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Lignin Structural Variation in Hardwood Species

Ricardo B. Santos, Ewellyn A. Capanema,† Mikhail Yu. Balakshin,† Hou-min Chang,* and Hasan Jameel

Department of Forest Biomaterials, North Carolina State University, Raleigh, North Carolina 27606, United States

ABSTRACT: A comprehensive lignin structure analysis of ten industrially relevant hardwood species is presented. Milled wood lignin (MWL) was isolated from each species using a modified protocol and all milled wood lignin preparations were analyzed through quantitative ^{13}C NMR spectroscopy, elemental analysis, methoxyl analysis, sugar analysis, and nitrobenzene oxidation. Nitrobenzene oxidation and ozonation were carried out on extractive-free wood, alkali-extracted wood, milled wood lignin, and alkali-extracted lignin. Milled wood lignin isolated by the modified protocol was found to be representative of the total lignin in alkali-extracted wood. Significant variations in lignin structures, such as syringylpropane/guaiacylpropane ratio (S/G ratio), arylglycerol-β-aryl ether (β-O-4), degree of condensation, and elemental and methoxyl contents, were found among the hardwood species studied. These structural variations among species appear to be correlated to a single factor, the syringyl/guaiacyl ratio. A new method to predict the S/G ratio of total lignin in wood was developed, using a calibration line established by the syringaldehyde/vanillin (S/V) ratio (nitrobenzene oxidation) and the S/G ratio (^{13}C NMR) of milled wood lignin (MWL).

KEYWORDS: *S/G ratio, S/V ratio, lignin, hardwoods, ^{13}C NMR*

INTRODUCTION

Lignin, a naturally occurring aromatic polymer, is found in every vascular plant on earth. It is derived from three lignin precursors, *p*-coumaryl, coniferyl, and sinapyl alcohols (Figure 1), via an enzyme-initiated dehydrogenative polymerization.

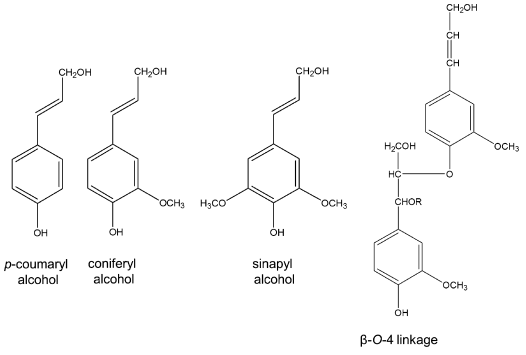

Figure 1. The lignin precursors and the most abundant linkage in lignin: arylglycerol-β-aryl ether (β-O-4).

Lignin can be divided into three classes according to their structural elements. Guaiacyl lignin, which occurs in almost all softwoods, is largely a polymerization product of coniferyl alcohol. Guaiacyl-syringyl lignin, typical of hardwoods, is a copolymer of coniferyl and sinapyl alcohols. Although small amounts of *p*-hydroxyphenylpropane units derived from incorporation of *p*-coumaryl alcohol, are found in both softwood and hardwood lignins, substantially more are found in monocot lignin, which is a copolymer of all three lignin precursors.[1] While softwood lignin appears to vary little between species,[2,3] there is increasing evidence that the structure of hardwood lignin varies greatly from one species to another. The major difference is the ratio of syringylpropane to guaiacylpropane units (S/G ratio). Early studies on nitrobenzene oxidation of hardwoods clearly showed wide variation in syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde) to vanillin (4-hydroxy-3-methoxybenzaldehyde) ratio (S/V ratio) among different species.[3−7] Milled wood lignin isolated from different hardwood species also showed considerable variation in methoxyl content and S/G ratio among several species of West Coast hardwoods.[3] These variations were confirmed later by ultraviolet microscopy.[8] The ultraviolet microscopy also demonstrated morphological differences in S/G ratio among various cell types and morphological regions. While guaiacyl lignin was found in vessels and cell corners, syringyl-guaiacyl lignin was found in the secondary wall of fibers and rays, where the S/G ratio varied from species to species among hardwoods. Other studies demonstrated that S/G ratio varies not only between species,[2,9] but also between species of the same genus[4,10,11] and between clones of the same species.[12]

The most abundant lignin linkage is the arylglycerol-β-aryl ether (β-O-4) linkage (Figure 1). It consists of two diastereomers, the erythro and threo forms. While softwood lignin has about an equal amount of the two forms, the erythro form is predominant in hardwood lignin. The ratio and total amount of the erythro and threo forms can be determined by ozonation, which gives erythronic (E) and threonic (T) acids, respectively.[13,14] Using the ratio [E/(E + T)] and the total amount (E + T), it clearly showed a wide variation in the amount of β-O-4 structure among hardwoods.[2] A correlation was also established between the β-O-4 structure and the S/G ratio, indicating that the syringyl/guaiacyl composition is the key factor governing the proportion of erythro and threo forms of β-O-4 structure in hardwood lignin.

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A major problem associated with the study of hardwood lignin structure is the absence of a simple and accurate method for determining S/G ratio, as noted in the literature.[10] Most previous studies used nitrobenzene oxidation, thioacidolysis, and pyrolysis/GC/MS. These methods only obtain degradation products from uncondensed units. It is well-known that syringyl units are less condensed than guaiacyl units and hence these methods tend to give higher S/G ratios. Permanganate oxidation, when proceeded by a kraft cook or cupric oxide oxidation, oxidizes both condensed and uncondensed units and should give a good S/G ratio. Unfortunately, the yields of uncondensed products (methyl esters of vanillic acid and syringic acid) are lower than the vanillin and syringyaldehyde obtained from nitrobenzene oxidation, respectively, indicating degradation of aromatic nuclei during permanganate oxidation. Ozonation yields, among other degradation products, erythronic (E) and threonic (T) acids from both condensed and uncondensed β-*O*-4 structures.[2] A method using the methoxyl content of Klason lignin to evaluate the S/G ratio of hardwood was proposed.[14−16] However, a recent study showed that methoxyl contents of Klason lignin from different species correlated poorly with both ozonation results, E/(E + T) and E + T, or nitrobenzene oxidation results, S/(S + V).[2] Interestingly, an excellent correlation was obtained between E/(E + T) and S/(S + V). On the basis of the ratio of the uncondensed and the condensed oxidation products of permanganate oxidation, earlier study developed an empirical equation for conversion of the S/V of nitrobenzene oxidation to the S/G ratio.[10] Good correlation between the corrected S/G and lignin content was obtained for 13 poplars. Whether the formula can be used for different hardwood species remains unknown.

In this report, a comprehensive lignin structure analysis of 10 industrially relevant hardwood species was performed with loblolly pine used as a softwood reference. A method was developed to determine the S/G ratio in wood, using a modified nitrobenzene oxidation that is calibrated with the S/G ratio obtained by ^{13}C NMR.

MATERIALS AND METHODS

Raw Material. Wood samples of *Eucalyptus nitens*, *Eucalyptus globulus*, *Eucalyptus urograndis*, birch (*Betula pendula*), red alder (*Alnus rubra*), acacia (*Acacia mangium*) and, cottonwood (*Populus trichocarpa*) were received as chips from different pulp and paper mills from around the world. Sweet gum (*Liquidambar styraciflua*), red maple (*Acer rubrum*), red oak (*Quercus rubra*), and loblolly pine (*Pinus taeda*) were received as logs from the Experiment Forest of North Carolina State University and were chipped after debarking in our pilot plant. The air-dried chips were ground in a Wiley mill and sieved, and the fraction that passed through a 40 mesh screen but was retained by a 60 mesh screen was collected. The wood meal was Soxhlet extracted for 24 h with benzene−ethanol 2:1 (v/v), dried, and used for lignin study.

Isolation of Milled Wood Lignin (MWL) and Alkali-Extracted Lignin (AEL). MWL was isolated according to the modified protocol for all 10 hardwood species.[17] The extracted wood meal (20 g) was refluxed with 1 L of 0.3% NaOH for 1 h under N_2 to remove tannins. The alkali-extracted wood meal was washed thoroughly with deionized water, air-dried, and finally dried in a vacuum desiccator. Alkali-extracted wood meal (2 g) was ball milled in a Pulverisette 7 planetary ball mill (Fritsch, Idar-Oberstein, Germany) for 6 h at 600 rpm, using a silicon nitride bowl (45 mL) and zirconium oxide balls (9 balls, 1 cm in diameter). The ball milled wood was extracted with 96% aqueous dioxane (freshly distilled over sodium), in accordance with the method of Bjorkman.[18] However, no purification was performed since the MWL prepared from alkali-extracted wood contained low amounts of carbohydrates.[17] With the exception of ball milling, which was carried out in a planetary ball mill for 15 h, MWL from loblolly pine was isolated according to the method of Bjorkman.[18] The yield of the crude MWL from loblolly pine was 75% and that of the purified MWL was 56%, all based on the lignin content of wood.

Lignin dissolved during the alkali extraction with 0.3% NaOH (AEL) was isolated by acidifying the filtrate to pH 2.5 with HCl. The precipitated lignin was centrifuged, washed free of acid, and freeze-dried in water suspension. Klason lignin and acid-soluble lignin were determined for each species using the extractive free wood meal, according to the method of Dence.[19] The sum of Klason lignin and acid-soluble lignin is reported as total lignin content.

Carbohydrate Content and Sugar Composition of MWL. Due to the limited amount of isolated MWL, a modified protocol for sugar analysis was developed. Lignin sample (10 mg) was swollen in 0.214 mL of 72% H_2SO_4 at room temperature for 2 h. The sample was then diluted with 8.0 mL of deionized water and placed in the 105 °C oven for 3.5 h. After completion of the reaction, sample was cooled down and the internal standard (fucose) was added. Acid in the sample was neutralized by passing through an OnGuard IIA cartridge from Dionex. The neutralized sample was injected into a high-performance anion-exchange chromatograph with pulsed amperometric detection (HPAE-PAD) on a Dionex IC-3000 chromatography system, using a guard column (Carbo-Pac PA1, 2 mm × 50 mm i.d.) and an analytical column (Carbo-Pac PA1, 2 mm × 250 mm i.d.) connected in series. Water was used as eluent at the flow rate of 1.0 mL/min and the column temperature was maintained at 18 °C. The postcolumn used 40 mM NaOH at a flow rate of 1.0 mL/min to improve detection by pulsed amperometry.

Nitrobenzene Oxidation. Nitrobenzene oxidation for S/V ratio determination was performed according to Chen.[20] OD wood meals (200 mg, 40−60 mesh) were reacted with 7 mL of 2 N NaOH (aq) and 0.4 mL of nitrobenzene in a stainless steel bomb at 170 °C for 2.5 h. After 2.5 h, the hot stainless steel bomb was cooled down immediately by ice water, and 1 mL of 5-iodovanillin (80 mg in 5 mL dioxane) was added as internal standard. The reaction mixture was extracted with CH_2Cl_2 four times and the organic phase (CH_2Cl_2 phase) was discarded. The remaining water phase (alkali solution) was then acidified with 2 N HCl to pH 2−3. The acidified solution was further extracted again with CH_2Cl_2 three times, and this organic phase (CH_2Cl_2 phase) was collected for analysis. The CH_2Cl_2 phase was dried over Na_2SO_4(s) and the volume was adjusted to 100 mL. One milliliter of this solution was dried by rotatory evaporation at 30 °C. The dried product was dissolved in 50 μL of pyridine, and 50 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was added, and the solution was shaken for 15 min at 50 °C. The derivatized mixture was then directly injected (2 μL) onto the GC. Quantitative GC analysis was carried out on a HP 6890 GC equipped with a flame ionization detector and HP-1 column (30 m × 0.32 mm i.d., 0.25 μm). The injection temperature was 200 °C, the detector temperature was 270 °C, and the column flow rate was 2 mL of helium/min. The column was held for 3 min at 120 °C, raised at 5 °C/min to 200 °C, followed by 10 °C/min to 260 °C, and kept isothermal at 260 °C for 5 min. S/V was determined by the molar ratio of the amount of syringaldehyde divided by the sum of vanillin and vanillic acid. Syringic acid was not included, since the amount was negligible in all cases.

NMR Spectra. NMR analysis was carried out for all the species using the isolated MWL. The analysis[21] was performed using quantitative ^{13}C NMR spectroscopy of the acetylated and non-acetylated lignin preparations in DMSO-d_6 using a Shigemi NMR microtube. ^{13}C NMR spectra were acquired on a Bruker AVANCE 300 MHz spectrometer equipped with a 5 mm QNP probe using an inverse-gated proton decoupling (IGD) sequence at 300 K using a 90° pulse width, 1.2 s acquisition time, and 1.7 s relaxation delay. Sample concentration was ca. 25%. Chromium(III) acetylacetonate (0.01 M) was added to the NMR tube prior to quantitative ^{13}C NMR acquisition to provide complete relaxation of all nuclei. A total of 20 000 scans were collected. The data set was processed using an

exponential multiplication (EM) window function, with zero-filling and line broadening of 10 Hz.

Ozonation. Ozonation was carried out according to the method of Akiyama et al.[2] A 50 mg portion of extractive-free wood meals (OD) with particle size smaller than 100 mesh was suspended in 30 mL of ozonation solvent, acetic acid/water/methanol (80/15/5, v/v/v). Oxygen containing ca. 3% ozone was bubbled into the solution with stirring at the rate of 0.5 L/min for 2 h at 0 °C. After 2 h, the residual ozone was removed by continuous oxygen bubbling for about 15 min. The reaction mixture was further reduced with 300 μL of 0.1 M sodium thiosulfate (reductive post treatment). The reaction solvent of the mixtures was evaporated using rotatory evaporation at 40 °C. The residual acetic acid was removed by rotavaporation with water. The ozonation products were saponified with 0.1 M NaOH (20 mL) at room temperature and left overnight. One milliliter of 5 mM erythritol was added as an internal standard. The solution was then passed through a column filled with ca. 10 mL of cation-exchange resin, Dowex-50W-X8, $NH4^+$ form, and the column was washed with water until the pH of eluent was 7−8, resulting a total volume of 100 mL. Two mL from this eluent was dried by rotatory evaporation at 40 °C. The dried product was silylated in 300 μL of dimethyl sulfoxide (DMSO), with 200 μL of hexamethyldisilazane (HMDS), and 100 μL of trimethylchlorosilane (TMCS) at 60 °C for 30 min. The reaction phase separates and the upper phase was used for GC analysis. Quantitative GC analysis was carried out on a HP 6890 GC equipped with a flame ionization detector and HP-1 column (30 m × 0.32 mm i.d., 0.25 μm). The injection temperature was 250 °C, the detector temperature was 280 °C, and the column flow was 2 mL of helium/min. The column was held for 5 min at 120 °C, increased to 170 at 4 °C/min and then at 10 °C/min to 280 °C and kept isothermal at 280 °C for 5 min.

Elemental and Methoxyl Content Analyses. Elemental analyses (C, H, and N) were performed by the Department of Soil Science at North Carolina State University, Raleigh, NC, using a Perkin-Elmer 2400 CHN elemental analyzer. Methoxyl content analyses were performed according to the procedure of Goto et al.[22]

RESULTS AND DISCUSSION

Isolation of MWL and AEL. For some hardwood species, alkali extraction is necessary to remove tannins even after the benzene−ethanol extraction.[17] In the present study, alkali extraction was performed for all species of hardwood, and MWL of each species was isolated from the alkali-extracted wood. Lignin content, yield of wood after alkali extraction, yields of MWL, and AEL (both expressed as a percentage of the total lignin content in wood), and carbohydrate content of MWL are given in Table 1, for all 10 hardwood species studied. Lignin content varied from 21.5% to 27.7% among the 10 hardwood species. While the yield of wood after alkali extraction and yield of AEL varied widely among hardwood species, there was no correlation between the two. Yield of MWL from alkali-extracted wood was relatively constant, varying only from 24% to 29%, and was independent of the lignin content or yield of AEL. These yields of MWL were consistent with the finding that the yield of MWL depends only on the extent of ball milling.[23] Also shown in Table 1 are the carbohydrate contents of MWL, which range from 2 to 3%. It should be noted that the MWL here is isolated without the purification procedure of Bjorkman,[18] yet the carbohydrate contents of all these MWL samples are lower than that of the purified MWL isolated by the normal Bjorkman protocol. MWL of sweet gum, for example, contains 2.5% carbohydrates without any purification (Table 1), whereas that isolated previously according to the procedure of Bjorkman contained 3.6% carbohydrates after purification.[25] Another example can be found in our recent work with birch.[25] MWL isolated according to the procedure of Bjorkman[18] has a carbohydrate content of 22.2% and 8.7% before and after purification, respectively,[25] whereas MWL isolated from the same birch wood by the modified procedure gave a carbohydrate content of 2.4% without purification (Table 1). These results indicate that the modified procedure[17] used in the present study (alkali extraction prior to ball milling) is the preferred method of isolating MWL from hardwood for structural studies.

Table 1. Total Lignin Content, Yield of Wood after Alkali Extraction, Yield of Dissolved Lignin in Alkali (AEL), and Yield of MWL

		% yield			
wood species	% total lignin content	wood after NaOH extraction	AEL[a]	MWL[a]	% carbohydrate in MWL
E. globulus	23.9	82.0	3.8 (0.9)[b]	26.2	2.4
E. nitens	25.5	78.6	5.6 (1.4)	25.4	2.5
E. urograndis	26.6	91.4	9.4 (2.5)	24.3	3.0
red oak	27.7	77.5	29.6 (8.2)	25.5	2.1
cottonwood	21.5	83.5	20.5 (4.4)	24.9	3.0
sweet gum	27.2	86.5	11.2 (3.1)	26.3	2.5
acacia	26.8	89.5	10.5 (2.8)	25.0	2.0
birch	22.8	83.6	7.0 (1.6)	27.1	2.4
red alder	24.4	85.8	16.4 (4.0)	27.6	2.5
maple	25.9	86.5	14.5 (3.8)	28.5	2.4

[a]On the basis of the original lignin content. [b]Values in parentheses are based on wood.

Since various amounts of lignin are extracted by alkali, it is important to be assured that the lignin remaining in the alkali-extracted wood is representative of the total lignin in wood. Thus, nitrobenzene oxidation was carried out for both original and alkali-extracted wood. As shown in Figure 2A, an excellent linear correlation is found for the ratio S/V between AEW and

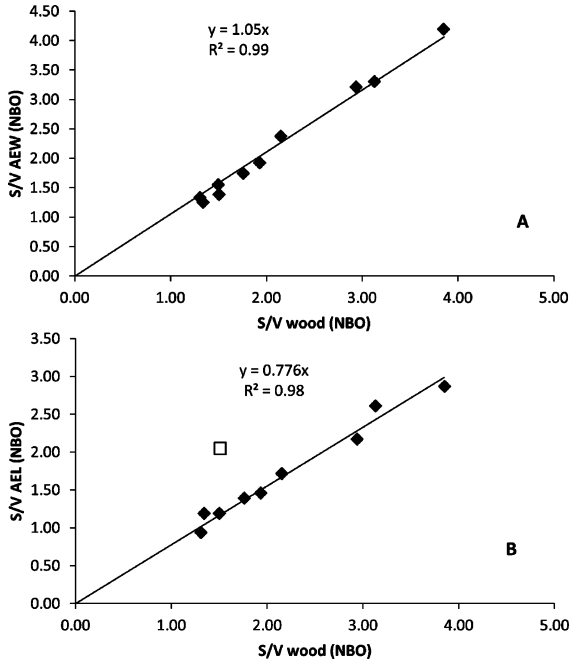

Figure 2. S/V ratios of (A) alkali-extracted wood (AEW) and (B) alkali-extracted lignin (AEL) (B) versus that of the original wood. The unfilled square in plot B represents cottonwood.

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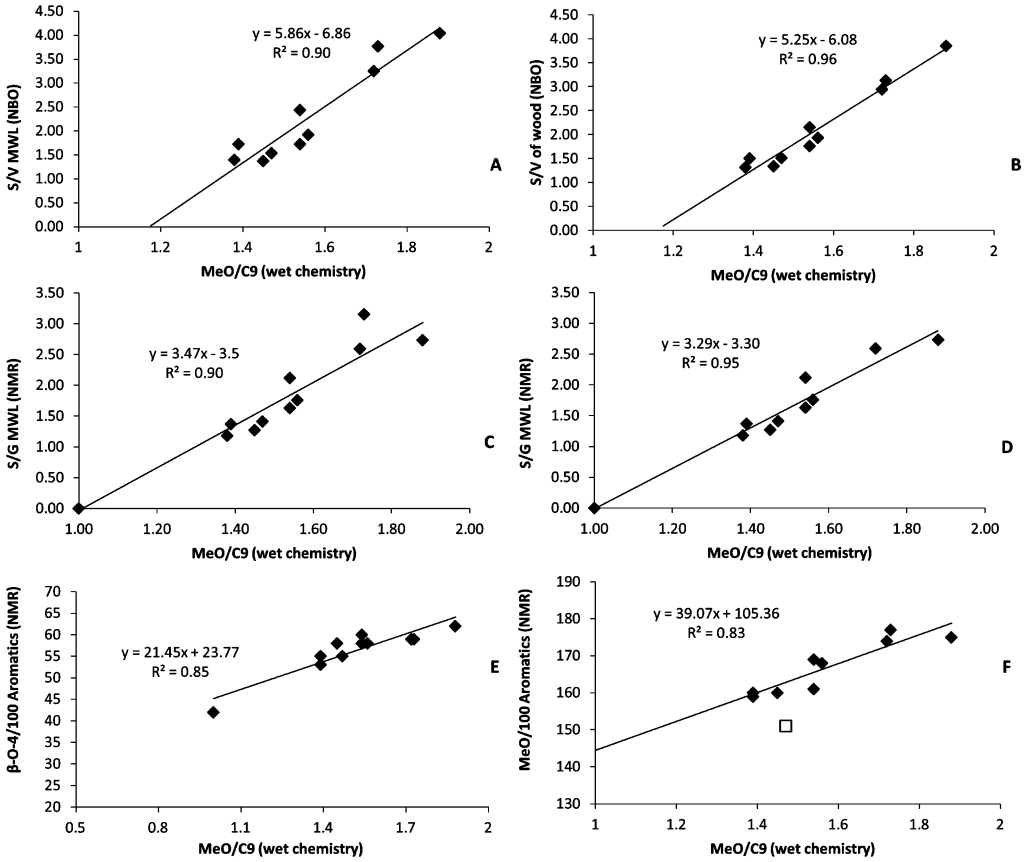

Figure 3. Ratios of S/V (A), S/V of wood (B), S/G (C, D), *β-O*-4/100 aromatics (E), and MeO/100 aromatics (F) of MWL versus MeO/C_9 of MWL. Plot D has one point removed (birch) compared to plot (C). The unfilled square (F) represents cottonwood.

original wood. The slope of the line indicated that AEW has a 5% higher S/V ratio than the original wood. In order to confirm these results, nitrobenzene oxidation was carried out for the alkali-extracted lignin (AEL), the lignin that is precipitated from alkali extraction liquor by acidification. The results are plotted against the S/V of the original wood as shown in Figure 2B. Again, with the exception of cottonwood, a linear correlation was found between the S/V ratio of alkali-extracted lignin (AEL) and that of the original wood. The results also showed that AEL gave 22% lower S/V than the original wood. These results are in good agreement with those of the previous study showing that alkali extraction removes lignin enriched in the G (guaiacyl-propane) unit.[17] The exception shown by cottonwood in Figure 2B may due to the fact that species of Salicaceae family contains significant amounts of *p*-hydroxybenzoic acid,[26] which is known to incorporate in lignin by, in addition to *γ*-ester,[26] carbon to carbon linkages. The latter are presumably condensed with guaiacyl units, which would lower the yield of vanillin and hence raise S/V.

The excellent correlations among the S/V ratios of the original wood, the alkali-extracted wood, and the alkali extracted lignin as shown in Figure 2A,B are totally unexpected since the amount of alkali extracted lignin (AEL) varies widely among species (Table 1). The results suggest that S/V is the key factor controlling lignin structure in hardwood.

Characterization of MWL. Elemental composition and methoxyl content of MWL were calculated as the C_9 normalized formula. MeO/C_9 varies from 1.39 to 1.88 among the 10 hardwood species. The methoxyl contents of red alder and sweet gum MWL are in good agreement with those reported in earlier studies.[8,24] To the best our knowledge, the 1.88 MeO/C_9 of *E. globulus* is the highest value ever found for MWL of any hardwood species.

In Figure 3A,B, the S/V ratios of MWL and wood are plotted against MeO/C_9. Both give good linear correlation. Good correlation between the S/V ratio of MWL and the MeO/C_9 appears to be expected, since both are determined from MWL. However, while the S/V ratio from nitrobenzene oxidation gives the S/G ratio of the uncondensed phenylpropane units, the methoxyl content of MWL is from both uncondensed and condensed units. Consequently, the good correlation suggests that the degree of condensation in MWL is a function of the methoxyl content. The good correlation between the S/V ratio of wood and MeO/C_9 gives further support to the above and suggests that MWL is representative of the lignin in wood and that there is a good correlation between the S/V ratio of MWL and that of wood. Indeed, the latter is found to be the case, as shown in Figure 4A.

An excellent linear correlation exists between the S/V ratio of MWL and the S/V ratio of wood. The slope of the line indicates that MWL of all species has 9% higher S/V ratio (molar ratio) than the total lignin in wood. The fact that MWL has higher S/G, S/V, and MeO/C_9 than the total lignin in wood is expected and can be attributed to two reasons. First, MWL was isolated after alkali extraction, which removed lignin of higher guaiacyl content (Figure 2A). Second, MWL represents the less condensed portion of lignin in wood and hence should have higher S/G, S/V, and MeO/C_9. It should be noted that these MeO/C_9 values are for the MWL. The MeO/

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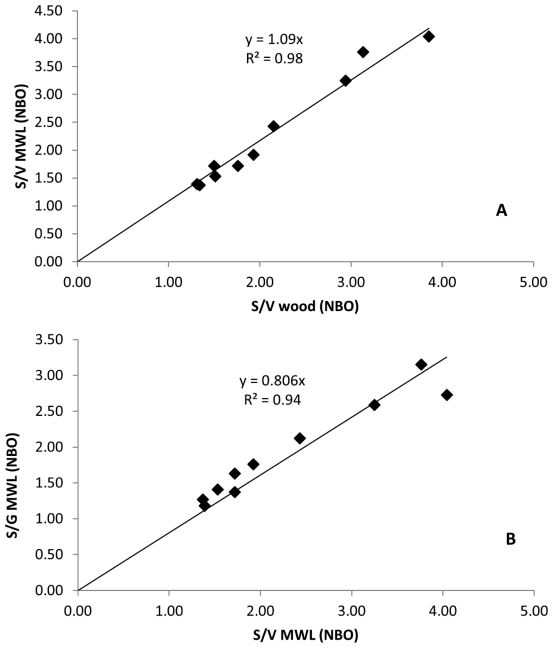

Figure 4. Plots of S/V of MWL versus of S/V of wood (A) and S/G ratio of MWL versus S/V ratio of MWL (B).

C_9 for the total lignin should be lower than these values for the same reasons discussed above.

The objective of alkali extraction of wood is to remove tannin from wood. Various amounts of wood were extracted, ranging from 8.6 to 22.5% in weight based on starting material. AEL, which consist almost exclusively of lignin on the basis of carbohydrate analysis, accounts only for a small portion of the extracts, 1−8% based on wood (Table 1). In addition to tannin, alkali extraction is expected to extract some hemicelluloses and hydrolyze alkali-labile linkages in lignin−carbohydrate complex (LCC),[17] including γ-esters and possibly some phenyl glycosides. The hydrolysis of alkali-labile linkages in LCC is presumably attributed to the low carbohydrate contents in both AEL and MWL isolated from AEW. Both types of lignin have around 2−3% carbohydrate content without purification, whereas normal MWL prepared according to the procedure of Bjorkman[18] has 5−10% carbohydrate, even after purification. The present results support the earlier report that a significant amount of carbohydrates are attached to lignin through alkali-labile linkages of ester type.[17] A recent study clearly showed that birch has much lower benzyl ether type and higher glycoside and ester type lignin−carbohydrate linkages than loblolly pine.[25] In addition to the low carbohydrate content, the sugar compositions are significant. Xylan and glucan are the main residual sugars in MWL and their amounts appear to vary little among the 10 hardwood species, suggesting little variation in the alkali-stable lignin−carbohydrate linkages among these species. Furthermore, these alkali-stable lignin−carbohydrate linkages are attached mainly to xylose and glucose.

NMR spectroscopy has been a powerful tool facilitating investigations into complex lignin structure.[25,27] The low carbohydrate content of MWL isolated with alkali pre-extraction should provide substrates for quantitative assessment of the structure feature in MWL according to the published method.[27] Figure 5 shows acetylated and no-acetylated ^{13}C NMR spectra of *E. globulus* and acacia. The S/G ratio of MWL, as determined by NMR, is plotted against MeO/C_9 (Figure 3C), and a significant correlation does exist. However, NMR appears to overestimate the S/G ratio of birch. If birch is not included in the plot, a better correlation is observed, as shown in Figure 3D. Similarly, correlations are found between β-*O*-4 per 100 aromatic rings and MeO/C_9 (Figure 3E) and between MeO per 100 aromatic rings and MeO/C_9 (Figure 3F). Again, β-*O*-4 and MeO per 100 aromatic rings are estimated from

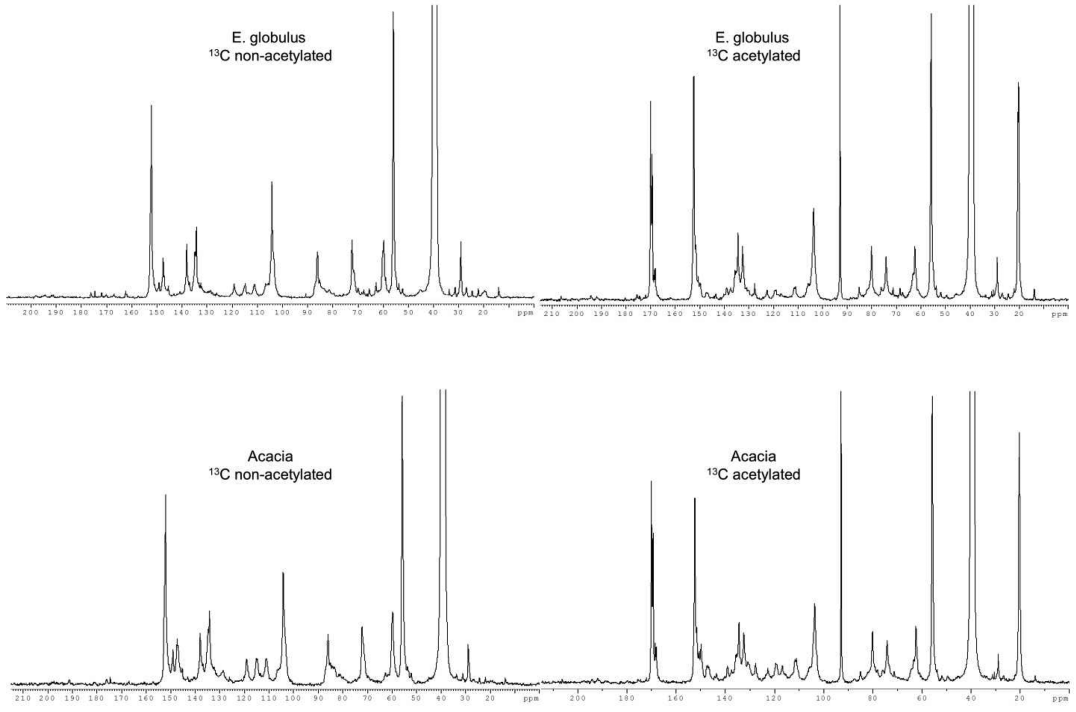

Figure 5. Acetylated and nonacetylated ^{13}C NMR spectra of MWL from *E. globulus* and acacia.

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quantitative ^{13}C and 2D NMR[27] whereas MeO/C_9 is calculated on the basis of elemental and methoxyl analyses.

It has been shown previously[17] that quantitative ^{13}C NMR spectroscopic analysis showed that little structural difference existed between the two MWL samples of different yields (24.8% and 56.0%). These lignin samples were isolated from *Eucalyptus grandis* after alkali extraction under two difference balling conditions. It was concluded that the MWL isolated from the alkali extracted hardwood is representative of the total lignin in the alkali-extracted wood. Our present results on nitrobenzene oxidation of MWL and AEW for all 10 wood species clearly shows (Figure 6) that there is no difference in S/V ratios between MWL and AEW, thus giving a strong support to the previous conclusion that MWL isolated from alkali-extracted wood is representative of the total lignin in alkali-extracted wood, regardless of yield.

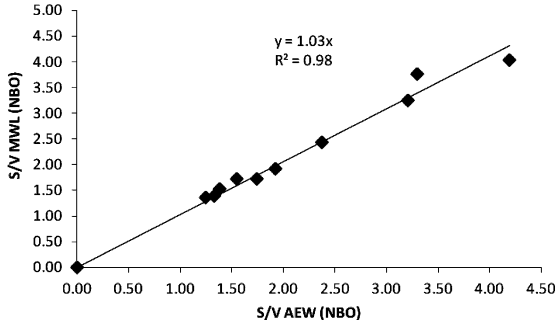

Figure 6. S/V ratios of MWL and AEW, indicating MWL is representative of the lignin in AEW.

Since MWL is representative of the total lignin in alkali-extracted wood, the structure of AEL, the portion of lignin extracted by alkali, is of particular interest. AEL represents 4–30% of the total lignin in hardwood (Table 1). It produced about 22.4% lower S/V ratio than that of wood upon nitrobenzene oxidation (Figure 1b), indicating that AEL contains higher guaiacyl units (G-units) than the rest of lignin. A hypothesis has been proposed that lignin in hardwood consist of two fractions: the "easily soluble lignin" (AEL) enriched in G-units and the "bulk lignin".[17] The results shown in Figure 1 give strong support to the hypothesis. Furthermore, the results also show that the S/V ratio of AEL is proportional to the S/V ratio of wood (Figure 2b). For structural characterization of lignin in hardwood, both AEL and MWL should be characterized to represent the total lignin in wood. MWL isolated by the modified method (alkali extraction prior to ball milling) is representative of the total lignin in alkali-extracted wood, the structure of which will not depend on the yield of MWL,[23,28] as opposed to the MWL isolated by the method conventional method.

Variation in Structures of Hardwood Lignin. The existence of variations in the S/G and S/V ratios among the lignin of hardwood species is well-documented.[2,12] The present study confirms that a wide range of variation in S/G and S/V ratios exist among the 10 hardwood species, as shown in Figures 2–6 as well as FTIR (Fourier transform infrared, not shown) and ^{13}C NMR of MWL (Figure 5). However, it is totally unexpected that all variations in major lignin structures are correlated to the S/V ratio of wood. As powerful as NMR spectroscopic methods are, they can only determine structures of isolated lignin that are soluble in dimethyl sulfoxide (DMSO). Elemental and methoxyl analyses can only be done meaningfully with isolated lignin. Ozonation can be carried out on wood or isolated lignin and gives an indication of the relative amount of *β-O*-4 structure in lignin from both condensed and uncondensed units.[2,13] Nitrobenzene oxidation can be done with either wood or isolated lignin but gives the S/V ratio directly and hence an indication of the S/G ratio from the uncondensed units of lignin only.[20] Thus, all these analytical methods determine different structural features of lignin. The fact that structural variations of lignin in the 10 hardwoods as determined by these analytical methods are all correlated to the S/V ratio clearly points to the inevitable conclusion that all major lignin structures in hardwood is correlated to a single factor, the S/G ratio of lignin. The S/G ratio is also the key factor affecting the rate of kraft pulping of hardwoods.[11,29] These results have practical implications for tree breeding and energy plantation, as the S/G ratio in lignin can be manipulated by genetic transformation. Ozonation of wood gives quantitative estimation of *β-O*-4 linkage in lignin from both condensed and uncondensed structures.[2,13] The *β-O*-4 content of lignin can be deduced by the total yield (E + T) and the ratio [E/T or E/(E + T)] of erythronic (E) and threonic (T) acids liberated by ozonation.[2,13] The amount of *β-O*-4 linkage of MWL can also be estimated independently by NMR.[17,21,27] The amount of *β-O*-4 linkage in lignin has been shown to correlate well to the S/G ratio in hardwood lignin.[2,12]

While ozonation of wood gives quantitative estimation of *β-O*-4 linkage in lignin from both condensed and uncondensed structures,[2,13] nitrobenzene oxidation gives the S/V ratio of lignin from only uncondensed units.[20] However, good correlations exist when E/(E + T) is plotted against S/(S + V) and E/T is plotted against S/V, all of extractive-free wood, as shown in Figure 7A,B. These results are in total agreement with early study by Akiyama et al.[2] and imply that not only the *β-O*-4 structure but also the degree of condensation in lignin are closely related to the S/G ratio. These results are also

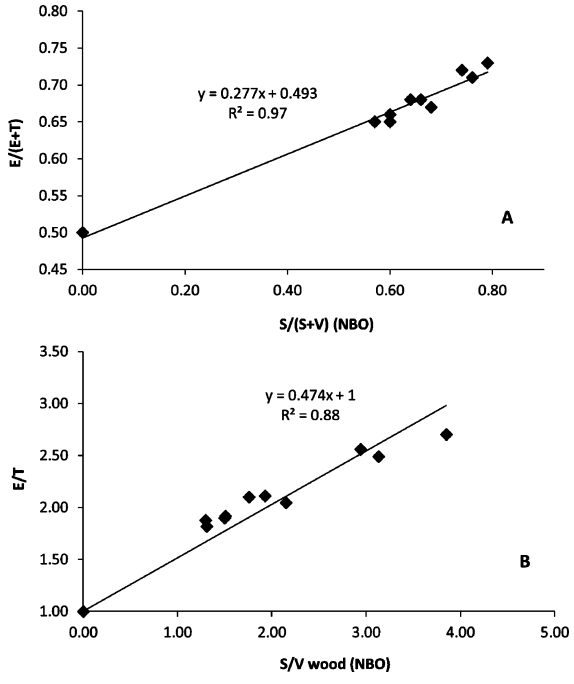

Figure 7. Plots of E/(E + T) (A) and E/T (B) of wood as a function of S/(S + V) and S/V of wood, respectively.

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supported by ^{13}C NMR spectra in which the amount of β-*O*-4 structure and degree of condensation can be estimated for MWL.

Prediction of S/G Ratio of Lignin in Hardwood. Unlike other methods, NMR gives S/G from both uncondensed and condensed units. The S/G of MWL obtained by NMR is plotted against the S/V of MWL obtained by nitrobenzene oxidation for all 10 hardwood species and loblolly pine as shown in Figure 4B. The plot shows a straight line with excellent correlation. The straight line has a slope of 0.806 and passes through the origin. These results indicate that the S/G of lignin can be predicted for any hardwood lignin by multiplying the S/V value by 0.806. The prediction described here is credible since MWL has been shown to represent the total lignin in alkali-extracted wood (Figure 6) and since the S/V of AEL is also correlated to the S/V of wood (Figure 2B). The predicted S/G, the S/G of MWL, and the S/V of MWL and wood are given in Table 2 for all 10 hardwood species.

Table 2. Predicted S/G of Wood by S/V of Wood and the Calibration Line (Predicted S/G = 0.806 × S/V)

wood species	S/G MWL by NMR	S/V MWL	S/V wood	predicted wood S/G
E. globulus	2.73	4.04	3.85	3.10
E. nitens	2.59	3.25	2.94	2.37
E. urograndis	1.76	1.92	1.93	1.56
red oak	2.12	2.43	2.15	1.73
cottonwood	1.41	1.53	1.51	1.22
sweet gum	1.63	1.72	1.76	1.42
acacia	1.18	1.39	1.31	1.06
birch	3.15	3.76	3.13	2.52
red alder	1.37	1.72	1.50	1.21
maple	1.27	1.40	1.30	1.08

Since nitrobenzene oxidation is relatively simple and the protocol is well-established, the calibration line gives a simple way of predicting S/G in any hardwood lignin. This method uses S/G from NMR as a reference for conversion of S/V ratio from NBO to S/G ratio. A recent study used permanganate oxidation results to derive an empirical equation to convert S/V ratio obtained from NBO to S/G ratio.[10] The correction factor of 0.694 (S/G = S/V × 0.694) was derived on the basis of the findings that uncondensed fractions were 96% and 66.6% for the syringyl units and guaiacyl units, respectively.[10,30] The S/G ratios predicted by the two methods for the 10 hardwood species studied are plotted against each other as shown in Figure 8A. The results of this study predict 17% higher S/G ratio for every species compared to the earlier study of Bose et al.[10] The lower prediction of S/G ratios, based on the permanganate oxidation, could be due to the fact that permanganate is a strong oxidant and may preferentially oxidize more syringyl units. Oxidation of aromatic nuclei during permanganate oxidation is known as oxidation of model compound and showed that, on the average, only 60% of the aromatic nuclei can be recovered as aromatic acids. Another source of errors in the correction factor could be attributed to the assumption of the uncondensed fractions in hardwood lignin. If the uncondensed fractions of other published data (84.4% and 61% for the syringyl units and guaiacyl units, respectively) were used, the correction factor became 0.722.[30] Our method based on NMR could also suffer from some overlaps when signals were integrated, resulting in some

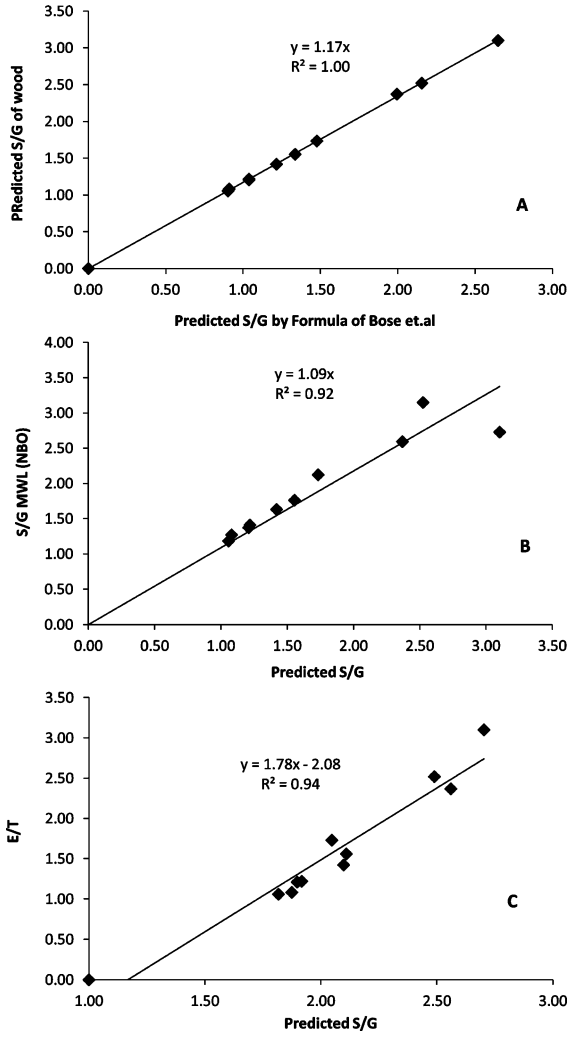

Figure 8. Plots of predicted S/G of wood lignin by NBO/NMR versus predicted S/G by Bose et al.[10] (A) and S/G ratio of MWL (B) and E/T (C) as a function of predicted S/G ratio of wood.

variations. However, regardless of the difference, both methods provide simple ways to obtain the relative S/G ratios of hardwood samples by nitrobenzene oxidation. When the S/G of MWL is plotted against the predicted S/G ratio of wood, the S/G ratio of MWL is 9% higher than that of the lignin in wood (Figure 8B). The fact that MWL has higher S/G and S/V ratios than the total lignin in wood is expected, as MWL was isolated after alkali extraction, which removed lignin of higher guaiacyl content (Figure 2A,B), and as MWL represents the less condensed portion of lignin in wood, it should have higher S/G and S/V, as discussed earlier. The amount of β-*O*-4 linkage in lignin has been shown to correlate well to the S/G ratio in hardwood lignin.[2,12] As shown in Figure 8C, E/T correlates well with the predicted S/G ratio of wood.

Overall, this work has revealed that MWL isolated with alkali extraction prior to ball milling is representative of the total lignin in alkali-extracted wood. The S/V ratio of the alkali-labile lignin (AEL) from a given species, which amounts to 4−30% of the total lignin in wood, was found to have linear correlation with the S/V ratio of wood of the same species, albeit enriched in guaiacyl units as compared with the bulk of lignin. Structural differences in hardwood lignin as determined by nitrobenzene oxidation, ozonation, quantitative ^{13}C NMR, and elemental and

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methoxyl analyses are all correlated to a single factor, the S/V or S/G ratio, suggesting that major structural features of hardwood lignin, such as β-*O*-4 linkage, degree of condensation, and methoxyl content, are controlled by the S/G ratio.

AUTHOR INFORMATION

Corresponding Author

*Tel: +1 9l9 515-7712. Fax: +1 919 515-6302:. E-mail: hchang@ncsu.edu.

Present Address

[†]Lignol Innovations Ltd., Unit 101, 4705 Wayburne Drive, Burnaby, BC, Canada V5G 3L1.

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Notes

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ABBREVIATIONS USED

S/G, syringyl/guaiacyl ratio; β-*O*-4, arylglycerol-β-aryl ether; E, erythronic acid; T, threonic acid; S/V, syringaldehyde/vanillin ratio; MWL, milled wood lignin; AEL, alkali-extracted lignin; AEW, alkali-extracted wood; NBO, nitrobenzene oxidation

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