Comparative Study of Three Lignin Fractions Isolated from Mild Ball-Milled *Tamarix austromogoliac* and *Caragana sepium*

Feng Xu,[1,2] Run-Cang Sun,[1,3,4] Mei-Zhi Zhai,[4] Jin-Xia Sun,[4] Jian-Xin Jiang,[1] Guang-Jie Zhao[1]

[1]*College of Material Science and Technology, Beijing Forestry University, Beijing 100083, China*
[2]*College of Light and Textile Industry, Qiqihar University, Wenhua Street, Qiqihar, China*
[3]*State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, Guangzhou 510641, China*
[4]*College of Forestry, North-Western University of Agricultural and Forest Sciences and Technology, Yangling 712100, China*

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ABSTRACT: Six lignin fractions from mild ball-milled *Tamarix austromogoliac* (TA) and *Caragana sepium* (CS) were sequentially isolated with 80% dioxane containing 0.05*M* HCl at 75°C for 4 h, 50% aqueous ethanol containing 1*M* triethylamine at 70°C for 4 h, and 8% aqueous NaOH at 45°C for 3 h. The results showed that the successive treatments made it possible to isolate lignin from wood with a high yield and purity, in which 89.4 and 90.6% of the original lignin from TA and CS were released, respectively. The lignin fractions isolated with the three-step method were analyzed with Fourier transform infrared, ^{1}H- and ^{13}C-NMR, alkaline nitrobenzene oxidation, and gel permeation chromatography. It was found that the three lignin fractions isolated from TA were rich in syringyl units, and the molar ratio of the relatively total moles of vanillin, vanillic acid, and acetovanillin to the relatively total moles of syringaldehyde, syringic acid, and acetosyringone decreased from 1 : 2.6 to 1 : 3.2 to 1 : 3.6 in the lignin preparations, whereas this ratio in the corresponding lignin fractions isolated from CS was found to be 1.4 : 1, 1.1 : 1, and 1 : 1.4, respectively. More importantly, the results revealed that the sequential extractions of the mild ball-milled TA and CS with 80% acidic dioxane, 50% alkaline ethanol, and 8% aqueous NaOH under the conditions used did not significantly cleave the β–*O*-4 and α–*O*-4 linkages in lignin macromolecules. J Appl Polym Sci 108: 1158–1168, 2008

Key words: fractionation of polymers; FTIR; gel permeation chromatography (GPC); molecular weight distribution/molar mass distribution

INTRODUCTION

Lignin is an amorphous, three-dimensional copolymer of phenylpropanoid units linked through ether and carbon–carbon bonds such as β–*O*-4, 4–*O*-5, β–β, β–1, β–5, and 5–5′.[1] In addition, lignin is covalently linked to polysaccharides, forming a lignin–hemicellulose network made up of benzyl–ether,[2–4] benzyl–ester,[5–7] and phenyl–glycoside bonds.[8,9] It provides mechanical support for plants, facilitates the transport of nutrients, and defends against attack from microorganisms.[10] Because of this complex structure, quantitative and complete isolation of the natural lignin polymer has proven to be impossible.[11] Early lignin preparation techniques used strong mineral acids to reach high lignin yields.[12] Such drastic conditions, however, were found to cause irreversible reactions that severely alter the structure of the isolated lignin polymers.

To isolate the total lignin from wood in a chemically unaltered form, the most currently used techniques are based on the extraction of ball-milled wood by neutral[13,14] or mildly acidic[15–17] solvents. Milled wood lignin (MWL) is extracted from finely milled wood with neutral dioxane,[13] and cellulolytic enzyme lignin (CEL) uses cellulolytic enzymes to remove most of the polysaccharides before aqueous dioxane extraction of ball-milled wood meal.[14] In this case, the structure of lignin is modified by the milling processes, although the extent of structural changes has not been verified.[11] In general, the more severe the milling conditions are, the

Correspondence to: R.-C. Sun (bcs00a@bangor.ac.uk).
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higher the yields are that are achieved by such isolation processes. However, concerns exist over the similarity between MWL and native lignin based on the low yields (25–50% protolignin) and structural alterations due to ball milling.[18] It has been found that substantial lignin depolymerization via the cleavage of uncondensed β–aryl ether linkages may take place under severe mechanical action.[16,17] As a result, relatively minor structural features may be merely artifacts of lignin isolation.[11] Furthermore, it has been shown that the nature of lignin in wood is heterogeneous, and the layers of the cell walls are not proportionally represented in MWL.[19] Therefore, it would be preferable to characterize the structural features of lignin in different fractions. Interestingly, a novel procedure using the combination of enzymatic and mild acidolysis [enzymatic mild acidolysis lignin (EMAL)] was recently proposed by Wu and Argyropoulos[15] to isolate lignin that may be more representative of the total lignin present in milled wood. In this case, mild acid hydrolysis can liberate lignin from milled wood with a high yield and purity. It has been reported that the yield of EMAL from Norway spruce is about 4 times greater than that of the corresponding MWL and about 2 times greater compared to that of CEL isolated from the same batch of milled wood.[16] More importantly, in comparison with MWL and CEL, no extent of structural changes has been verified in EMAL, and this indicates that EMAL is released by cleavage of lignin–hemicellulose bonds rather than other linkages within the lignin macromolecule.[17]

In this study, three lignin fractions were therefore isolated by sequential treatments of mild ball-milled *Tamarix austromogoliac* (TA) and *Caragana sepium* (CS) with dioxane–H_2O (80/20 v/v) containing 0.05*M* HCl at 75°C for 4 h, 50% ethanol containing 1*M* triethylamine (TEA) at 70°C for 4 h, and 8% aqueous NaOH at 45°C for 3 h. The solubilized lignin fractions were analyzed to characterize their chemical composition, physicochemical properties, and linkages between units by the use of a degradation method such as alkaline nitrobenzene oxidation and by the use of nondegradation techniques such as ultraviolet (UV), Fourier transform infrared (FTIR), and ^{1}H- and ^{13}C-NMR as well as gel permeation chromatography (GPC). Moreover, we used the virtues of such lignin isolation protocols to better understand the inhomogeneities in the chemical structure of lignin in the cell walls of wood. The isolated lignins can be commercially used in a wide range of products. Some of these applications are materials for automotive brakes, wood panel products, phenolic resins, biodispersants, polyurethane foams, epoxy resins for printed circuit boards, and surfactants.[20,21]

EXPERIMENTAL

Materials

TA and CS, 5 years old, were harvested in October 2004 in Mongolia, China, with an average stem height of 3.1 m. The leaves and the bark were removed, and the trunks were chipped into small pieces. After drying at 60°C for 16 h in an oven, the chips were then ground to pass a 0.8-mm-size screen, and the powder was dewaxed with toluene–ethanol (2 : 1 v/v) in a Soxhlet instrument for 6 h. The dewaxed sample was further dried in a cabinet oven with air circulation at 60°C for 16 h and stored at 5°C before ball milling. The major components (% w/w) of TA and CS were cellulose (39.8 and 40.6%), hemicelluloses (31.0 and 30.5%), and lignin (19.8 and 19.2% on a dry weight basis, respectively); this was determined by the method for measuring the chemical composition of wheat straw described previously.[22] All weights and calculations were made on an oven-dried basis. All chemicals were purchased from Sigma Chemical Co. (Beijing, China).

Fractional isolation of lignins

Rotary ball milling was performed in a 2-L porcelain jar in the presence of 68 porcelain balls (15 mm in diameter), which occupied 20% of the active jar volume. The extractive-free powder was loaded into the jar, creating a porcelain ball to sample weight ratio of 25.3. The mild milling process was conducted at room temperature for up 78 h with a rotation speed of 70 rpm. The ball-milled sample was first submitted to mild acid hydrolysis with aqueous dioxane (80 : 20 v/v dioxane/water containing 0.05*M* HCl) under a nitrogen atmosphere at 75°C for 4 h with a solid-to-liquid ratio of 1 : 20 (g/mL). The resulting suspension was filtered, and the residue was washed with 80% aqueous dioxane twice. The combined supernatant was concentrated with a rotary evaporator under reduced pressure to about 80 mL and then mixed with 3 volumes of 95% ethanol (2 h, 22°C) for the isolation of dioxane-soluble hemicellulosic polymers. The precipitated hemicellulosic fraction was filtered and washed with 70% ethanol at room temperature and was then dried in air. The 80% acidic dioxane-soluble lignin fraction was obtained by reprecipitation at pH 1.5–2.0 from the corresponding supernatants after the evaporation of ethanol and remaining dioxane. It was washed with acidified water (pH 2.0), freeze-dried overnight, and kept at 5°C until analysis. The 80% acidic dioxane-soluble lignins from TA and CS were labeled lignin fractions L_{1T} and L_{1C}, respectively. The residue free of 80% acidic dioxane solubles was successively treated with 50% ethanol containing 1*M* TEA at 70°C for 4 h and 8% aqueous NaOH at 45°C for 3 h. The insolu-

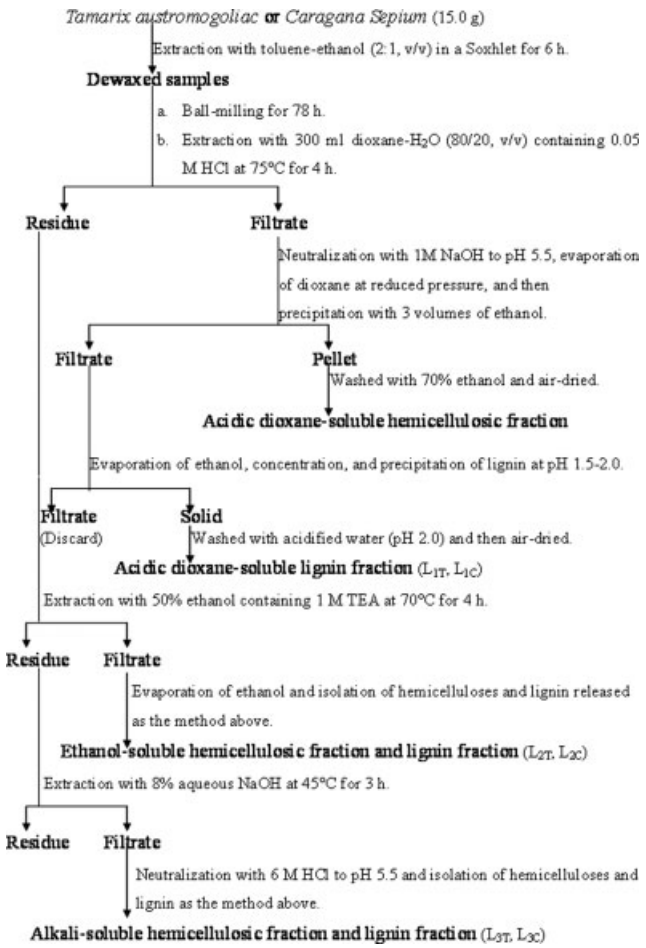

Figure 1 Scheme for fractionation of lignins from mild ball-milled TA and CS.

ble residue was collected as crude cellulose by filtration, washed with distilled water, and then dried at 60°C for 16 h. Each of the supernatant fluids was adjusted to pH 5.5–6.0 and then concentrated under reduced pressure. The hemicellulosic polymers released were precipitated by the pouring of the concentrated supernatant fluid into 3 volumes of 95% ethanol. The solubilized lignins were then obtained from the corresponding supernatants by precipitation at pH 1.5–2.0, purified by washing with acidified water (pH 2.0), and freeze-dried. Note that the lignins solubilized during the sequential treatments of the 80% acidic dioxane extracted TA residue with 50% ethanol containing 1*M* TEA and 8% aqueous NaOH were labeled lignin fractions L_{2T} and L_{3T}, and the lignins released during the corresponding 50% alkaline ethanol and 8% aqueous NaOH treatments of the 80% acidic dioxane extracted CS residue were coded lignin fractions L_{2C} and L_{3C}, respectively. The scheme for the sequential processes of TA and CS and the fractional isolation of the lignins solubilized is illustrated in Figure 1. All experiments were performed at least in duplicate. Yields of the fractional lignins are given on a dry weight basis related to the starting material (Table I). The standard errors or deviations were observed to be lower than 5.6%.

Characterization of the lignin fractions

The hemicellulosic moieties associated with the lignin fractions were determined by hydrolysis with dilute sulfuric acid. A 20-mg sample of lignin was hydrolyzed with 2 mL of 6% H_2SO_4 for 2.5 h at 100°C. The mixture was filtered, and the filtrate containing monosaccharides was analyzed by gas chromatography of their alditol acetates.[23] The total content of uronic acids in the lignin fractions was colorometrically determined as 4-*O*-methyl-D-glucopyranosyluronic acid.[22] The analyses were run at least twice, and the error was less than 0.05% for all of the lignin fractions. The monomeric composition of the noncondensed monomeric units of the six lignin fractions was analyzed by nitrobenzene oxidation, and an analysis of the resulting aromatic aldehydes and acids by high-performance liquid chromatography has been reported previously.[24] All nitro-

TABLE I
Yields of Hemicelluloses and Lignin (% Dry Matter) Obtained by Sequential Extractions of Mild Ball-Milled TA and CS with Dioxane–H_2O (80/20 v/v) Containing 0.05*M* HCl at 75°C for 4 h, 50% Aqueous Ethanol Containing 1*M* TEA at 70°C for 4 h, and 8% Aqueous NaOH at 45°C for 3 h

Lignin fraction	Yield (% dry matter) TA	CS
Total solubilized lignin in acidic dioxane treatment	9.5	7.7
Acid-insoluble lignin	8.2 (L_{1T})[a]	5.8 (L_{1C})[a]
Lignin associated in isolated hemicelluloses	0.12	0.11
Acid-soluble lignin	1.2	1.8
50% ethanol-soluble lignin	5.4	6.4
Acid-insoluble lignin	4.3 (L_{2T})[b]	4.8 (L_{2C})[b]
Lignin associated in isolated hemicelluloses	0.08	0.19
Acid-soluble lignin	1.0	1.4
8% aqueous NaOH-soluble lignin	2.8	3.3
Acid-insoluble lignin	2.1 (L_{3T})[c]	2.3 (L_{3C})[c]
Lignin associated in isolated hemicelluloses	0.10	0.17
Acid-soluble lignin	0.60	0.86
Total solubilized lignin	17.7	17.4

[a] L_{1T} and L_{1C} represent the acid-insoluble lignin fractions solubilized during the extraction with dioxane–H_2O (80/20 v/v) containing 0.05*M* HCl at 75°C for 4 h from the mild ball-milled TA and CS, respectively.

[b] L_{2T} and L_{2C} represent the acid-insoluble lignin fractions released during the sequential extraction with 50% ethanol containing 1*M* TEA at 70°C for 4 h from the 80% dioxane-treated TA and CS, respectively.

[c] L_{3T} and L_{3C} represent the acid-insoluble lignin fractions obtained by extraction with 8% aqueous NaOH at 45°C for 3 h from the 50% ethanol-treated TA and CS, respectively.

benzene oxidation results represent the mean of triplicate samples, and each oxidation mixture was chromatographed twice. The standard deviations were observed to be 5.8–13.0%. The method for measuring the molecular weights of the lignin fractions has been described in a previous article.[25] The average relative deviation was found to be 3.5–5.8%.

Ultraviolet–visible (UV–vis) spectra were recorded on a Hewlett–Packard 8425A diode array spectrophotometer. The FTIR spectra of the lignin fractions were recorded from a KBr disc containing 1% finely ground samples on a Nicolet 750 FTIR spectrophotometer in the range of 4000–400 cm^{-1}. The solution-state ^{1}H-NMR and ^{13}C-NMR spectra were obtained on a Bruker (Ettlingen, Germany) MSL300 spectrometer operating in the FT mode at 74.5 MHz. The lignin sample (25 mg for ^{1}H, 250 mg for ^{13}C) was dissolved in 1 mL of dimethyl sulfoxide-d_6 (99.8% D). The ^{13}C-NMR spectrum was recorded at 25°C after 30,000 scans. A 60° pulse flipping angle, a 3.9-μs pulse width, an 0.85-s acquisition time, and a 1.2-s relaxation delay time were used.

RESULT AND DISCUSSION

Lignin yield and purity

Based on the standard technique for isolating MWL, rotary milling under an N_2 atmosphere with porcelain balls was performed at room temperature for 7 days.[11] Under severe conditions, the milling process was conducted at room temperature for up to 28 days.[16] In this light, intensive milling protocols should be considered with caution because ball milling reduces the degree of polymerization, creating new free-phenolic hydroxyl groups through cleavage of β–aryl ether linkages and increasing α-carbonyl groups via side-chain oxidation.[26–28] In addition, some condensation reactions may occur during the ball-milling process.[26] Therefore, to isolate the lignin that is more representative of the natural lignin in wood, wood meal was milled for only 78 h in this experiment. More interestingly, Wu and Argyropoulos[15] proposed a novel lignin isolation procedure composed of an initial mild enzymatic hydrolysis of milled wood followed by a mild acid hydrolysis stage. In this procedure, the mild acidolysis is designed to cleave the remaining lignin–hemicellulose bonds, liberating lignin in a high yield and purity.[16] To improve yields while minimizing damage to the lignin structure, the extent of mechanical action during milling was reduced by ball milling at a low severity (for only 78 h), and mild acid hydrolysis (80% dioxane containing 0.05*M* HCl) was used to isolate the lignins, which may have facilitated the isolation of less modified lignin from milled wood. As the data given in Table I show, treatment of the mild ball-milled TA and CS with 80% aqueous dioxane containing 0.05*M* HCl at 75°C for 4 h released 48.0 and 40.1% of the original lignin (w/w; based on the amount of Klason lignin of the starting wood and the isolated lignin), respectively, in which acid-insoluble lignin was predominant, accounting for 86.3 and 75.3% of the total solubilized lignins from TA and CS, respectively. On the other hand, the acid-soluble lignin and lignin associated with the isolated hemicelluloses accounted for only 6.1 and 0.60% of the original lignin from TA and 9.4 and 0.57% from CS. These data reveal that the concerted effect of mild ball milling action and mild acid hydrolysis offered significant yield improvements over the traditional procedure for isolating lignin from wood without mild acid hydrolysis.

The Organosolv treatment has proved to be a promising process for achieving complete utilization of lignocellulosics without an impact on the environment.[29] In this case, a considerable extraction of lignin could be also achieved with aqueous organic solvents under acidic or alkaline conditions. The solvent primarily acts on the promotion of vegetal tissue impregnation and the solubilization of the lignin fragments so produced.[30] In this study, posttreatment of 80% acidic dioxane extracted TA and CS with 50% aqueous ethanol containing 1*M* TEA at 70°C for 4 h resulted in 27.3 and 33.3% removal of the original lignin, respectively. As expected, the yield of acid-insoluble lignin (4.3% from TA and 4.8% from CS) was much higher than that of the acid-soluble lignin (1.0% from TA and 1.4 from CS), indicating a slight degradation reaction during the posttreatment with 50% aqueous ethanol under the organic alkali condition (1*M* TEA) because these acid-soluble phenolic compounds of low molecular weight were released as a result of lignin degradation. However, a much higher yield of free lignin (acid-insoluble lignin plus acid-soluble lignin, 5.3% from TA and 6.2% from CS) than that of the lignin associated with the released hemicelluloses (0.08% from TA and 0.19% from CS) verified that the linkages between hemicelluloses and lignin in the cell walls of the two materials were significantly cleaved under the 50% alkaline ethanol posttreatment condition given.

To isolate the remaining lignins, 8% aqueous NaOH was used in the third stage of this experiment, in which any ester bonds present between hemicelluloses and lignin were simultaneously saponified. It was found that a further treatment of the 50% ethanol–1*M* TEA extracted residues of TA and CS with 8% aqueous NaOH at 45°C for 3 h solubilized 15.8 and 19.1% of the original lignin, respectively. It should be noted that the yields of acid-soluble lignin (21.4% of the total lignin solubilized during the third stage of 8% NaOH treatment from TA and 19.1% from CS) and the lignin associated

with the isolated hemicelluloses (3.6% of the total lignin solubilized during the third stage of 8% NaOH treatment from TA and 5.2% from CS) were higher than those obtained during the first and second treatments with 80% acidic dioxane and 50% alkaline ethanol (Table I). This indicated that a significant degradation of the lignin macromolecules occurred during the third-stage treatment with a relatively high concentration of alkali, and the lignin molecules were strongly linked to the polysaccharides in the cell walls of 50% alkaline ethanol treated resides. Taken together, the sequential three-step treatment of the mild ball-milled TA and CS released 89.4 and 90.6% of the original lignin, respectively. Apparently, the acidic dioxane-soluble lignin was the major fraction, comprising 53.7 and 44.3% of the total solubilized lignins from TA and CS, respectively. This result revealed that the acidic dioxane treatment under the condition given significantly liberated lignin by cleaving the ether linkages between lignin and hemicelluloses from the cell walls of mild ball-milled TA and CS. These results were consistent with the findings obtained by Guerra et al.[16] for Norway spruce. The authors showed that the yield of EMAL was about 4 times greater than that of the corresponding MWL and about 2 times greater compared to that of CEL isolated from the same batch of milled wood. In other words, the EMAL protocol was found to offer much higher gravimetric lignin yields and purities than those of the corresponding MWL and CEL isolated from the same batch of milled wood.[17] In comparison, a higher yield of the lignin fraction from TA (53.7%) than from CS (44.3%) implied that the lignin in the cell walls of TA was more easily dissolved than the lignin in CS during the first treatment with 80% acidic dioxane.

UV spectroscopy has been used to semiquantitatively determine the purity of lignin or monitor the lignin distribution among various tissues of gymnosperm and dicotyledonous angiosperm with respect to the concentration.[31] In this study, UV–vis absorption measurements of the acid-insoluble lignin fractions were carried out with a dioxane/water mixture, which solubilized the lignins but was limited to wavelengths above 240 nm. Figure 2 illustrates the UV spectra of acid-insoluble lignin fractions L_{1T}, L_{2T}, and L_{3T} [Fig. 2(a)] and L_{1C}, L_{2C}, and L_{3C} [Fig. 2(b)]. As shown in the spectra, the six lignin fractions exhibit the basic UV spectra typical of lignins, which have a maximum at $\sim$ 274–280 nm, originating from noncondensed phenolic groups in lignin. The presence of a second characteristic region of L_{2C} lignin absorption around 320 nm indicated that the lignin fraction, isolated with 50% ethanol containing 1*M* TEA from the 80% acidic dioxane treated CS, contained noticeable amounts of hydroxycinnamic acids such as ferulic and *p*-coumaric acids.[32] Remarkably,

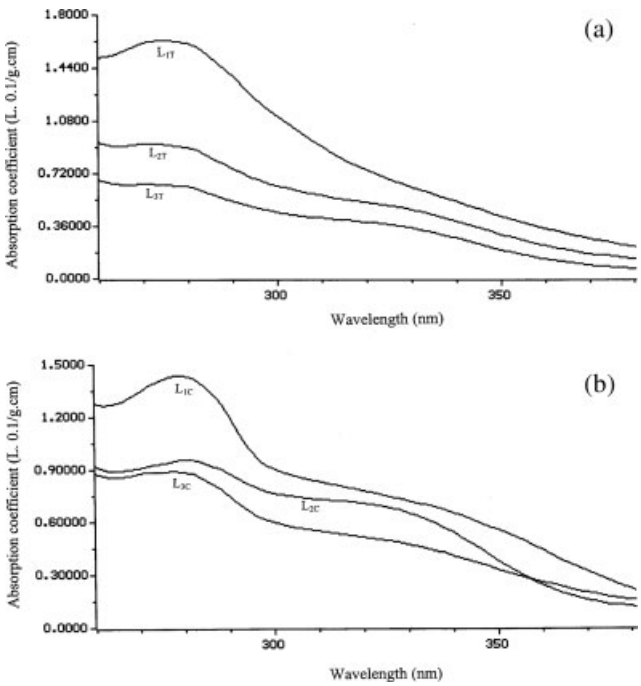

Figure 2 UV spectra of the acid-insoluble lignin fractions: (a) L_{1T}, L_{2T}, and L_{3T} and (b) L_{1C}, L_{2C}, and L_{3C}.

in comparison with lignin fractions L_{1T} and L_{1C}, isolated with 80% acidic dioxane from the mild ball-milled wood, the lower absorption coefficient of lignin fractions L_{2T}, L_{3T}, L_{2C}, and L_{3C}, solubilized during the sequential treatments with 50% ethanol containing 1*M* TEA and 8% aqueous NaOH, was undoubtedly due to the higher amounts of nonlignin materials, such as ash and salt. This higher purity of lignin fractions L_{1T} and L_{1C} demonstrated that most of the nonlignin contaminants (hemicelluloses) were readily removed by the mild acidolysis with 80% dioxane containing 0.05*M* HCl under the condition given. Moreover, the shift of the maximum wavelength from 274 (L_{1T}, L_{2T}, and L_{3T}) to 280 nm (L_{1C}, L_{2C}, and L_{3C}) suggested a higher content of syringyl (S) units in lignin fractions L_{1T}, L_{2T}, and L_{3T} isolated from the mild ball-milled TA because S units exhibit the bands at somewhat shorter wavelengths, specifically at 268–277 nm.[31]

The composition of associated hemicelluloses in the six isolated acid-insoluble lignin fractions was determined by their neutral sugar and uronic acid contents, and the analytical results are shown in Table II. Clearly, all the lignin preparations contained rather low amounts of bound hemicelluloses as shown by the 2.2–8.0% neutral sugar content. This result revealed that the sequential treatments with acidic 80% dioxane, alkaline 50% ethanol, and 8% aqueous NaOH under the conditions used significantly cleaved the ether bonds between lignin and hemicelluloses in the cell walls of TA and CS. As the

TABLE II
Content of Neutral Sugars (% Dry Sample) in the Acid-Insoluble Lignin Fractions

Sugar	Lignin fraction[a]					
	L_{1T}	L_{2T}	L_{3T}	L_{1C}	L_{2C}	L_{3C}
Arabinose	0.4	0.6	0.6	0.3	0.7	0.6
Xylose	0.4	0.7	0.8	0.4	0.8	0.8
Mannose	—[b]	0.1	0.1	—[b]	0.1	0.1
Galactose	0.1	0.2	0.3	0.2	0.4	0.3
Glucose	0.2	0.4	0.4	0.3	0.4	0.4
Uronic acids	6.9	0.8	Trace	2.6	0.5	T
Total	8.0	2.8	2.2	3.8	2.9	2.2

[a] Corresponding to the acid-insoluble lignin fractions in Table I.
[b] Not detectable.

data show in Table II, the two lignin samples of L_{1T} and L_{1C} extracted by acid hydrolysis had much less neutral sugars (1.1–1.2%) but more uronic acids, mainly 4-*O*-methoxylglucuronic acid (2.6–6.9%), whereas the four lignin fractions, isolated by 50% alkaline ethanol and 8% aqueous NaOH, contained 2 times more neutral sugars with small amounts of uronic acids. It is very likely that uronic acids are closely associated with lignin macromolecules by esterification in the cell walls of TA and CS, and these linkages are resistant to being cleaved during the acid hydrolysis process used. In short, these results indicated that the mild acidolysis with 80% dioxane containing 0.05*M* HCl made it possible to isolate lignin from the mild ball-milled wood with a high yield and purity. This significantly low hemicellulose contamination was also an important advantage of the acidolysis method over the enzymatic method because lignins isolated by enzymatic methods contained up to 10% polysaccharides.[33] The data in Table II show that xylose and arabinose were the two major sugar components. Galactose and glucose were verified in minor quantities, and mannose appeared in a trace amount.

Composition of phenolic acids and aldehydes

Nitrobenzene oxidation is a standard analytical technique for lignin monomer determination in wood.[34] During the oxidation, the three constitutive monomeric lignin units [*p*-hydroxyphenyl (H), guaiacyl (G), and S] produce the corresponding *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde. To elucidate the differences in the structures of the fractions, the six lignin fractions were submitted to alkaline nitrobenzene oxidation at 165°C for 3 h, and their phenolic monomers are listed in Table III. The major lignin oxidation products were vanillin and syringaldehyde, which were 79.4–93.1% of the total phenolic monomers. The presence of less *p*-hydroxybenzoic acid (0.10–0.38%) and *p*-hydroxybenaldehyde (0.19–0.74%) was considered most likely to be indicative of noncondensed H units, indicating the incorporation of *p*-hydroxycinnamyl or *p*-coumaryl alcohol in the lignins. Vanillic acid (0.33–1.51%), syringic acid (0.58–2.28%), acetovanillin (0.11–0.73%), and acetosyringone (0.35–1.50%) occurred in small amounts. In comparison, as shown in Table III, the lower yield of nitrobenzene oxidation of lignin fraction L_{1C} (17.2%) suggested the more condensed lignin fraction isolated with 80% acidic dioxane from mild ball-milled CS. In contrast, a slightly lower degree of condensation was observed in all three lignin fractions from TA and the other two lignin fractions from CS.

There are significant differences in the chemical structure of lignin depending on its morphological origin,[35] and these differences can be elucidated by the ratio of the relatively total moles of vanillin, vanillic acid, and acetovanillin (*g*) to the relatively total moles of syringaldehyde, syringic acid, and acetosyringone (*s*) in lignin preparations. It is also well known that lignin in the secondary wall (S2) and lignin in the middle lamella (CML) have different chemical structures.[36] Lignin in the CML has a lower

TABLE III
Yields (% Dry Sample, w/w) of Phenolic Acids and Aldehydes from Alkaline Nitrobenzene Oxidation of the Acid-Insoluble Lignin Fractions

Phenolic acid or aldehyde	Lignin fraction[a]					
	L_{1T}	L_{2T}	L_{3T}	L_{1C}	L_{2C} L_{3C}	
p-Hydroxybenzoic acid	0.18	0.16	0.26	0.40	0.38	0.35
p-Hydroxybenzaldehyde	0.33	0.19	0.41	0.75	0.74	0.49
Vanillic acid	0.33	0.33	1.02	0.50	1.51	0.66
Vanillin	5.06	5.19	3.09	7.69	11.73	7.06
Syringic acid	0.73	0.75	1.42	0.58	2.28	0.70
Syringaldehyde	16.23	20.59	17.50	6.52	11.68	12.33
Acetovanillone	0.14	0.11	0.73	0.33	0.52	0.23
Acetosyringone	0.35	0.40	1.50	0.39	0.63	0.36
Total	23.35	27.72	25.93	17.16	29.47	22.18
g/*s*	1 : 2.6	1 : 3.2	1 : 3.6	1.4 : 1	1.1 : 1	1 : 1.4

[a] Corresponding to the acid-insoluble lignin fractions in Table I.

methoxyl content, is richer in H units and has a higher degree of condensation than the lignin in S2.[37] Furthermore, it has been reported that the majority of lignin in wood is in the S2.[38] As can be seen in Table III, the relative molar ratios of *g* to *s* decreased from 1 : 2.6 in L_{1T} to 1 : 3.2 in L_{2T} to 1 : 3.6 in L_{3T}. Similarly, the *g*/*s* ratio in the three lignin fractions L_{1C}, L_{2C}, and L_{3C} obtained from the mild ball-milled CS decreased from 1.4 : 1 to 1.1 : 1 to 1 : 1.4, respectively. Obviously, the lignin from TA has a higher *s*-unit content than the lignin from CS. Taking into consideration these earlier findings and our current results, we can deduce that lignin isolated during the first treatment with 80% acidic dioxane from the mild ball-milled wood may originate mainly from lignin in CML and partially from lignin in S2, and the two L_{2T} and L_{2C} lignin fractions isolated during the sequentially second treatment with 50% alkaline ethanol qualitatively represent the lignin in both the CML and S2 regions of the cell wall, whereas the L_{3T} and L_{3C} lignin fractions extracted during the last stage with 8% aqueous NaOH dominated the lignin from S2.

Molecular weight

The question of whether the mild acidolysis in the 80% dioxane containing 0.05*M* HCl, 50% alkaline ethanol, and 8% aqueous NaOH treatments caused some lignin depolymerization was addressed by the investigation of the GPC elution curves for the six lignin samples. Table IV illustrates the weight-average (M_w) and number-average (M_n) molecular weights and polydispersity (M_w/M_n) of the acid-insoluble lignin fractions; a distinctly higher molecular weight was found for L_{2T} and L_{2C} (6120–6910 g/mol) than for L_{1T}, L_{1C}, L_{3T}, and L_{3C} (4260–4920 g/mol). These results revealed that the concerted effect of mild ball-milling action and mild alkaline with 50% alkaline ethanol under the conditions used did not cause significant depolymerization of the lignin macromolecules. It must be emphasized, however, that the possibility of such degradation of the lignins during the mild acidolysis with 80% dioxane containing 0.05*M* HCl and alkaline hydrolysis with 8% aqueous NaOH under the conditions given cannot be ruled out. This is also surprising because of the acidic conditions of the dioxane lignin isolation procedure. The reason for this was probably the slightly higher concentration of HCl (0.05*M*) used in this study, which may seriously compromise the structure of the isolated lignin. On the other hand, these different values of M_w in the six lignin fractions can be explained by the various lignin compositions. It is well known that the β–*O*-4 ether bond is the most common linkage in all lignin types; however, some quantities of carbon–carbon bonds between the lignin units are also important, of which those involving C-5 of the aromatic ring are the most abundant.[39] G-type units are able to form this kind of bond, whereas this is not possible in S-type units as they have the C-5 position substituted by a methoxy group. It should be noted that these C—C bonds are not cleaved during conditions such as those of a severe alkali or acid treatment because of their higher stability. As a result, lignins strictly composed of G units are expected to have a higher molecular weight than those presenting high contents of S units.[40] This seems to be valid for the six lignin fractions isolated from the mild ball-milled TA and CS in this study. As shown in Table IV, the M_w values of the three lignin samples isolated from CS (M_w = 4400–6910 g/mol) were higher than those of the corresponding three lignin fractions extracted from TA (M_w = 4260–6120 g/mol), and this correlated with their G content.

TABLE IV
M_w, M_n, and M_w/M_n Values of the Hemicellulosic Fractions

	Lignin fraction[a]					
	L_{1T}	L_{2T}	L_{3T}	L_{1C}	L_{2C}	L_{3C}
M_w	4260	6120	4520	4400	6910	4920
M_n	1720	2230	1740	1710	2400	1460
M_w/M_n	2.5	2.7	2.6	2.6	2.9	3.4

[a] Corresponding to the acid-insoluble lignin fractions in Table I.

FTIR spectra

The FTIR spectrum of all lignin samples showed bands at 1598, 1508 or 1510, and 1420 or 1422 cm^{-1} (Figs. 3 and 4), corresponding to aromatic ring vibrations of the phenylpropane skeleton. These typical spectra of lignin revealed that the core of the lignin structure did not change significantly during the mild ball milling and acidolytic and alkaline hydrolysis processes. However, the changes in the carbonyl absorption region might enable the evaluation of the effects of various treatments. The bands in the range of 1636–1648 cm^{-1} and at 1708 cm^{-1} are assigned to the presence of conjugated and nonconjugated carbonyl groups in the lignin structure.[24] Obviously, a remarkable increase of carbonyl absorption at 1708 cm^{-1}, observed in spectrum 3, implies that noticeable oxidation of the lignin structure did occur during the sequential extraction with 8% aqueous NaOH. A wide absorption band focused at 3444 cm^{-1} originated from the O—H stretching vibration in aromatic and aliphatic OH groups, whereas bands at 2920 and 2852 cm^{-1} and at 1467 or 1464 cm^{-1} arose from the C—H stretching and asymmetric vibrations of CH_3 and CH_2, respectively.[40]

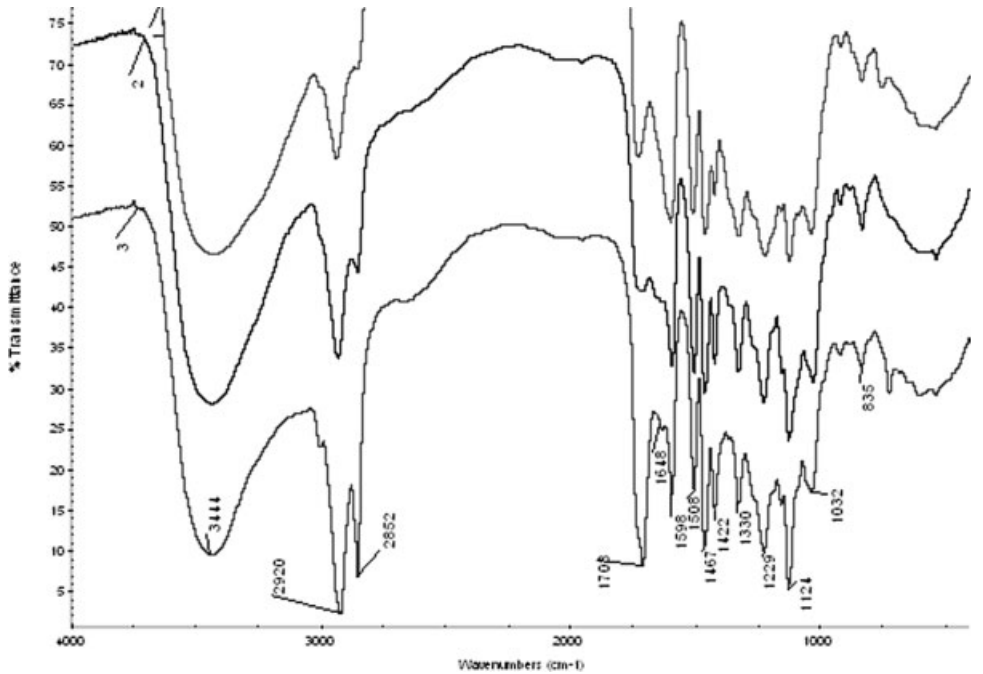

Figure 3 FTIR spectra of the lignin fractions: (1) L_{1T}, (2) L_{2T}, and (3) L_{3T}.

It is well know that in softwoods the lignin network is mainly composed of G moieties with small amounts of S and only traces of H-type units.[41] On the other hand, in hardwoods and dicotyl crops, different G/S ratios have been reported.[42] These characteristics become visible in the spectra of the six studied lignins. As illustrated in Figure 3, the low intensive ratio of 1508 to 1467 cm^{-1} decreased from L_{1T} to L_{2T} to L_{3T}, indicating that the three lignin fractions isolated from TA were rich in S units, which increased from the L_{1T} to L_{2T} to L_{3T} lignin preparations, corresponding to the results obtained from alkaline nitrobenzene oxidation. In contrast, as can be seen in Figure 4, the intensive ratio of 1508 to 1467 cm^{-1} was higher (>1) in L_{1C}, equal in L_{2C} ($\sim$ 1), and lower in L_{3C} (<1), and this implied that the ratio of G units to S units in the lignin fractions isolated from CS was in the order of $L_{1C} > L_{2C} > L_{3C}$, corresponding to the results obtained by alkaline nitrobenzene oxidation in Table IV.

Interestingly, as the spectra show in Figures 3 and 4, the band at 1360 cm^{-1} for nonetherified phenolic OH groups in lignin, resulting from cleavage of β–*O*-4 and α–*O*-4 linkages, occurs as a shoulder, and this reveals that the sequential extractions of the mild ball-milled TA and CS with 80% acidic dioxane, 50% alkaline ethanol, and 8% aqueous NaOH under the conditions used did not significantly cleave the β–*O*-4 and α–*O*-4 linkages in lignin macromolecules. A noticeable band at 1032 cm^{-1} in Figure 3 and at 1041 cm^{-1} in Figure 4 is attributed to the C—O stretching vibration in the first-order aliphatic C—OH and ether linkages (C—O—C).[40] The C—O stretching vibration in S units gives a band region of 1328–1330 cm^{-1}. The absorption in the region of 1223–1229 cm^{-1} is due to the C—O stretching vibration in the aromatic ring. Aromatic C—H in-plane deformation vibration in G and S units exhibits a sharp peak at 1124–1125 cm^{-1}.

^{1}H and ^{13}C spectra

In comparison with the structural features of lignins between TA and CS, the chemical structure of lignin fractions L_{1T} and L_{1C} was investigated with ^{1}H and ^{13}C spectrometry, and their spectra are shown in Figures 5 and 6. The integral of all signals between 6.0 and 8.0 ppm belongs to aromatic protons in G and S

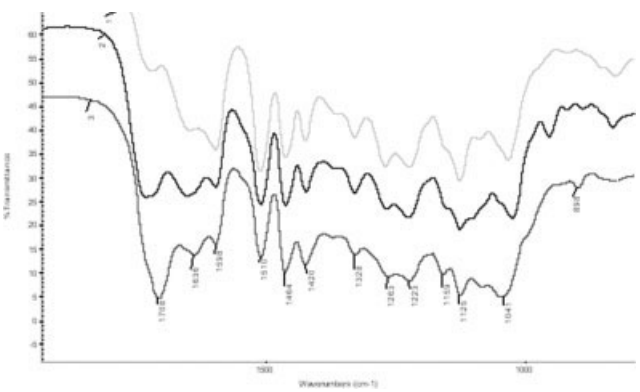

Figure 4 FTIR spectra of the lignin fractions: (1) L_{1C}, (2) L_{2C}, and (3) L_{3C}.

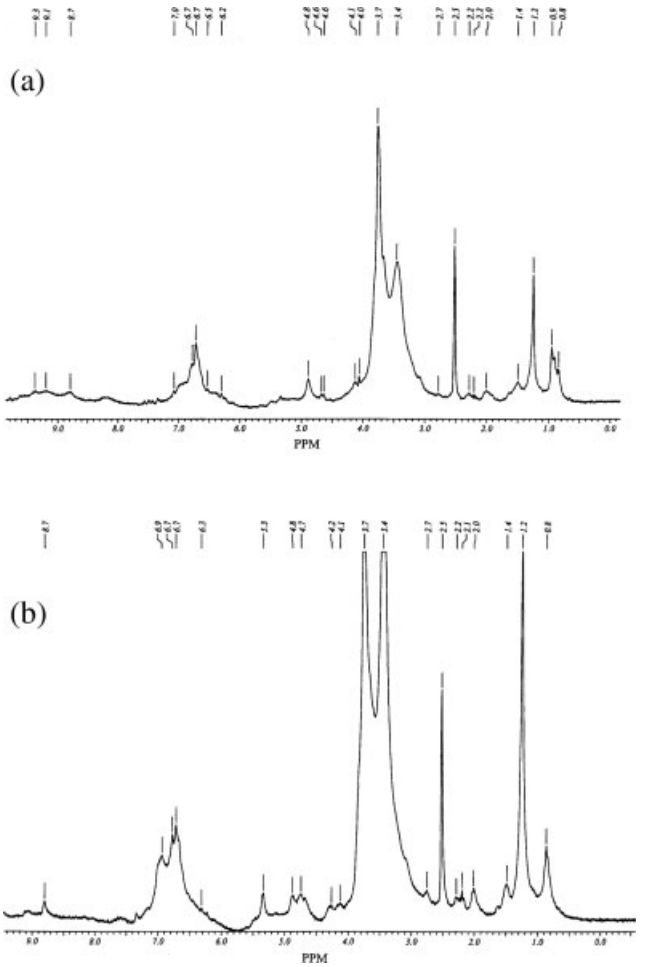

Figure 5 ^{1}H-NMR spectra of the lignin fractions: (a) L_{1T} and (b) L_{1C}.

units, whereas those between 0.8 and 1.4 ppm are due to the aliphatic moiety in the lignin.[40] In particular, the signals at 7.0 or 6.9 and 6.7 ppm are attributed to the aromatic protons in G and S units, respectively;[43] a quite weak signal at 7.0 ppm in Figure 5(a) reveals that lignin fraction L_{1T}, isolated with 80% acidic dioxane, contained only small amounts of G units, corresponding to the results obtained by alkaline nitrobenzene oxidation, as shown by a *g*/*s* ratio of 1 : 2.6. The signals in the region of 4.6–4.7 and 4.0–4.5 ppm originated from H_β and H_γ of β–*O*-4 structures, respectively.[19] Methoxyl protons ($-OCH_3$) give two strong signals at 3.7 and 3.4 ppm. The signal at 4.8 ppm represents H_α in β–β linkages. A sharp signal at 2.5 ppm is related to the protons in dimethyl sulfoxide. The peaks at 2.0, 2.1, and 2.2 ppm arose from the methyl protons adjacent to double bonds or carbonyl groups.[44] The small signals at 8.7, 9.1, and 9.3 ppm are attributed to the C=O groups in aldehydes such as cinnamaldehyde and benzaldehyde structures. These results indicate that β–aryl ether bond (β–*O*-4) and β–β carbon–carbon linkages are the abundant interunit linkages in the lignin structure.

^{13}C-NMR spectroscopy is one of the most powerful tools in lignin chemistry. To gain a more complete understanding of the structures in the isolated lignins, the ^{13}C-NMR spectra of 80% acidic dioxane-soluble lignin fractions L_{1T} and L_{1C} are illustrated in Figure 6. In the region of the aromatic part of the lignin, G units were identified by signals at 149.3 and 149.1 (C-3, G etherified), 147.8 and 147.4 (C-4, G etherified), 134.2 (C-1, G etherified), 119.0 (C-6, G), 114.8 and 114.6 (C-5, G), and 110.9 ppm (C-2, G). The S units were verified by signals at 152.0 (C-3/C-5, S), 147.8 and 147.4 (C-3/C-5, S nonetherified), 134.2 (C-1, S etherified), and 104.1 ppm (C-2/C-6 S). The H units were detected at 127.6 ppm (C-2/C-6, H). The sharp peak at 129.4 ppm (C-2/C-6, PC ester) is characterized by the presence of the esterified *p*-coumaric acid, indicating that isolated lignin fraction L_{1T} contained noticeable amounts of esterified *p*-coumaric acid.

The β–*O*-4 structure was also revealed by the ^{13}C-NMR spectrum. As can be seen in Figure 6(a), the resonance of C—β, C—α, and C—γ in β–*O*-4 linkages produces signals at 85.9, 72.1, and 59.5 ppm,

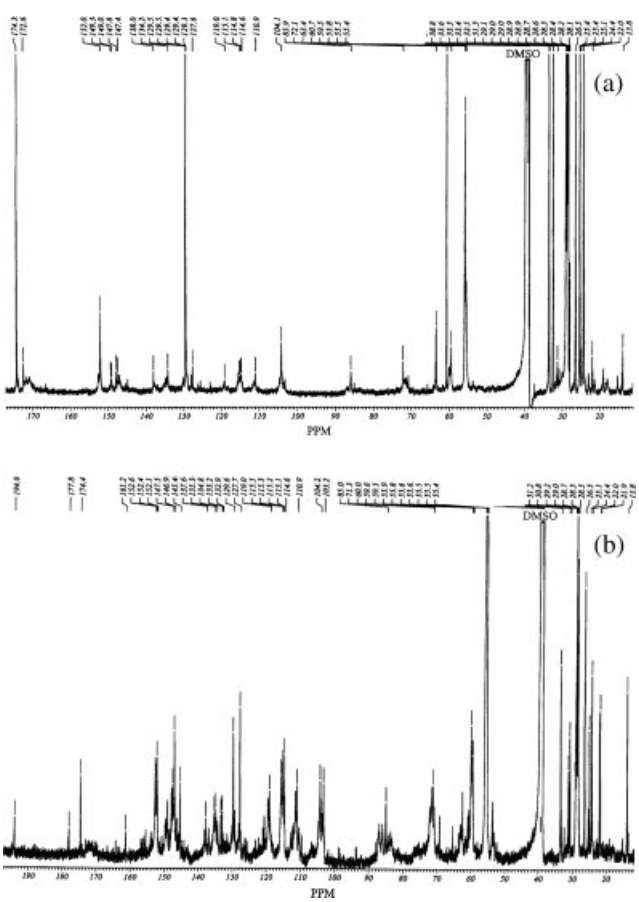

Figure 6 ^{13}C-NMR spectra of the lignin fractions: (a) L_{1T} and (b) L_{1C}.

respectively, confirming that the mild ball milling followed the acidolytic hydrolysis under the condition given did not cleave the β–aryl ether structure to a significant extent. The common carbon–carbon linkages such as β–β (C—γ in β–β units, 71.3 ppm, C—β in β–β units, 54.1 ppm, data not shown) and β–5 (C—γ in β–5, 66.2 ppm, data not shown) were also observed.[45] The signal at 125.9 ppm (data not shown) is indicative of C-5/C-5′ in 5–5′ structures. These observations show that lignin fraction L_{1T}, isolated with 80% acidic dioxane from mild ball-milled TA, is mainly composed of β–*O*-4 ether bonds together with small amounts of β–β, β–5, and 5–5′ carbon–carbon linkages. The strong signals at 55.8, 55.5, and 55.4 ppm arose from OCH_3 in S and G units. The signals representing the γ-methyl and α- and β-methylene groups in *n*-propyl side chains appear in the spectrum at 13.8 ppm and in the region of 22.0–33.6 ppm, respectively.

During the milling process, radical generation can lead to oxidation reactions, resulting in C=O groups.[11] As shown in Figure 6(b), the signal at 194.0 ppm is assigned to carbon atoms of carbonyl groups in cinnamyl aldehyde and α–C=O in β–*O*-4 moieties. The peak at 177.8 ppm in the spectrum of L_{1C} is due to C-4 in spirodienone structures of the S type.[46] On the other hand, stronger signals in the region of 172.6–174.4 ppm in the spectrum of L_{1T} [Fig. 6(a)] indicated that the content of carboxylic groups in the 80% acidic dioxane-soluble lignin fraction isolated from TA (L_{1T}) was higher than that of the corresponding lignin preparation extracted from CS (L_{1C}). The signals at 174.3 and 60.7 ppm represent C-6 in methyl urinates and the 4-*O*-methoxyl group of glucuronic acid residue, respectively.[25] These two intensive bands for uronic acids and esters suggest that the uronic acids are closely associated with lignin macromolecules in the cell walls of TA. Similarly, an intensive peak at 63.4 ppm in Figure 6(a) corresponds to the C-5 in xylose from the contaminated hemicelluloses. Furthermore, lignin fraction L_{1T} in Figure 6(a) also shows another small signal at 172.6 ppm resulting from the aliphatic carboxyl groups.

In comparison with the spectrum of L_{1T} isolated from TA, G units identified by signals at 147.5, 146.9, 145.4, 135.3, 133.2, 129.6, 119.0, 114.6, and 110.9 ppm gave a higher intensity in the spectrum of L_{1C} isolated from CS. This observation indicated that lignin fraction L_{1C} contained higher amounts of G units than S units, which corresponded to the higher *g*/*s* molar ratio of 1.4 : 1 in Table IV. In contrast, a lower intensity of the signals for G units in lignin fraction L_{1T} implied that the 80% acidic dioxane-soluble lignin fraction isolated from the mild ball-milled TA was rich in S units, which corresponded to the lower *g*/*s* molar ratio of 1 : 2.6 obtained by alkaline nitrobenzene oxidation.

CONCLUSIONS

The results show that the sequential treatments of mild ball-milled TA and CS with 80% acidic dioxane, 50% alkaline ethanol, and 8% aqueous NaOH made it possible to isolate lignin with a better yield than acidolysis alone and with a higher degree of purity than after enzymatic hydrolysis alone. Physicochemical characterization of six lignin fractions was performed, and the results were related to their different origins and different isolation procedures. FTIR spectroscopy and ^{13}C-NMR spectrometry revealed that the three lignin fractions isolated sequentially from the mild ball-milled TA with 80% acidic dioxane, 50% alkaline ethanol, and 8% aqueous NaOH (L_{1T}, L_{2T}, and L_{3T}) were mainly composed of S units, whereas the corresponding three lignin preparations isolated sequentially from the mild ball-milled CS (L_{1C}, L_{2C}, and L_{3C}) showed both G and S structural units. The two lignin fractions isolated during the first treatment with 80% acidic dioxane (L_{1T} and L_{1C}) may have originated mainly from lignin in the CML and partially from lignin in the S2, and the two lignin fractions isolated during the sequentially second treatment with 50% alkaline ethanol (L_{2T} and L_{2C}) qualitatively represented the lignin in both the CML and S2 regions of the cell wall, whereas the lignin fractions extracted during the last stage with 8% aqueous NaOH (L_{3T} and L_{3C}) dominated the lignin from S2. In addition, it should be noted that noticeable amounts of 4-*O*-methoxyl-glucuronic acid and *p*-coumaric acid were esterified in the lignin fraction isolated with 80% acidic dioxane from the mild ball-milled wood, and this was particularly true in the L_{1T} fraction. Furthermore, studies also showed that lignin fraction L_{1T}, isolated with 80% acidic dioxane from mild ball-milled TA, was mainly composed of β–*O*-4 ether bonds together with small amounts of β–β, β–5, and 5–5′ carbon–carbon linkages.

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