Determination of Lignin-Carbohydrate Complexes Structure of Wheat Straw using Carbon-13 Isotope as a Tracer

Lan Yao,[a,b,c] Congxin Chen,[a] Xing Zheng,[a] Ziqian Peng,[d] Haitao Yang,[a,*] and Yimin Xie [a,*]

To maximize the use of wheat straw as a feedstock for biofuels and other biorefinery products, the structure of lignin-carbohydrate complexes (LCCs) was characterized by injection of ^{13}C isotope-labeled xylose into living wheat straw. Afterwards, lignin-carbohydrate complexes were extracted from the harvested straw by the Björkman method. The extracted LCCs were chemically characterized by Fourier transform-infrared spectroscopy (FT-IR), sugar composition, molecular weight analysis, ^{13}C-NMR, and HSQC. The results showed that LCCs in wheat straw were particularly enriched with xylan and exhibited narrow polydispersity (M_w/M_n < 1.5). NMR analysis showed that the lignin was linked with the carbohydrates through *γ*-ester, phenyl glycoside, and benzyl ether bonds. In addition to S, G, and H lignin units, *p*-coumarate and ferulate were also in the LCCs. The substructures in lignin were *β*-*O*-4', *β*-*β*', and *β*-5'. Quantitative data analysis of ^{13}C-NMR combined with HSQC showed that the lignin in the LCCs of wheat straw contained guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) units in the proportion of 5:4:1 (S:G:H). The main lignin substructure, *β*-*O*-4', comprised 71.64% of the isolated lignin. The total LCC linkages (the sum of phenyl glycoside, *γ*-ester and benzyl ether bonds) were 210.86 /100C_9 in ^{13}C-LCC, which was dominated by phenyl glycoside linkages, followed by *γ*-ester bonds and minor amounts of benzyl ether bonds. Lignin and xylan in the LCCs of wheat straw were mainly linked by benzyl ether bonds and phenyl glycoside linkages.

Keywords: Isotope-labeled; Wheat straw; Lignin-carbohydrate complexes (LCCs); ^{13}C-NMR; 2D-HSQC

Contact information: a: School of Pulp & Paper Engineering, Hubei University of Technology, 430068, Wuhan, China; b: State Key Laboratory of Pulp and Paper Engineering, South China University of Technology , Guangzhou, 510640 China; c: Key Laboratory of Pulp and Paper Science & Technology of Ministry of Education of China, Jinan, 250353; d: NO.1 Middle School Affiliated to Central China Normal University, 430223, Wuhan, China;
** Corresponding authors: pphtyang1979@aliyun.com; ppymxie@scut.edu.cn*

INTRODUCTION

Bioethanol production from lignocellulosic materials has drawn worldwide attention due to concern about the exhaustion of fossil fuels. In China, agricultural residues can be used as the feedstock to produce ethanol fuel because of its abundance, high carbohydrate content, and low cost (Yao *et al*. 2010). Agricultural residue is mainly comprised of cellulose, hemicellulose, and lignin, which is considered for second generation biofuel production (Wörmeyer *et al*. 2011). Wheat straw is the most attractive low cost feedstock for production of bioethanol because of its abundance (nearly 600

million tons/year), renewability, and low lignin content (*ca.* 15%) (Buranov and Mazza 2008). The bioconversion of lignocellulosic materials to ethanol involves four steps: pretreatment, enzymatic hydrolysis, fermentation, and concentration (Hendriks and Zeeman 2009). Pretreatment removes some hemicelluloses and lignin or breaks the linkages connecting lignin to the carbohydrates (Chen *et al*. 2009). Previous studies have shown that lignin is important during enzymatic hydrolysis, as it reduces the effective cellulase activity (Berlin *et al*. 2006; Pan 2008; Nakagame *et al*. 2011; Hu *et al*. 2012). The existence of lignin-carbohydrate complexes (LCCs) in the plant cell wall makes it more difficult to reduce the lignin content of the material during pretreatment. Recent studies revealed that LCCs also impact glucose recovery. There is a correlation between LCCs and enzymatic saccharification, such that LCCs hinder lignocellulosic biodegradation (Min *et al*. 2014a, b). The structural changes of lignin and LCCs in corn stover after alkaline pretreatment have been examined for their effects on enzymatic saccharification (Min *et al*. 2014c). As an abundant biomass grown in China, it is particularly important to study the LCC linkages of wheat straw to develop more environmentally compatible technologies for bioethanol production.

Traditional chemical degradation methods have been used to study the chemical structure of LCCs, but these methods degrade most native LCC linkages (Watanabe and Koshijima 1988). Solution-state NMR is a powerful tool to characterize the structure of lignin in biomass and in isolated LCC structures (Lu and Ralph 2003; Yelle *et al*. 2008; Mansfield *et al*. 2012; Cheng *et al*. 2013). Information about lignin structures and linkages connecting lignin to carbohydrates can be obtained using one-dimensional ^{1}H- and ^{13}C-NMR and two-dimensional heteronuclear single-quantum coherence (2D-HSQC) NMR spectroscopy. Recently, combined quantitative ^{13}C-NMR and ^{1}H-^{13}C HSQC NMR techniques have been developed (Zhang and Gellerstedt 2007) and effectively applied to measure lignin and LCCs (Balakshin *et al*. 2011; Wen *et al*. 2013a, 2013b, 2013c). ^{13}C isotope labeling is a useful tool to study the structure of lignin and LCCs (Yang *et al*. 2013, 2014; Yao *et al*. 2015) as it amplifies the signal of ^{13}C-labeled lignin or carbohydrates in the NMR spectra for easier analysis.

In this study, ^{13}C isotope-labeled xylose was injected into living wheat straw to obtain ^{13}C-labeled LCCs, which were isolated using the Björkman technique. FT-IR, HPLC, GPC, and NMR were used to analyze the LCC structures. A more in-depth and complete characterization of the LCCs of wheat straw was acquired to maximize the use of wheat straw as a feedstock for the production biofuels and other biorefinery products.

EXPERIMENTAL

Administration of ^{13}C Isotope-Labeled Xylose to Wheat Straw

A conventional variety of wheat straw (*Triticum aestivum* E-mai 352) was chosen. A solution containing the ^{13}C isotope-labeled xylose (2 mg/mL), L-2-aminooxy-3-phenylpropionic acid (AOPP, 0.005 mol/L), and coniferin (2 mg/mL) was injected into the internode sections of the wheat straw over 30 days. For the control samples, a solution containing xylose (2 mg/mL), L-2-aminooxy-3-phenylpropionic acid (AOPP, 0.005 mol/L), and coniferin (2 mg/mL) was injected into the wheat straw. After the injections, the wheat straw was allowed to grow for another 20 days.

Preparation of ^{13}C Isotope-Labeled Xylose Wheat Straw Powder

The air-dried, ^{13}C isotope-labeled wheat straw culms were cut into small pieces, ground, and passed through 80- to 100-mesh screens to obtain a sieved powder. This sieved powder was subjected to Soxhlet extraction with benzene/ethanol (2:1, v/v) for 6 h and then washed with hot water to remove water-insoluble and water-soluble impurities. The extractive-free wheat straw powder was freeze-dried.

Preparation of ^{13}C Isotope-Labeled LCCs

The dried wheat straw powder (extractive-free) was ground in a vibratory ball mill for 72 h. Afterwards, the wheat straw powder was used to obtain LCCs using the Björkman (1957) procedure. LCCs extracted from wheat straw control (injected with xylose) and ^{13}C-labeled were called LCC and LCC-^{13}C.

Component Analyses of LCCs

Lignin and carbohydrate contents were determined according to the method described by NREL (2006). The procedure uses a two-step acid hydrolysis to fractionate the biomass into two forms that are more easily quantified. The lignin fractionates were the acid-insoluble material. Monomeric sugars in the hydrolysis liquid were determined by high performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) with a refractive index detector (Shimadzu) on an Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA) running at a flow rate of 0.6 ml/min at 65 °C, with pure water as the eluent. Chromatography-grade D-glucose, D-xylose, D-galactose, D-mannose, and L-arabinose were used as calibration standards to quantify the polysaccharide content of the LCCs.

FT-IR Spectroscopy

Infrared spectra were determined using a Nicolet Nexus FT-IR (Thermo Scientific, Waltham, USA). Dried preparations (1 to 2 mg) were milled into a powder with a diameter less than 1 mm. The powder was dispersed in spectroscopic grade KBr and subsequently pressed into disks using 10 tons of pressure for 1 min. A total of 32 scans with a 4 cm^{-1} resolution were collected and averaged. The wavenumber range scanned was 4000 to 500 cm^{-1}.

Molecular Weight Determination

Gel permeation chromatography (GPC) was determined by HPLC (Shimadzu, Kyoto, Japan) using a refractive index detector (Shimadzu) and using a Shim-pack GPC-803D column (Shimadzu) operating at a flow rate of 0.6 mL/min at 40 °C with dimethylformamide (DMF) as the eluent. A sample (2 mg) was dissolved in 1 mL DMF, and 20 μL of this solution was injected into the GPC. Monodisperse polystyrene standards with average molecular weights of 2900; 6100; 47,100; 10,700; and 19,800 g mol^{-1} were used as calibration standards for determining the molecular weight of lignin-carbohydrate complexes. LC solution software (Shimadzu, Kyoto, Japan) was used for the data analysis.

NMR Spectroscopy

All NMR spectra were recorded on a Bruker AVIII 400 MHz spectrometer (Fällanden, Switzerland) that was operated at 25 °C utilizing DMSO-d_6 as the solvent. For quantitative ^{13}C NMR, 125 mg of the LCC was dissolved in 0.5 mL of DMSO-d_6. Quantitative ^{13}C NMR spectra were recorded in the FT mode at 100.6 MHz. An inverse-

gated decoupling sequence, which allows quantitative analysis and comparison of the signal intensities, was used with the following parameters: 90° pulse angle; 1.4 s acquisition time; 2 s relaxation delay; 64,000 data point acquisitions; and 30,000 scans. Chromium (III) acetylacetonate (0.01M) was added to the sample solution to provide complete relaxation of all nuclei.

For heteronuclear single quantum coherence (HSQC), LCC (60 mg) was dissolved in 0.5 mL of DMSO-d_6. HSQC NMR spectra were recorded. The spectral widths were 5000 and 20,000 Hz for the ^{1}H and ^{13}C dimensions, respectively. The number of collected complex points was 1024 for the ^{1}H dimension with a recycle delay of 1.5 s. The number of transients was 64, and 256 time increments were recorded in the ^{13}C dimension. The $^{1}J_{CH}$ used was 145 Hz. Prior to Fourier transformation, the data matrices were zero filled to 1024 points in the ^{13}C dimension. MestReNova software (Mestrelab Research, Santiago de Compostela, Spain) was used for the integration with the acquired 2D HSQC data.

RESULTS AND DISCUSSION

Molecular Weight and Component Determination

The molecular weight distributions of the LCCs were studied with gel permeation chromatography (GPC) connected to a refractive index (RI) detector, using dimethylfomamide (DMF) as the eluent and polystyrene standards for molecular weight calibrations (Table 1). No LCC samples had wide polydispersity, as shown by $M_w/M_n < 1.5$. The molecular weights of the LCC samples were between 30,000 to 50,000 g·mol^{-1}. The M_n of the LCC and LCC-^{13}C was almost the same, at about 35,000 g·mol^{-1}. Compared with LCCs in *Arundo donax* L., the molecular weights of wheat straw LCCs were higher. This result was attributed to the different species and different mobile phase used with GPC (You *et al*. 2015). The M_w of the LCC-^{13}C was slightly higher than that of the LCC, indicating that there were more saccharides connected to the lignin in the LCC-^{13}C than in the LCC sample (Yang *et al*. 2013a); this result corresponded to the results obtained from the carbohydrate analysis (discussed below).

Table 1. Average Molecular Weight of LCCs

Isolated Component	M_n	M_w	M_w/M_n
LCC	34,434	38,773	1.13
LCC-^{13}C	34,944	46,399	1.33

The LCCs were composed of lignin and carbohydrate. The sugar and lignin content of wheat straw LCCs are shown in Table 2. Most of the LCCs were carbohydrate, with about 20% lignin. The LCC samples were also analyzed by HPLC for their sugar composition. The results indicated that the isolated wheat straw LCCs were primarily composed of four different monosaccharides, namely, xylose (62.66%), glucose (6.88%), arabinose (7.23%), and mannose (1.17%); there were negligible amounts of galactose. The Ara/Xyl ratio reflects the branching degree of hemicelluloses. The Ara/Xyl ratio of the isolated LCCs was 0.115, which was lower than that of the hemicelluloses isolated from wheat straw (Sun *et al*. 2005). This observation suggests that the side-chains of the hemicelluloses of LCCs could be degraded during their extraction. The results implied that

arabinoxylans are the major hemicellulose fraction in wheat straw LCCs. The glucan content suggested that glucans were associated with the xylans in the LCCs. Generally, there were insignificant differences between the LCC and the LCC-^{13}C data.

Table 2. Chemical Component Analyses of LCCs (%)

Isolated Component	Glucose	Xylose	Arabinose	Mannose	Acid Insoluble Lignin	Total
LCC	10.07	56.38	7.02	1.64	16.03	91.14
LCC-^{13}C	6.88	62.66	7.23	1.17	20.69	98.63

FT-IR Determination of LCC

FT-IR spectra of the LCC-^{13}C and LCC are shown in Fig. 1. Strong signals from the aromatic rings were observed at 1515 cm^{-1} and 1421 cm^{-1} (Faix 1991), which indicated lignin in the sample. The strong absorption at 1046 cm^{-1} was from stretching vibrations of C-O and C-C, which indicated the presence of xylans (Sun *et al*. 2005). Additionally, signals from C=O stretching were observed at 1731 cm^{-1}, which was attributed to esterified phenolic groups and acetyls connected to the hemicelluloses (You *et al*. 2015). The weak signal at 1630 cm^{-1} was assigned to the uronic acid group from hemicelluloses. The C-O stretching of syringyl lignin units (S) was observed at 1322 cm^{-1} (Sun *et al*. 2005). Signals at 1246 cm^{-1} were assigned to guaiacyl lignin units (G). The two signals showed that G and S were the main lignin substructures in wheat straw LCCs. The band at 1162 cm^{-1} is characteristic of carbonyl groups comprising esters, which indicated the presence of ester-linked *p*-counaric or ferulic acids (Sun *et al*. 2011). A characteristic peak from a *β*-glucosidic bond between the xylose in the hemicelluloses was observed at 897 cm^{-1} (Sun *et al*. 2005).

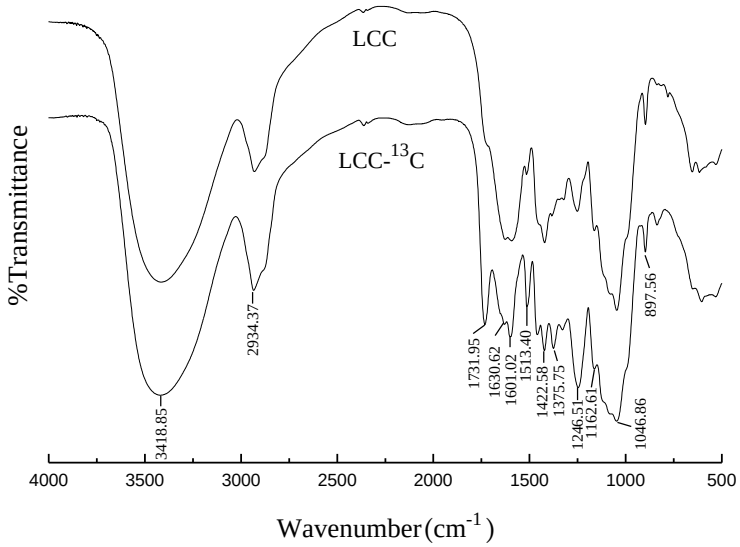

Fig. 1. FT-IR spectra of LCCs from wheat straw

There were insignificant differences when comparing the two IR spectra. Thus, the injection of exogenous ^{13}C-xylose did not interfere with the normal metabolism of wheat straw. These observations are in agreement with an earlier study with AOPP and conferin (Yang *et al*. 2014), where xylose-^{13}C was successfully introduced into the wheat plant.

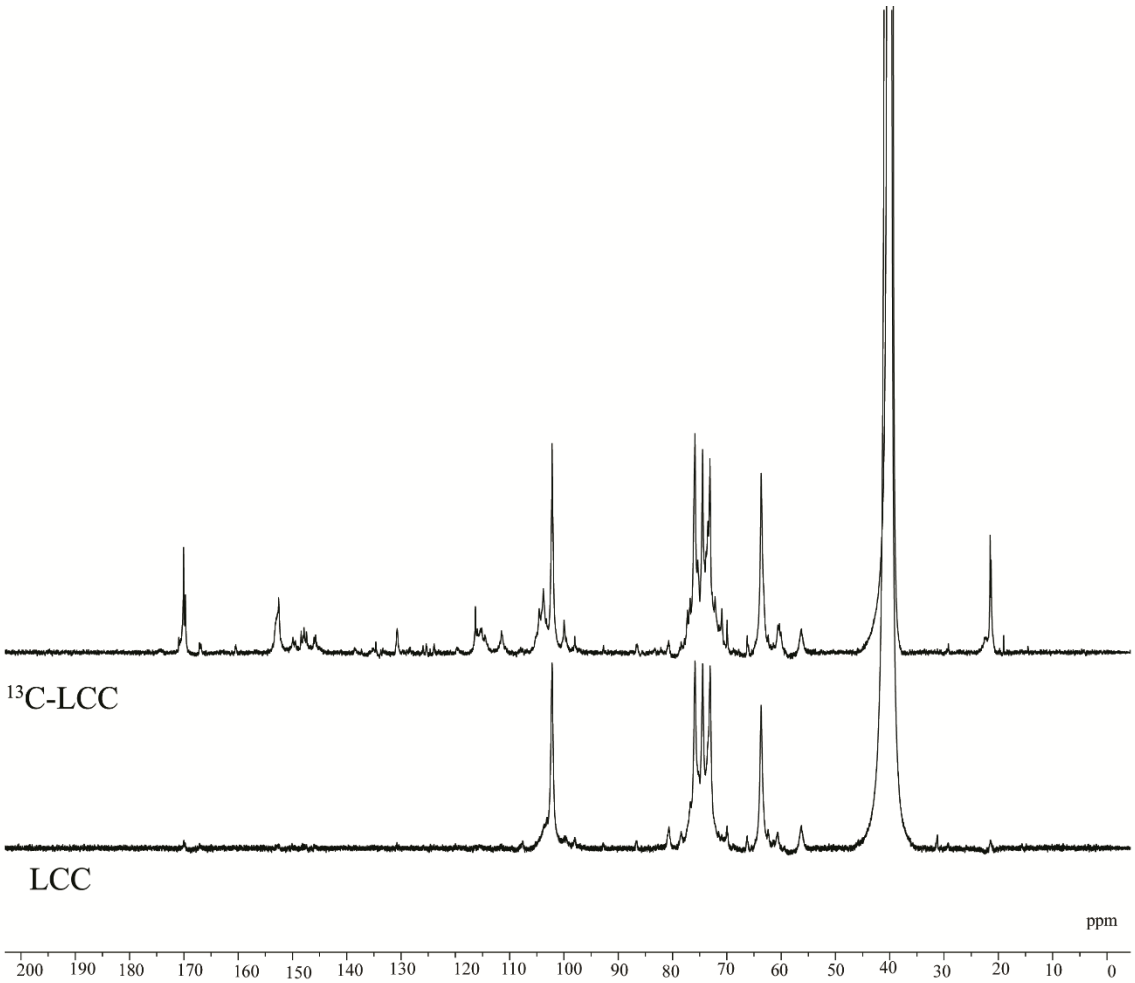

Fig. 2. ^{13}C-NMR spectra of LCCs from wheat straw

^{13}C-NMR Determination of LCC

The ^{13}C-NMR spectra of the LCC and ^{13}C-LCC are shown in Fig. 2. The assignments of important signals are listed in Table 3. The region between 160 and 103 ppm was used as a reference to compare the other signal shifts.

The ^{13}C-NMR signals from the C_1 to C_5 of the xylose cyclic ring were observed at 102.2 ppm, 73.1 ppm, 74.5 ppm, 75.9 ppm, and 63.7 ppm; all signals were intensified in the ^{13}C-LCC, which indicated the success of the injections of exogenous ^{13}C-xylose into the wheat straw (Yang *et al*. 2014). There were deviation of signals from C_1-C_5 of xylan when compared with purified xylan, which were at 101.8 ppm (C_1), 72.5 ppm (C_2), 74.0 ppm (C_3), 75.4 ppm (C_4), and 63.0 ppm (C_5) (Cheng 2011), indicating that most of the carbons in xylan were connected with lignin by certain linkages in LCC from wheat straw. Additionally, a cross-peak at 169.7 ppm was assigned to an acetyl group (Sun *et al*. 2005). The strong signal at 73.1 ppm is characteristic of the C_3 atom in 4-*O*-methyl-D-glucuronic acid groups of xylans.

Table 3. Analysis of ^{13}C-NMR Spectra of LCC from Wheat Straw

δ_C（ppm）		Assignments
LCC	LCC-^{13}C	
169.9	170.1	-C=O in acetyl group
152.5	152.6	C3/C5 in syringyl, etherified
150.0	149.9	C3 in guaiacyl, etherified
-	148.4	C3 in guaiacyl
145.8	145.7	C4 in guaiacyl, non-etherified
128.4	128.5	C2/C6 in p-hydroxyphenyl
115.5	115.9	C5 in guaiacyl
111.5	111.5	C2 in guaiacyl
-	104.6	C2/C6 in syringyl
-	103.8	C1 in Glc, C2/C6 in S non-etherified
102.2	102.2	C1 in β-D-Xyl
100.3	100.1	Phenyl glycoside linkage
98.3	98.0	(1-4)-β-D-Xyl
87.4	87.5	Cα in β-5'
86.5	86.5	C_β in S type β-O-4'
82.3	82.2	C_β in G type β-O-4'
80.7	80.8	Cα benzyl etherified to carbohydrate, C4 in 4-O-MeGlcA
75.9	75.9	C4 in β-D-Xyl
74.4	74.5	C3 in β-D-Xyl
73.1	73.1	C2 in acetyl-β-D-Xyl
72.3	72.2	Cα in β-aryl ether, C2 in β-D-Xyl
70.9	70.9	Cγ(resinol), C2 in Man
69.9	69.9	C5 in 4-O- MeGlcA
63.7	63.7	C5 in β-D-Xyl, Cγ in γ-acylated β-aryl ether
60.7	60.3	Cγ in β-aryl ether
56.3	56.3	-OCH_3
21.3	21.7	-CH_3 in acetyl group

2D-HSQC Determination of LCC

2D-HSQC is commonly used to analyze the structures of lignin and LCCs, and it provides evidence for the existence of various linkages between lignin and carbohydrates. This analytical technique can deconvolute the overlapping signals from lignin and carbohydrate in ^{13}C-NMR spectra. The 2D-HSQC spectra of the LCC-^{13}C and LCC are shown in Fig. 3. The main linkages and structural units identified are shown in Fig. 4.

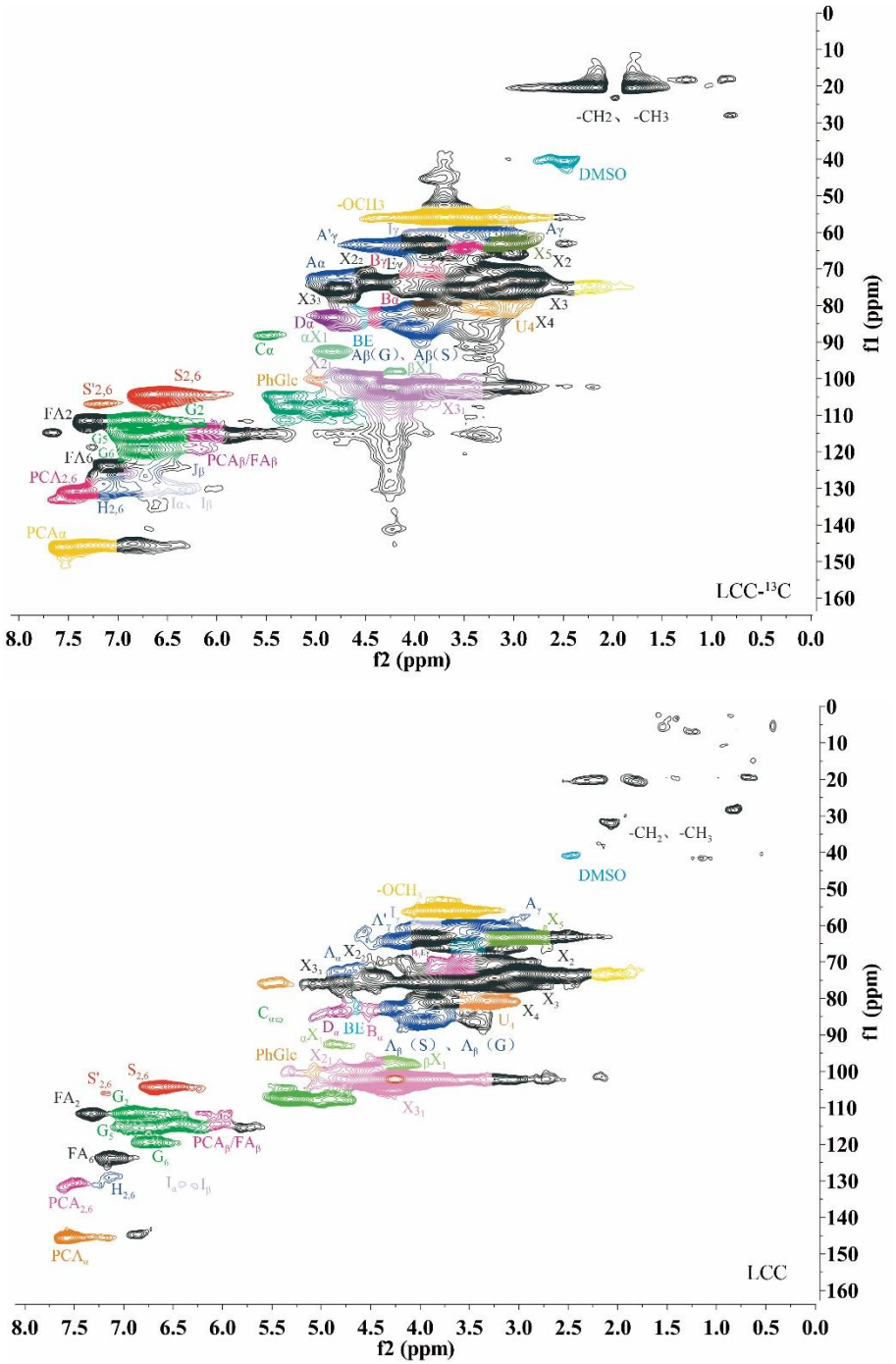

Fig. 3. HSQC spectra of LCCs from wheat straw (f1 and f2 refer to ^{13}C and ^{1}H in the axis)

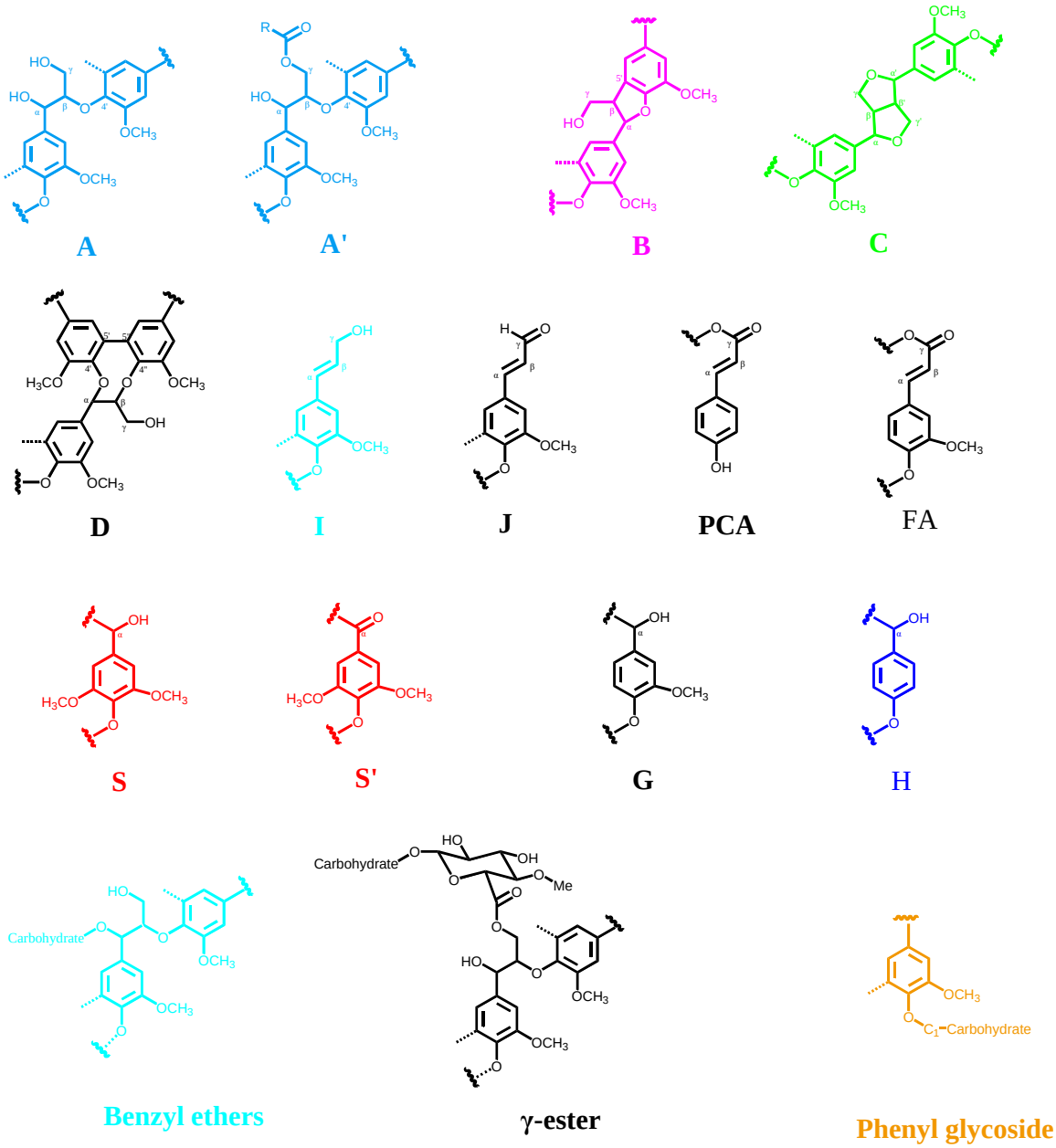

Fig. 4. Major structures and LCC linkages present in the LCCs of wheat straw: (A) *β-O*-4' alkyl-aryl ethers; (A') *β-O*-4' alkyl-aryl ethers with acetylated *γ-OH*; (B) phenylcoumarane; (C) resinols; (D) dibenzodioxocin; (I) cinnamyl alcohol end-groups; (J) cinnamyl aldehyde end-groups; (PCA) *p*-coumarates; (FA) ferulates; (H) *p*-hydroxyphenyl units; (G) guaiacyl units; (S) syringyl unit; and (S') oxidized syringyl units with a C_α ketone

Benzyl ethers (BEs) in LCC linkages

The amount of benzyl ethers in the LCCs was obtained from the signal of $C_\alpha H$ in the LCC structure. Benzyl ether structures are composed of linkages between the C_α position of lignin with the primary OH groups (BE1) and secondary OH groups (BE2) of the carbohydrates (Balakshin *et al*. 2011). Previous studies indicated cross-peaks at 80 to

81/4.4 to 4.6 ppm and 80 to 81/5.0 to 5.2 ppm, respectively (Min *et al*. 2014c). The linkages between lignin and primary OH groups of carbohydrates primarily occur with the C_6 position of glucan, galactan, and mannan, and with the C_5 position of arabinan, while linkages between lignin and secondary OH groups of carbohydrates was mostly formed between lignin and xylan (Yuan *et al*. 2011). It is worth noting that the BE1 signal might overlap *γ*-acetylated *β-O*-4' substructures linked to a G lignin unit. The signals of BE2 always overlap the signals of *β*-1' lignin substructures. However, these kinds of signals are not observed in the 2D-HSQC spectra.

In the present study, the C_α-H_α correlations in the benzyl ethers of LCC structures were found at *δ*C/*δ*H 82.1/5.0 ppm in the HSQC spectra; this signal suggests a bond between the C_α position of lignin with secondary OH groups of carbohydrates. Signals for benzyl ether bonds at about *δ*C/*δ*H 80 to 81/4.4 to 4.6 ppm from the C_α position of lignin with primary OH groups of carbohydrates were not detected. This observation indicated that the benzyl ethers (BEs) LCC linkages in wheat straw are principally formed between lignin and xylan.

The formulae used to calculate the varying amounts of identified LCC moieties have been proposed by Yuan *et al*. (2011). To obtain the absolute values from corresponding signals, the resonance for the total aromatic carbons (163.0 to 103.0 ppm in ^{13}C NMR) is assigned a value of 600. The integral values for selected internal standard references and other structural moieties are expressed per 100 aromatic units (Ar). To quantify the amount of LCC linkages, three clusters at *δ*C 103 to 96/*δ*H 5.5 to 3.8 ppm, *δ*C 90 to 78/*δ*H5.7 to 3.0 ppm and *δ*C 65 to 58/*δ*H5.0 to 2.5 ppm were integrated in the ^{13}C NMR spectra; these clusters corresponded to phenyl glycoside, benzyl ether, and *γ*-esters, respectively (Min *et al*. 2014c) (Table 4). The number of BEs in the LCC and ^{13}C-LCC were 2.12 and 11.84 per 100 Ar. These values correspond to the values previously reported for the LCC linkages of *A. donax* L. (You *et al*. 2015). The difference of BE2 in the LCC and ^{13}C-LCC was due to the ^{13}C-labeling, which indicated that part of the lignin and xylan in wheat straw were connected by benzyl ether bonds.

Esters in LCC linkages

There was no signal detected at *δ*C/*δ*H 75/6.1 ppm for a benzyl ester (*α*-ester) (You *et al*. 2015). Signals from C_γ in the *γ*-ester were found in the range of *δ*C/*δ*H 65 to 62/4.0 to 4.5 ppm. Signals from lignin, which was *γ*-acetylated, overlap the *γ*-ester; thus, it was not feasible to assign this type of signal to the *γ*-ester linkages (Del *et al*. 2012a). However, this issue could be resolved by a cryoprobe, as the signals in the range of *δ*C/*δ*H 62 to 63/4.0 to 4.5 ppm and 63 to 64/4.0 to 4.5 ppm were ascribed to *γ*-acetylated lignin and C_γ in the LCC *γ*-ester, respectively (Balakshin *et al*. 2011). After the analysis, the *γ*-ester (including *γ*-acetylated *β-O*-4' aryl ether substructures) in the LCC and ^{13}C-LCC were 13.95 and 15.28 per 100 Ar, respectively. These results are consistent with the report by Min *et al*. (2014c) examining the LCCs of corn stover. The similarity of *γ*-ester linkages in the LCC and ^{13}C-LCC suggested that the number of this kind linkage between lignin and xylan in wheat straw was slight.

Phenyl glycoside (PhGly) in LCC linkages

Phenyl glycoside produced signals at *δ*C/*δ*H 100 to 103/4.0 to 4.8 ppm (Min *et al*. 2014c). The classic Björkman LCC extraction method is an appropriate analysis for these linkages. A cross-peak from C_1-H_1 of the carbohydrates connected to lignin by PhGly

linkages occurred at δC/δH 101.9/4.25 ppm. The phenyl glycoside in the LCC and ^{13}C-LCC were 50.17 and 183.74 per 100 Ar, respectively. Compared with the analysis of You *et al*. (2015), which examined the LCCs of *A. donax*, the amount of phenyl glycoside the linkages seen here, 251 per 100 monomeric lignin units, was lower. This result was attributed to the higher amount of glucan (28.9%) in the LCCs of *A. donax*. The amount of PhGly linkages in the ^{13}C-LCC was three times higher than LCC, which indicated that part of lignin and xylan in the wheat straw were connected by PhGly linkages. Based on the HSQC data of LCC model compound (Miyagawa *et al*. 2014), signals at δC/δH 100.1/5.01, 100.3/4.96 and 102.1/5.04 ppm were assigned to *p*-hydroxybenzaldehyde (G, H and S, respectively) derivative β-xylosides. Signals attributed to monolignol β-xylosides and dihydromonolignol β-xylosides were not detected, which indicated that those compounds had been destroyed during the ball milling process to extract LCC.

Associated carbohydrates

Carbohydrate signals in the LCC were also noteworthy. The strong signals from the C_1, C_2, C_3, C_4, and C_5 positions in the β-D-xylose were at δC/δH 101.9/4.24 ppm, 72.8/3.05 ppm, 74.1/3.24 ppm, 75.6/3.49 ppm, and 63.1/3.14 ppm, respectively (Wen *et al*. 2010). Signals from the C_2-H_2 position in 2-*O*-acetyl-β-D-xylopyranoside, as well as the C_3-H_3 and C_1-H_1 positions in 3-*O*-acetyl-β-D-xylopyranoside, were at δC/δH 73.5/4.49 ppm, 75.1/4.79 ppm, and 101.9/4.25 ppm, respectively (Peng *et al*. 2014). A signal at δC/δH 80.4/3.32 ppm was assigned to C_4-H_4 in 4-*O*-methyl glucuronic acid (You *et al*. 2015). The signal indicated that the side chains of most carbohydrates in the wheat straw LCCs are 4-*O*-methyl glucuronic acid, which was partially acetylated at the C_2 and C_3 positions. Additionally, cross-peaks at δC/δH 92.3/4.89 ppm and 98.1/4.26 ppm were ascribed to (1→4)-α-D-xylose (αX_1) and (1→4)-β-D-xylose (βX_1) (Peng *et al*. 2014).

Major lignin structures

In the region of δC/δH 50 to 90/2.5 to 6.0 ppm, signals from -OCH_3 (δC/δH 55.7/3.72 ppm) and β-*O*-4' were detected. Signals from C_γ-H_γ and acylated C_γ-H_γ positions in the β-*O*-4' were at δC/δH 60.9/3.56 and 63.2/3.86 ppm, respectively (Rencoret *et al*. 2009). Signals at δC/δH 71.9/4.74, 86.2/3.96 and 82.4/4.20 ppm were assigned to C_α-H_α and C_β-H_β positions in the β-*O*-4' (Del *et al*. 2012a). In addition, signals from β-β', β-5' and 5-5' were detected. A signal at δC/δH 83.3/4.80 ppm was ascribed to the C_α-H_α position in the β-β'. A weak signal at δC/δH 71.4/3.84 ppm was assigned to the C_γ-H_γ position in the β-β' (Martínez *et al*. 2008). Signals from C_α-H_α in the β-5' were detected at δC/δH 87.4/5.52. A signal from the C_γ-H_γ position of β-5' overlapped that of the C_5-H_5 position in xylose. Signals assigned to C_α-H_α in the 5-5' were observed at δC/δH 83.8/4.90 ppm (Del *et al*. 2012a).

Signals in the region of δC/δH 100 to 135/5.5 to 8.5 ppm were principally from S, G, and H lignin units. Signals of C_2-H_2/C_6-H_6 aromatic positions in S and in oxidized S were at δC/δH 104.1/6.66 and 106.7/7.26, ppm, respectively (Wen *et al*. 2013). Signals at δC/δH 111.0/6.92, 114.2/6.80, and 119.1/6.75 ppm were from C_2-H_2, C_5-H_5, and C_6-H_6 aromatic positions in G lignin units. Signals assigned to C_2-H_2/C_6-H_6 aromatic positions in H units were at δC/δH 128.5/7.05 ppm. Additionally, a signal from C_2-H_2 in ferulic acid (FA_2) was at δC/δH 111.3/7.33 ppm (Wen *et al*. 2013). Signals from *p*-coumaric acid (PCA_2 and PCA_6) and end groups in *p*-coumaryl alcohol and cinnamaldehyde were also detected. Combining HSQC with ^{13}C-NMR is an analytical method to obtain the absolute

quantities of various substructures (Min *et al*. 2014a). The absolute contents of important signals were calculated from the spectra data and are summarized in Table 4. The analysis showed that wheat straw LCCs were mostly bonded by ferulate linkages, which was followed by *γ*-ester, phenyl glycoside, and benzyl ether linkages. Lignin was mostly connected by benzyl ether linkages to the xylans. The main substructure of the lignin was the *β*-*O*-4' (71.64%), which was followed by the *β*-*β*' (14.93%) and 5-5' (11.94%), with minor amounts of *β*-5' (1.49%). The S/G ratio was 1.28, which was the same as that observed from milled lignin from wheat straw (Yang *et al*. 2014). This observation implied that the structural units were the same in milled lignins and LCCs. However, these weak signals were attributed to the degradation of the lignin during the isolation process.

Table 4. Characteristics of LCCs from Quantitative NMR Method

Aliphatic Region	LCC[a]	^{13}C-LCC[a]
α-*O*H/*β*-*O*-4'	0.41	0.48
Phenylcoumaran	0.01	0.01
Resinols	0.08	0.10
Dibenzodioxocin	0.09	0.08
LCC Linkages		
BE_2	2.12	11.84
PhGlc	50.17	183.74
γ-Ester	13.95	15.28
Aromatic region		
Syringyl (S)	33.23	31.73
Oxidized Syringyl (S')	0.69	0.70
Guaiacyl (G)	26.45	24.71
p-Hydroxyphenyl (H)	6.44	5.92
p-Coumarate (PCA)	10.44	10.09
Ferulate (FA)	19.49	19.84

a: amount of specific interunit linkage was expressed as number per 100 Ar

CONCLUSIONS

1. HPLC and GPC analysis showed that the major polysaccharide in the LCCs was xylan. LCCs had narrow polydispersity. There were insignificant differences between the LCC and the LCC-^{13}C in sugar composition and molecular weight.
2. FT-IR and ^{13}C-NMR results showed that lignin and carbohydrate coexist in the LCCs. The injection of exogenous xylose was successfully introduced into the wheat plant.
3. Quantitative analysis showed that the total LCC linkages were 210.86 /100C_9 in ^{13}C-LCC, which was dominated by phenyl glycoside linkages, followed by γ-ester bonds and minor amounts of benzyl ether bonds. Compared with the HSQC data of LCC model compound, it was found that xylan and lignin were mainly connected as *p*-hydroxybenzaldehyde derivative β-xylosides. The main lignin substructures in the LCCs of wheat straw were *β*-*O*-4', with small amounts of *β*-*β*' and 5-5', and trace amounts of *β*-5'. In addition to S, G, and H lignin units, there were also certain amounts of *p*-coumarate and ferulate in the LCCs. The lignin structural features in the LCCs were quite similar to that of milled straw lignin.

ACKNOWLEDGMENTS

The authors are grateful for the support from the Natural Science Foundation of China (No. 31500496), the China Scholarship Council (No. 2011842330), the State Key Laboratory of Pulp and Paper Engineering (No. 201510), and the Foundation (KF201409) of Key Laboratory of Paper Science & Technology of the Ministry of Education.

REFERENCES CITED

Buranov, A. U., and Mazza, G. (2008). "Lignin in straw of herbaceous crops," *Ind. Crop. Prod.* 28(3), 237-259. DOI: 10.1016/j.indcrop.2008.03.008

Balakshin, M., Capanema, E., Gracz, H., Chang, H.-m., and Jameel, H. (2011). "Quantification of lignin-carbohydrate linkages with high-resolution NMR spectroscopy," *Planta* 233(6), 1097-1110. DOI: 10.1007/s00425-011-1359-2

Berlin, A., Balakshin, M., Gilkes, N., Kadla, J., Maximenko, V., Kubo, S., and Saddler, J. (2006). "Inhibition of cellulase, xylanase and *β*-glucosidase activities by softwood lignin preparations," *J. Biotechnol.* 125(2), 198-209. DOI: 10.1016/j.jbiotec.2006.02.021

Björkman, A. (1957). "Studied on finely divided wood. Part 3. Extraction of lignin-carbohydrate complexes with neutral solvent," *Sven. Papperstidn.* 60(3), 243-251. DOI: 1010.1007/BF00392053

Chen, M., Zhao, J., and Xia, L. M. (2009). "Comparison of four different chemical pretreatments of corn stover for enhancing enzymatic digestibility," *Biomass Bioenerg.* 33(10), 1381-1385. DOI: 10.1016/j.biombioe.2009.05.025

Cheng, H. L. (2011). "Isolation, characterization and sulfation of corn stover hemicellulose," Doctoral Thesis, South China University of Technology, 90-91. (Chinese)

Cheng, K., Sorek, H., Zimmermann, H., Wemmer, D. E., and Pauly, M. (2013). "Solution-state 2D NMR spectroscopy of plant cell walls enabled by a dimethylsulfoxide-d_6/1-ethyl-3-methylimidazolium acetate solvent," *Anal. Chem.* 85(6), 3213-3221. DOI: 10.1021/ac303529v

del Río, J. C., Prinsen, P., Rencoret, J., Nieto, L., Barbero, J. J., Ralph, J., Martínez, A. T., and Gutierrez, A. (2012a). "Structural characterization of the lignin in the cortex and pith of elephant grass (*Pennisetum purpureum*) stems," *J. Agric. Food. Chem.* 60(14), 3169-3634. DOI: 10.1021/jf300099g

del Río, J. C., Rencoret, J., Prinsen, P., Martínez, A. T., Ralph, J., and Gutierrez, A. (2012b). "Structural characterization of wheat straw lignin as revealed by analytical pyrolysis, 2D-NMR, and reductive cleavage methods," *J. Agric. Food. Chem.* 60(23), 5922-5935. DOI: 10.1021/jf301002n

Faix, O. (1991). "Classification of lignins from different botanical origins by FT-IR spectroscopy," *Holzforschung* 45(s1), 21-27. DOI: 10.1515/hfsg.1991.45.s1.21,

Hendriks, A. T. W. M., and Zeeman G. (2009). "Pretreatments to enhance the digestibility of lignocellulosic biomass," *Bioresour. Technol.* 100(1), 10-18. DOI: 10.1016/j.biortech.2008.05.027

Hu, F., Jung, S., and Ragauskas, A. (2012). "Pseudo-lignin formation and its impact on enzymatic hydrolysis," *Bioresour. Technol.* 117(Aug. 2012), 7-12 DOI: 10.1016/j.biortech.2012.04.037

Lam, T. B. T., Iiyama, K., and Stone, B. A. (1992). "Cinnamic acid bridges between cell wall polymers in wheat and phalaris internodes," *Phytochem.* 31(4), 1179–1183. DOI: 101016/0031-9422(92)80256-E

Lu, F., and Ralph, J. (2003). "Non-degradative dissolution and acetylation of ball-milled plant cell walls: high-resolution solution-state NMR," *Plant J.* 35(4), 535-544. DOI: 10.1046/j.1365-313X.2003.01817.x

Mansfield, S. D., Kim, H., Lu, F., and Ralph, J. (2012). "Whole plant cell wall characterization using solution-state 2D NMR," *Nat. Protoc.* 7(9), 1579-1589. DOI: 10.1038/nprot.2012.064

Martínez, A. T., Rencoret, J., Marques, G., Gutierrez, A., Ibarra, D., Barbero, J. J., and del Río, J. C. (2008). "Monolignol acylation and lignin structure in some nonwoody plants: A 2D NMR study," *Phytochemistry* 69(16), 2831-2843. DOI: 10.1016/j.phytochem.2008.09.005

Min, D.-y., Yang, C., Chiang, V., Hasan, J., and Chang, H.-m. (2014a). "The influence of lignin-carbohydrate complexes on the cellulose-mediated saccharification II: Transgenic hybrid poplars (*Populus nigra* L. and *Populus maximowiczii* A.)," *Fuel* 116(15 Jan.2014), 56-62. DOI: 10.1016/j.fuel.2013.11.047

Min, D.-y., Li, Q., Chiang, V., Jameel, H., Chang, H.-m., and Lucia, L. (2014b). "The influence of lignin-carbohydrate complexes on the cellulase-mediated saccharification I: Transgenic black cottonwood (western balsam poplar, California poplar) *P. trichocarpa* including the xylan down-regulated and the lignin down-regulated lines," *Fuel* 119(1 March 2014), 207-213. DOI: 10.1016/j.fuel.2013.11.047

Min, D.-y., Jameel, H., Chang, H.-m., Lucia, L., Wang, Z. G., and Jin, Y. C. (2014c). "The structural changes of lignin and lignin-carbohydrate complexes in corn stover induced by mild sodium hydroxide treatment," *RSC Adv.* 4(21), 10845-10850. DOI: 10.1039/C3RA47032F

Miyagawa, Y., Mizukami, T., Kamitakahara, H., and Takano, T. (2014). "Synthesis and fundamental HSQC NMR data of monolignol β-glycosides, dihydromonolignol β-glycosides and *p*-hydroxybenzaldehyde derivative β-glycosides for the analysis of phenyl glycoside type lignin-carbohydrate complexes (LCCs)," *Holzforschung* 68(7), 747-760.

Nakagame, S., Chandra, R. P., Kadla, J. F., and Saddler, J. N. (2011). "The isolation, characterization and effect of lignin isolated from steam pretreated Douglas-fir on the enzymatic hydrolysis of cellulose," *Bioresour. Technol.* 102(6), 4507-4517. DOI: 10.1016/j.biortech.2010.12.082

Pan, X. (2008). "Role of functional groups in lignin inhibition of enzymatic hydrolysis of cellulose to glucose," *J. Biobased Mater. Bioenerg.* 2(1), 25-32. DOI: 10.1166/jbmb.2008.005

Peng, H., Luo, Q., Ruan, R., Zhang, J. S., and Liu, Y. H. (2014). "Structural features of lignin and lignin-carbohydrate complex from bamboo (*Phyllostachys pubescens* Mazel)," *BioResources* 9(1), 1276-1289. DOI: 10.15376/biores.9.1.1276-1289

Ralph, J. (2010). "Hydroxycinnamates in lignification," *Phytochem. Rev.* 9(1), 65-83. DOI: 10.1007/s11101-009-9141-9

Rencoret, J., Marques, G., Gutierrez, A., Nieto, L., Santos, J. I., Jimenez, B. J., and del Rio, J. C. (2009). "HSQC-NMR analysis of lignin in woody (*Eucalyptus globulus* and *Picea abies*) and non-woody (*Agave sisalana*) ball-milled plant materials at the gel state," *Holzforschung* 63(6), 691-698. DOI: 10.1515/HF.2009.070

NREL. (2006). "Biomass Program, Standard Method for Determination of Total Solids in Biomass," (http://www.nrel.gov/biomass/pdfs/42618.pdf).

Sun, R.-C., Xiao, B., and Lawther, J. M. (1998). "Fractional and structural characterization of ball-milled and enzyme lignins from wheat straw," *J. Appl. Polym. Sci.* 68(10), 1633-1641. DOI: 10.1002/(SICI)1097-4628(19980606)68:10<1633::AID-APP12>3.0.CO;2-Y

Sun, R.-C., Sun, X.-F., and Zhang, S.-H. (2001). "Quantitative determination of hydroxycinnamic acids in wheat, rice, rye, and barley straws, maize stems, oil palm frond fiber, and fast-growing poplar wood," *J. Agric. Food Chem.* 49(11), 5122-5129. DOI: 10.1021/jf010500r

Sun, X.-F., Sun, R.-C., Fowler, P., and Baird, M S. (2005). "Extraction and characterization of original lignin and hemicelluloses from wheat straw," *J. Agric. Food Chem.* 53(4), 860-870. DOI: 10.1021/jf040456q

Watanabe, T., and Koshijima, T. (1988). "Evidence for an ester linkage between lignin and glucuronic acid in lignin-carbohydrate complexes by DDQ-*O*xidation," *Agric. Biol. Chem*. 52(11), 2953-2955. DOI: 10.1080/00021369.1988.10869116

Wen, J.-L., Sun, Z., Sun, Y.-C., Sun, S.-N., Xu, F., and Sun, R.-C. (2010). "Structural characterization of alkali-extractable lignin fractions from bamboo," *J. Biobased Mater. Bioenerg.* 4(4), 408–425. DOI: 10.1166/jbmb.2010.1111

Wen, J.-L., Sun, S.-L., Xue, B.-L., and Sun, R.-C (2013a). "Quantitative structures and thermal properties of birch lignins after ionic liquid pretreatment," *J. Agric. Food Chem.* 61(3), 635-645. DOI: 10.1021/jf3051939

Wen, J.-L., Sun, S.-L., Xue, B.-L., and Sun, R.-C. (2013b). "Recent advances in characterization of lignin polymer by solution-state nuclear magnetic resonance (NMR) methodology," *Materials* 6(1), 359-391. DOI: 10.3390/ma6010359

Wen, J.-L., Xue, B.-L, Xu, F., Sun, R.-C., and Pinkert, A. (2013c). "Unmasking the structure features and property of lignin from bamboo," *Ind. Crop Prod.* 42(March 2013), 332-343. DOI: 10.1016/j.indcrop.2012.05.041

Wörmeyer, K., Ingram, T., Saake, B., Brunner, G., and Smirnova, I. (2011). "Comparison of different pretreatment methods for lignocellulosic materials. Part II: Influence of pretreatment on the properties of rye straw lignin," *Bioresour. Technol.* 102(5), 4157-4164. DOI: 10.1016/j.biortech.2010.11.063

Yao, L., Yue, J., Zhao, J., Dong, J., Li, X., and Qu, Y. (2010). "Application of acidic wastewater from monosodium glutamate process in pretreatment and cellulose production for bioconversion of corn stover-feasibility evaluation," *Bioresour. Technol.* 101(22), 8755-8761. DOI: 10.1016/j.biortech.2010.04.104

Yao, L., Yang, H., Xu, J., Zhang, N., Chen, Y., and Xie, Y. (2015). "Elucidation of the structure of lignin and lignin carbohydrate complex of *Gramineae*," *Cell. Chem. Technol.* 49 (3-4), 259-266.

Yang, D., Zhong, L.-X., Yuan, T.-Q., Peng, X.-W., Sun, R.-C. (2013a). "Studies on the structural characterization of lignin, hemicelluloses and cellulose fractionated by ionic liquid followed by alkaline extraction from bamboo," *Ind. Crop Prod.* 43(May 2013), 141-149. DOI: 10.1016/j.indcrop.2012.07.024

Yang, H., Zheng, X., Yao, L., Gu, R., and Xie, Y. (2013b). "Elucidation of the bonds between cellulose and dehydrogenation polymer with carbon-13 isotopic tracer method," *Cell. Chem. Technol.* 47(3-4), 143-151.

Yang, H., Zheng, X., Yao, L., and Xie, Y. (2014). "Structural changes of lignin in the Soda-AQ pulping process studied using the carbon-13 tracer method," *BioResources* 9(1), 176-190. DOI: 10.15376/biores.9.1.176-190

Yelle, D. J., Ralph, J., and Frihart, C. R. (2008). "Characterization of nonderivatized plant cell walls using high-resolution solution-state NMR spectroscopy," *Magn. Res. Chem.* 46(6), 508-517. DOI: 10.1002/mrc.2201

You, T.-T., Zhang, L.-M., Zhou, S.-K., and Feng, X. (2015). "Structural elucidation of lignin-carbohydrate complex (LCC) preparations and lignin from *Arundo donax* Linn," *Ind. Crop Prod.* 71(Sept. 2015), 65-74. DOI: 10.1016/j.indcrop.2015.03.070

Yuan, T.-Q., Sun, S.-N., Xu, F., and Sun, R.-C. (2011). "Characterization of lignin structures and lignin-carbohydrate complex (LCC) linkages by quantitative ^{13}C and 2D HSQC NMR spectroscopy," *J. Agric. Food Chem.* 59(19), 10604-10614. DOI: 10.1021/jf2031549

Zeng, J., Helms, G. L., Gao, X., and Chen, S. (2013). "Quantification of wheat straw lignin structure by comprehensive NMR analysis," *J. Agric. Food Chem.* 61(46), 10848−10857. DOI: 10.1021/jf4030486

Zhang, A., Lu, F., Sun, R., and Ralph, J. (2009). "Ferulate-coniferyl alcohol cross-coupled products formed by radical coupling reactions," *Planta* 229(5), 1099-1108. DOI: 10.1007/s00425-009-0894-6

Zhang, L., and Gellerstedt, G. (2007). "Quantitative 2D HSQC NMR determination of polymer structures by selecting suitable internal standard references," *Magn. Res. Chem.* 45(1), 37-45. DOI: 10.1002/mrc.1914

Zhang, X. M., Meng, L. Y., Xu, F., and Sun, R.-C. (2010). "Pretreatment of partially delignified hybrid poplar for biofuels production: Characterization of organosolv hemicelluloses," *Ind. Crop Prod.* 33(2), 310-316. DOI: 10.1016/j.indcrop.2010.11.016

Article submitted: February 23, 2016; Peer review completed: May 22, 2016; Revised version received and accepted: May 31, 2016; Published: June 28, 2016.
DOI: 10.15376/biores.11.3.6692-6707