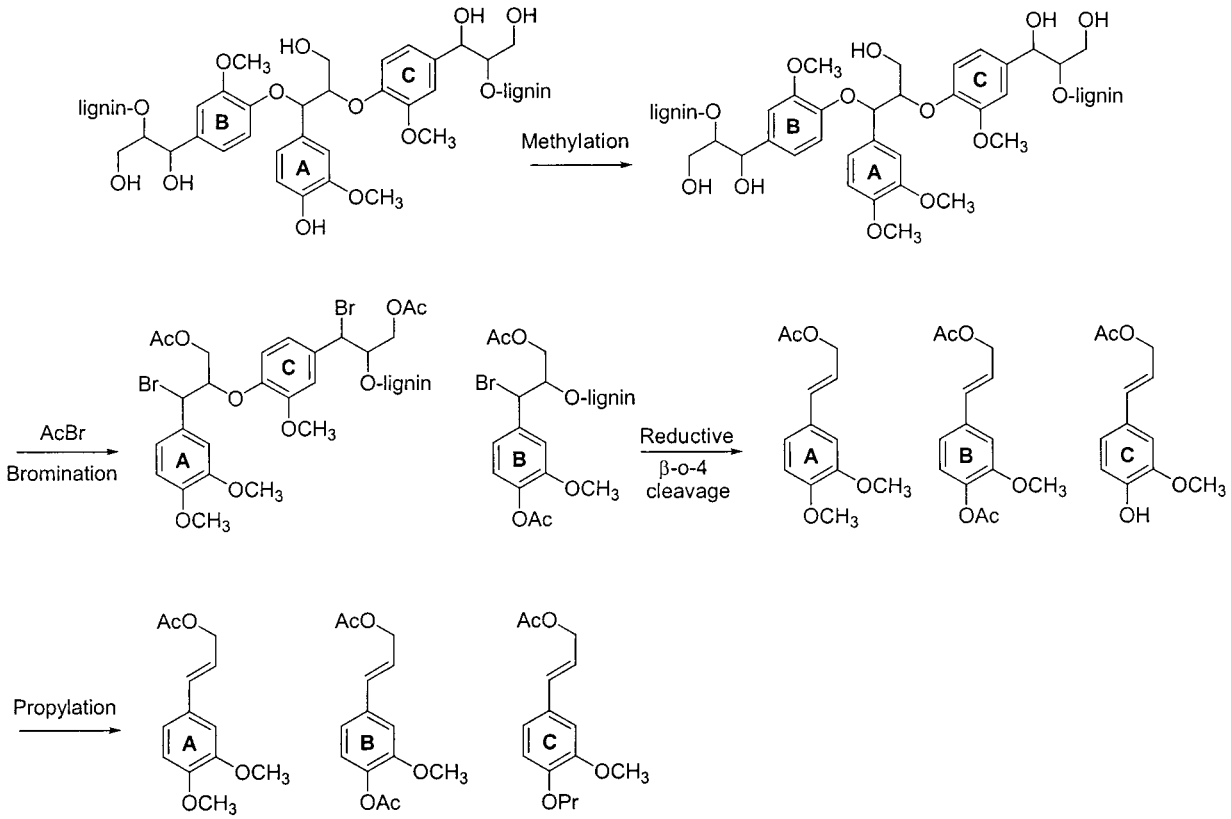

Figure 2. Schematic representation of the modified DFRC method: unit A, from phenolic β-*O*-4 units; unit B, from α-*O*-4 units; unit C, from etherified β-*O*-4 units.

the UV difference spectra. In addition, ^{1}H NMR analysis of acetylated methylated lignin showed no phenolic acetate group (*26*). In the MWL (1.0 g) and CEL (1.0 g), the samples were dissolved in dioxane/methanol (20 mL; 2:1, v/v) and reacted for 1 h with diazomethane (5 mL). Methylation with diazomethane was repeated 3 times; then the samples were concentrated, dissolved in dioxane/methanol (5 mL; 2:1, v/v), precipitated into ether (100 mL), and collected by filtration.

Modified DFRC Method. Milled wood (50 mg), MWL (25 mg), CEL (25 mg), and REL (25 mg) were reacted with acetic acid/acetyl bromide (5 mL; 4:1, v/v) with tetracosane (5 mL) as a internal standard and stirred for 3 h at 50 °C. The reaction was terminated by removal of the solvent under vacuum; the residue was then immediately dissolved in dioxane/acetic acid/water (5 mL; 5:4:1, v/v) and reacted with zinc (250 mg) while stirring for 30 min at room temperature. The zinc dust was removed by filtration, and the solution was extracted two times with dichloromethane (20 mL). The organic layer was dried with anhydrous sodium sulfate and removed by evaporation. The residue was dissolved in pyridine/propionic anhydride (2 mL; 1:1, v/v) and stirred for 40 min at room temperature. The reaction was stopped by removing the solvent, and the residue was dissolved in dichloromethane (0.5 mL) and analyzed by gas chromatography. To ensure that the results obtained for the propionate derivatives (unit C) were not the result of transesterification with the acetyl group of unit B, coniferyl diacetate was reacted with a large excess of propionic anhydride/pyridine at room temperature. GC analysis of the reaction showed no change in the coniferyl diacetate concentration even after 24 h of reaction at room temperature, indicating no transesterification occurred. Vibratory wood (dry) and MWL (dry) samples were repeated 4 times, and the total yields were 13.6 $\pm$ 0.71 and 16.9 $\pm$ 0.62 mol %, respectively (*27*).

Gas Chromatography Analysis. Monomeric products produced from the DFRC treatments were quantitatively determined by gas chromatography (GC) (Hewlett-Packard 6890). The analytical column was a Hewlett-Packard HP-1, (30 m $\times$ 0.32 mm i.d.). The carrier gas was helium with a flow rate of 2.0 mL/min. The GC conditions were as follows: injection temperature 220 °C with a split ratio of 10:1; FID detector temperature 310 °C; column temperature 100 °C, which was held for 1 min, raised 3 °C/min to 240 °C, held for 1 min, raised 30 °C/min to 300 °C, and held for 5 min. The amounts of individual monomers were determined by using response factors derived from pure compounds relative to tetracosane as the internal standard. Response factors of 6.69 (unit A, cis and trans forms, in Figure 2) and 2.86 (unit B and unit C, cis and trans forms, in Figure 2) were used.

Nitrobenzene Oxidation. Nitrobenzene oxidation was performed according to the method of Leopold (*28*).

RESULTS AND DISCUSSION

Modified DFRC Method. In the original DFRC method, three steps are involved as shown in Figure 1. The first step involves acetylation of all free hydroxyl groups, including both phenolic and aliphatic hydroxyl groups, and bromination of the α-position in both α-hydroxyl and α-ether structures. In the second step, the β-*O*-4 structures are reductively cleaved with zinc followed by acetylation of the newly released phenolic hydroxyl groups with acetic anhydride. Thus, a single monomeric product, coniferyl alcohol diacetate, is produced from the trilignol as shown in Figure 1 (units A, B, and C). Both the cis and trans isomers of the diacetate are formed and quantitatively determined by GC.

In our modified DFRC method, a new step is added to first methylate of the free-phenolic hydroxyl groups in the original lignin. This is followed by the same acetyl bromide treatment and zinc reduction steps as in the original DFRC procedure. In the final step, propionic anhydride, instead of acetic anhydride, is used to esterify the newly generated phenolic hydroxyl groups. Thus, the modified DFRC method, as shown in Figure 2, allows the quantitative determination of three different monomeric units, the uncondensed phenolic β-*O*-4 (unit A), the uncondensed α-*O*-4 (unit B), and the uncondensed etherified β-*O*-4 (unit C) structures in lignin.

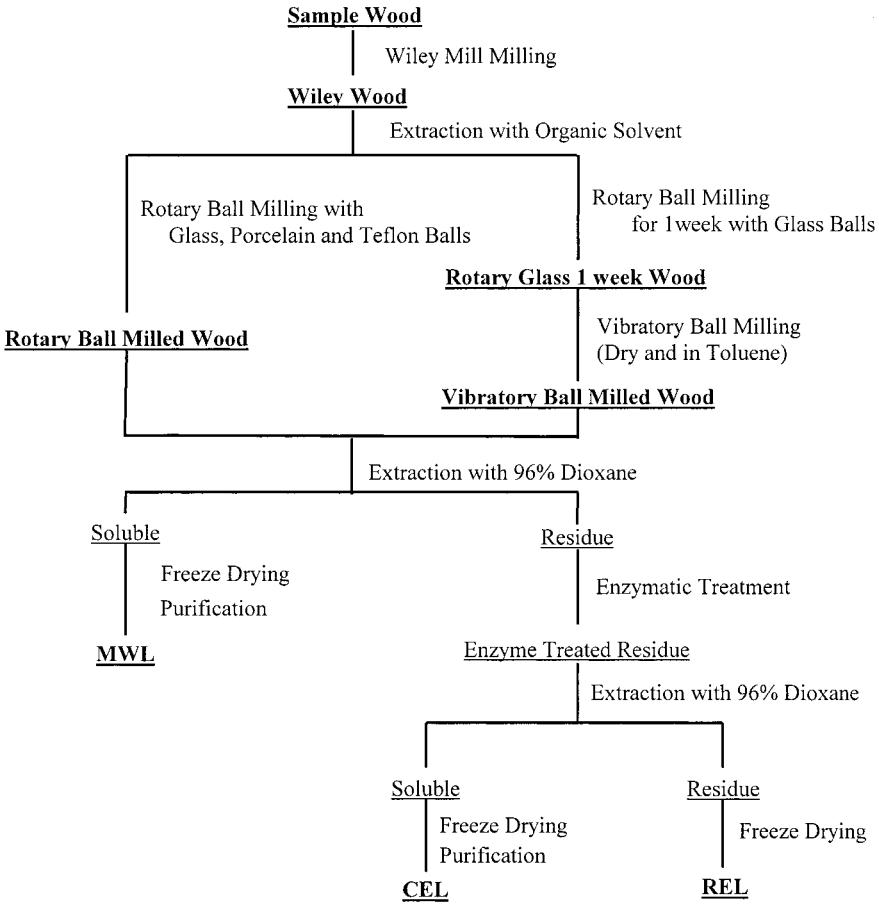

Figure 3. Isolation procedure for milled woods, MWL, CEL, and REL.

In this study with loblolly pine, DFRC monomeric products derived from both guaiacylpropane and *p*-hydroxyphenylpropane moieties were found, whereas no syringylpropane structures were observed. The yield of monomers from guaiacylpropane structures accounted for over 95% of the total monomer yield, therefore only the yields from guaiacylpropane structures were reported.

Effect of MWL Isolation on Lignin Structure. A typical procedure used in the isolation of MWL is to first Wiley mill the wood and then subject it to vibratory milling in toluene for 48 h (*7*). The resulting wood meal is then extracted to yield MWL. The progressive mechanical treatments facilitate the disruption of the wood structure to enable the lignin to be removed by extraction. We first analyzed the various steps in the MWL isolation procedure to determine whether MWL is representative of lignin in its native state. Wiley Wood, rotary glass 1week wood, vibratory wood (dry and toluene), and MWL that was isolated from vibratory wood (vibratory dry MWL and vibratory in toluene MWL) were analyzed using the original and our modified DFRC methods. The results are given in Table 1. Good agreement was obtained for the total yield of monomeric products detected from each sample by the original and modified DFRC methods. Furthermore, the values obtained for dry MWL (16.9 wt % modified, 17.8 wt % original) were very close to the results reported by Lu and Ralph (*19*) for loblolly pine MWL (17.1 wt %). These results clearly indicate the efficacy of the modified DFRC method.

Comparing DFRC results between the rotary glass 1 week wood and the original Wiley wood shows little change in the amount of the three different monomeric units. There was a slight increase (3.4 wt %) in the phenolic β-*O*-4 structures (unit A, Table 1) and a slight decrease (6.4 wt %) in the etherified β-*O*-4 structures (unit C, Table 1). Thus, rotary ball milling appears to have minimal effect on the structure of lignin. The vibratory wood (dry), which was produced from the rotary glass 1 week wood by grinding in a vibratory ball mill, without toluene, for 48 h showed a dramatic decrease in the DFRC monomeric products. The total yield of monomeric products decreased by one-third. However, in the toluene vibratory ball milled wood, no decrease in monomer yield was observed. As the monomeric products originate exclusively from the uncondensed units in lignin, the results suggest that vibratory ball milling under nitrogen atmosphere (dry ball milling) may cause some condensation reactions in lignin. In fact, the α-*O*-4 aryl ether content increased substantially as a result of dry vibratory

Table 1. Yields of Monomeric Products from Original and Modified DFRC Methods

	unit composition (mol %)[a]			total yield[b]	
sample	unit A	unit B	unit C	wt %	mol %[c]
Wiley wood	21.6	6.8	71.6	19.6	13.2
original				20.2	13.8
rotary glass 1 week wood	25.0	9.7	65.3	20.3	13.6
original				20.7	14.1
vibratory dry wood	23.9	12.5	63.6	13.6	9.2
original				12.5	8.5
vibratory dry MWL	40.9	10.0	49.1	16.9	11.7
original				17.8	12.2
vibratory in toluene wood	26.7	7.8	65.5	21.5	14.8
vibratory in toluene MWL	41.3	7.7	51.0	21.0	14.5

[a] Based on monomeric products (total of units A, B, and C in Figure 2). [b] Based on Klason lignin. [c] Based on a C9 unit molecular weight of 182.

milling (unit B, Table 1). In addition, the total yield of DFRC monomeric products decreased by over 30 wt %. This is not surprising in view of the report by Hon and Glasser (*29*) that ball milling of lignocellulosics results in mechanical depolymerization involving free radicals. In contrast, the vibratory milling in the presence of toluene showed almost no difference in monomeric linkages or yield as compared to the original Wiley wood.

MWL was isolated from the vibratory wood in a yield of 22.8 wt % (dry milling) and 18.0 wt % (toluene milling) based on the Klason lignin content of the original wood. As shown in Table 1, total monomeric DFRC products of the yield of the vibratory MWL dry (16.9 wt %) was lower than that of the Wiley wood (19.6 wt %) and the rotary glass 1 week wood (20.3 wt %), but was higher than that of the vibratory wood (13.6 wt %). In contrast, the total monomeric DFRC products of the vibratory MWL toluene (21.0 wt %) were the same. The distribution of the three monomeric products was quite different between the vibratory MWL (dry and toluene) and the corresponding vibratory wood. The vibratory MWLs had higher phenolic β-*O*-4 (40.9 wt % and 41.3 wt %) and lower etherified β-*O*-4 (49.9 wt % and 51.0 wt %) structures than the vibratory wood (23.9 wt % and 26.7 wt %, and 63.6 wt % and 67.0 wt %, respectively) consistent with the recent findings of Onnerud and Gellerstedt (*30*) using thioacidolysis. It appears that structural changes occur during vibratory ball milling, whether under dry conditions or in the presence of toluene, resulting in depolymerization of the lignin. In addition to this is the selective extraction of specific lignin structures in the isolation of the corresponding MWL. Thus, vibratory MWL (dry and toluene) may represent the lower-molecular-weight and less-condensed fraction and may not be representative of the total lignin, representing instead that of the secondary wall lignin as presented by Whiting and Goring (*10*) and Terashima et al. (*31*). Furthermore, it is clear that dry vibratory ball milling is not as good a method as vibratory ball milling in toluene.

Effect of Ball Milling on Lignin Structure. Rotary ball milling, 1 week, has minimal impact on the yield and structure of the lignin units detectable by DFRC. However, to isolate MWL in appreciable yields, more extensive milling, namely vibratory milling, is required. To minimize the effect of structural changes associated with vibratory milling, and to increase MWL yields, longer rotary ball milling stages were investigated. Milling times were increased to 4 weeks (rotary glass 4 week wood) and 6 weeks, (rotary glass 6 week wood). In addition, rotary ball milling with porcelain balls and Teflon balls (which have higher densities than glass balls) were performed at 4 weeks (rotary porcelain 4 week wood), and 6 weeks (rotary porcelain 6 week wood and Teflon 6 week wood). All rotary ball milling was dry milling under a nitrogen atmosphere. MWL, CEL, and REL were isolated from the various ball milled woods, and subjected to modified DFRC analysis. Included for comparison are the results obtained from the classical Björkman method.

The yields of MWL and CEL are given in Table 2. As expected, yields increased with increasing milling time. Ball milling with porcelain balls gave higher MWL and CEL yields than milling with glass balls. The higher density of the porcelain balls inflicts a larger mechanical impact on the wood meals, enabling more lignin to be extracted in the subsequent dioxane/water extraction stage. Ball milling with Teflon balls, also of higher density than glass balls, gave much lower MWL and CEL yields than milling with glass balls. In this case, the low surface energy of the Teflon balls does not enable enough friction and mechanical action to be imposed on the wood meal, limiting the effective milling. To obtain MWL and CEL yields comparable to those produced from 1 week rotary ball milling followed by 48 h vibratory milling (conventional MWL isolation), 6 weeks of ball milling with porcelain balls is required.

Table 2. Yields of MWL and CEL

sample		yield (%)[a]	lignin (%)[b]
rotary glass 4 week	MWL	3.7	87.3
	CEL	5.3	68.3
rotary porcelain 4 week	MWL	11.1	87.1
	CEL	7.9	64.2
rotary glass 6 week	MWL	13.1	83.2
	CEL	7.6	87.5
rotary porcelain 6 week	MWL	18.1	87.7
	CEL	12.3	84.4
rotary Teflon 6 week	MWL	0.9	71.8
	CEL	1.0	66.5
vibratory in toluene	MWL	18.0	91.1
	CEL	11.0	84.7
vibratory (dry)	MWL	22.8	87.7

[a] Based on Klason lignin of wood (ash free). [b] Klason lignin content.

Table 3. Yields of Monomeric Products from Modified DFRC

		unit composition[a]			total yield[b]	
sample		unit A	unit B	unit C	wt %	mol %[c]
rotary glass 4 week	wood	25.7	7.6	66.7	19.3	13.1
	MWL	38.2	3.8	58.0	20.6	14.2
	CEL	34.4	5.8	59.8	21.0	14.4
	REL	27.7	5.1	67.2	17.0	11.6
rotary porcelain 4 week	wood	33.6	6.7	59.7	22.9	15.8
	MWL	37.8	4.7	57.5	20.5	14.2
	CEL	32.5	6.2	61.3	21.4	14.8
	REL	27.8	4.1	68.1	13.4	9.2
rotary glass 6 week	wood	26.6	5.5	67.9	19.3	13.2
	MWL	46.1	4.8	49.1	23.0	16.3
	CEL	39.0	6.3	54.7	22.0	15.1
	REL	23.7	5.0	71.3	15.9	10.8
rotary porcelain 6 week	wood	30.5	6.8	62.7	18.1	12.5
	MWL	44.0	4.7	51.3	22.0	15.5
	CEL	42.3	5.4	52.3	20.6	14.4
	REL	24.5	5.8	69.7	18.2	12.4
vibratory in toluene	wood	26.7	7.8	65.5	21.5	14.8
	MWL	41.3	7.7	51.0	21.0	14.5
	CEL	36.6	6.5	56.9	21.0	14.5
	REL	25.2	5.3	69.5	19.0	12.9

[a] Based on monomeric products (total of units A, B, and C in Figure 2). [b] Based on Klason lignin. [c] Based on a C9 unit molecular weight of 182.

Modified DFRC analysis of the various milled samples is shown in Table 3. Regardless of the milling time, the monomeric product yield of each milled wood preparation was about 20 wt %. These yields are in good agreement with that of the Wiley wood (19.6 wt %, Table 1). Unit A (phenolic β-*O*-4) composition of all the milled Wood was higher than that of the Wiley wood (Table 1), whereas unit C (etherified β-*O*-4) compositions were lower. This can be explained by depolymerization of the lignin macromolecule during milling, where etherified β-*O*-4 linkages are cleaved producing new phenolic β-*O*-4 lignin structures.

In the MWL and CEL preparations, unit A compositions were much higher than the respective milled woods and Wiley wood, whereas unit C compositions were lower. As was observed with the MWL isolation results shown in Table 1, phenolic rich lignin structures are more easily and preferentially isolated in the extraction procedure utilized for both MWL and CEL isolation. This selective fractionation explains the much lower unit A composition, and higher unit C composition for the REL preparation. More significantly, the results obtained indicate that

Table 4. Yield of Vanillin from Nitrobenzene Oxidation and Coniferyl Diacetate from DFRC Analysis

sample	nitrobenzene oxidation vanillin yield (%)[a]	DFRC monomer yield (%)[a]
wiley wood	24.0	20.2
vibratory wood	24.1	21.5
MWL	26.6	21.0
CEL	27.0	22.0
REL	21.6	19.0

[a] Based on Klason lignin.

the fraction of lignin linked to carbohydrates (CEL) is structurally the same as the lignin free of carbohydrate linkages (MWL). Thus, MWL and CEL may be combined to further increase the yields of isolated lignin. Together, the dioxane/water soluble fraction (CEL) and the dioxane/water insoluble fraction (REL) for the rotary porcelain 6 week wood, when added together and averaged, have unit A, B, and C values (33.4 wt %, 5.6 wt %, and 61 wt %) very close to those obtained for the milled wood (30.5 wt %, 6.8 wt %, and 62.7 wt %). Furthermore, the total monomeric yield from DFRC analysis of MWL, CEL, and Wiley milled wood are practically identical, indicating that both MWL and CEL are representative, structurally, of the majority of lignin in wood, with the exception of higher phenolic hydroxy content and presumably lower molecular weight (*7*, *8*). Further evidence for the similarity between MWL, CEL, and the lignin in Wiley milled wood can be seen from the nitrobenzene oxidation data, Table 4. Finally, in all cases, unit B compositions of MWL, CEL, and REL were lower than that of the Wood. Thus, cleavage of α-*O*-4 bonds occurs during the respective isolation procedures.

Comparison of the modified DFRC results for the vibratory in toluene wood and the rotary porcelain 6 week wood showed only slight changes in the lignin structure versus the rotary glass 1 week wood. However, the porcelain marble rotary milling took 6 weeks, whereas the vibratory milling was completed in 9 days (1 week rotary ball milling and 2 days vibratory ball milling). Therefore, the preferred milling process is 1 week rotary ball milling followed by 2 days vibratory ball milling in toluene. Nevertheless, if a vibratory ball mill is not available, satisfactory wood meal can be obtained by prolonging the ball milling time and using porcelain balls.

DFRC Analyses and the Significance of the Results Obtained. A distinct advantage of the DFRC method for lignin structure analysis is its relatively simple protocol and applicability to all types of woody tissues (*18*, *21*, *32*). Through a simple protocol modification, outlined above, identification of the various aryl ether structures can be obtained. However, only ∼20 wt % (∼14 mol %) of the lignin, based on Klason lignin content, is analyzed by DFRC using GC. Although this number is in good agreement with values reported by Lu and Ralph, it does seem low, particularly when compared to yields reported for thioacidolysis (*14*). DFRC and thioacidolysis selectively degrade β-*O*-4 inter unit linkages, therefore these methods should yield comparable results. However, our DFRC results, as well as those in the literature, are 5−10 mol % lower than those reported from thioacidolysis (*14*). Furthermore, even repeated applications of DFRC to our various lignin samples did not increase monomer yields. Therefore, further research into increasing monomer yields from DFRC, and understanding the differences between DFRC and thioacidolysis is needed, and is presently underway in our laboratory.

DFRC results using this new modified method show that MWL and CEL have a much higher phenolic unit content, ∼50% more phenolic β-*O*-4 units and ∼25% less etherified β-*O*-4 units, than that of the initial milled wood. It appears that phenolic-rich lignin structures are preferentially isolated in the 96% dioxane extraction step, as DFRC results of the vibratory milled wood are consistent with that of the original wood. MWL and CEL are structurally identical, and indicate that the portion of lignin linked to carbohydrates and that not linked to carbohydrates are structurally the same. Vibratory ball milling under nitrogen atmosphere (dry milling) appears to cause substantial changes in lignin structure. Rotary ball milling with glass balls and porcelain balls, and vibratory ball milling in toluene, caused no significant structural changes. However, extensive milling times were required for the dry ball milling (greater than 6 weeks) to obtain MWL and CEL yields comparable to rotary ball milling for 1 week followed by vibratory ball milling in toluene for 48 h (9 days total).

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