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Structural Transformation of *Miscanthus* × *giganteus* Lignin Fractionated under Mild Formosolv, Basic Organosolv, and Cellulolytic Enzyme Conditions

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ABSTRACT: Detailed chemical structural elucidation of lignin fractions from *Miscanthus* × *giganteus* was performed by several analytical techniques. Mild formosolv, basic organosolv, and cellulolytic enzyme treatments were applied to isolate three lignin fractions (AL, BL, and CL, respectively), and their structural characterization was comparatively evaluated. Both non-destructive techniques [e.g., Fourier transform infrared (FTIR) spectroscopy, size-exclusion chromatography (SEC), and two-dimensional (2D) nuclear magnetic resonance (NMR)] and degradation methods [e.g., acidic hydrolysis, derivatization followed by reductive cleavage (DFRC), and thioacidolysis] were used. The analysis revealed that a certain amount of carbohydrates (12.8%) was associated with CL and partially led to its increased molecular weight determined by SEC before acetylation. β-O-4 linkages were determined to be the predominant interunits (82%), but also, extensively acylated structures were observed. Alkaline organosolv treatment significantly improved the purity of the lignin fraction (carbohydrate content of 1.0%) and basically kept the original structure of the lignin macromolecule. Under acidic conditions, not only the portion of aryl alkyl ether bonds were cleaved but also new carbon−carbon bonds were formed by condensation reactions, resulting in an increment of the lignin molecular weights. Guaiacyl units were more reactive toward condensation than syringyl units, which was evidenced by an increasing S/G ratio from 0.7 (CL) to 1.7 (AL).

KEYWORDS: *Lignin, organosolv, cellulolytic enzyme, fractionation, structural characteristic, Miscanthus* × *giganteus*

INTRODUCTION

Lignin is an essential wood component accounting for up to 30% of the organic carbon in the biosphere.[1] It is the most complex and irregular heteropolymer present in the cell walls of vascular plants and is mainly built from three basic monolignols (*p*-coumaryl, coniferyl, and sinapyl alcohols). They are connected by various bonds, such as several types of ether (e.g., β-*O*-4, α-*O*-4, and 4-*O*-5) and carbon−carbon (e.g., β−β, β−5, and 5−5) linkages, mediated by laccases and peroxidases.[2−4] With the increase of biomass use, lignin is considered as a potential starting material for manufacturing adhesives, epoxy and phenolic resin, and polyolefins because of its polyphenolic chemical structure.[5−7] Therefore, investigations devoted to the elucidation of its structure, purity, and properties are being under way. Traditionally, the first step to characterize lignin is the extraction of a representative portion from lignocellulosic material. Generally, milled wood lignin (MWL) is considered to have preserved the original structure of the native lignin from biomass.[8] However, MWL usually gives a relatively low yield and is characterized by a high content of carbohydrates. Whiting and Goring[9] pointed out that MWL might not be representative of the whole lignin because it primarily originates from the secondary cell wall of the wood. Increasing the ball-milling time could improve the yield of MWL by dissociating lignin from carbohydrates, but it also results in depolymerization reactions. In 1975, Chang et al.[10] reported a new method combining the enzymatic degradation of cellulose and subsequent dioxane extraction. This so-called cellulolytic enzyme lignin (CEL) showed increased yield but kept similar characteristics compared to MWL.[11,12] Organosolv processes were also extensively investigated in respect to the removal of lignin and the enzymatic hydrolyzability of the remaining carbohydrates.[13−16] Historically, ethanol is the most common solvent in the organosolv process, normally operating at high-temperature (>150 °C) or high-pressure (>15 bar) with or without the addition of catalysts. The hydrolyzed lignin dissolves in the organophilic phase and is recovered as a filtrate via a precipitation reaction, and the organic solvents are easily recovered by distillation and recycled. Organic acids, as solvent in the organosolv process, were proven to be promising processes for the efficient fractionation of biomass into cellulose, hemicelluloses, and lignin under mild conditions (<100 °C).[17,18] The mild alkaline ethanol process was extensively employed and described in previous papers.[19−21] It has the advantages of a mild operation condition, low alkaline consumption, high lignin yield, and efficient recovery of hemicelluloses. Prior to the determination of the individual advantages of these fractionation processes and guidance of the various kinds of practical use of

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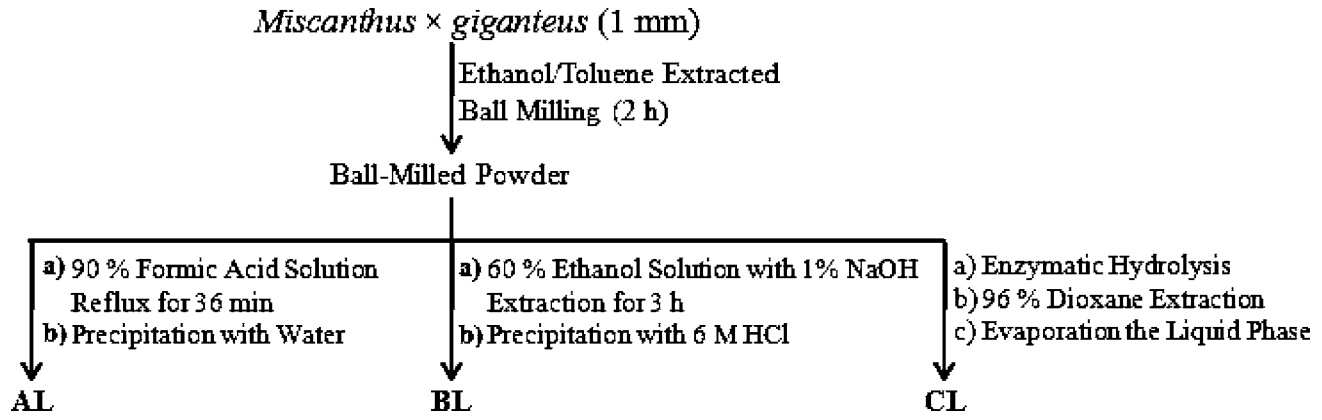

Figure 1. Simple acidic (formosolv) lignin (AL), basic ethanol organosolv lignin (BL), and cellulolytic enzyme lignin (CL) fractionation procedures.

the lignin component, the evaluation of the characteristic changes of the obtained lignin is needed in terms of their purity, chemical structure, and properties.

Among biomass feedstocks, *Miscanthus* × *giganteus* has attracted considerable interest recently.[22] *M.* × *giganteus* is a C_4 perennial grass with several advantages, such as little nitrogen or herbicide requirement,[23] long productive lifetime (10−15 years), low moisture content at harvest, and low susceptibility to pests and diseases.[24] It is even capable of producing in England at 52° N a peak biomass of 30 megatons ha^{-1} $year^{-1}$ and harvestable biomass of 20 megatons ha^{-1} $year^{-1}$, the highest yield recorded for a cool temperate climate.[25] With the increasing interest in second-generation biofuels, biorefinery of this energy crop into high-value co-products is critical to improve the economics of commercial application.

In the present work, lignin from *M.* × *giganteus* was isolated by mild formosolv (acidic condition, AL), basic organosolv (BL), and cellulolytic enzyme (CL) processes, which are all environmentally friendly processes and, in general, do not extensively degrade the overall chemical structure of lignin. The obtained lignin samples were characterized by size-exclusion chromatography (SEC), derivatization followed by reductive cleavage (DFRC), thioacidolysis, Fourier transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR), and residual carbohydrate analysis. We aimed to maximize the use of this interesting grass for the various applications by enriching the knowledge of the structure of the most complicated lignocellulosic component, the lignin polymer.

MATERIALS AND METHODS

Materials. *M.* × *giganteus* was provided by the Blaschek Research Group, University of Illinois at Urbana−Champaign. It was ground using a Thomas-Wiley mill to pass a 1 mm sieve and stored in a closed container until further use. The composition of *Miscanthus* was 42% glucan, 20% xylan, 2% arabinan, 22% Klason lignin, 3% acetyl group, and 4% ash. Cellulase (1.5 L) and Novozyme 188 were purchased from Sigma. All other reagents and chemicals of analytical grade were purchased from either Sigma−Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise stated.

Fractionation of Lignin. The simple scheme for fractionating lignin preparations AL, BL, and CL is shown in Figure 1. In brief, AL was obtained according to the formosolv process, as reported by Villaverde et al.,[26] boiling the mixtures of *Miscanthus*, formic acid (90%, w/w), water, and hydrochloric acid (0.1%, w/w) for 36 min with a liquid/solid ratio of 12. This lignin sample was obtained from the same batch of *Miscanthus* and kindly provided by Dr. Joe Binder. The fractionation of BL was performed in 60% alkaline ethanol solution (60 mL of ethanol and 1 g of NaOH filled to 100 mL with water) with a solid/solvent ratio of 1 g to 20 mL. After refluxing at 80 °C for 3 h, the pH of the supernatant was adjusted to pH 6 with 6 M HCl and 95% ethanol was added (3× the volume of the supernatant). The precipitated hemicelluloses were removed by centrifugation (3250*g*). Ethanol in the supernatant was removed by rotary evaporation (40 °C), and the dissolved lignin was precipitated by acidifying to pH ∼ 2 with 6 M HCl. After centrifugation (3250*g*), the solids were washed with acidified water (pH ∼ 2, adjusted by HCl), freeze-dried, and labeled BL. The CL isolation process described by Chang et al.[10] was followed in this study. Ball-milled *Miscanthus* (1 g) was suspended in citric acid buffer (100 mL, 0.1 M, pH 5.0), with the loading of cellulase (20 FPU/g) and β-glucosidase (40 IU/g). The reaction mixture was incubated at 50 °C in a rotary shaker (200 rpm) for 48 h. The solution was centrifuged, and the residue was extensively washed with water, freeze-dried, and then incubated with 96% dioxane for 48 h at room temperature under stirring. The liquid phase was collected with filtration and then evaporated to dry under vacuum. No further purification was performed to preserve all structural features of the isolated CL.

Associated Polysaccharide Analysis. The composition of structural carbohydrates was determined using the National Renewable Energy Laboratory (NREL) protocol[27] and analyzed by high-performance anion-exchange chromatography (HPAEC) (Dionex, ISC 3000, Sunnyvale, CA) on a CarboPac PA 20 analytical column (4 × 250 mm) with pulsed-amperometric detection.

SEC Analysis. Lignin samples were acetylated by incubation in a mixture of acetic acid and acetyl bromide (92:8, v/v) at 50 °C for 2 h.[28] Both derivatized and non-derivatized lignin samples were dissolved in tetrahydrofuran (THF) and filtered [0.45 μm polytetrafluoroethylene (PTFE) membrane syringe filter, Sartorius Stedim Biotech, Germany] before injection. SEC runs were carried out on a PL-GPC 50 Plus (Polymer Laboratories, Amherst, MA) equipped with an ultraviolet (UV) detector (280 nm) and a series of two connected MesoPore columns (300 × 7.5 mm) with a mobile phase of tetrahydrofuran (THF) (1 mL/min). The molecular weight was calibrated with polystyrene standards (Polymer Laboratories, Amherst, MA).

Table 1. Yield and Monosaccharide Content of Isolated Lignin Samples from *Miscanthus*

	yield (%)[a]		neutral sugars (%)[b]					
sample	with sugars	without sugars	Rha	Ara	Gal	Glc	Xyl	total
AL	65.2	64.0	ND[c]	0.8	T[d]	0.4	0.7	1.9
BL	43.1	42.7	T	0.2	0.1	0.1	0.6	1.0
CL	25.8	22.5	0.7	0.9	0.1	4.4	6.6	12.8

[a]On the basis of the Klason lignin of *Miscanthus*. [b]Rha, rhamnose; Ara, arabinose; Gal, galactose; Glc, glucose; Xyl, xylose. [c]ND = not detected. [d]T = trace.

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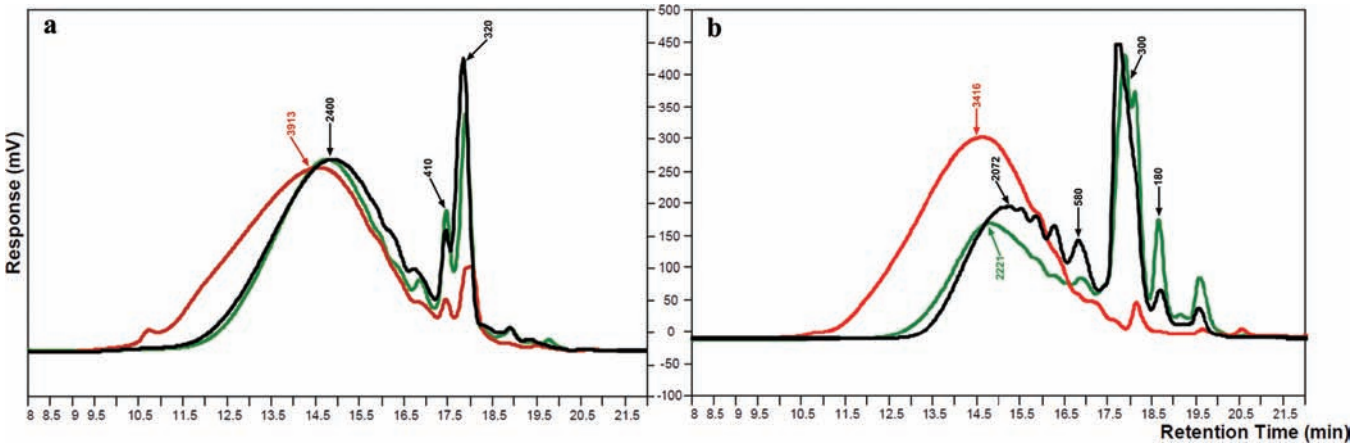

Figure 2. Molecular-weight distributions of AL (red line), BL (black line), and CL (green line) (a) with and (b) without acetobromination derivatization.

Table 2. Weight Average (M_w) and Number Average (M_n) Molecular Weights and Polydispersity (DP) of Fractionated Lignin Samples from *Miscanthus*

	acetylated			non-acetylated		
	AL	BL	CL	AL	BL	CL
M_w (Da)	3643	2051	1959	2679	1196	1365
M_n (Da)	1301	753	751	1564	1039	1050
DP	2.8	2.7	2.6	1.7	1.2	1.3

DFRC Analysis. The DFRC method was reported by Lu and Ralph[29] and applied to selective cleavage of the arylglycerol-β-aryl (β-*O*-4) ether linkages in lignin. A modified DFRC process as described by Ikeda et al.[30] was used to identify the residues and quantify the β-*O*-4 structure.

Thioacidolysis. Thioacidolysis was performed according to the procedure by Rolando et al.[31]

Infrared Spectroscopy. FTIR spectra were recorded on a FTIR spectrophotometer (Nicolet 6700, Thermo Scientific, Waltham, MA) using KBr disk. Spectra were recorded in the absorption mode from 2000 to 800 cm^{-1}.

$^1H-^{13}C$ Two-Dimensional (2D) Correlation NMR Spectroscopy. Two-dimensional NMR spectra were recorded at room temperature on a Bruker AVANCE 600 MHz using a z-gradient triple-resonance probe. The lignin sample (50 mg) was dissolved in 0.5 mL of dimethylsulfoxide (DMSO)-d_6. Heteronuclear single-quantum coherence (HSQC) settings: The spectral widths were 5000 and 25 625 Hz for the $^1H-^{13}C$ dimensions, respectively. The number of collected complex points was 2048 for the ^1H dimension with a recycle delay of 5 s. The number of transients was 64, and 256 time increments were always recorded in the ^{13}C dimension. A squared cosine-bell apodization function was applied in both dimensions. Prior to Fourier transform, the data matrixes were zero-filled up to 1024 points in the ^{13}C dimension. Signals were assigned by comparison to literature spectra.[32−37]

Table 3. Structural Characteristic (Content of β-*O*-4′ Interunit Linkages and S/G Ratio) in AL, BL, and CL Observed from the HSQC Spectra, DFRC, and Thioacidolysis

	β-*O*-4′ linkage (%)		S/G ratio		
samples	HSQC[a]	DFRC[b]	HSQC[c]	DFRC	thioacidolysis
AL	56	52	1.0	1.0	1.7
BL	83	60	1.1	1.1	1.8
CL	82	54	0.9	0.7	0.7

[a]Relative percentage of the integration area of side chains (interunit linkages) in panels a, c, and e of Figure 4. [b]Relative percentage of the propionate derivatives to the whole esterified products. [c]Ratio of the integration area of S and G units in panels b, d, and f of Figure 4.

RESULTS AND DISCUSSION

Yield and Composition of Associated Neutral Sugars. In comparison to MWL, CL (CEL) was proposed to isolate lignin in a much higher yield with a minimal structural alteration. However, to maximally obtain the lignin for further use, AL and BL were obtained by acidic and basic organosolv processes, respectively. The yields of AL, BL, and CL were 64.0, 42.7, and 22.5% of the Klason lignin, respectively (Table 1). Clearly, AL and BL lignin samples contained lower levels of associated polysaccharides (1.9% for AL and 1.0% for BL, respectively) (Table 1). In comparison, CL had a much higher carbohydrate content (12.8%), suggesting that a certain amount of carbohydrates was co-extracted by dioxane or was still linked to the lignin in CL. Xylose was found to be the major sugar component in all cases; however, the ratio of xylose and arabinose was much lower in AL (0.9) and BL (3.0) compared

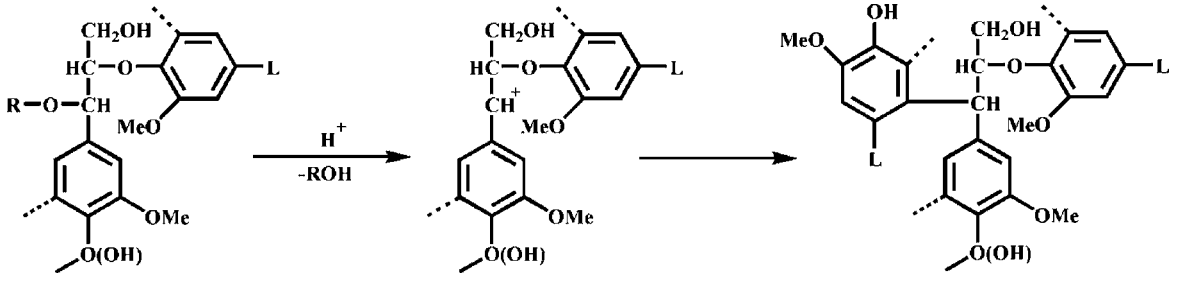

Figure 3. Proposed mechanism of condensation reactions in lignin under an acidic condition.[43]

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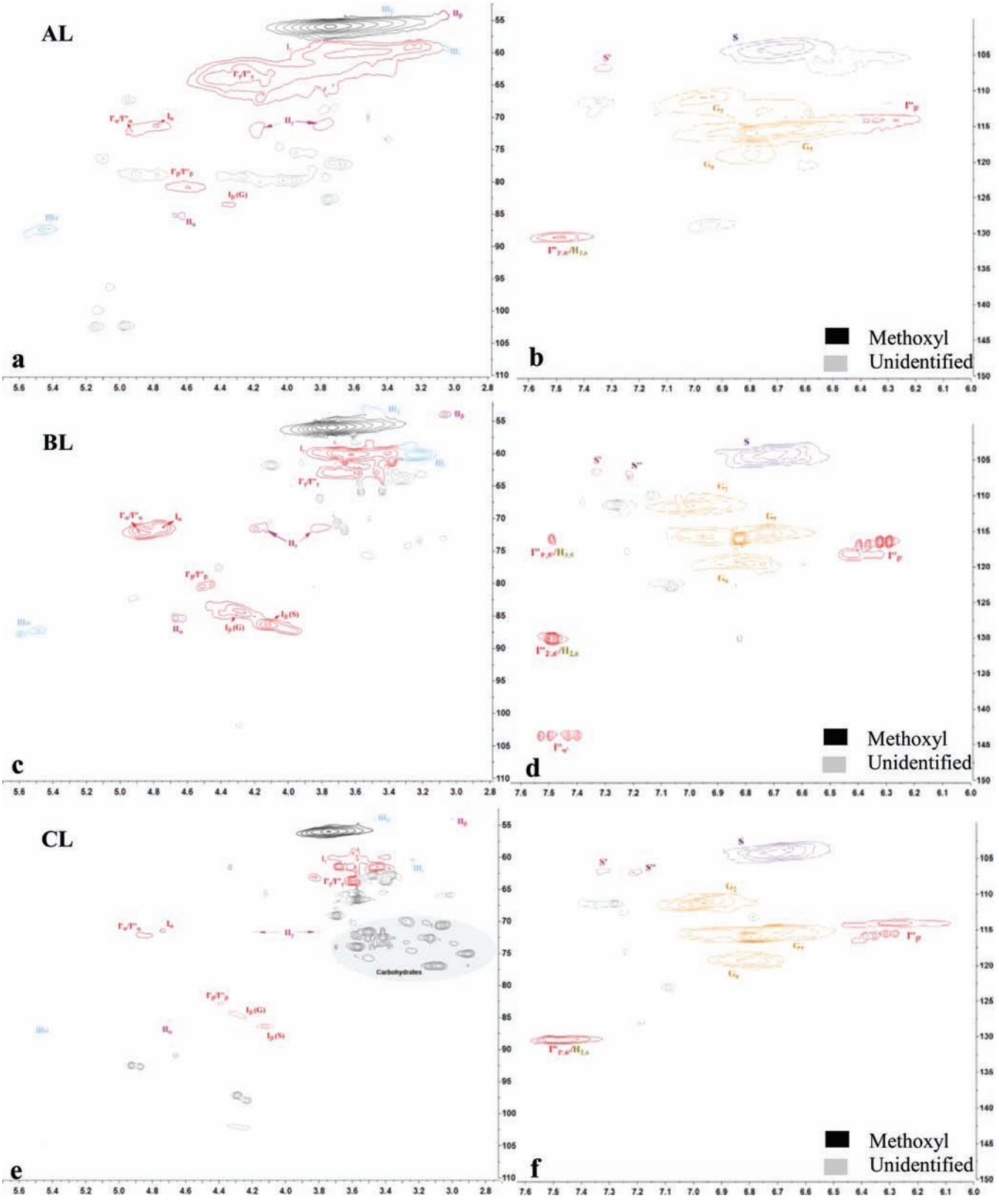

Figure 4. HSQC NMR spectra of (a and b) AL, (c and d) BL, and (e and f) CL: expanded side-aliphatic region δ_C/δ_H 50−110/2.8−5.7 (a, c, and e) and expanded aromatic region δ_C/δ_H 100−150/6.0−7.8 (b, d, and f). Colored peaks are derived from respective structures of the same color in Figure 5.

to CL (7.3), indicating that a high percentage of xylose was removed during the acidic and basic organosolv fractionation processes. It is known that *p*-coumaryl and feruloyl groups are cross-linking lignin and xylan polymers with ester linkages at the arabinose side chains of xylan. Because CL was isolated under the least drastic conditions, most ester linkages were

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preserved with longer xylan unit chains attached. Under a basic condition, a considerable amount of lignin−carbohydrate ester bonds could be broken,[38,39] resulting in the obvious decrease in total carbohydrate content and the xylose/arabinose ratio of BL. In comparison, acid hydrolysis could significantly cleave the glycosidic bonds of polysaccharides, shortening the xylan chains but cleaving the ester bonds only to a small degree. Therefore, it was not surprising to observe the lowest xylose/arabinose ratio in AL, because arabinose is mainly linked to lignin via the *p*-coumaric or ferulic acid esters. Clear effects of acidic and basic conditions on the preparation of lignin with a higher purity were observed.

Molecular-Weight Distribution. Lignin samples have usually low solubility in the solvents commonly used for SEC. The absorption on the gel (hydrophilic or hydrophobic), ionic interactions, or intermolecular association could lead to an underestimation of the molecular weight.[40] It has been suggested that hydrogen bonding or hydrophobic interactions between lignin molecules can lead to the formation of association complexes and bimodal elution curves in SEC.[41] Therefore, SEC of lignin is comparatively performed with and without acetobromination derivation. It is clear that all of the SEC chromatograms shifted to the shorter elution time region after the derivation process (Figure 2). Correspondingly, the values of weight average (M_w) and number average (M_n) molecular weight and polydispersity were increased (Table 2). Lawoko et al.[42] compared the size-exclusion chromatograms of MWL pure (carbohydrate <1%), MWL impure, and enzymatically treated MWL impure with a cocktail of xylanase and mannanase (EMWL impure) and concluded that the presence of poly(oligo)saccharide chains bonded to lignin led the molecular-mass distribution shift to a shorter retention time. The acidic acetobromination derivatization procedure not only improved the solubility of lignin in THF but also partially cleaved the linkages between lignin and carbohydrates and, therefore, reduced the observed molecular weight. Hence, it can be speculated that the molecular weight and polydispersity index values of CL after acetylation are more accurate, and the lowest value (1959 g/mol) was probably due to the partial fractionation of the lignin with a low molecular weight in dioxane aqueous solution after enzymatic treatment.

In comparison, AL had the largest molecular weight and polydispersity index in both acetylated (3643 g/mol and 2.8) and non-acetylated (2679 g/mol and 1.7) samples, suggesting a relatively condensed structure. Under an acidic condition, condensation reactions can occur besides acidic depolymerization reactions. As proposed by Li et al.,[43] the C_α of the side chain is prone to form a carbon cation, which can then bind with an electron-rich carbon atom in the aromatic ring of another lignin unit (Figure 3). The formation of new carbon−carbon bonds, thereby, gives an increase in the heterogeneity of the resulting lignin. The same phenomenon was also observed by Villaverde et al.[26] after SEC analysis of MWL and AL, where the adsorption peaks of AL obviously distributed in the higher molecular-weight region after thioacidolysis. Although the molecular weight and polydispersity index values of BL were smaller than those of CL in the non-acetylated form, it was believed that BL had a slightly larger macromolecule than CL, because the molecular weight of CL was overestimated in the non-acetylated form, and therefore, a relatively higher molecular weight of BL than that of CL in the acetylated form should be observed. In short, the data in the current study were much lower than those reported in a previous paper.[26] It is probably due to the differences in the source of raw material (area, growth time, etc.) and analytical instrument (SEC system and column). However, it is reasonable to comparably investigate the relative variation of the lignin macromolecule structure in different fractionation processes.

Table 4. Adsorption Assignments in the Region of 800−2000 cm^{-1} of the FTIR Spectra of the Lignin Samples[44]

assignments	wavenumbers (cm^{-1})
C−H out-of-plane in positions 2 and 6 of S units and in all positions of H units	835
C−H out-of-plane in positions 2, 5, and 6 of G units	870
aromatic C−H in-plane deformation, G > S; C−O deformation in primary alcohols	1032
C−O deformation in secondary alcohols and aliphatic ethers	1086
aromatic C−H in-plane deformation, typical for S units	1126
C═O stretch in conjugated ester groups, such as *p*-coumaric acid	1166
C−C, C−O, and C═O stretch	1226
G-ring breathing with C═O stretch	1268
condensed S and G rings (substituted in one of the positions in the ring)	1330
aliphatic C−H stretch in $-CH_3$	1370
aromatic skeletal vibration combined with C−H in-plane deformation	1425
C−H asymmetric deformation in $-CH_3$ and $-CH_2-$	1460
aromatic skeletal vibration	1505
aromatic skeletal vibration	1600
C═O stretch in conjugated *p*-substituted aryl ketones	1636
C═O stretch in conjugated aldehydes and carboxylic acids	1695
C═O stretch in unconjugated ketones, carbonyls, and ester groups	1725

Table 5. Assignments of $^{13}C-^{1}H$ Correlation Signals in the HSQC NMR Spectra of the Isolated Lignin from *Miscanthus*

δ_C/δ_H (ppm)	assignments
53.5/3.5	$C_\beta-H_\beta$ in **III**
54.0/3.1	$C_\beta-H_\beta$ in **II**
60.4/3.2	$C_\gamma-H_\gamma$ in **III**
60.4/3.4−4.0	$C_\gamma-H_\gamma$ in **I**
63.9/3.7−4.4	$C_\gamma-H_\gamma$ in **I′** and **I″**
71.4/4.7	$C_\alpha-H_\alpha$ in **I**
71.5/3.8 and 71.5/4.2	$C_\gamma-H_\gamma$ in **II**
72.1/4.9	$C_\alpha-H_\alpha$ in **I′** and **I″**
80.3/4.5	$C_\beta-H_\beta$ in **I′** and **I″**
84.1/4.3	$C_\beta-H_\beta$ in G units
85.3/4.7	$C_\alpha-H_\alpha$ in **II**
86.3/4.1	$C_\beta-H_\beta$ in S units
87.3/5.4	$C_\alpha-H_\alpha$ in **III**
104.2/6.7	$C_{2,6}-H_{2,6}$ in S units
106.6/7.3	$C_{2,6}-H_{2,6}$ in S′ units
107.1/7.2	$C_{2,6}-H_{2,6}$ in S″ units
111.4/7.0	C_2-H_2 in G units
116.0/6.8	C_5-H_5 in G units
116.0/7.5	$C_{3',5'}-H_{3',5'}$ in **I″** and H units
116.6/6.3	$C_{\beta'}-H_{\beta'}$ in **I″**
119.1/6.8	C_6-H_6 in G units
130.0/7.5	$C_{2',6'}-H_{2',6'}$ in **I″** and H units
143.7/7.5	$C_{\alpha'}-H_{\alpha'}$ in **I″**

Structural Characteristic of Lignin Fractions. Lignin is a polyphenolic amorphous material containing several types of ether and carbon−carbon linkages mediated by laccases and/or

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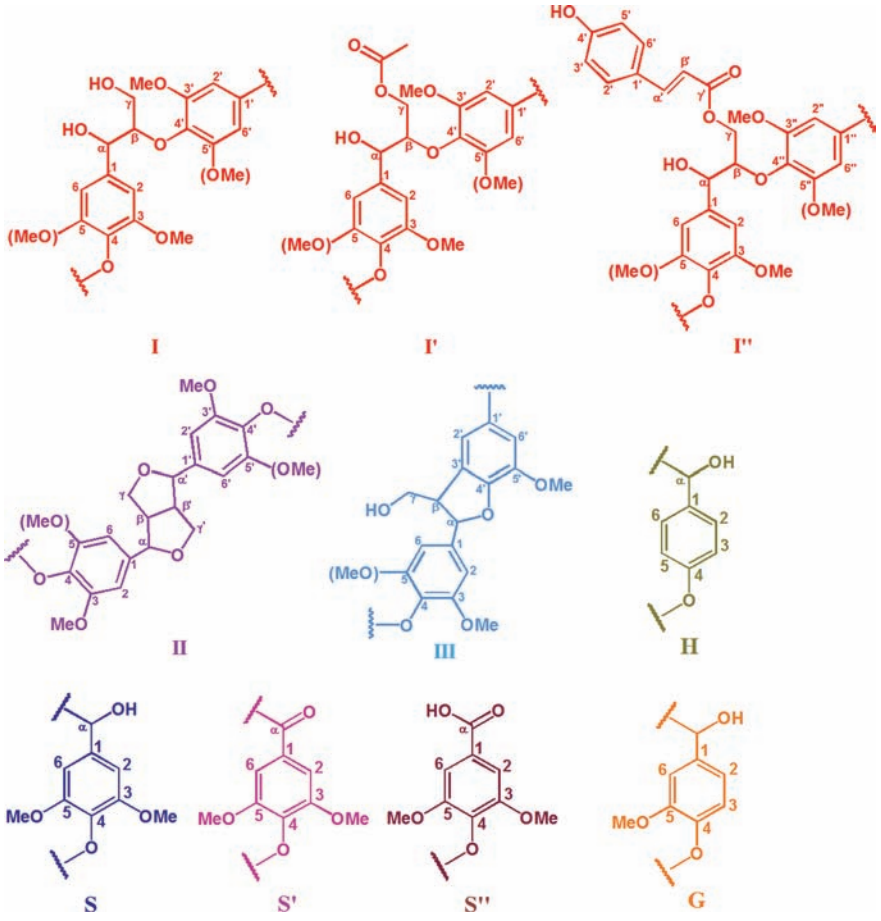

Figure 5. Main substructures identified in *Miscanthus* lignin: (**I**) *β*-*O*-4′, (**I′**) *γ*-acetylated *β*-*O*-4′, (**I″**) *γ*-*p*-coumaroylated *β*-*O*-4′, (**II**) resinol, formed by *β*−*β*′ coupling and *α*-*O*-*γ*′ and *γ*-*O*-*α*′ bonding during quinone methide rearomatization, (**III**) phenylcoumaran, formed by *β*−5′ coupling and subsequent *α*-*O*-4′ bonding, (S) syringyl unit, (S′) oxidized syringyl units with a C_α ketone, (S″) oxidized syringyl units with a C_α carboxyl group, (G) guaiacyl unit, and (H) *p*-hydroxyphenyl unit.

peroxidases.[44] Many degradative methods have been developed to investigate the bonding pattern of lignin macromolecules, of which thioacidolysis and DFRC treatments are considered to be the most effective diagnostic processes. The modified DFRC method allows for the quantitative determination of uncondensed etherified *β*-*O*-4 linkages, which derived from the ratio of the propionate derivatives to all esterified products (Table 3).[30] Recently, 2D HSQC NMR spectroscopy was applied to acquire main information on the structural characteristics of lignin.[32,36] The C_α−H_α correlation was used to estimate the relative abundance of interunit linkages, and the S/G ratio was calculated by the $C_{2,6}$−$H_{2,6}$ correlations from S units and the sum of C_2−H_2 and C_6−H_6 correlations from G units.[44] The HSQC spectra of AL, BL, and CL were divided into a side-aliphatic region δ_C/δ_H 50−110/2.8−5.7 and an aromatic region δ_C/δ_H 100−150/6.0−7.8 (Figure 4). The main lignin cross-signals were assigned and listed (Table 5), correlating to the main ether and C−C bonds existing in the lignin (Figure 5).

CL preparation is an effective method to isolate wood lignin with the original structure and in a higher yield than MWL.[10] No further purification process was performed to avoid any loss of lignin; however, a significant amount of carbohydrates was observed in the 2D NMR spectrum and sugar analysis (Figure 4). The prevalent occurrence of the side-chain signals from various *β*-*O*-4 linkages indicated the outstanding characteristics of the lignin, which contains a high proportion of the *β*-*O*-4 ether bonds (82%). This is in accordance with the generally accepted data for lignin from non-woody plants,[36,44] although it was slightly less than the previously reported data.[26] The DFRC method has been successfully applied to the quantitative analysis of lignin model compounds and the isolated lignin products.[29,45,46] A *cis* orientation of the bromine and *β*-*O*-4 ether oxygen is required for the degradation process. Because of the nature of the derivatization reaction, this geometry is not achieved at all bonds. Therefore, a portion of the *β*-*O*-4 linkages might not be degraded, and the value of the *β*-*O*-4 ether content (54%) was obviously lower than that calculated from the HSQC spectrum (82%). The S/G ratios (0.7−0.9) obtained from DFRC (0.7), thioacidolysis (0.7), and the HSQC spectrum (0.9) were fairly close to each other and consistent with the reported data from *M.* × *giganteus* MWL (0.7),[26] indicating the relatively higher amount of guaiacyl units. This was further confirmed by the FTIR spectrum (Figure 6, CL), which revealed a remarkable absorption at 870 cm^{-1} assigned to the G units (Table 4, in accordance with ref 47). Other functional groups were also detected in the spectrum typical of lignin, including aromatic ring vibration (1600, 1505, and 1425 cm^{-1}), aromatic ring breathing (1330 and 1268 cm^{-1}), aromatic in-plane C−H deformation (1032 and 1126 cm^{-1}), and out-of-plane C−H bending (835 and 870 cm^{-1}). The abundant peak around 1700 cm^{-1} underlined the assumption that CL had a high amount of carbonyl groups,

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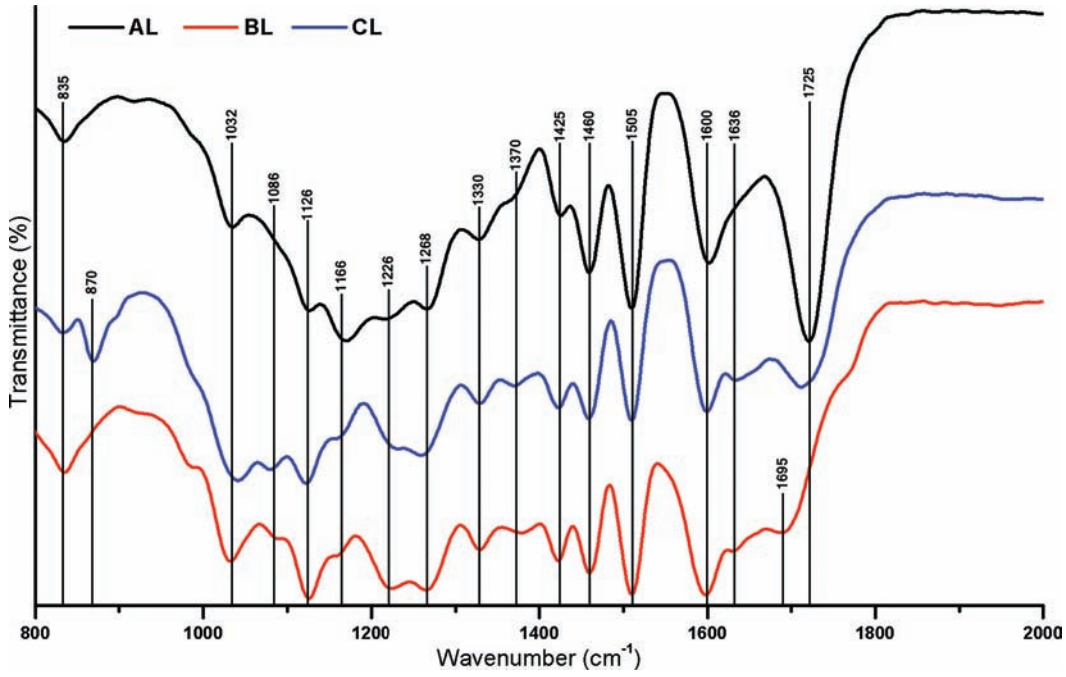

Figure 6. FTIR spectra of isolated lignin samples.

confirming previously reported results that *M.* × *giganteus* native lignin (MWL) was highly acylated with *p*-coumaryl and/or acetyl groups at C_γ.[26] This was further confirmed by the prominent signals in the HSQC 2D spectrum (Figure 4), assigned to the substructures **I′** and **I″** (Figure 5).

Fractionation under an alkaline condition (BL) preserved the original structure of *M.* × *giganteus* lignin. The *β*-*O*-4 substructures were still the most prominent in BL, and the content was 83 and 60% calculated by HSQC and DFRC, respectively. This difference was also ascribed to the inefficient cleavage of *β*-aryl ether bonds during the DFRC protocol. The alkaline reagent at the low concentration (1%) was obviously sufficient to cleave the ester or ether linkages present in the lignin−carbohydrate complex (LCC), assisting the isolation of more pure lignin, but was mild enough not to destroy the ether linkages between the structural monomers in lignin. The S/G ratio in BL (1.1−1.8) was remarkably increased in comparison to CL (0.7−0.9). The monomeric products from the DFRC process showed a S/G ratio of 1.1, which was in accordance with the results from HSQC analysis (1.1) but significantly lower than the results calculated from the thioacidolysis method (1.8) (Table 3). It is suspected that incomplete cleavage of *β*-*O*-4 bonds during the DFRC process and overlapping signals in the HSQC spectrum probably lead to inaccurate estimation, whereas the result obtained from thioacidolysis was considered more accurate because a more complete breakdown of *β*-*O*-4 bonds can be expected. This increasing trend in the S/G ratio was also observed in the FTIR spectrum (Figure 6), where the relative intensity of the adsorption assigned to aromatic in-plane C−H deformation for S units (1126 cm^{-1}) was enhanced.

In comparison, the formosolv process (AL) led to substantial structural changes, evident by a significantly lower intensity of the *β*-*O*-4 correlation signals measured by HSQC (56%) and DFRC (52%), as compared to BL or CL (Table 3). The unconjugated aryl ketol substructure was extensively formed during the fragmentation reactions,[48] resulting in the abundant peak at 1725 cm^{-1} in the FTIR spectrum (Figure 6). Similarly, the S/G ratio was increased, which was observed in the characteristic peaks of G and S units (1032 and 1126 cm^{-1}) in the FTIR spectrum, as well as after monomeric product analysis using degradation processes (Table 3). Unlike BL, the increment of the S/G ratio in AL (1.7 from thioacidolysis), as compared to CL (0.7 from thioacidolysis), was most likely due to acidic condensation reactions discussed above. The carbonium ion initiated under an acidic condition normally located at the C_α position of the side chain, and the free C_5 position in G units was the most electron-rich carbon atom and easiest to form C−C bonds. As a result, more G units were involved in the C−C bond formation and, therefore, were not susceptible to degradation to monomeric compounds by thioacidolysis or DFRC. Thus, lignin molecules with a more complex and heterogeneous structure were formed, which are more difficult to break down. Consequently, this would also make it more difficult for further use of this lignin as a starting material for high-value-added products.

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