Influence of Isolation Condition on Structure of Milled Wood Lignin Characterized by Quantitative ^{13}C Nuclear Magnetic Resonance Spectroscopy

Dou-yong Min,[a] Sarah Waters Smith,[b] Hou-min Chang,[a] and Hasan Jameel [a,]*

Milled wood lignin (MWL) was widely characterized to demonstrate the structure of native lignin by liquid state ^{13}C NMR. As an isolated lignin, the structure of MWL was influenced by the isolation procedure performed. In this article, hardwood (sweetgum) and softwood (loblolly pine) were subjected to various isolation conditions to elucidate the effect of extracting temperature and milling time on the structure of MWL. Purification was also carried out on the crude MWL. The structure of the crude MWL and the purified MWL was identified and quantified by ^{13}C NMR. Based on the yield and the lignin content of the crude MWL, the optimal isolation was achieved with 8 h milling and 20 °C extracting for hardwood. For softwood, the optimal isolation condition for crude MWL was 12 h milling and 20 °C extracting.

Keywords: Quantitative ^{13}C NMR; Milled wood lignin; Isolation procedure; Sweetgum; Loblolly pine

Contact Information: a. Department of Forest Biomaterials, North Carolina State University, P. O. Box 8005, Raleigh, NC 27695 USA; b. Caudill and Kenan Laboratories, Department of Chemistry, University of North Carolina at Chapel Hill, P. O. Box 3290, Chapel Hill, NC 27599 USA;
** Corresponding author: jameel@ncsu.edu*

INTRODUCTION

As the second most abundant natural polymer, lignin is poised to play an important raw material role as the production of bioproducts rapidly increases. Large amounts of lignin are produced each year by the pulp and paper industry as by-products of delignification and the bio-refinery industry during the pretreatment process. This degraded lignin is then employed in low-added value applications and energy production. Lignin continues to attract more attention because of its numerous potential applications. It is however more difficult for lignin to be widely used before its structure is fully characterized. Structural characterization of lignin is required for bio-ethanol production since it accounts for the recalcitrance of biomass to bio-degradation.

There are three main kinds of lignin in plants that vary depending on the species. Typically, softwood lignin is composed almost entirely of guaiacylpropane units (Obst and Landucci 1986). Hardwood lignin is more complicated because it contains varying ratios of syringylpropane (S) and guaiacylpropane (G) units. Grass lignin is even more compli-cated than woody species because it contains substantial amounts of *p*-hydroxyphenyl-propane (H) units; in addition, it involves *p*-coumaric and ferulic acids, which make it more difficult to characterize because of the structural similarity between the *p*-coumaric and H units and between the ferulic acid and G units. Numerous analytical methods have been used to resolve the composition and structure of lignin. Wet chemistry methods involving lignin degradation can enable precise characterization of specific functional groups and structural moieties. Those methods, however, give only

limited information and hardly provide a complete picture of lignin structure. Spectroscopic methods avoid-ing lignin degradation characterize lignin by direct detection of lignin moieties. Currently, nuclear magnetic resonance (NMR) spectroscopy is regarded as the most powerful technique for characterizing lignin structure due to its high resolution and specificity (Danielle 1982; Kanitskaya *et al.* 1993; Fukagawa *et al.* 1991; Ralph *et al.* 1999). The characterization of milled wood lignin structure by NMR does, however, typically include ball-milling in order to make the lignin soluble in solvents such as dimethyl sulfoxide-d_6. It has been confirmed that such milling anatomically destroys the plant cell walls (Fengel *et al.* 1978; Maurer and Fengel 1992; Fujimoto *et al.* 2005). Although bonds are cleaved and structures are altered somehow by milling, a relatively complete picture of milled wood lignin structure can still be demonstrated by NMR spectroscopy. Thus, milled wood lignin is still believed to be the most representative form of isolated native lignin (*i.e*. protolignin).

In this article, sweetgum (hardwood) and loblolly pine (softwood) were used to elucidate the influence of milling time and extracting temperature on the structure of milled wood lignin. After the application of various isolation conditions to milled wood lignins, the structures were identified and characterized by quantitative ^{13}C NMR.

EXPERIMENTAL

Raw Materials and Chemicals

Sweetgum and loblolly pine wood chips received from a pulp mill (USA) were ground and passed through a 40-mesh screen with a Genetic Electric Wiley mill. The sawdust between 40- and 60-mesh was collected as raw material. All of the samples were extracted with a mixture of benzene and ethanol (2:1 v/v) for 8 h to remove extractives. The extracted substrate was then air-dried, homogenized in a single lot to maintain constant composition, and stored for further use. The moisture content of the sawdust was measured by oven drying at 105 °C.

1,4-Dioxane (Catalog No. D111-4), pyridine (Catalog No. P368-500), and acetic anhydride (Catalog No. A10-500) were purchased from Fisher Scientific USA. Dimethyl sulfoxide-d6 came from Cambridge Isotope Laboratories, Inc.

Compositional Analysis of Samples

The chemical composition of extractive-free samples and the crude milled wood lignins (MWLc) were quantified according to the TAPPI Standard Method T222 om-98. An air-dried sample (about 0.1 g) was briefly reacted with 1.5 mL of H_2SO_4 (72% wt.) at 20 °C for 2 h with stirring every 15 min. The slurry was then diluted with 56 mL of deionized water (new H_2SO_4 concentration: 3% wt.). It was transferred to a serum bottle and was autoclaved at 122 °C for 1.5 h. The monomeric sugar content (arabinose, rhaminose, galactose, glucose, xylose, and mannose) in the filtrate was determined by Dionex-IC (Dionex IC-3000; Dionex, USA). The Dionex-IC system was equipped with a guard column (carboPac PA1 2×50 mm) and an ion-exchange column (CarboPac PA1 2×250mm) in tandem, a pulsed amperometric detector that had a gold electrode, and a Spectra AS 300 autosampler. Prior to injection, samples were filtered through 0.2 µm Nylon filters (Millipore) whereupon a volume of 5 µL was ultimately loaded. The column was pre-equilibrated with 250 mM NaOH and eluted with Milli-Q water at a flow rate of 0.3 mL/min.

Isolation of the Crude Milled Wood Lignin

The extractive-free sample (around 2 g) was subjected to 12, 8, 4, and 2 h of milling at 600 rpm using ZrO_2 bowls and 17 ZrO_2 balls in a planetary ball milling instrument (Pulverisette 7, Fritsch, Germany). During the milling process, the mill ran in 15 min increments, pausing for 15 min in between each to keep the jar temperature below 50 °C.

The wood meal was extracted with 30 mL of a mixture of 1,4-dioxane and H_2O (96% volume concentration) for 24 h at 50 °C and 25 °C, respectively. After separation by centrifugation, the upper layer liquor was collected and another extraction was performed on the residue.

The extraction was replicated three times, and the combined extraction liquor was filtered to remove suspended fine powders. 1,4-dioxane was evaporated under reduced pressure at 35 °C with a rotary evaporator. Several drops of deionized water were applied to wash the residue and remove traces of 1,4-dioxane; this was repeated several times, as necessary, until no trace of 1,4-dioxane remained. The final product (MWLc) was dried in a vacuum oven at 35 °C. The yield of MWLc ranged from approximately 10% to 60% (Fig. 1) based on lignin depending upon the milling time, the extracting temperature, and the species.

Acetylation of the Milled Wood Lignin

Acetylation of the milled wood lignin was carried out according to a published procedure (Adler *et al.* 1987). The acetylated lignin was recovered by the evaporation of the acetylation mixture (pyridine/Ac_2O) with ethanol. This was in contrast to typical precipitation in ice water (Chen and Danielle 1988), which can result in the loss of material, and therefore, lignin fractionation. To further avoid material loss, no purification of the acetylated preparation was performed.

NMR Characterization

The acetylated milled wood lignin (about 40 mg) was dissolved in 180 µL DMSO-d_6. About 20 µL chromium (III) acetylacetonate (0.01 M) was then applied to the lignin solution to completely relax all nuclei. Then the solution was transferred to the Shigemi microtube and characterized at 25 °C. Quantitative ^{13}C spectra were acquired on a Bruker AVANCE 300 MHz spectrometer equipped with a 5 mm QNP probe that may be characterized by its inverse gated proton decoupling sequence (Capanema *et al.* 2004). The acquisition parameters were a 90° pulse width, a relaxation delay of 1.7 s, and an acquisition time of 1.2 s. A total of 20,000 scans were collected.

RESULTS AND DISCUSSIONS

Chemical Composition of the Extractives Free Substrates

The compositions of sweetgum and loblolly pine are summarized in Table 1. Generally, there were more carbohydrate (67.8%) and less lignin (23.3%) in sweetgum compared with loblolly pine (60.8% and 28.9%, respectively). With respect to carbohydrate, more glucan (50.9%) and xylan (15.3%) were generated from sweetgum, while more mannan (10.3%) was produced from loblolly pine.

Table 1. Composition of Extractive-Free Samples (average ± standard deviations)

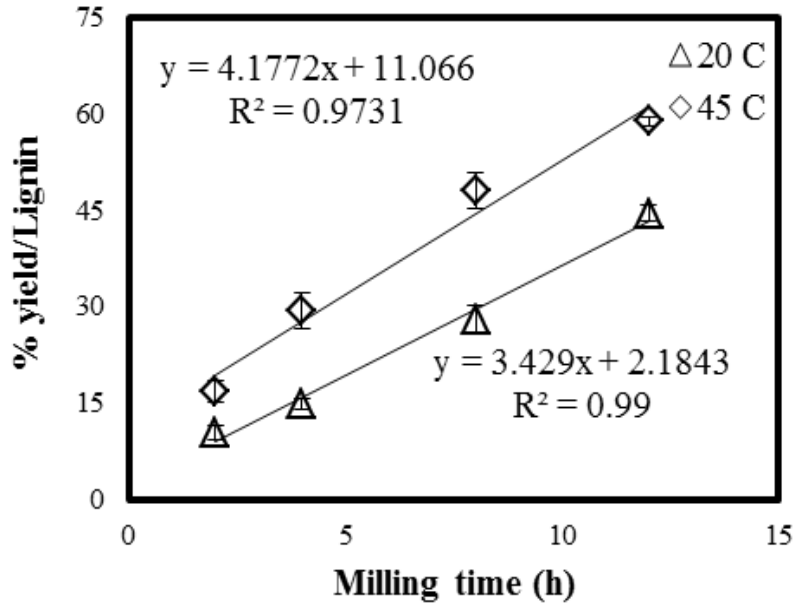
Sample	Arabinan	Rhamnan	Galactan	Glucan	Xylan	Mannan	TC*	TL*	TB*
SG	0.7 ±0.04	N/D	N/D	50.9 ±0.08	15.3 ±0.48	0.9 ±0.02	67.8 ±0.41	23.3 ±0.24	91.1 ±0.17
PINE	1.4 ±0.01	N/D	1.3 ±0.02	44.6 ±1.54	6.6 ±0.06	10.3 ±0.43	60.8 ±1.04	28.9 ±0.08	93.7 ±0.96

Note: Composition is expressed as % (w/w) of original extracted free substrate. TC*: Total carbohydrates including arabinan, rhamnan, galactan, glucan, xylan, and mannan. TL*: Total lignin including acid insoluble lignin and acid soluble lignin. TB*: total material balance.

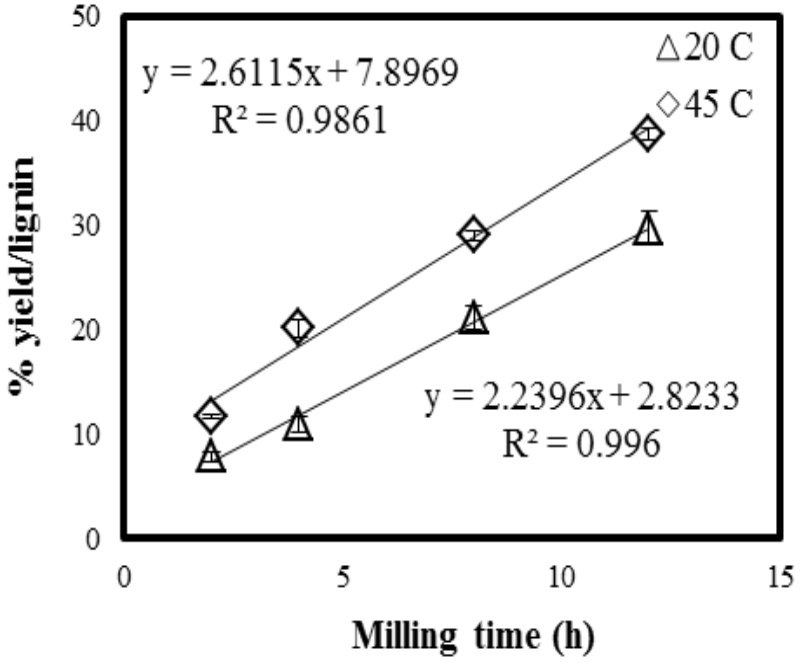
Isolation of the Crude Milled Wood Lignin

The influence of extracting temperature and milling time on the yield of crude milled wood lignin is shown in Fig. 1. Irrespective of wood species, the yield of crude milled wood lignin increased with increasing milling time due to the decrease of particle size and bond cleavage (Björkman 1956). With constant milling time, the sample exhibited a high yield of MWLc at high extracting temperature (Fig. 1). The lignin content of MWLc was semi-quantified by ultraviolet (UV) spectroscopy at 280 nm wavelength (Fig. 2). Sweetgum and loblolly pine exhibited the same trend, with lignin concentration decreasing with increased milling time. With milling time held constant, higher lignin concentration could be expected at a lower extracting temperature (Fig. 2). Taking into account the yield, the absolute value of lignin was higher with both longer milling time and higher extracting temperature; concentration was, however, lower. The result was confirmed by the Klason lignin method (TAPPI T222 om-98). The lignin content of MWLc determined from UV was higher for sweetgum and loblolly pine than that determined by the Klason lignin method.

Technically, 30% yield of MWLc based on lignin is optimal to represent the whole lignin because a more severe structural change of lignin induced by conditions is required to obtain higher yield. In fact, another 40% yield of cellulase-hydrolyzed lignin (CEL) based on lignin can be obtained from the residue. The total yield of isolated lignin (MWLc and CEL) is more than 70% of lignin, which is representative of lignin. Based on the yield and the lignin content of MWLc, the optimum isolation procedure was 8 h of milling for hardwood and 12 h of milling for softwood, with both exhibiting optimal extracting temperatures of 20 °C. The lignin content overestimation of sweetgum was exceptionally high due to the interference of conjugated α-carbonyl group (Sarkenen *et al.* 1967). In the MWLc, various levels of carbohydrates remained (Table 2), which could have potentially interfered with the characterization of lignin structure (Obst and Landucci 1986). It was surprising that little xylan was left in MWLc of sweet gum; however, a significant amount of xylan remained in MWLc of loblolly pine. Thus, the purification of two MWLc samples (sweetgum 8 h, 45 °C and loblolly pine 12 h, 45 °C) was carried out to eliminate interferences due to any contaminating carbohydrates. MWLc was briefly dissolved in 90% AcOH and then precipitated drop-wise into water. The precipitate was washed, freeze-dried, and dissolved in dichloroethane ethanol (volume ratio 2:1) mixture; afterwards, the solution was precipitated drop-wise into ether. The precipitate was filtered and washed with ether and petroleum ether, then dried to obtain the purified milled wood lignin (MWLp). After the purification, there were few carbohydrates left, typically 1 to 2% of softwood and 3 to 4% of hardwood which were comparable to the result reported by Balakshin *et al.* (2011).



(a)

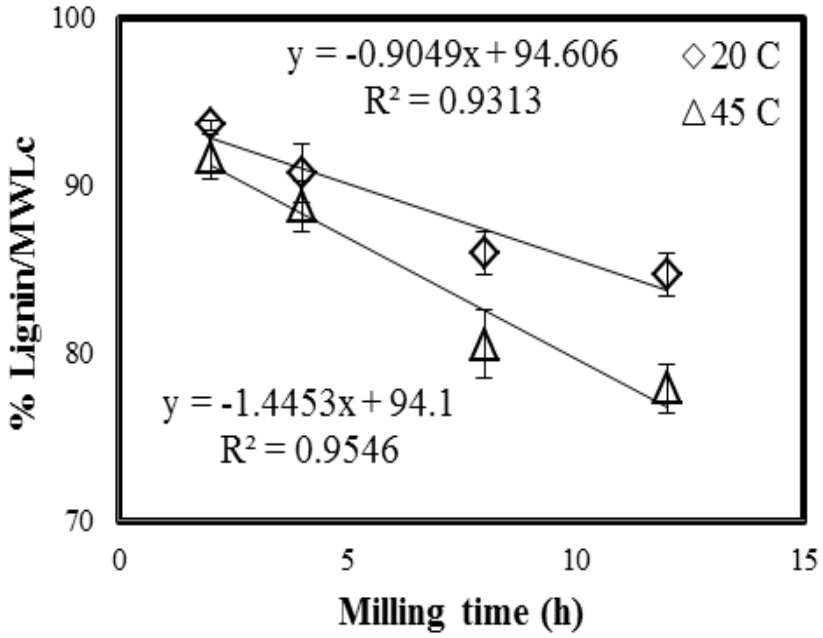


(b)

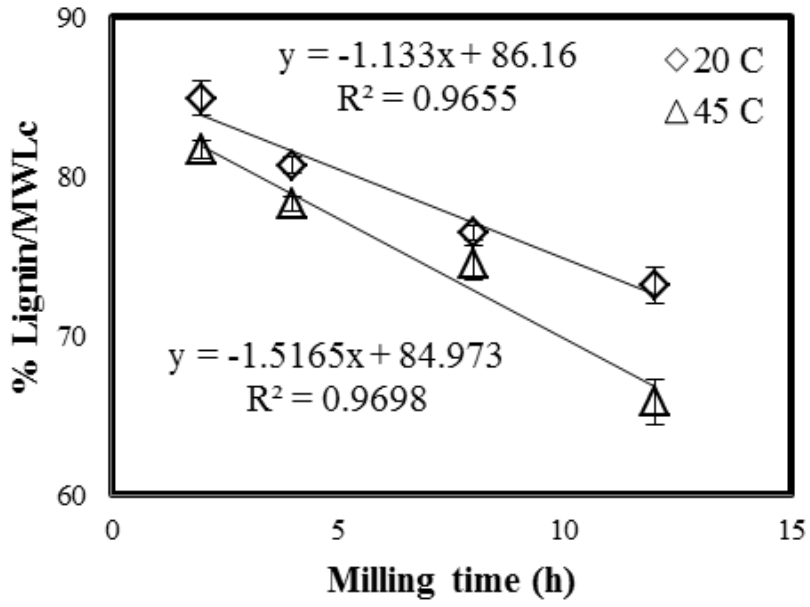
Fig. 1. Yield of milled wood lignin based on lignin (a) loblolly pine (b) sweetgum (average ± standard deviations)

Structure Characterization of MWL by ^{13}C NMR

Figures 3 and 4 illustrate the peak clusters of acetylated sweetgum and loblolly pine MWL quantified by use of ^{13}C NMR spectra. Each cluster was separated from the adjoining one by a sufficient amount of baseline to allow precise valuation. It was found that an ample baseline at both limits of the integral is essential for accurate phasing and reproducibility (Landucci 1985).



(a)



(b)

Fig. 2. Lignin content of milled wood lignin (a) loblolly pine, (b) sweetgum (average ± standard deviations)

Various lignin moieties could be detected in the spectra according to the well-known databases (Balakshin *et al.* 2003; Ibarra *et al.* 2007; Ralph *et al.* 2006; Yelle *et al.* 2008; Zhang *et al.* 2006). The integral at 160 to 102 ppm was set as the reference for sweetgum, assuming that it includes six aromatic carbons. The aliphatic signal at about 101 ppm was assigned to the C-1' of arabinofuranosides (Minor 1984). However, for the loblolly pine MWL, the integral ranging from 164 to 108 ppm was used as the reference to eliminate interferences of the contaminate carbohydrate.

Table 2. Chemical Components of the Crude Milled Wood Lignin (%)

T	No.	ISL	ASL	TL	Ara.	Rha.	Gal.	Glu.	Xyl.	Man.	TC	Balance
20 °C	SG12h	60.0	5.5	65.5	1.1	0.0	0.0	8.8	0.0	0.0	9.8	75.3
	SG8h	61.7	5.4	67.1	1.0	0.0	0.0	7.6	0.0	0.0	8.6	75.6
	SG4h	63.3	5.2	68.5	1.2	0.0	0.0	5.9	0.0	0.0	7.1	75.6
	SG2h	64.3	5.1	69.4	1.1	0.0	0.0	5.4	0.0	0.0	6.5	75.9
45 °C	SG12h	57.0	5.8	62.8	1.3	0.0	0.0	10.8	0.0	0.0	12.1	74.9
	SG8h	58.4	5.1	63.5	1.3	0.0	0.0	10.3	0.0	0.0	11.5	75.1
	SG4h	62.6	5.3	68.0	1.3	0.0	0.0	7.3	0.0	0.0	8.6	76.6
	SG2h	63.7	5.1	68.8	1.2	0.0	0.0	5.8	0.0	0.0	7.0	75.8
45 °C	PINE12h	77.7	1.7	79.3	0.0	2.0	1.2	1.8	1.6	1.8	8.3	87.6
	PINE8h	78.9	1.7	80.6	0.0	1.9	1.0	1.6	1.4	1.8	7.7	88.4
	PINE4h	77.4	2.2	79.6	0.0	1.6	0.7	1.6	1.1	1.5	6.5	86.0
	PINE2h	80.5	2.5	83.0	0.0	1.0	0.0	1.1	1.0	1.0	4.0	87.0
20 °C	PINE12h	77.6	1.8	79.4	0.0	2.0	1.0	2.0	1.5	1.9	8.5	87.9
	PINE8h	77.4	1.9	79.2	0.0	1.8	0.9	1.7	1.2	1.6	7.2	86.5
	PINE4h	80.1	2.0	82.1	0.0	1.5	0.7	1.3	1.0	1.2	5.4	87.5
	PINE2h	81.8	2.2	84.0	0.0	1.1	0.5	1.0	0.7	0.8	4.1	88.1

Notes: % values were based on the crude milled wood lignin; ISL, acid insoluble lignin; ASL, acid soluble lignin; TL, total lignin; TS, total carbohydrates.

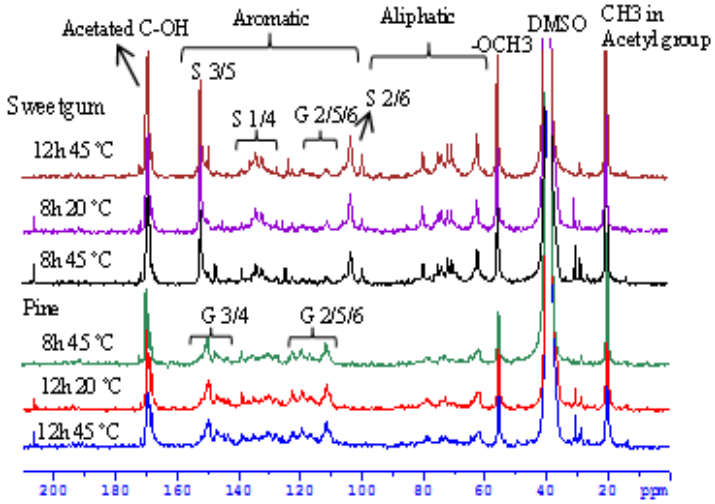


Fig. 3. ^{13}C NMR spectrum of acetylated sweetgum and loblolly pine milled wood lignin (different milling times and extraction temperatures)

The aliphatic signal at *ca.* 6 ppm was probably due to the C-1' of xylan (Toikka and Brunow 1999). In addition, the number of vinyl carbon atoms in cinnamyl alcohol and cinnamyl aldehyde structures in the MWL varied according to species. Typically, the correction is not made on the hardwood because the contribution to the integral at 160 to 102 ppm is so small; the correction was, however, applied on softwood due to the remarkable contribution (Fengel *et al.* 1978; Adler *et al.* 1987; Chen 1998). The integral values for other structural moieties (Table 3 and 4) were expressed per aromatic ring (Ar).

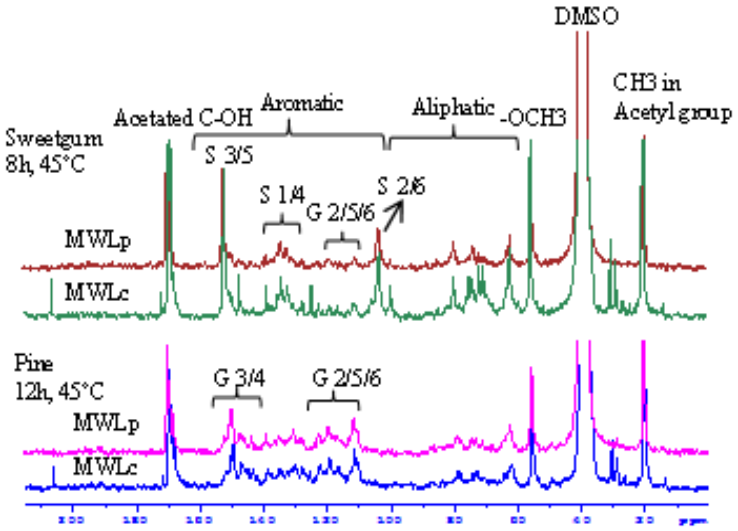


Fig. 4. ^{13}C NMR spectrum of acetylated sweetgum and loblolly pine milled wood lignin (MWLc and MWLp)

Table 3. Quantitative ^{13}C NMR of MWL of Sweetgum

Shift (ppm)	Assignment	20°C 8h	45°C 8h	45°C 8h purified	45°C 12h
172-169	primary aliphatic OH	1.16	1.16	1.05	1.31
169-168	secondary aliphatic OH	0.45	0.44	0.35	0.43
168-166	phenolic OH, conjugated COOR	0.27	0.31	0.27	0.23
125-101	CAr-H	2.16	2.07	2.06	2.04
109-101	S-2,6; R-2,6	1.21	1.16	1.18	1.14
90-77	Alk-O-Ar, α-O-Alk	0.99	0.99	1.06	0.86
66-58	OHprim	1.03	1.08	0.91	1.20
58-54	OMe	1.55	1.57	1.56	1.56
23-18	CH3 in acetyl group	1.77	1.87	1.77	2.25
Calculated	S/G	1.25	1.27	1.22	1.27

Note: All values are based on a C_9 lignin unit.

Table 4. Quantitative ^{13}C NMR of MWL of Loblolly Pine

Shift (ppm)	Assignment	20°C 12h	45°C 12h	45°C 12h purified	45°C 8h
172-169	primary aliphatic OH	0.97	0.93	0.80	0.88
169-168	secondary aliphatic OH	0.44	0.48	0.38	0.31
168-166	phenolic OH, conjugated COOR	0.17	0.19	0.23	0.17
125-101	CAr-H	2.37	2.37	2.41	2.30
109-101	S-2,6; R-2,6	0.28	0.39	0.39	0.20
90-77	Alk-O-Ar, α-O-Alk	0.64	0.68	0.79	0.55
66-58	OHprim	0.77	0.75	0.61	0.64
58-54	OMe	0.94	0.92	0.95	0.95
23-18	CH3 in acetyl group	1.39	1.34	1.34	1.49

Note: All values are based on a C_9 lignin unit.

Structural level information of the lignin can be obtained through the analysis of the various types of inter-unit linkages and functional groups presenting (Figs. 3 and 4). The amount of methoxyl groups (OMe) was estimated from the integral at 58 to 54 ppm

as 1.56/Ar for sweetgum and 0.95/Ar for loblolly pine, which was comparable to the result of previous research (Obst and Landucci 1986). Other minor moieties, which can be included in this integral (Capanema *et al.* 2004), were not considered because their amounts were in the range of the integration error (≤ 3%). The methoxyl content of softwood could be influenced not only by contamination from non-lignin aromatic extractives, but also by compression wood lignin, which contains a large amount of *p*-hydroxyphenyl units (Nimz 1981). The amount of hydroxyl groups was estimated in the acetylated MWL from the region of 172 to 166 ppm (Figs. 3 and 4). The amount of primary OH groups of sweetgum MWL ranged from 1.05 to 1.31/Ar depending on the technical samples, whereas the value for secondary OH groups ranged from 0.35 to 0.45/Ar. The maximum amount of Alk-O-Ar moieties was estimated from the region of 90 to 77 ppm. Useful information pertaining to lignin structure was obtained from ratios of the areas of peak clusters. For example, the syringyl-to-guaiacyl ratio of sweetgum MWLc can be estimated at about 1.27. The sweetgum MWLc contained about 6.5% to 12% carbohydrate, depending on the isolation condition, which was mainly xylan. After acetylation, all MWLc were soluble in DMSO-d_6. When the sweetgum MWLc was purified to give a nearly carbohydrate-free MWLp, there was little change in the methoxyl content and S/G ratio because the contribution of carbohydrate to the aryl integral was eliminated. So far, the milled wood lignin is still accepted as the best representative of native lignin of biomass despite comprising only 30 to 60% of the entire lignin (Fig. 1). The structure of MWL was determined by the severity of the extraction process (Table 3 and 4). For example, *β*-O-4', the most abundant linkage in lignin, was sensitive to factors such as milling speed and time (Ikeda *et al.* 2002). Carbohydrate influence on MWL structure could be distinguished as well (Table 3 and 4). A similar effect of the isolation condition on lignin structure was seen in loblolly pine (Table 4). While no definitive experiment was set up to unambiguously determine the origin of milled wood lignin (Capanema *et al.* 2004; Whiting and Goring 1981; Adler 1977; Musha and Goring 1975; Kolar *et al.* 1982), a more accurate and complete picture of native lignin structure was elucidated through quantitative ^{13}C NMR of MWL.

CONCLUSIONS

Firstly, liquid state quantitative ^{13}C NMR enabled detailed identification and quantification of various lignin moieties of the milled wood lignin (methoxyl content, S/G ratio, *etc.*). Most side-chain moieties of MWL could be estimated based on the structural level.

Secondly, the structure of the milled wood lignin was affected significantly by the severity of isolation such as the milling time and speed, and extracting temperature. The S/G ratio was derived and was comparable with previous studies.

Thirdly, information on the quantity of various functional groups and inter-unit linkages was relatively similar among the milled wood lignin if the same isolation procedure was carried out. The milled wood lignin is still the optimal candidate to represent the characteristics of the native lignin, despite slight differences, depending on isolation procedures. In general, the optimal isolation process for hardwood based on yield and lignin content of MWLc was judged to be 8 h of milling for hardwood, and 12 h for softwood, with both exhibiting optimal extracting temperatures of 20 °C.

REFERENCES CITED

Adler, E. (1977). "Lignin chemistry – past, present, and future," *Wood Sci. Technol.* 11(3), 169-218.

Adler, E., Brunow, G., and Lundquist, K. (1987). "Investigation of the acid catalyzed alkylation of lignin by means of NMR spectroscopic methods," *Holzforschung* 41(4), 199-207.

Balakshin, M. Y., Capanema, E. A., Chen, C.-L., and Gracz, H. (2003). "Elucidation of the structures of residual and dissolved pine kraft lignin using an HMQC technique," *J. Agric. Food. Chem.* 51(21), 6116-6127.

Balakshin, M. Y., Capanema, E. A., Gracz, H., Chang, H.-M., and Jameel, H. (2011). "Quantification of lignin–carbohydrate linkages with high-resolution NMR spectroscopy," *Planta* 233(6), 1097-1110.

Björkman, A. (1956). "Studies on finely divided wood. Part I. Extraction of lignin with neutral solvents," *Sven. Papperstidn* 59(13), 477-485.

Capanema, E. A., Balakshin, M. Y., and Kadla, J. F. (2004). "A comprehensive approach for quantitative lignin characterization by NMR spectroscopy," *J. Agric. Food Chem.* 52(7), 1850-1860.

Chen, C.-L., and Danielle, R. (1988). "Characterization of lignin by ^{1}H and ^{13}C NMR Spectroscopy," In: *Methods in Enzymology*, Wood, W. A., and Kellogg, S. T. (eds.), Academic Press, New York, Vol. 161B, pp. 137-174.

Chen, C.-L. (1998). "Characterization of milled wood lignins and dehydrogenative polymerisates from monolignols by ^{13}C NMR spectroscopy," In: *Lignin and Lignan Biosynthesis*, Lewis, N., Sarkanen, S. (eds.), ACS Symposium Series 697; American Chemical Society, Washington, DC, pp. 255-275.

Danielle, R. (1982). "Quantitative analysis of lignins by ^{13}C NMR analysis," In: *Extended Abstracts: Canadian Wood Chemistry Symposium*, Chemical Institute of Canada / Technical Section, CPPA, Niagra Falls, Ontario, Canada, Sept. 13-15, pp. 63-66.

Fengel, D., Stoll, M., and Wegener, G. (1978). "Studies on milled wood lignin from spruce Part II. Electron microscopic observations on the milled wood," *Wood Sci. Technol.* 12(2), 141.

Fukagawa, N., Meshitsuka, G., and Ishizu, A. (1991). "A two-dimensional NMR study of birch milled wood lignin," *J. Wood Chem. Technol.* 11(3), 373-396.

Fujimoto, A., Matsumoto, Y., Chang, H. M., and Meshitsuka, G. (2005). "Quantitative evaluation of milling effects on lignin structure during the isolation process of milled wood lignin," *J. Wood Sci.* 51, 89-97.

Ibarra, D., Chavez, M. I., Rencoret, J., Del Rio, J. C., Gutierrez, A., Romero, J., Camarero, S., Martinez, M. J., Jumenez-Barbero, J., and Martinez, A. T. (2007). "Lignin modification during *Eucalyptus globulus* kraft pulping followed by totally chlorine-free bleaching: A two dimensional nuclear magnetic resonance, Fourier transform infrared, and pyrolysis-gas chromatography/mass spectrometry study," *J. Agric. Food Chem.* 55(9), 3477-3490.

Ikeda, T., Holtman, K., Kadla, J. F., Chang, H. M., and Jameel, H. (2002). "Studies on the effect of ball milling on lignin structure using a modified DFRC method," *J. Agr. Food Chem.* 50(1), 129-135.

Kanitskaya, L. V., Medvedeva, S. A., Zakazov, A. N., Rokhin, A. V., Babkin, V. A., and Kalabin, G. A. (1993). "Quantitative ^{1}H, ^{13}C NMR spectroscopy of lignin," In:

Proceedings of the 7th International Symposium on Wood and Pulping Chemistry, Beijing, China, May 25-28, Vol. III, pp. 489-494.

Kolar, J. J., Lindgren, B. O., and Treiber, E. (1982). "The distribution of lignin between fiber wall and middle lamella," *Svensk Papperstidn* 85(3), R21-26.

Landucci, L. L. (1985). "Quantitative ^{13}C NMR characterization of lignin," *Holzforschung* 39(6), 355-359.

Maurer, A., and Fengel, D. (1992). "On the origin of milled wood lignin. Pt. 1: The influence of ball-milling on the ultrastructure of wood cell walls and the solubility of lignin," *Holzforschung* 46(5), 417.

Minor, J. L. (1984). "Composition and modification of polymers in pine kraft black liquors," In: *1984 International Chemical Congress of Pacific Basin Societies*, December 16-21, Honolulu, Hawaii.

Musha, Y., and Goring, D. A. I. (1975). "Distribution of syringyl and guaiacyl moieties in hardwoods as indicated by ultraviolet microscopy," *Wood Sci. Technol.* 9(1), 45-58.

Nimz, H. (1981). "The occurrence of non-cyclic benzyl ether bonds in lignin," *Wood Sci. Technol.* 15(4), 311-316.

Obst, R. J., and Landucci L. L. (1986). "Quantitative ^{13}C NMR of lignins – Methoxyl : aryl ratio," *Holzforschung* 40(1 / Suppl.), 87-92.

Ralph, J., Akiyama, T., Kim, H., Lu, F., Schatz, P. F., Marita, J. M., Ralph, S. A., Reddy, M. S. S., Chen, F., and Dixon, R. A. (2006). "Effects of coumarate 3-hydroxylase down-regulation on lignin structure," *J. Biol. Chem.* 281(13), 8843-8853.

Ralph, J., Marita, J., Ralph, S. A., Hatfield, R. D., Lu, F., Ede, R. M., Peng, J., Quideau, S., Helm, R. F., Grabber, J. H., Kim, H., Jimenez-Monteon, G., Zhang, Y., Jung, H. J. G., Landucci, L. L., MacKay, J. J., Sederoff, R. R., Chapple, C., and Boudet, A. M. (1999). "Solution-state NMR of lignins," In: *Advances in Lignocellulosics Characterization*, Argyropoulos, D. S. (ed.), TAPPI Press, Atlanta, GA, pp. 55-108.

Sarkenen, K. V., Chang, H.-m., and Allan, G. G. (1967). "Species variation in lignins. II. Conifer lignins," *Tappi* 50(12), 583-587.

Toikka, M., and Brunow, G. (1999). "Lignin-carbohydrate model compounds. Reactivity of methyl 3-O-(α-L-arabinofuranosyl)-β-D-xylopyranoside and methyl β-D-xylopyranoside towards a β-O-4-quinone methide," *J. Chem. Soc., Perkin Trans.* 1, 1877-1883.

Whiting, P., and Goring, D. A. I. (1981). "The morphological origin of milled wood lignin [*Picea mariana*]," *Sven. Papperstidn*. 84(15), R120-Rl22.

Yelle, D. J., Ralph, J., and Frihart, C. R. (2008). "Characterization of nonderivatized plant cell walls using high-resolution solution-state NMR spectroscopy," *Magn. Res. Chem.* 46(9), 508-517.

Zhang, L., Gellerstedt, G., Lu, F., and Ralph, J. (2006). "NMR studies on the occurrence of spirodienone structures in lignins," *J. Wood Chem. Technol.* 26(1), 65-79.

Article submitted: November 16, 2012; Peer review completed: February 10, 2013; Revised version received: February 12, 2013; Accepted: February 17, 2013; Published: February 19, 2013.