Molecular Changes in Corn Stover Lignin Resulting from Pretreatment Chemistry

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Lignin is an amorphous polymer that limits the enzymatic conversion of polysaccharides to fermentable sugars. Thus, a pretreatment that can enhance the accessibility of carbohydrates is a key step of successful biofuel conversion schemes. In this study, corn stover was fractioned into stem, cob, and leaf because their lignin is different. To elucidate the lignin changes, autohydrolysis, diluted acid, and alkali pretreatments were applied on the samples, followed by the isolation of cellulolytic enzyme lignin preparations. Alkaline nitrobenzene oxidation, ^{13}C-Nuclear Magnetic Resonance (NMR), and ^{1}H-^{13}C heteronuclear single quantum coherence NMR were used to profile the lignin changes. The results indicated that corn stover lignin is a *p*-hydroxyphenyl-guaiacyl-syringyl-type lignin that incorporates *p*-coumarate and ferulate esters. The β-aryl-ether was the most abundant inter-unit linkage, followed by condensed linkages, *e.g.* pino-/syringaresinol, phenylcoumaran, and spirodienone. As for the non-pretreated samples, leaf lignin was more condensed than stem lignin and cob lignin. More lignin was removed by the alkali pretreatment due to more cleavage of β-aryl-ether linkages. As a comparison, more condensed linkages were generated by the acidic pretreatments. The decrease of the syringyl/guaiacyl ratio indicated that the residual lignin became more condensed and confirmed that guaiacyl and *p*-hydroxyphenyl units were more stable than syringyl units during the pretreatment.

Keywords: Corn stover; Pretreatments; Cellulolytic Enzyme Lignin; Structure; ^{1}H-^{13}C HSQC NMR

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INTRODUCTION

Lignocellulosic biomass is the most abundant renewable resource and is mainly composed of cellulose, hemicelluloses, and lignin. However, humans only use approximately 3% of its annual global production (estimated billions of tons) (Lucia 2008). Thus far, a biological-based platform is considered an extremely promising way to convert lignocellulosics to fuels and chemicals due to advantageous economics and high efficiencies. However, the largest challenge of the biological-based platform is how to achieve a high amount of fermentable sugars *via* enzymatic saccharification. Technically, successful saccharification can be achieved by "opening up" the ultrastructure of the lignocellulosics through pretreatment or some other manner of pre-enzymatic treatment (Mansfield *et al.* 1999; Chandra *et al.* 2008). However, hurdles remain that must be overcome to ensure a successful rate and extent of substrate enzymatic hydrolysis, *inter*

alia, lignin structure and distribution, cellulose crystallinity, the degree of cellulose polymerization, particle size, and pore volume (Mooney *et al.* 1998; Hall *et al.* 2010). Hence, an improved understanding of biomass morphology and structural considerations after pretreatment, especially with respect to lignin, will enable advances in the field. Traditionally, wet chemistry techniques, such as nitrobenzene oxidation (NBO), thioacidolysis (TA), and Derivatization-Followed-by-Reductive-Cleavage (DFRC), have been employed to determine lignin structural features (Lai and Sarkanen 1971; Lu and Ralph 1997a; Lu and Ralph 1997b). However, a complete lignin structure is lacking despite the availability of these methods. Therefore, various spectroscopic and chromatographic methods, including Fourier transform infrared spectroscopy (FT-IR), nuclear magnetic resonance (NMR), gas chromatography mass spectrometry (GC-MS), and gel permeation chromatography (GPC) have been applied. The NMR can profile the entire lignin structure and detect lignin moieties directly, unlike other methods, while ^{13}C-NMR and two-dimensional heteronuclear single quantum coherence NMR (^{1}H-^{13}C HSQC NMR) can identify and quantify inter-unit linkages and functionalities in the lignin (Capanema *et al.* 2004; Yelle *et al* 2008; Balakshin *et al.* 2011). However, a new approach using a ^{1}H-^{13}C HSQC NMR has been developed and is widely used to characterize lignin (Zhang and Gellerstedt 2007).

As an abundant feedstock, corn stover has been pretreated for the fermentable sugar production by enzymatic hydrolysis (Lee *et al.* 2015). Autohydrolysis, dilute sulfuric acid, and alkali treatments are generally used as pretreatment to enhance the enzymatic hydrolysis of lignocellulosic biomass. However, the changes of lignin induced by the pretreatments were not extensively characterized. This paper attempts to profile structural alterations of lignin from pretreatments through alkaline NBO and a combination of ^{13}C-NMR and ^{1}H-^{13}C HSQC NMR.

EXPERIMENTAL

Materials

Corn stover was obtained from Ames, Iowa (USA) and separated into three fractions: stem, cob, and leaf. The air-dried fraction was ground using a Wiley mill, Thomas Model 4 (General Electric, Swedesboro, NJ, USA) to pass 40-mesh sieves. The portion between 40 mesh and 60 mesh was collected, and all adventitious contaminants were extracted for 8 h by use of a mixture of benzene and ethanol (2:1 v/v). The composition of samples was determined according to Laboratory Analytical Procedures developed by the NREL.

Pretreatments

Autohydrolysis, diluted sulfuric acid, and alkali treatments were applied consecutively to the biomass as the "pretreatment." All pretreatments were performed in a stirred reactor (Series 4520-1, Parr Instrument Company, Moline, IL USA). The sample was first suspended in a glass beaker to give 250 g/L consistency. The slurry was then loaded into the stainless steel reactor jar. The reactor jar had a volume of 1 L and was filled with 400 mL of slurry to give free space for liquid expansion. Briefly, the reactor was preheated to the designated temperature. Then, the pretreatment was completed for the residence time. Approximately 5 min were required as the heat-up time once the

sample was loaded. The detailed reaction conditions related to the pretreatment are summarized in Table 1.

Table 1. Conditions for All of the Pretreatments Performed

Pretreatments	Chemicals	S:L*	Temperature (°C)	Heat-up Time (min)	Residence Time (min)
Autohydrolysis	H_2O	1:4	180	5	30
Diluted Acid	1% H_2SO_4	1:4	170	4	20
Alkaline	6% NaOH	1:4	170	4	20

Note: S: L*, solid to liquor ratio

The autohydrolysate and the dilute acid prehydrolysate were collected for the quantification of sugars and inhibitors. The monomeric sugars generated in the prehydrolysate were quantified by Ion Chromatography (Dionex IC-3000, Dionex Corporation, Sunnyvale, USA). Oligomeric sugars solubilized in the prehydrolysate were hydrolyzed by 3% H_2SO_4 into monomeric sugars.

Alkaline nitrobenzene oxidation

Alkaline NBO was performed according to the same method used in the authors' previous study (Min *et al*. 2014). Approximately 100 mg of oven-dried sample was suspended in 7 mL of 2N NaOH (aq) and reacted with 0.4 mL of nitrobenzene in a stainless bomb at 170 °C for 2.5 h. The derivatized products (aldehydes) were quantified with gas chromatography (Agilent GC 7890, Agilent Technologies Inc., Santa Clara, USA).

Isolation and acetylation of cellulolytic enzyme lignin

Enzymatic hydrolyses of pretreated samples were done according to previously reported procedures (Min *et al*. 2014). As for the non-pretreated sample, the cellulolytic enzyme lignin (CEL) was obtained using a standard procedure (Bjorkman 1956; Chang *et al*. 1975). The residues from the enzymatic hydrolysis of the pretreated samples were washed and air-dried, after which the CEL was obtained by a previously reported procedure (Min *et al*. 2014). In brief, the sample was treated with cellulase (from *Trichoderma viride*, 4.7 U mg^{-1} solid, Sigma; loading: 500 U g^{-1} sample) in an acetate buffer solution (pH 4.5) at 50 °C for 24 h, and repeated three times to remove the carbohydrates as much as possible. Lignin was acetylated according to a previously reported method (Lundquist 1992).

Methods

^{13}C NMR and ^{1}H-^{13}C HSQC NMR acquisition

The CEL (c.a. 60 mg) was dissolved in 200 µL dimethyl sulfoxide (DMSO)-d_6, and transferred to the Shigemi micro-tube and characterized at 25 °C. A quantitative ^{13}C spectrum was recorded on a Bruker AVANCE 500 MHz spectrometer (Zurich, Switzerland) with a 5 mm BBO probe. A chromium (III) acetylacetonate solution (0.01 M) was applied to expedite the relaxation of all nuclei. The acquisition parameters were: a 90° pulse width, a relaxation delay of 1.7 s, an acquisition time of 1.2 s, and a total of 20,000 scans. In the ^{1}H-^{13}C HSQC NMR acquisition, CEL (*c.a*. 60 mg) was also dissolved in 200 µL DMSO-d_6 and scanned on a Bruker AVANCE 500 MHz

spectrometer with a 5 mm BBI probe. The acquisition parameters were: 160 transients (scans per block) acquired using 1,000 data points in the F2 (^{1}H) dimension, an acquisition time of 151 ms, 256 data points in the F1 (^{13}C) dimension with an acquisition time of 7.68 ms, and a coupling constant $^{1}J_{C–H}$ of 147 Hz. The ^{1}H-^{13}C HSQC data were processed with 1,000 ×1,000 data points using a Qsine function in both dimensions.

Statistical analysis

All of the experiments were duplicated and the numbers in the tables of this work were the average values.

RESULTS AND DISCUSSION

The solid yields following the pretreatment along with the chemical compositions of the samples are summarized in Table 2. Compared to stem and cob, the leaf fraction displayed the lowest solid yield after pretreatments. Meanwhile, the chemical compositions of the samples were modified according to specific pretreatments. Both autohydrolysis and the dilute acid pretreatment were able to remove a significant amount of xylan *via* an acid catalytic mechanism. Comparable results were previously observed by Jin *et al*. (2016a) and Fan *et al.* (2016). Table 2 shows that an abundance of xylan was hydrolyzed while very little lignin was removed from the stem and cob fractions after the acidic pretreatments. However, the removal of lignin was achieved from the leaf fraction by the acidic pretreatments, which indicated that its lignin was potentially different from those of the stem and cob. Because an additional catalyst "acid" was applied, the dilute acid pretreatment removed more xylan from the samples as compared to autohydrolysis. By contrast, the alkali pretreatment removed more lignin while maintaining more carbohydrate (especially xylan). This observation was similar to the previous study (Jin *et al.* 2016b).

Table 2. Chemical Composition Analysis of Samples

Sample	Pretreatment	Yield	Glucan	Xylan	TC*	TL*	Ash	MB*	Extractives
Stem	Original	100.0	36.6	25.8	65.7	21.7	2.7	87.4	4.5
	Autohydrolysis	60.2	29.4	6.3	35.7	18.1	--	53.8	--
	Diluted Acid	52.4	27.4	3.9	31.3	17.2	--	48.5	--
	Alkali	73.4	32.9	13.6	48.4	15.6	--	64.0	--
Cob	Original	100.0	33.6	29.5	68.5	16.7	1.3	86.4	2.0
	Autohydrolysis	58.8	29.0	5.5	34.5	15.1	--	49.6	--
	Diluted Acid	49.5	28.4	1.2	29.8	14.4	--	48.5	--
	Alkali	74.5	25.8	22.6	51.2	12.4	--	63.6	--
Leaf	Original	100.0	35.9	24.8	67.0	20.4	3.1	88.3	1.4
	Autohydrolysis	54.4	32.5	2.8	35.4	17.2	--	52.6	--
	Diluted Acid	45.9	26.4	1.6	28.2	14.2	--	42.4	--
	Alkali	68.7	33.9	15.0	51.8	9.3	--	61.1	--

Note: all average values are percentages based on the original raw sample; TC*, total carbohydrates; TL*, total lignin; MB*, mass balance; and --: not checked

Nitrobenzene oxidation of lignin not only characterizes lignin by providing the relative amounts of the uncondensed *p*-hydroxyphenyl-(H), guaiacyl-(G), and syringyl-

propane(S) units of lignin, but it can also provide information on the taxonomy of vascular plants (Chen 1992). The variation of the yield of aldehydes, syringyl aldehyde to vanillin (S/V) ratio, and *p*-hydroxyphenylaldehyde to vanillin (H/V) ratio (Table 3) revealed structural variations in the lignin biopolymer. Based on the yield of aldehydes, leaf lignin had the most condensed lignin, followed by cob and stem. Meanwhile, it was confirmed by NBO analysis that pretreatments induced structural changes in lignin. For example, decreases in the yield of aldehydes and S/V indicated that the residual lignin became more condensed. However, decreases in aldehydes and S/V were potentially induced by the removal of *p*-coumarate ester, ferulate ester, and non-condensed lignin that was mainly composed of S units. Therefore, if *p*-coumarate and ferulate esters are subunits in lignin, NBO can characterize lignin structure. Otherwise, it is essential to remove *p*-coumarate esters and ferulate esters before NBO analysis (Min *et al*. 2014b).

Table 3. Yields of Aldehydes, S/V Ratios, and H/V Ratios

Sample	Pretreatment	Yield	S/V	H/V
Stem	Original	27.8	1.1	0.9
	Autohydrolysis	27.3	1.5	1.2
	Diluted Acid	26.8	1.5	1.2
	Alkali	25.1	1.3	0.7
Cob	Original	24.7	0.6	0.8
	Autohydrolysis	23.8	1.0	0.9
	Diluted Acid	21.4	1.0	1.2
	Alkali	9.9	0.8	0.7
Leaf	Original	17.2	0.7	0.6
	Autohydrolysis	16.2	1.0	0.9
	Diluted Acid	16.5	1.1	0.8
	Alkali	10.6	0.9	0.5

Note: The average yield of aldehydes was based on lignin; S/V and H/V were molar ratios

The ^{13}C – NMR spectra of acetylated and non-acetylated stem CELs are shown in Figs. 1 A and B, respectively. The assignments of signals were done using a literature precedent and model compounds (Chen 1998; Villaverde *et al*. 2009; Del Rio *et al*. 2012). Although signals of some inter-unit linkages were overlapped in Fig. 1, it was still possible to quantify inter-unit linkages by the combination analysis of nonacetylated and acetylated CELs. A good correlation between the nonacetylated and acetylated CELs was confirmed by their similar values for integrals in methoxyl of signal at δ_C54-56 ppm and the other three main clusters of signals at δ_C90 – 77 ppm, δ_C77 – 66 ppm, and δ_C65 – 57.5 ppm. The ^{13}C – NMR spectra of non-acetylated samples showed that corn stover lignin is of *p*-hydroxycinnamyl-guaiacyl-syringyl type. Briefly, the presence of guaiacylpropane units was evidenced by strong signals at δ_C149.5 ppm and 149.2 ppm, which corresponded to C-3 and C-4 of guaiacyl rings, respectively. The presence of syringylpropane units was revealed by strong signals at ~δ_C104 ppm that corresponded to C-2 and C-6 of syringyl rings. The presence of *p*-hydrocinnamylpropance units was demonstrated by a moderate signal at δ_C167.3 ppm that corresponded to the C-4 of the *p*-hydrocinnamyl rings. The presence of *p*-coumarate esters and ferulate esters in corn

stover lignin was indicated by strong signals at δ_C167.2 ppm, 125.6 ppm, and 115.7 ppm assigned to the carbons of two esters. The presence of these two esters was also confirmed by the corresponding contours in the ^{1}H-^{13}C HSQC NMR spectra (Fig. 3). A small amount of carbohydrate that remained in the CELs was evidenced by the signals of non-acetylated sample at δ_C179.5 ppm, 101.6 ppm, 76 – 73 ppm, and 20.9 ppm, which were assigned to O-acetyl C=O, C-1, and C-2 of xylan, C-3 and C-4 of xylan, and O-acetyl CH_3, respectively. The structural changes of lignin from the pretreatments are clearly shown in Fig. 1.

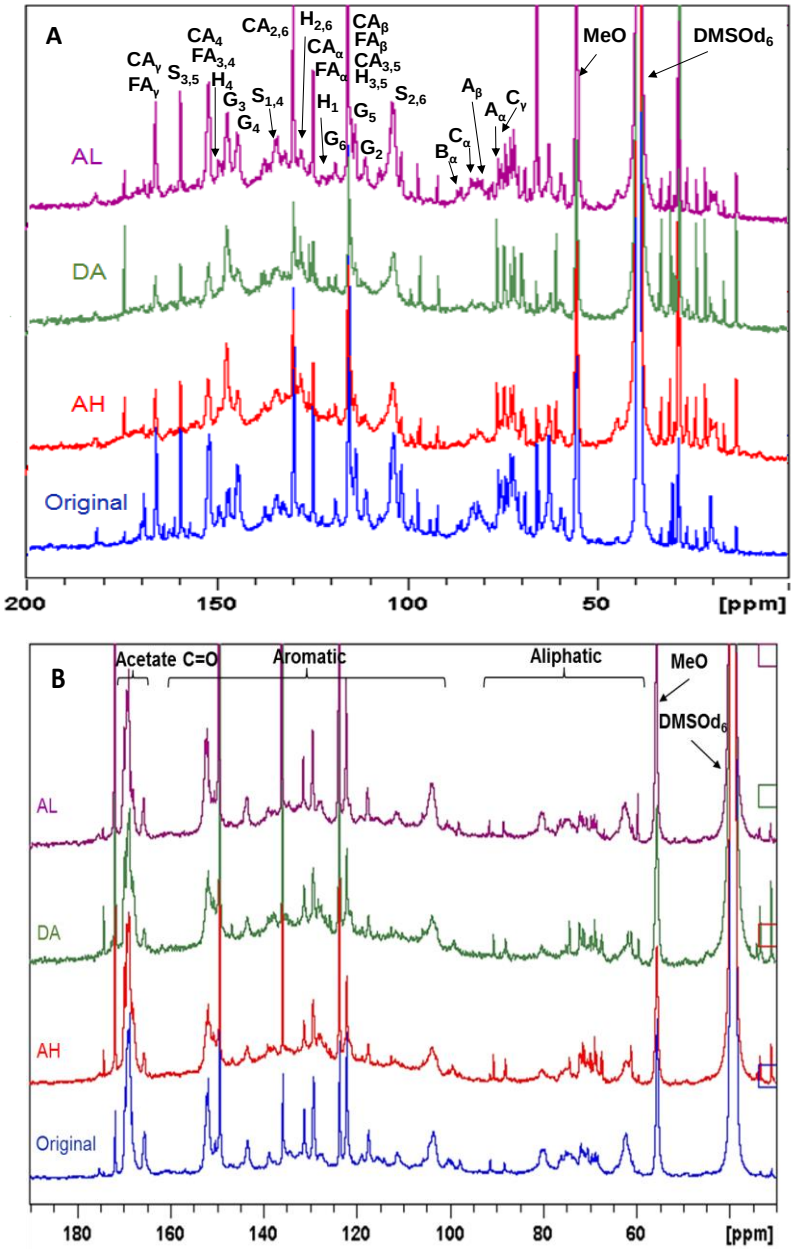


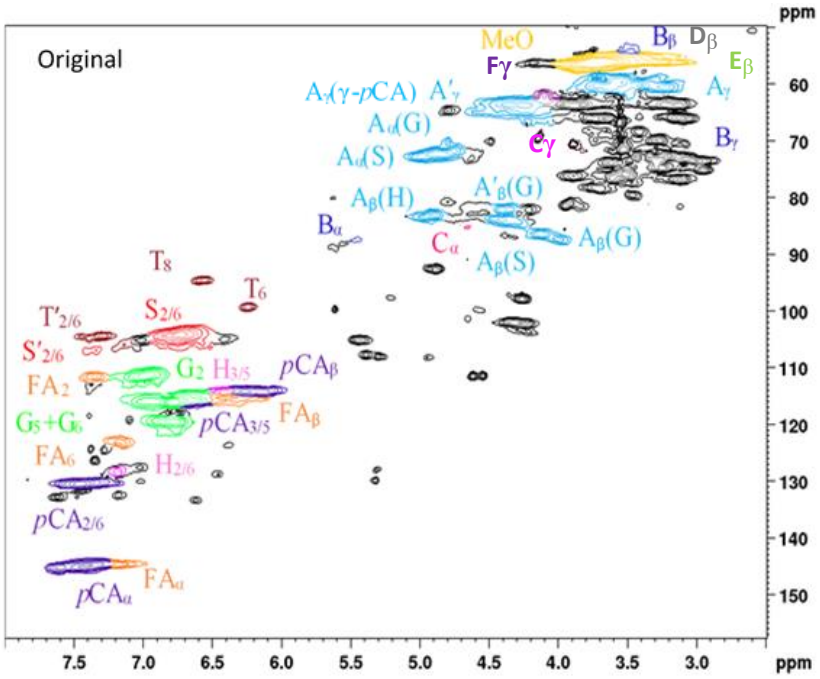
Fig. 1. ^{13}C-NMR spectra of stem: A non-acetylated CEL; B acetylated CEL; AH: autohydrolysis; DA: dilute acid pretreatment; and AL: alkali pretreatment

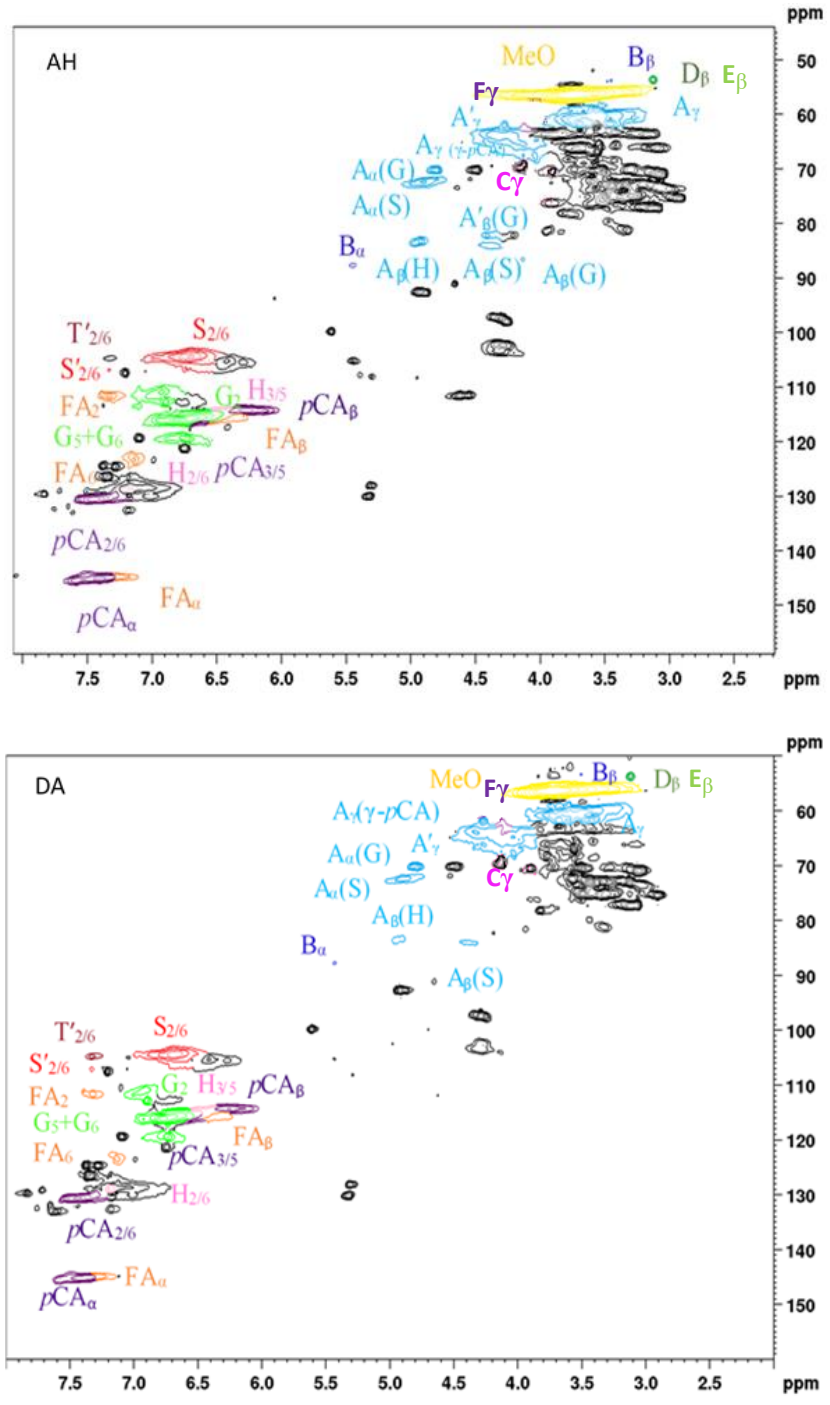
As for the non-acetylated samples, the integral of δ_C160 – 102 ppm of the predominantly aromatic region was set as the internal standard for the ^{13}C-NMR quantitative analysis. The signals in this range encompass those from 6.12 of aromatic and alkenyl carbons (Chen 1998). Thus, it follows that the integral value divided by 6.12 is equivalent to one aromatic ring. Most of the peaks assignments to different inter-unit linkages overlapped to some extent (Fig. 1). Therefore, it was impossible to quantify all major inter-unit linkages of lignin by only using ^{13}C – NMR of non-acetylated samples. Whereas the peak of the phenyl coumaran (β-5′) was well-resolved at approximately δ_C 87 ppm in the spectrum of CEL-Ac (Fig. 1 B), the integration gave a value of 0.09/Ar, which was used as the reference in the ^{1}H – ^{13}C HSQC NMR analysis. Thus, the combination of ^{13}C – NMR and ^{1}H – ^{13}C HSQC NMR characterized the structural changes in lignin induced by pretreatments.

The CELs isolated from the original and pretreated samples were characterized by ^{1}H – ^{13}C HSQC NMR, and results are shown in Fig. 2. The characteristic regions of ^{1}H – ^{13}HSQC NMR spectra were divided into oxygenated aliphatic side chains and aromatic ^{1}H – ^{13}C correlations. The oxygenated aliphatic side chain at δ_C/δ_H 50 – 95/2.5 – 5.5 ppm revealed different inter-unit linkages of lignin. The contour signals from methoxyls (δ_C/δ_H 55.5/3.71 ppm) and β-O-4′ linkage were most prominent. Other intense contour signals over δC/δH 62.5/3.95 – 4.62 ppm and δ_C/δ_H 60.0/3.12-3.83 ppm were assigned to γ-acylated units and hydroxylated γ-carbons in β-O-4′ linkages, which indicated that corn stover lignin was extensively acylated at the γ-position of the lignin side chain. Other low intensive contour signals assigned to β-5′, β-β′, β-1′ (spirodienone) were also identified. The major contour signals at the aromatic regions were assigned to different lignin aromatic units and *p*-hydroxycinnanate units (Ralph *et al.* 1994; Sun *et al.* 2002; Buranov and Mazza 2008). The intensive contour signals at δ_C/δ_H 103.5/6.72 ppm, δ_C/δ_H 110.5/6.95 ppm, and δ_C/δ_H 118.2/6.71 ppm were assigned to $S_{2/6}$, G_2, G_5 and G_6, respectively. A contour signal identified at δ_C/δ_H 128.1/7.20 ppm was assigned to $H_{2/6}$. The additional contours of H units and G units (G_5 and $H_{3,5}$) that overlapped at chemical shifts at δ_C/δ_H 114 – 116/6.7 – 6.9 ppm were not differentiated. This observation confirmed that corn stover lignin was of G/S/H type lignin. Meanwhile, the intensive contour signals that corresponded to *p*-coumarate esters, ferrulate esters, and tricin (a flavonoid) were identified in the aromatic region. The signals at tδ_C/δ_H 130.2/7.38 ppm, δ_C/δ_H 115.1/6.35 ppm, δ_C/δ_H 145.0/7.48 ppm, and δ_C/δ_H 113.2/6.21 ppm were assigned to pCA2/6, pCA3/5, pCAα, and pCAβ, respectively. The signals at δC/δH 110.2/7.35 ppm and δ_C/δ_H 123.2/7.12 ppm were assigned to FA_2 and FA_6, respectively. The signals at δ_C/δ_H 93.3/6.52 ppm and δ_C/δ_H 98.2/6.21 ppm were assigned to T_8 and T_6, respectively (Wen *et al*. 2013).

Because ^{13}C, ^{1}H – correlated (HSQC, HMQC) spectroscopic techniques provide higher resolution and non-overlapped spectra compared to ^{13}C – NMR, they have been widely used to quantitatively analyze complex samples. Each lignin inter-unit bonding spin system showed three different signals in the ^{1}H – ^{13}C HSQC NMR spectra due to CH and CH_2 groups in the α, β, and γ positions of the side chain. The T_2 of each signal was dependent on carbon substitution; thus, only the CH signals were taken into consideration for quantitative analyses. Specifically, the α signals for the β-aryl ether (β-O-4′) and phenyl coumaran (β-5′), and β signals for the resinol (β-β′) dibenzodioxocin (5-5′-O-4) diphenylethane (β-1′), and spirodienone (SD) lignin inter-unit bonds were considered. It

is known that the G_2 signals (C_2-H) and the combination of $S_{2,6}$ and G_2 signals (S C_2-H, C_6-H, and G C_2-H) can be used as internal standards for the quantitative lignin analyses of gymnosperms and angiosperms, respectively. However, these methods cannot be applied for non-woody lignins because the contours of *p*-coumarate and ferulate esters partially overlap with *p*-hydroxyphenyl $C_{2,6}$-H in the $^{1}H - ^{13}C$ HSQC NMR spectrum. To overcome this issue, a comprehensive approach using a combination of quantitative ^{13}C – NMR and $^{1}H - ^{13}C$ HSQC NMR techniques was applied. For example, the absolute value of the phenyl coumaran substructure (β-5′ linkage) could be directly quantified from the ^{13}C – NMR spectrum. The integral of the phenyl coumaran substructure was integrated from $^{1}H - ^{13}C$ HSQC NMR. As a result, the internal reference from the 2D NMR could be deduced from the phenylcoumaran substructure. Eventually, the deduced internal reference can be used to quantify the contents of other inter-unit linkages.

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Bβ
Dβ Eβ
Fγ
Aγ
A′γ
Aγ (γ-pCA)
Aα(G)
Cγ
Aα(S)
A′β(G)
Bα
Aβ(H)
Aβ(S)
Aβ(G)
T′2/6
S2/6
S′2/6
FA2
G2 H3/5
pCAβ
G5+G6
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FAα
pCAα
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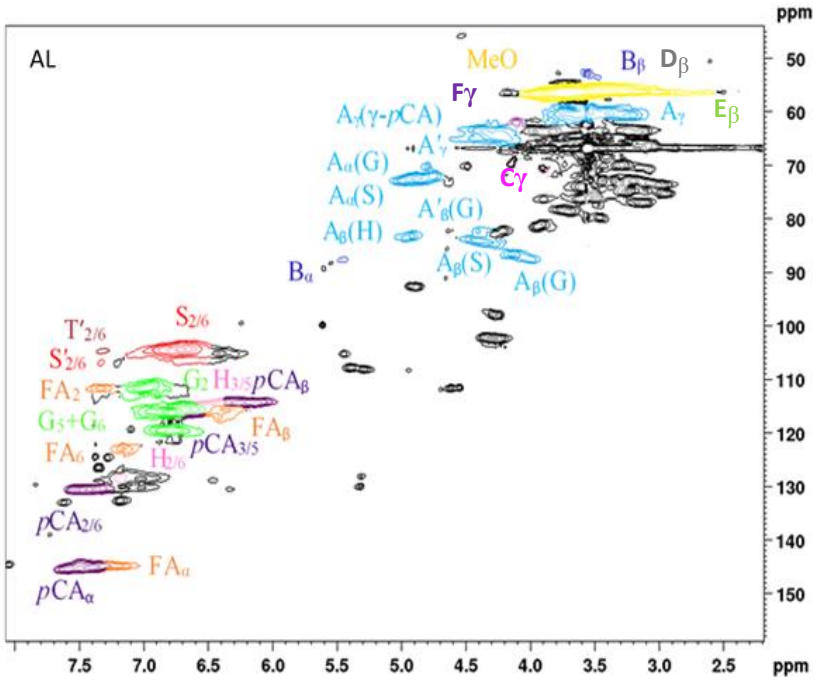

Fig. 2. ^{1}H-^{13}C HSQC NMR spectra of stem with pretreatments; Original: original stem, AH: autohydrolysis, DA: dilute acid, and AL: alkali

The structural alterations of lignin induced by the pretreatments were observed and are shown in Fig. 2. Generally, the contour assigned to tricin was noticeably reduced in the samples by the pretreatments. Unlike the reported results, small amounts of *p*-coumarate and ferulate esters were removed by the alkali pretreatments, possibly because the ester bonds could not be cleaved completely in short residence time. In contrast to the alkali pretreatment, autohydrolysis and dilute acid removed more carbohydrates (the black signal contours that corresponded to carbohydrates were not assigned). Eventually, the details on lignin alternations were quantitatively assigned through the combination of ^{13}C – NMR and ^{1}H-^{13}C HSQC NMR.

The major inter-unit linkages, S/G ratio, and H/G ratio of the samples are summarized in Table 4. The results indicated that corn stover lignin contained a preponderance of β-aryl ether (β-O-4′) lignin linkages with low amounts of "condensed" substructures, *e.g.* β-5', β-1′, and spirodienone. It was noteworthy that leaf lignin was shown to be more condensed than stem lignin and cob lignin because it has reduced amounts of β-aryl ether and a low S/G ratio. It could be considered that a high S/G ratio lignin possessed a more linear structure because of the limited availability of the C-5 position in the aromatic ring to adopt cross-linked structures (condensed lignin). Furthermore, a similar lignin structure can be deduced from the original stem and original cob because of their similar contents of inter-unit linkages and S/G ratio.

Table 4. Lignin Structure Determined by $^{1}H - ^{13}C$ HSQC NMR

Unit/ $100C_9$	Stem				Cob				Leaf			
	Ori	AH	DA	AL	Ori	AH	DA	AL	Ori	AH	DA	AL
β-O-4'	45.7	30.7	28.4	16.6	42.5	35.7	32.2	27.8	35.3	32.4	26.9	11.7
β-5'	2.1	2.9	2.7	1.9	2.6	4.0	3.8	2.5	6.6	7.3	7.4	5.8
β-β′	1.5	3.1	2.9	1.3	1.1	1.7	1.3	1.2	1.6	2.1	2.0	1.5
β-1′	N.D.	0.8	0.5	N.D.	0.5	N.D.	N.D.	N.D.	0.9	1.2	1.4	1.1
SD	0.5	N.D.	N.D.	N.D.	0.6	N.D.	N.D.	N.D.	0.8	N.D.	N.D.	N.D.
S/G	1.1	0.9	0.9	0.7	1.2	1.0	1.1	0.7	0.9	0.7	0.7	0.4
H/G	0.1	0.3	0.4	0.2	0.2	0.2	0.4	0.2	0.1	0.1	0.3	0.4

Note: all values are based on $100C_9$; S/G and H/G are the molar ratios; SD: spirodienone; N.D.: not detectable, less than $0.5/100C_9$

The structural alterations of lignin induced by the pretreatments are also shown in Table 4. For example, it was found that the content of β-aryl ether decreased $10/100C_9$, while the contents of "condensed" substructures, including β-5′ and β-β′, increased when the stem was pretreated by autohydrolysis or the dilute acid. These condensation patterns were previously observed by Shimada *et al.* (1997) while performing acidolysis on syringyl and guaiacyl model compounds. By contrast, the alkali pretreatment cleaved more β-aryl ether in the stem that decreased from $45.7/100C_9$ to $16.6/100C_9$, while maintaining "condensed" substructures that were more specifically the alkali-stable C-C linkages, which was consistent to the mechanism proposed by Kleinert (1966). The reduction of β-aryl ether indicated that the acidic pretreatments (autohydrolysis and the dilute acid) could cleave β-O-4′ linkages. Yet, the increase of "condensed" substructures suggested that lignin was partially cross-linked under the acidic condition. Moreover, more cleavage of the β-O-4′ linkage by the alkali pretreatment was explained by the fact that more lignin was removed when the alkali condition was applied. The S/G ratios of the original stem, cob, and leaf were 1.1, 1.2, and 0.9, respectively, which suggested that stem lignin and cob lignin were more linear than leaf lignin. Technically, the impact of pretreatments on lignin was also elucidated by the changes of the S/G ratios. The decrease of the S/G ratio revealed that the residual lignin became more condensed after the pretreatments. For example, the S/G ratio of stem lignin decreased from 1.1 to 0.7 after the alkali pretreatment, which indicated that the pretreated sample contained more G units and H units that further indicated the "condensed" lignin, mainly composed of G units and H units, was more stable during pretreatment. Therefore, the results taken as an aggregate showed that the lignin structure had a large impact on the delignification of the sample during the pretreatment and a large impact on the enzymatic hydrolysis of a pretreated sample. Thus, it is important to characterize the changes in the lignin structure resulting from pretreatment to be able to correlate them to downstream applications such as enzymatic saccharification.

CONCLUSIONS

1. Corn stover lignin is an S/G/H-type lignin that incorporates *p*-coumarate esters and ferulate esters with different substructures within the stem, cob, and leaf samples.

2. The β-O-4′ was confirmed as the most dominant inter-unit linkage followed by β-5′, β-1′, and spirodienone. Leaf lignin was more condensed than stem lignin and cob lignin because of less β-O-4′ linkages and lower S/G ratios.
3. The acidic pretreatment, *e.g.* autohydrolysis and dilute acid, decreased β-O-4′ by cleaving β-aryl ether linkages, while they increased "condensed" substructures through forming C-C linkages such as β-5′, β-β′, and β-1′. However, the alkali pretreatment cleaved β-aryl ether linkages while didn't generate new "condensed" C-C linkages.
4. The S/G ratios decreased after the pretreatments, which indicated that the pretreated samples contained comparatively more G units and H units, and further supported the finding that the G units and H units were stable.

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