Physicochemical Characterization of Lignin Fractions Sequentially Isolated from Bamboo (*Dendrocalamus brandisii*) with Hot Water and Alkaline Ethanol Solution

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ABSTRACT: Nine lignin fractions from bamboo (*Dendrocalamus brandisii*) were sequentially isolated with hot water at 80, 100, and 120°C for 3 h and 60% aqueous ethanol containing 0.25, 0.5, 1.0, 2.0, 3.0, and 5.0% NaOH at 80°C for 3 h. Molecular weight and purity analysis revealed that the lignin fractions isolated by hot water (L_1, L_2, and L_3) had lower weight-average molecular weights (between 1350 and 1490 g mol^{-1}) and contained much higher amounts of associated hemicelluloses (between 9.26 and 22.29%), while the lignin fractions isolated by alkaline aqueous ethanol (L_4, L_5, L_6, L_7, L_8, and L_9) had higher weight-average molecular weights (between 2830 and 3170 g mol^{-1}) and contained lower amounts of associated hemicelluloses (between 0.63 and 1.66%). Spectroscopy (UV, FTIR, ^{13}C-NMR, and HSQC) analysis showed that the bamboo (*Dendrocalamus brandisii*) lignin was typical grass lignin, consisting of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units. The major interunit linkages presented in the alkaline aqueous ethanol extractable bamboo lignin were β-O-4′ aryl ether linkages (about 74.3%), followed by β-β′ resinol-type linkages and β-1′ spirodienone-type linkages (both for 7.8%), together with small amounts of β-5′ phenylcoumaran (6.8%) and *p*-hydroxycinnamyl alcohols end groups (3.1%). In addition, a small percentage (1.0%) of the lignin side-chain was found to be acetylated at the γ-carbon, predominantly over syringyl units. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 125: 3290–3301, 2012

Key words: bamboo; NMR; biopolymers; lignin; structure

INTRODUCTION

Lignin, accounting for about one-fourth of the lignocellulosic biomass, is the third most abundant biopolymer after cellulose and hemicelluloses. Unlike most natural polymers, which consist of a single intermonomeric linkage, 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] Besides, lignin is covalently linked to hemicellulosic polysaccharides, forming a lignin–hemicelluloses network made up of benzyl–ether, benzyl–ester, and phenyl–glycoside bonds.[2–6] As one of the most potential biomass feedstock for chemical industry, lignin can be refined or transformed into various high-value-added green chemicals.

Using lignin as a biomass feedstock for chemical production requires an initial step, to separate it and elucidate its chemical structural properties. A major problem in native lignin structure elucidation has been in trying to isolate as much of the lignin as possible while minimizing the extent of chemical modification.[7] A mild and widely used method for lignin isolation was proposed by Björkman,[8] based on extensive grinding of plant material followed by extraction with dioxane/water. The lignin obtained by this procedure has been considered as the standard preparation to perform most of the chemical and biological studies. Nevertheless, concerns exist over the similarity between milled wood lignin (MWL) and native lignin based on the low yields (25–50% of protolignin) and structural alterations due to ball milling.[9] Further improvements in the yield of lignin isolated from ball-milled wood have arisen through the use of cellulolytic enzymes. Pew and Weyna[10] treated ball milled wood with cellulolytic enzymes

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and obtained an insoluble residue containing almost all of the lignin presented in spruce and aspen woods. Nevertheless, the residue had as much as 12% of polysaccharides. Aqueous alkali treatment has been considered as a suitable method for fractional isolation and structural characterization of *Gramineae* plants due to the high solubility of lignin.[11] The possible mechanisms for the solubilization of *Gramineae* lignin by alkali include the release of phenolic acids from cell walls and the release of lignin resulting from cleavage of ester linkages in lignin–polysaccharide complexes.

Bamboo *Dendrocalamus brandisii*, belonging to *Bambusoideae* of *Gramineae*, with strong and abundant woody stems, is mainly distributed in southeast Asia, including the southwest region of China. Due to its easy propagation, fast growth, and high productivity, *D. brandisii* is considered as one of the most potential non-wood forest resources to replace wood resources. Various studies have been concerned with lignin of bamboo. Ujiie and Yoshikawa[12] found differences in the chemical structure of lignin from juvenile and mature tissue of *Sasa senanensis*. The presence of *p*-coumaric ester groups in bamboo lignin was described by Higuchi and coworker.[13] Fengel and Shao[14] found that the lignin isolated from *Phyllostachys makinoi* was rich in syringyl unit. Faix et al.[15] compared the molecular weights and molecular weight distributions of the lignin fractions of some bamboo species. In a preceding publication, we reported that lignin extracted from *Neosinocalamus affinis* consisted of *p*-hydroxyphenyl, guaiacyl, and syringyl type lignins with minor cinnamate units.[16]

Unfortunately, to the best of our knowledge, the detailed physicochemical properties of lignin polymer presented in *D. brandisii* have not been reported in the literature prior to this article. The aim of this research was to elucidate the physicochemical properties of lignin fractions extracted from *D. brandisii* culm with less modification under mild conditions. In this work, bamboo samples were sequentially isolated by hot water and alkaline aqueous ethanol solution, and the lignin fractions were characterized by a series of degradative and spectroscopic techniques.

EXPERIMENTAL

Materials

Bamboo (*D. brandisii*), 3-years old, was obtained from Yunnan Province, China. It was first dried in sunlight and then chipped into small pieces. The air-dried pieces of bamboo were ground and screened to obtain a 40–60 mesh fraction. This fraction was subjected to extraction with toluene/ethanol (2 : 1, v/v) in a Soxhlet apparatus for 6 h, and then the dewaxed powder was further dried in an oven under circulated air at 60°C for 16 h before use. The major compositions of *D. brandisii* were 72.96% holocellulose, 27.08% lignin on dry weight basis. All standard chemicals, such as monosaccharides and chromatographic reagents, were purchased from Sigma Chemical Company (Beijing, China).

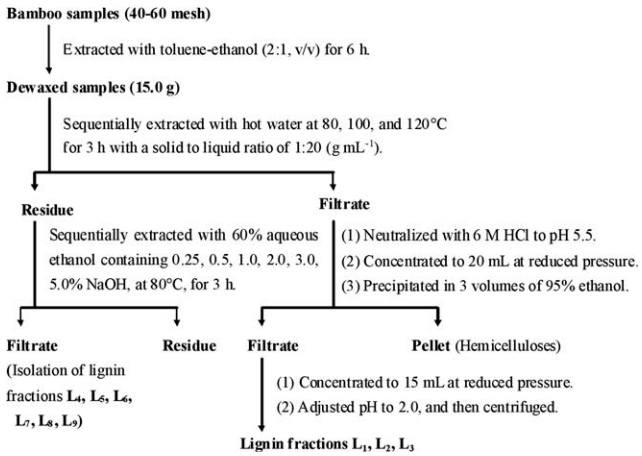

Figure 1 Scheme for separation of lignin fractions from bamboo *D. brandisii*.

Isolation of lignin fractions

A scheme for separation of bamboo (*D. brandisii*) lignin is shown in Figure 1. The dewaxed bamboo sample (15 g) was firstly treated with distilled water at 80°C for 3 h under stirring with a solid to liquid ratio of 1 : 20 (g mL^{-1}). After filtration, the residue was washed repeatedly with distilled water, and then oven-dried. The filtrate was neutralized with 6*M* HCl to pH 5.5, and concentrated to about 20 mL with a rotary evaporator under reduced pressure. Then, the concentrated filtrate was slowly poured into three volumes of 95% ethanol to precipitate hemicelluloses. The hemicellulose fraction obtained was centrifuged and washed with 70% ethanol at room temperature and then freeze-dried. After that, the combined solutions were further concentrated to about 15 mL, adjusted pH to 2.0, and centrifuged to isolate lignin fraction L_1. The residue free of the hot water-solubles was successively treated with hot water at 100 and 120°C for 3 h under stirring with a solid to liquid ratio of 1 : 20 (g mL^{-1}), and the hot water-soluble lignin fractions (L_2 and L_3) were obtained by the same method mentioned above. It should be noted that the bamboo sample and water were transferred into an autoclave when extracted with 120°C hot water to obtain the lignin fraction L_3. The bamboo sample after 120°C hot water extraction was then sequentially extracted with 60% aqueous ethanol containing 0.25, 0.5, 1.0, 2.0, 3.0, and 5.0% NaOH at 80°C for 3 h with a solid to liquid ratio of

1 : 20 (g mL^{-1}), and the corresponding lignin fractions isolated by the same method mentioned above were labeled as L_4, L_5, L_6, L_7, L_8, and L_9, respectively. All the experiments were performed at least in duplicate. Yields of the lignin fractions are calculated on dry weight basis related to the dewaxed bamboo samples. The relative standard deviation was observed to be lower than 4.8%.

Characterization of the lignin fractions

The hemicellulosic moieties associated with the lignin fractions were determined by hydrolysis with dilute sulfuric acid. That is, 4–6 mg sample of lignin was hydrolyzed with 1.475 mL of 6.1% H_2SO_4 for 2.5 h at 105°C. After hydrolysis, the mixture was filtered, and the filtrate containing the liberated neutral sugars was analyzed by high-performance anion exchange chromatography (HPAEC) system (Dionex ICS-3000, Sunnyvale, CA, USA) with pulsed amperometric detector and an ion exchange Carbopac PA-1 column (4 × 250 mm). Neutral sugars were separated in 18 m*M* NaOH (carbonate-free and purged with nitrogen) with postcolumn addition of 0.3*M* NaOH at a rate of 0.5 mL min^{-1}. Run time was 45 min, followed by 10 min elution with 0.2*M* NaOH to wash the column and then a 15 min elution with 18 m*M* NaOH to re-equilibrate the column. Calibration was performed with standard solutions of L-rhamnose, L-arabinose, D-glucose, D-galactose, D-mannose, D-xylose, glucuronic acid, and galacturonic acid. The analyses were run twice, and the average values were calculated for all of the lignin fractions.

The weight-average ($\overline{Mw}$) and number-average ($\overline{Mn}$) molecular weights of the lignin fractions were determined by gel permeation chromatography (GPC, Agilent 1200, Palo Alto, CA, USA) with a refraction index detector on a PL-gel 10 μm Mixed-B 7.5 mm ID column, calibrated with PL polystyrene standards. Four milligrams of the sample was dissolved in 2 mL tetrahydrofuran, and 20 μL sample in solution was injected. The column was operated at ambient temperature and eluted with tetrahydrofuran at a flow rate of 1.0 mL min^{-1}.

UV spectra were recorded on an ultraviolet/visible spectrophotometer (Tech comp, UV 2300). Lignin sample (5 mg) was dissolved in 95% (v/v) dioxane aqueous solution (10 mL). A 1 mL aliquot was diluted to 10 mL with 50% (v/v) dioxane aqueous solution and the absorbance between 250 and 400 nm was measured. The FTIR spectra of the lignin fractions were obtained on an FTIR spectrophotometer (Bruker Tensor 27) using a KBr disc containing 1% finely ground samples. Thirty-two scans were taken of each sample recorded from 4000 to 400 cm^{-1} at a resolution of 2 cm^{-1} in the transmission mode.

The solution-state ^{13}C-NMR spectroscopy was carried out on a Bruker AV III 400 MHz NMR spectrometer. The sample (80 mg) was dissolved in 0.5 mL of dimethyl sulfoxide-d_6 (DMSO, 99.8%), and the spectrum was recorded at 25°C after 30,000 scans. A 30° pulse flipping angle, a 9.2 μs pulse width, 1.89 s delay time, and 1.36 s acquisition time between scans were used. 2D HSQC spectra were acquired on the same spectrometer in the HSQC GE experiment mode. The spectral widths were 1800 Hz and 10,000 Hz for the ^1H- and ^{13}C-dimensions, respectively. A 128 data points, a 2.6 s delay time between transients, and a 1.5 s relaxation time were used. The $^1J_{C-H}$ used was 145 Hz. The central solvent (DMSO) peak was used as an internal chemical shift reference point (δ_C 39.5; δ_H 2.49 ppm). Data processing was performed with standard Bruker Topspin-NMR software.

Thermogravimetric analysis of lignin fractions was performed using thermogravimetric analysis (TGA) and differential thermal analysis (DTA) on a simultaneous thermal analyzer (DTG-60, Shimadzu, Japan). Samples of approximately 10 mg weight were heated in an aluminum crucible from room temperature to 600°C at a heating rate of 10°C min^{-1} while the apparatus was continually flushed with a nitrogen flow of 20 mL min^{-1}.

RESULTS AND DISCUSSION

Fractional yield and purity

The yield of lignin fractions was expressed as a percentage of dry starting material. Table I shows that successive treatments of the dewaxed bamboo sample with hot water at 80, 100, and 120°C for 3 h, and with 60% aqueous ethanol containing 0.25, 0.5, 1.0, 2.0, 3.0, and 5.0% NaOH at 80°C for 3 h, resulted in a dissolution of 0.9, 0.4, 0.1, 3.2, 11.8, 0.8, 0.6, 0.7, and 0.6% of the bamboo lignin, corresponding to 3.3, 1.5, 0.4, 11.9, 43.7, 3.0, 2.2, 2.7, and 2.2% of the original lignin, respectively. Altogether, there was 70.8% soluble lignin extracted from bamboo samples during the three steps of the hot water extraction and six steps of the alkaline aqueous ethanol treatments.

The composition of associated hemicelluloses in the nine isolated acid-insoluble lignin fractions was determined by their sugar and uronic acid contents, and the analytical results are also listed in Table I. As expected, the lignin fractions L_1, L_2, and L_3, isolated by hot water at 80, 100, and 120°C for 3 h, contained much higher contents of associated hemicelluloses, 22.9%, 17.13%, and 9.26%, respectively. As a comparison, the acid-insoluble lignin fractions L_4, L_5, L_6, L_7, L_8, and L_9, extracted by 60% aqueous ethanol with 0.25, 0.5, 1.0, 2.0, 3.0, and 5.0% NaOH, contained only 0.63, 1.23, 1.28, 1.66, 1.47, and 1.50%

TABLE I
The Yields (% dry Bamboo Sample, w/w) and the Neutral Sugar Content of the Isolated Bamboo Lignin Fractions

	Lignin fractions[a]								
	L_1	L_2	L_3	L_4	L_5	L_6	L_7	L_8	L_9
Yield (%)[b]	0.9	0.4	0.1	3.2	11.8	0.8	0.6	0.7	0.6
Rhamnose	0.06	0.12	0.14	0.01	Tr[c]	Tr	0.01	Tr	Tr
Arabinose	0.52	1.33	1.88	0.01	0.11	0.14	0.15	0.08	0.11
Galactose	0.28	0.29	0.15	0.03	0.04	0.08	0.06	0.03	0.04
Glucose	20.31	13.11	5.43	0.36	0.77	0.46	0.34	0.47	0.11
Xylose	0.76	2.08	1.54	0.22	0.27	0.52	1.01	0.83	1.17
Mannose	0.27	0.02	N.D[d]	N.D	N.D	N.D	N.D	N.D	N.D
GlcA	0.04	0.06	0.04	N.D	0.03	0.03	0.03	0.01	0.01
GalA	0.06	0.12	0.09	N.D	0.01	0.04	0.07	0.05	0.04
Total[e]	22.29	17.13	9.26	0.63	1.23	1.28	1.66	1.47	1.50

[a] Represents the bamboo lignin fractions isolated by hot water at 80, 100, and 120°C for 3 h, and aqueous ethanol containing 0.25, 0.5, 1.0, 2.0, 3.0, and 5.0% NaOH at 80°C for 3 h.
[b] Represents the yield of the bamboo lignin fractions (% dry matter, w/w).
[c] Tr, trace.
[d] N.D, not detectable.
[e] Represents the total associated hemicelluloses in the isolated bamboo lignin fractions (% dry lignin sample, w/w).

associated hemicelluloses, respectively. Glucose and xylose were identified as the major sugar components in all of the lignin fractions, implying that more mixed-linked glucans and xylans were extracted during the treatments.

Molecular weight

Weight-average ($\overline{Mw}$) and number-average ($\overline{Mn}$) molecular weights, and polydispersity ($\overline{Mw}/\overline{Mn}$) of the nine acid-insoluble lignin preparations computed from their GPC curves are given in Table II. It is important to mention here that the molecular weights discussed throughout this work were obtained from a calibration performed with monodisperse polystyrene standards. Therefore, these values should only be considered as relative molecular weights and not absolute.

Clearly, the three lignin preparations L_1, L_2, and L_3, isolated by hot water at 80, 100, and 120°C were shown to have a similar but much lower weight-average molecular weights ($\overline{Mw}$), ranging from 1350 to 1490 g mol^{-1}. Whereas, the lignin preparations L_4, L_5, L_6, L_7, L_8, and L_9, released during the alkaline aqueous ethanol treatment under the conditions given, appeared to have much higher weight-average molecular weights ($\overline{Mw}$) between 2830 and 3170 g mol^{-1}. That is to say, the bamboo lignin fractions, released during the alkaline aqueous ethanol treatments, had much higher molecular weights than those lignin fractions isolated with hot water. The results obtained in this study were in good comparison with the corresponding data of our previous study,[17] when the sweet sorghum stem was subjected to treatment with hot water at 80°C and 6% NaOH aqueous solution, the weight-average molecular weights ($\overline{Mw}$) of lignin fractions were 1270 and 2920 g mol^{-1}, respectively. Interestingly, as shown in Table II, both the three lignin fractions (L_1, L_2, and L_3) released during the hot water treatments, and the six lignin fractions (L_4, L_5, L_6, L_7, L_8, and L_9) released during the alkaline aqueous ethanol treatments, showed a relative comparable weight-average molecular weights ($\overline{Mw}$) and polydispersity

TABLE II
Weight-Average ($\overline{Mw}$) and Number-Average ($\overline{Mn}$) Molecular Weights and Polydispersity ($\overline{Mw}/\overline{Mn}$) of the Isolated Bamboo Lignin Fractions

	Lignin fractions[a]								
	L_1	L_2	L_3	L_4	L_5	L_6	L_7	L_8	L_9
$\overline{Mw}$	1350	1490	1420	3170	2830	3040	3060	2840	2850
$\overline{Mn}$	790	1010	910	1660	950	1510	1360	1310	1350
$\overline{Mw}/\overline{Mn}$	1.7	1.4	1.6	1.9	3.0	2.0	2.2	2.2	2.1

[a] Represents the bamboo lignin fractions isolated by hot water at 80, 100, and 120°C for 3 h, and aqueous ethanol containing 0.25, 0.5, 1.0, 2.0, 3.0, and 5.0% NaOH at 80°C for 3 h.

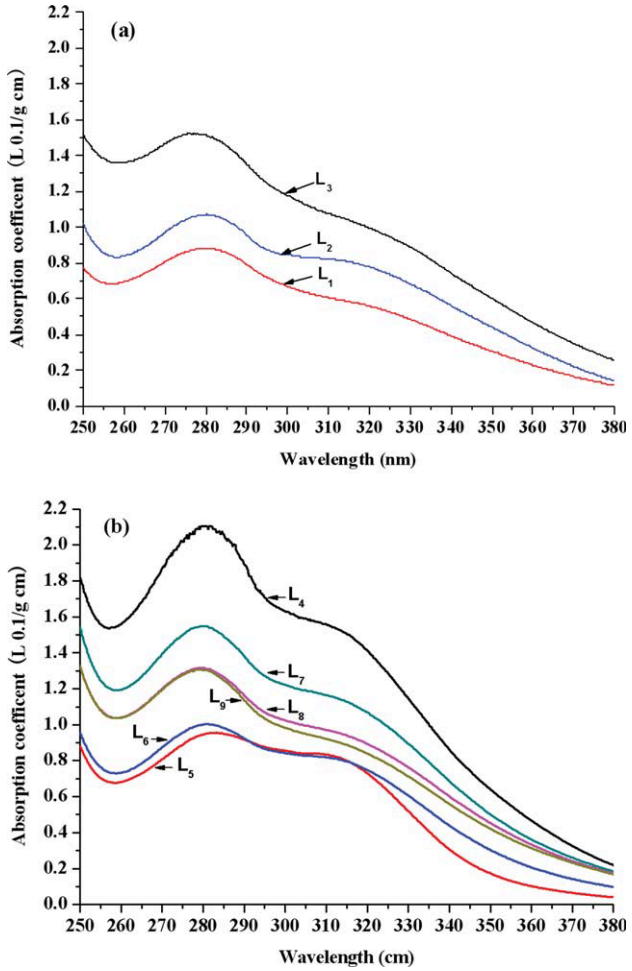

Figure 2 UV spectra of the nine isolated bamboo lignin fractions: (a) L_1, L_2, L_3, and (b) L_4, L_5, L_6, L_7, L_8, L_9. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

($\overline{Mw}/\overline{Mn}$), these probably opened the possibility of exploiting lignin-based composites due to the homogeneous lignin fractions.[18]

UV spectra

UV spectroscopy has been used to semi-quantitatively determine the purity of lignin or monitor the lignin distribution among various tissues of gymnosperm and dicotyledonous angiosperm with respect to the concentration. In this study, UV–vis absorption measurements of the nine lignin fractions were carried out with a dioxane/water mixture, which solubilized the lignins but was limited to wavelengths above 250 nm. Figure 2 illustrates the UV–vis spectra of the hot water-soluble lignin fractions L_1, L_2, and L_3 [Fig. 2 (a)], and alkaline aqueous ethanol-soluble lignin fractions L_4, L_5, L_6, L_7, L_8, and L_9 [Fig. 2 (b)]. Obviously, the spectra of the nine lignin fractions are similar except for the magnitude of the absorption coefficient representations. The maximum absorbance λ at 280 nm originated from non-conjugated phenolic hydroxyl groups in the lignin. The presence of the second characteristic region of lignin absorption around λ of 318 nm could be assigned to the presence of both ferulic acids and *p*-coumaric acids. As shown in Figure 2(b), the highest absorption coefficient occurred in L_4 preparation, suggesting that the most pure lignin preparation could be obtained when aqueous ethanol with 0.25% NaOH was used as a solvent. On the other hand, the lowest absorption coefficients of L_1 and L_5 fractions [in Fig. 2(a,b), respectively], released during the treatment with hot water at 80°C and aqueous ethanol containing 0.5% NaOH, respectively, were probably due to a high amount of associated hemicelluloses and other non-lignin materials, such as ash and inorganic salt. Moreover, the shift of the maximum wavelength from 280 nm to 276 nm implied that a higher content of syringyl (S) units in lignin fraction L_3 isolated by hot water at 120°C, since S units exhibit the bands at somewhat shorter wavelengths, specifically at 268–277 nm.[19]

FTIR spectra

To further investigate the heterogeneity between the lignin fractions, FTIR spectra have been recorded and the peaks were assigned by comparing their wavenumbers with previous literatures.[20–22] Figure 3 shows the FTIR spectra of lignin preparations isolated by hot water (L_1, L_2, and L_3). As can been seen from the diagram, the spectra showed minor changes in the peaks and the absorption intensities, indicating an analogous structure of the lignin fractions. The absorption at 3393 cm^{-1} is attributed to O—H stretching vibration in aromatic and aliphatic OH groups, and the absorption bands at 2936 and 2845 cm^{-1} are assigned to asymmetric and symmetrical vibrations of saturated CH_2 in side chain of lignin, respectively. The much weaker intensity of the unconjugated C=O stretch at 1725 cm^{-1} in L_2 and L_3 than that of L_1 indicate that much more ester groups were cleaved during the treatments of hot water with the increase of temperature from 80°C to 100°C and to 120°C. The band at 1659 cm^{-1} is attributed to conjugated carbonyl stretching in lignins. The aromatic skeleton vibration in the lignin fractions occurs at 1601, 1513, and 1422 cm^{-1}. Absorption at 1461 cm^{-1} indicates the C—H deformations and aromatic ring vibration. The weak signal of 1364 cm^{-1}, corresponding to free phenolic OH groups, indicates a partial cleavage of aryl-ether of lignin in the separation process. The 1331 and 1269 cm^{-1} bands are assigned to syringyl and guaiacyl ring breathing, respectively. The bands at 1124 cm^{-1} and 835 cm^{-1} and shoulder at 1152 cm^{-1} in lignin indicate a typical structure of lignin with *p*-hydroxy

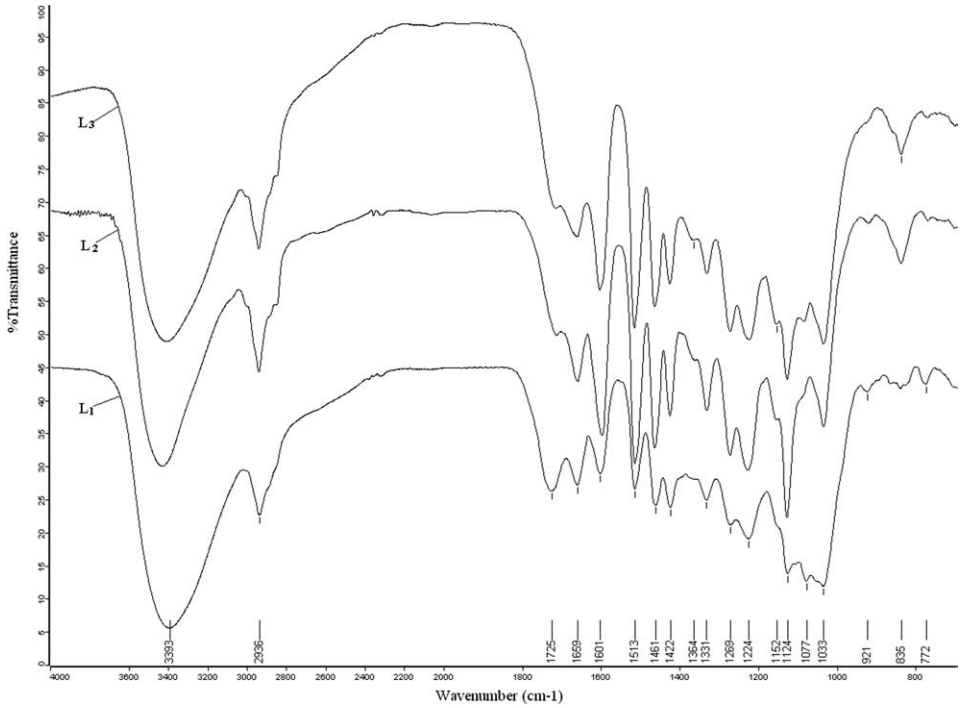

Figure 3 FTIR spectra of the bamboo lignin fractions isolated with hot water at 80, 100, and 120°C (L_1, L_2, and L_3).

phenylpropane (H), guaiacyl (G), and syringyl (S) units. The shoulder at 1152 cm^{-1}, corresponding to C=O in an ester group (conjugated), gives signals for typical GSH-lignin, while 835 cm^{-1} is assigned to C—H out of plane in positions of 2 and 6 of S and in all position of H units. In addition, the much weaker intensity of the aromatic skeleton vibration in L_1 is undoubtedly due to the serious contamination of non-lignin components, such as associated hemicelluloses, which corresponds to the results of sugar analysis in Table I.

Figure 4 illustrates the FTIR spectra of the six acid-insoluble lignin preparations (L_4, L_5, L_6, L_7, L_8, and L_9) isolated with alkaline aqueous ethanol. The spectra profiles and the relative intensities of the bands were rather similar in the six spectra, which

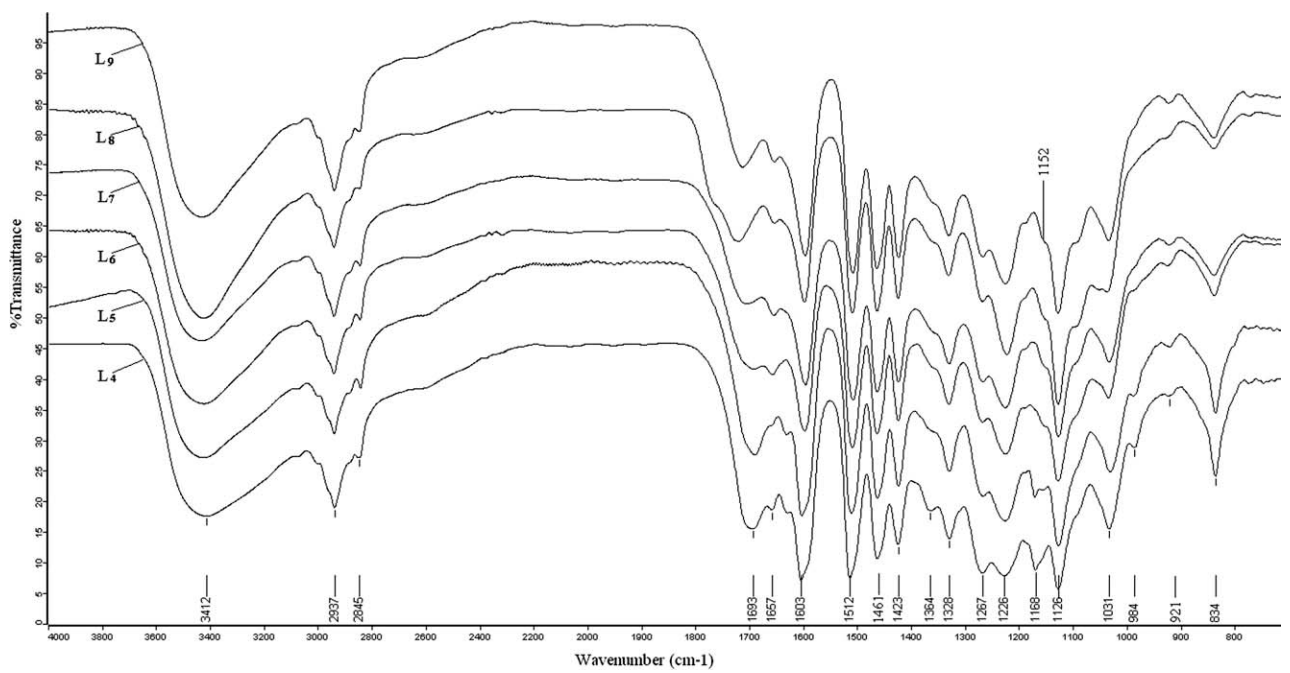

Figure 4 FTIR spectra of the bamboo lignin fractions isolated with alkaline aqueous ethanol (L_4, L_5, L_6, L_7, L_8, and L_9).

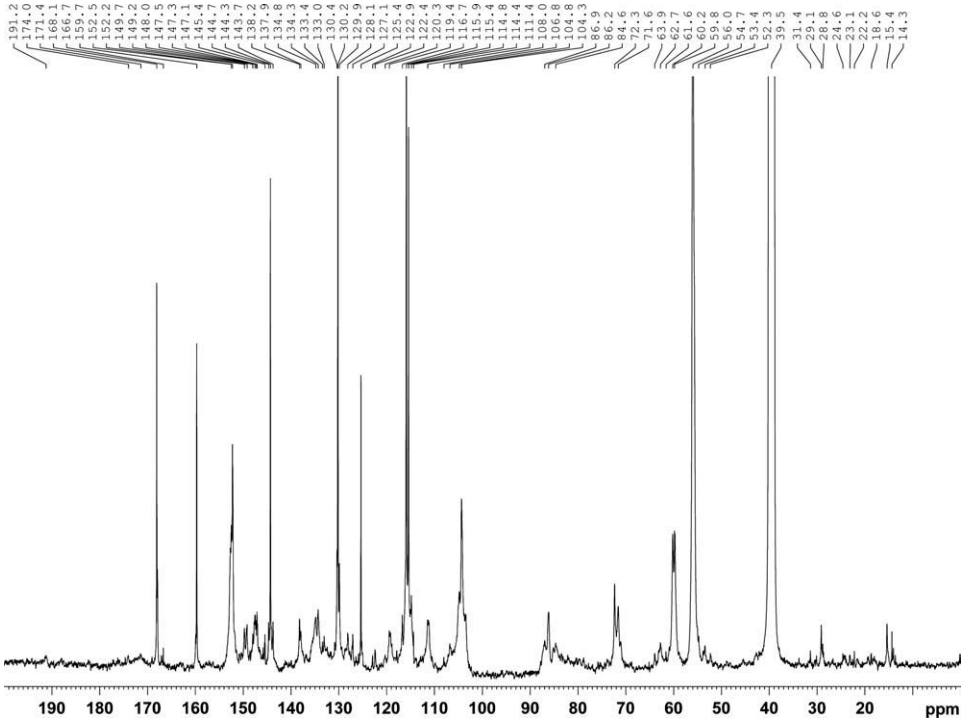

Figure 5 ^{13}C-NMR spectrum of the bamboo lignin fraction L_5 isolated with 60% aqueous ethanol containing 0.5% NaOH.

confirmed that the "core" of lignin structure did not change dramatically during the alkaline aqueous ethanol treatments. Clearly, all the lignins showed the spectral features of GSH type lignin, namely, the bands at 1126 cm^{-1} and 834 cm^{-1} and a small band at 1152 cm^{-1}. In addition, the absence of adsorption at 1725 cm^{-1} indicates that the ester groups of lignin were cleaved during the treatments with alkaline aqueous ethanol under the conditions given.

^{13}C-NMR spectrum

The solution-state ^{13}C-NMR analysis of the lignin fraction L_5 extracted with aqueous ethanol containing 0.5% NaOH at 80°C for 3 h (Fig. 5) has been conducted according to the classical signal assignments,[23–26] and its chemical shifts (δ, ppm) and the assignments are listed in Table III. As can be seen, the lignin fraction was almost absent of typical polysaccharide signals between 57 and 103 ppm, indicating that the lignin preparation contained only trace amount of associated polysaccharides, which corresponded to the results of sugar analysis of associated hemicelluloses in the lignin fractions (in Table I). The small signal at 191.2 ppm is characteristic of α-CHO in cinnamaldehyde, suggesting the appearance of cinnamaldehyde during the alkaline aqueous ethanol treatment. The carbonyl resonances from uronic acids and esters may contribute to signal at 174.0 ppm. A strong signal at 171.4 ppm arises from -COOH groups in aliphatic acids.

The region between 104 and 168 ppm in the ^{13}C-NMR spectrum represents the aromatic moiety of the lignin. The syringyl residues were indicated by signals at 152.2 (C-3/C-5, S), 138.2 (C-4, S etherified), 134.8 and 134.3 (C-1, S etherified), 133.0 (C-1, S non-etherified), 106.8 (C-2/C-6, S with α-C=O), and 104.3 ppm (C-2/C-6, S). The guaiacyl residues were indicated by signals at 149.7 and 149.2 (C-3, G etherified), 147.3 and 147.1 (C-4, G), 145.4 (C-4, G non-etherified), 134.8 and 134.3 (C-1, G etherified), 133.0 (C-1, G non-etherified), 119.4 (C-6, G), 114.8 (C-5, G), and 111.4 ppm (C-2, G). The *p*-hydroxyphenyl residues were identified by signals at 129.9 and 128.1 ppm (C-2/C-6, H). These signals confirmed that the lignin fractions isolated from bamboo species *D. brandisii* could be justified as GSH-lignin, as supported by aforementioned FTIR spectra in Figures 3 and 4.

The signals at 168.7 (C-γ, PC ester), 159.7 (C-4, PC ester), 144.7 (C-α and C-β, PC ester), 130.4 (C-2/C-6, PC ester), 125.9 and 125.4 (C-1, PC ester), 115.9 and 115.4 ppm (C-3/C-5, PC ester) indicated the presence of esterified *p*-coumaric ester (PC ester). Such strong signals suggested that the content of *p*-coumaric ester in the lignin fractions was relatively higher. This also revealed that ester linkages between the *p*-coumaric acid and lignin units were not completely cleaved after the treatment with alkaline aqueous ethanol. In addition, ether-linked

TABLE III
Chemical Shift Value (δ, ppm) and Signal Assignment of Bamboo Lignin Fraction L_5 Isolated with 60% Aqueous Ethanol Containing 0.5% NaOH

δ (ppm)	Assignment	δ (ppm)	Assignment
191.2	α-CHO in cinnamaldehyde	122.4	C-6, FE ether
174.0	C-6 in 4-O-MeGlcA	119.4	C-6, G; C-5, G
171.4	-COOH, aliphatic acids	116.7	C-3/C-5, PC ester
168.1	C-γ, FE ether	115.9, 115.4	C-β, PC ester
166.7	C-γ, PC ester	114.8	C-5, G units
159.7	C-4, PC ester	114.4	C-3/C-5, H etherified
152.5	C-3/C-5, S units	111.4	C-2, G units
149.7, 149.2	C-3, G etherified	106.8	C-2/C-6, S with α-C=O
148.0	C-3, G units	104.8, 104.3	C-2/C-6, S
147.5	C-4, G etherified	86.9	C-α, β-5
147.3, 147.1	C-4, G units	86.2	C-β, β-O-4′
145.8	C-4, G non-etherified	84.6	C-α, β-β′
145.1	C-4, G in β-5′ (non-etherified)	72.3	C-α, β-O-4′
144.7	C-α and C-β PC ester	71.6	C-γ, β-β
144.3	C-α, PE ether	62.7	C-γ, β-5
138.2	C-4, S etherified	60.2, 59.8	C-γ, β-O-4′
134.8, 134.3	C-1, S etherified; C-1, G etherified	56.0	OCH_3, G and S unit
133.4	C-1, S and G non-etherified	53.4	C-β, β-5′ units
133.0	C-1, S non-etherified	52.3	C-β, β-β′ units
130.4	C-2/C-6, PC ester	39.5	DMSO
129.9, 128.1	C-2/C-6, H units	31.4	CH_3 in ketones (conj) or in aliphatic side chains
125.4	C-1, PC ester	30.0–15.0	CH_3 or CH_2 group in saturated side chains
122.9	C-6, FE ester	14.3	γ-CH_3 in *n*-propyl side chains

G, guaiacyl unit; S, syringyl unit; H, *p*-hydroxylphenyl unit; PC ester, esterified *p*-coumaric acid; FE ester, esterified ferulic acid; FE ether, etherified ferulic acid.

ferulic acid gave signals at 168.1 (C-γ, FE ether) and 122.4 ppm (C-6, FE ether), whereas the esterified ferulic acid exhibited an intense signal at 122.9 ppm (C-6, FE ester). These signals implied that *p*-coumaric acids were linked to lignin by ester bonds, whereas ferulic acids were linked to lignin by both ether and ester bonds.

The weak signals presented in the spectrum between 14.3 and 31.4 ppm could be assigned to γ-methyl and α- and β-methylene groups in *n*-propyl side chains. Besides, the very strong signal at 56.0 ppm corresponds to -OCH_3 in syringyl and guaiacyl units. Moreover, one of the most important reactions for lignin degradation in alkaline media is the cleavage of β-O-4′ structures. In this study, the C-β, C-α, C-γ of β-O-4′ units in the lignin fractions could be distinguished by 86.2, 72.3, 60.2 ppm, respectively. These signals indicated that β-O-4′ linkages between the lignin structural units were not cleaved significantly under the alkaline condition given. As expected, the interunit C—C linkages such as β-β (C-γ in β-β, 71.6 ppm) and β-5 (C-α in β-5, 86.9 ppm; C-γ in β-5/β-O-4 units with α-C=O, 62.7 ppm) were found to be present in the bamboo lignin fraction (L_5).

2D-HSQC NMR spectroscopy

For an in-depth structural characterization, bamboo lignin (sample L_5) was subjected to 2D-HSQC NMR analysis. The HSQC NMR spectrum of alkaline ethanol extractable lignin fraction (L_5) exhibited three regions corresponding to aliphatic, side chain, and aromatic ^{13}C–^{1}H correlations. The aliphatic region showed signals without significant structural information and therefore were not discussed in this study. The side chain (δ_C/δ_H 40–100/2.5–6.0) and the aromatic (δ_C/δ_H 100–150/6.0–8.5) region of the HSQC spectrum of the lignin are shown in Figure 6. The main cross-signals of the lignin fraction in the HSQC spectrum are assigned in Table IV, and the potential main substructures are depicted in Figure 7.

The side-chain region of the spectrum gave important information about the different interunit linkages present in bamboo lignin. The HSQC spectrum showed prominent signals corresponding to β-O-4′ substructures (A). The C—H correlations in β-O-4′substructures were observed for α- and γ-C positions at δ_C/δ_H 72.3/4.83, 60.1/3.73 and 3.40 ppm, and for β-C positions at δ_C/δ_H 83.9/4.32 ppm in G and H type lignin, and 86.2/4.11 ppm in S type lignin. In addition to β-O-4′ aryl ether structure, other various interunit linkages were also observed in significant amounts. Strong signals for resinol (β-β′/α-O-γ′/γ-O-α′) substructures (B) were observed in the spectrum, with their C_α—H_α, C_β—H_β and the double C_γ—H_γ correlations at δ_C/δ_H 84.6/4.64, 53.9/3.04 and 71.6/3.83 and 4.16, respectively. The

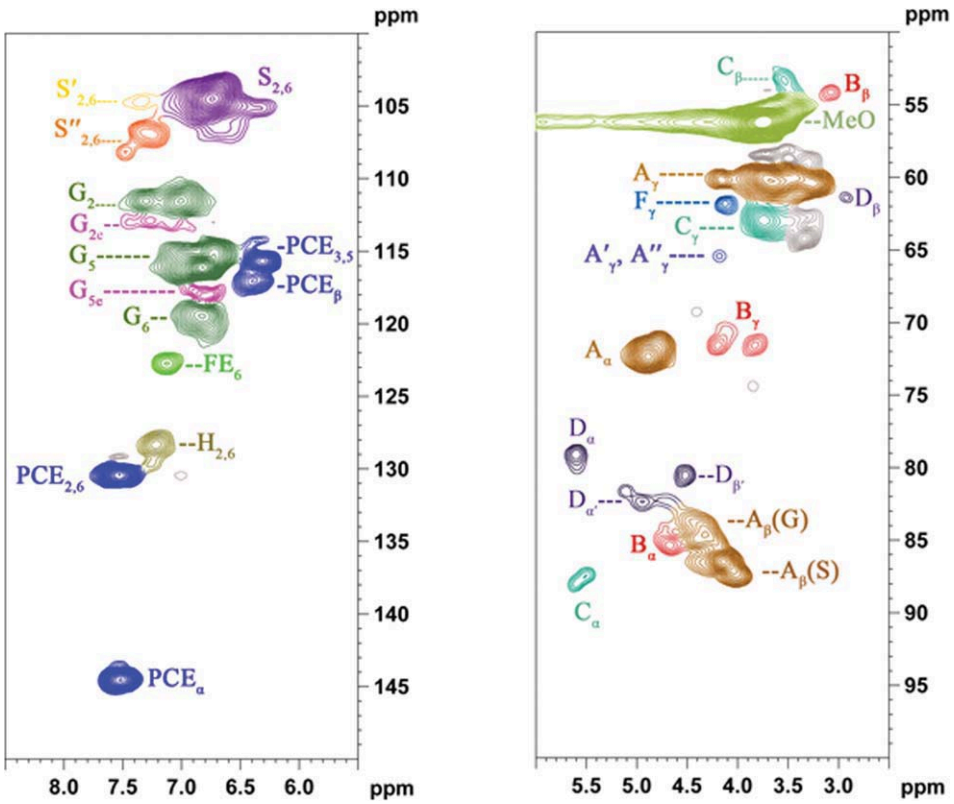

Figure 6 HSQC NMR spectrum of the bamboo lignin fraction L_5 isolated with 60% aqueous ethanol containing 0.5% NaOH. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

phenylcoumaran substructures (C) were also detected from the spectrum, and the signals for their C_α—H_α, C_β—H_β, and C_γ—H_γ correlations were observed at δ_C/δ_H 87.3/5.59, 53.4/3.46, and 62.7/3.78, respectively. Furthermore, very small signals corresponding to spirodienone (β-1′ and α-O-α′) substructures (D) could also be observed in the spectrum, their C_α—H_α, $C_{\alpha'}$—$H_{\alpha'}$, C_β—H_β, and $C_{\beta'}$—$H_{\beta'}$ correlations being at δ_C/δ_H 79.2/5.59, 82.4/4.96, 61.1/2.95, and 80.7/4.51, respectively. However, these interunit linkages are really rare in nature, which was the main reason for the weak signals in the spectrum. Other small signals in the side chain region of the HSQC spectrum of bamboo lignin corresponding to C_γ—H_γ correlations (at δ_C/δ_H 61.6/4.07) were assigned to *p*-hydroxycinnamyl (F) end groups.

The main cross-signals in the aromatic region of the HSQC spectrum corresponded to the aromatic rings of the different lignin units. Signals from syringyl (S) and guaiacyl (G) units and *p*-hydroxyphenyl (H) units were observed. The S lignin units showed a prominent signal for the $C_{2,6}$—$H_{2,6}$ correlation at δ_C/δ_H 104.3/6.68. The G lignin units showed different correlations for C_2—H_2 (δ_C/δ_H 111.4/6.95), C_5—H_5 (δ_C/δ_H 114.8/6.71 and 6.94), and C_6—H_6 (δ_C/δ_H 119.4/6.81). The multiple C_5—H_5 signals revealed some heterogeneity among the G units, especially affecting the C_5—H_5 correlation, probably due to different substituents at C_4 (e.g., phenolic or etherified in different substructures).[27] The structures with C_α carbonyl group (S′ and S″) gave signals at δ_C/δ_H 104.8/7.35 and 105.9/7.28, respectively. Besides, a significant amount of *p*-hydroxyphenyl (H) units was observed from $C_{2,6}$—$H_{2,6}$ correlations at δ_C/δ_H 128.1/7.16 ppm.

In addition, it was easy to identify correlations of esterified *p*-coumaric acid structures (A″) due to its very prominent signals as shown by ^{13}C-NMR and HSQC spectra. Aromatic ring cross-signals corresponding to correlations $C_{2,6}$—$H_{2,6}$ and $C_{3,5}$—$H_{3,5}$ in *p*-coumaric ester were observed at δ_C/δ_H 130.4/7.51 and 116.7/6.32 ppm, respectively. Side chain cross-signals corresponding to correlations C_α and C_β were revealed at 144.7/7.51 and 115.4/6.32, respectively.

To evaluate the relative abundances of the main interunit linkages, as well as the percentage of γ-acetylation and the molar S/G ratios, semi-quantitative analysis of the HSQC cross-signal intensities was performed, and their values are listed in Table V. The results showed that the major interunit linkages presented in the alkaline aqueous ethanol extractable bamboo lignin fraction (L_5) were β-O-4′

TABLE IV
Assignments of ^{13}C-^{1}H Correlation Signals in the HSQC Spectrum of the Isolated Bamboo Lignin Fraction (L_5)

Lables	δ_C/δ_H	Assignment
C_β	53.4/3.46	C_β—H_β in phenylcoumaran substructures (C)
B_β	53.9/3.04	C_β—H_β in β-β′ (resinol) substructures (B)
MeO	56.0/3.70	C—H in methoxyls
A_γ	60.2/3.40 and 3.73	C_γ—H_γ in β-O-4′ substructures (A)
D_β	61.1/2.95	C_β—H_β in spirodienone substructures (D)
F_γ	61.6/4.07	C_γ—H_γ in *p*-hydroxycinnamyl alcohol end groups (F)
C_γ	62.7/3.78	C_γ—H_γ in phenylcoumaran substructures (C)
A'_γ (A''_γ)	64.2/4.21	C_γ—H_γ in γ-acylated β-O-4′ substructures (A′ and A″)
B_γ	71.6/3.83 and 4.16	C_γ—H_γ in β′-β″ resinol substructures (B)
A_α	72.3/4.83	C_α—H_α in β-O-4′ substructures linked to a S unit (A)
D_α	79.2/5.59	C_α—H_α in spirodienone substructures (D)
$D_{\beta'}$	80.7/4.51	$C_{\beta'}$—$H_{\beta'}$ in spirodienone substructures (D)
$D_{\alpha'}$	82.4/4.96	$C_{\alpha'}$—$H_{\alpha'}$ in spirodienone substructures (D)
$A_{\beta(G)}$	83.9/4.32	C_β—H_β in β-O-4′ substructures linked to a G and H unit (A)
B_α	84.6/4.64	C_α—H_α in β-β′ (resinol) substructures (B)
$A_{\beta(S)}$	86.2/4.11	C_β—H_β in β-O-4′ substructures linked to a S unit (A)
$A_{\beta(S)}$	86.9/3.96	C_β—H_β in β-O-4′ substructures linked to a S unit (A)
C_α	87.3/5.59	C_α—H_α in phenylcoumaran substructures (C)
$S_{2,6}$	104.3/6.68	$C_{2,6}$—$H_{2,6}$ in etherified syringyl units (S)
$S'_{2,6}$	104.8/7.35	$C_{2,6}$—$H_{2,6}$ in oxidized (C_αOOH) syringyl units (S′)
$S''_{2,6}$	105.9/7.28	$C_{2,6}$-$H_{2,6}$ in oxidized (C_α=O) phenolic syringyl units (S″)
G_2	111.4/6.95	C_2—H_2 in guaiacyl units (G)
G_{2e}	112.8/7.24	C_2—H_2 in etherified guaiacyl units (G)
G_5	114.8/6.71 and 6.94	C_5—H_5 in guaiacyl units (G)
G_{5e}	117.9/6.85	C_5—H_5 in etherified guaiacyl units (G)
PCE_β	115.4/6.32	C_β—H_β, *p*-coumaroylated substructures (A″)
$PCE_{3,5}$	116.7/6.32	$C_{3,5}$—$H_{3,5}$, *p*-coumaroylated substructures (A″)
G_6	119.4/6.81	C_6—H_6, G units (G)
FE_6	122.9/7.06	C_6—H_6 in FE ester (FE)
$H_{2,6}$	128.1/7.16	$C_{2,6}$—$H_{2,6}$ in H units (H)
$PCE_{2,6}$	130.4/7.51	$C_{2,6}$—$H_{2,6}$, *p*-coumaroylated substructures (A″)
PCE_α	144.7/7.51	C_α—H_α, *p*-coumaroylated substructures (A″)

aryl ether linkages (74.3% of total side chains), followed by β-β′ resinol-type linkages and β-1′ spirodienone-type linkages (both for 7.8%), and lower amounts of β-5′ phenylcoumaran (6.8%), *p*-hydroxycinnamyl alcohols end groups (3.1%). This revealed that β-O-4′ aryl ether was reasonably stable under the alkaline condition. Moreover, a small percentage (1.0%) of the lignin side-chain was found to be acetylated at the γ-carbon, predominantly over syringyl units.

Thermal stability

For the purpose of comparing the thermal properties of the isolated lignin fractions, water soluble lignin fraction L_2 and alkaline aqueous ethanol soluble lignin fraction L_8 were studied by TGA and DTA. As illustrated in the Figure 8, the TGA curves of the two lignin fractions exhibited three stages during the pyrolysis process. At the first stage, the temperature below 200°C, the weight loss was due to the volatilization of moisture present in the lignin samples as well as some decomposition products with low molecular weight, such as carbon dioxide, carbon monoxide, and methane. At the second stage, which involved a wide range of temperature from 200 to 500°C, the main weight loss could be explained by the violent degradation of lignin. At the third stage, when the temperature was beyond 500°C, the weight loss was not very evident. At this stage, decomposition reactions often occurred in concurrence with condensation reactions of aromatic rings.

As can be seen from the Figure 8, there were still 31% and 39% solid residues left even at 600°C for lignin fractions L_2 and L_8, respectively. This was probably due to the higher thermal stability of these lignin subfractions, which require much higher temperatures for decomposition. Another reason for this higher content of residues at 600°C was also probably due to the end-products of the decomposition of lignin, which were carbonaceous residues formed in an inert atmosphere, along with the salts formed during the extraction processes.[28] The results demonstrated that the lignin fraction L_8 had a higher thermal stability than the lignin fraction L_2, which was in accordance with the higher molecular weight of L_8 than that of L_2.

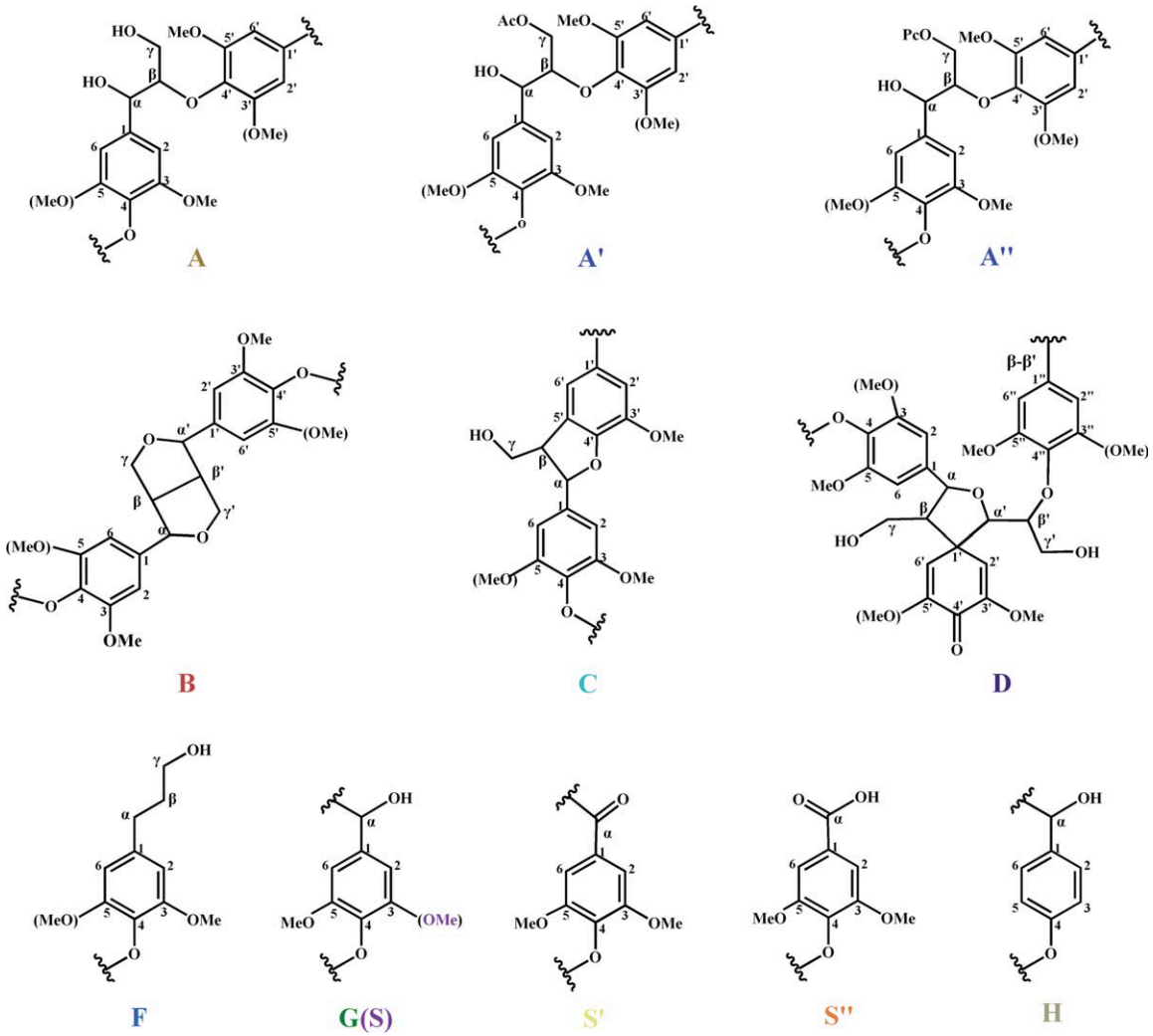

Figure 7 Main substructures presented in alkaline ethanol extractable bamboo lignin (L_5): (A) β-O-4′ linkages; (A′) γ-acetylated β-O-4′ substructures; (A″) γ-*p*-coumaroylated β-O-4′ linkages; (B) resinol structures formed by β-β′/α-O-γ′/γ-O-α′ linkages; (C) phenylcoumarane structures formed by β-5′/α-O-4′ linkages; (D) spirodienone structures formed by β-1′ linkages; (F) *p*-hydroxycinnamyl alcohol end groups; (G) guaiacyl unit; (S) syringyl unit; (S′) oxidized syringyl unit with a carbonyl group at C_α (phenolic); (S″) oxidized syringyl unit with a carboxyl group at C_α; (H) *p*-hydroxyphenyl unit. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE V
Structural Characteristics (Relative Sbundance of the Main Interunit Linkages, Percentage of γ-Acylation and S/G Ratio) from Integration of ^{13}C-^{1}H Correlation Signals in the HSQC Spectrum of the Isolated Bamboo Lignin Fraction (L_5)

Linkage relative abundance (% of side chains involved)	
β-O-4′ linked units (β-O-4′, A/A′/A″)	74.3
Resinols (β-β′, B)	7.8
Phenylcoumarans (β-5′, C)	6.8
Spirodienones (β-1′, D)	7.8
p-Hydroxycinnamyl alcohols (F)	3.1
Percentage of γ-acetylation	1.0
S/G ratio	1.56

CONCLUSIONS

The above results revealed that the content of associated carbohydrates in the lignin preparations obtained by alkaline aqueous ethanol were much lower than those of the lignin fractions isolated by hot water, indicating that the treatments with alkaline aqueous ethanol under the condition given significantly cleaved the linkages between hemicelluloses and lignin from the cell walls of bamboo (*D. brandisii*) stem, which made it possible to isolate lignin fractions with a high purity from the bamboo stem. In addition, spectroscopic analyses showed that the bamboo lignin was typical grass lignin, consisting of *p*-hydroxyphenyl, guaiacyl, and syringyl units. Furthermore, it was found that the alkaline aqueous ethanol soluble lignin fraction L_5 was

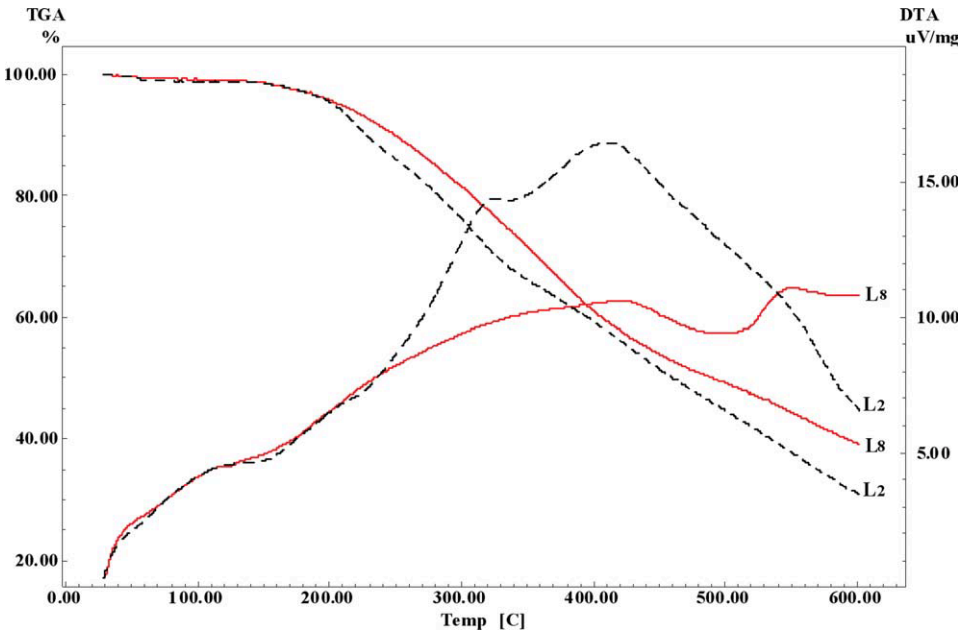

Figure 8 TGA/DTA curves of the isolated bamboo lignin fractions (L_2, L_8). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mainly composed of β-O-4′ aryl ether linkages, together with lower amounts of β-β′, β-1′, and β-5′ linkages. The results obtained have showed that these treatments under the conditions used did not change the lignin structure to a significant extent.

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