The Structure of Lignin of Corn Stover and its Changes Induced by Mild Sodium Hydroxide Treatment

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Corn stover is an abundant feedstock in the US that can be used for second generation bioethanol production. However, there is little useful data on structure of the lignin of corn stover. The following principal tasks will be addressed to profile the structure of corn stover: (1) separation of corn stover into stem, cob, and leaf; (2) isolation of cellulolytic enzyme lignins (CEL) from extractive-free and the alkali-treated fractions; (3) quantification of *p*-coumarate and ferulate of fractions by HPLC. The results of alkaline nitrobenzene oxidation and ^{1}H-^{13}C HSQC NMR indicated: (1) the structure of lignin varied in the fractions; (2) a remarkable amount of *p*-coumarate and ferulate was identified and determined; (3) the remarkable structural changes of lignin induced by alkaline treatment were elucidated.

Keywords: Corn stover; Lignin; Alkali-treatment; Nitrobenzene oxidation; ^{1}H-^{13}C HSQC NMR

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INTRODUCTION

Lignocelluloses are a general class of renewable materials composed of cellulose, hemicelluloses, lignin, extractives (fatty acids, resins, and other chemicals), and a trace amount of inorganics. They comprise the most abundant renewable resource on the planet with an estimated annual worldwide production in the hundreds of billions of tons, of which only 3% is used by humans (Lucia 2008). Among the many pathways for the transformation of lignocellulosics to fuels and chemicals, biological-based platforms are among the most promising because of promising economics and high efficiency. However, the key step to ensuring high conversion efficiency is enzymatic hydrolysis, the breakdown of the polysaccharides to fermentable sugars.

One of the most important considerations that has been universally recognized to address efficient enzymatic hydrolysis is "opening up" the ultrastructure of the lignocellulosics and thus increasing its accessibility to enzymatic penetration and activity. This can be done by pretreatment or other manner of pre-enzymatic treatment (Chandra *et al*. 2008; Mansfield *et al*. 1999). From the perspective of the properties of the sample, the rate and extent of its enzymatic hydrolysis is strongly influenced by lignin structure and distribution, cellulose crystallinity, the degree of cellulose polymerization, particle size, and pore volume (Hall *et al*. 2010; Mooney *et al*. 1998). Consequently, the characterization of lignin and LCCs is becoming more important both in the analysis of plant cell wall and in the process of ascertaining their industrial utility. Hence, an improved understanding of biomass characteristics especially lignin before and after

pretreatment will enable the development of a more efficient pretreatment or pre-enzymatic treatment process.

Despite major advances in analytical methods, most of the current knowledge of the composition and structure of lignin is derived from interpretations and extrapolations of data from wet chemistry methods such as thioacidolysis and nitrobenzene oxidation, which provide only a fraction of the whole macromolecular structure. NMR hardware has become increasingly sophisticated, and recent advances have improved both the sensitivity and the quality of data that can be acquired. All of these advances have paved the way for what would have been previously inconceivable—the acquisition of spectra from lignocelluloses without severe degradation. Therefore, through alkaline nitrobenzene oxidation and ^{1}H-^{13}C HSQC NMR, this study profiles the structure of lignin of extractive-free samples and reveals the structural changes of samples induced by the mild alkali-treatment.

EXPERIMENTAL

Raw Materials and Composition Analysis

Corn stover collected in the state of Iowa and provided by Novozymes (Franklinton, NC USA) was separated into three fractions: stem, cob, and leaf. All were air-dried and ground to pass 40-mesh sieves using a Wiley mill (General Electric, USA). The fraction between 40 and 60 meshes was collected, and all adventitious contaminants were extracted by a mixture of benzene and ethanol (2:1 v/v) for 8 h. The composition of samples was characterized according to TAPPI Standard Method T222 om-98.

Quantification of *p*-Coumarate and Ferulate

Each fragment of corn stover (around 30 g) was treated for 24 h with 1 N NaOH (300 mL) at room temperature to hydrolyze *p*-coumarate esters and ferulate esters. Then, 20 mL of the treatment liquor was acidified to pH 2 with 12 M HCl and extracted with 10 mL ethyl ester three times. Five milliliters of cinnamic acid (10 mg/mL) were added to a combination of three ethyl ester solutions as an internal standard. Quantitative HPLC analysis was carried out on a Shimadzu LC-20AT equipped with a SPD-20A UV/Vis detector (280 nm) and Agilent Zorbax SB-C8 column (5 μM pore size, 4.6 mm × 150 mm). The gradient solvent flow rate was 1.0 mL/min. Solvent A was H_2O (10 mM formic acid). Solvent B was ACN (10 mM formic acid).

Determination of the ratios of syringaldehyde to vanillin (S/V) and *p*-hydroxybenzaldehyde to vanillin (H/V)

Alkaline nitrobenzene oxidation (NBO) was performed according to Chen (1992). 100 mg of OD samples were reacted with 7 mL of 2 N NaOH (aq) and 0.4 mL of nitrobenzene in a stainless bomb at 170 ˚C for 2.5 h. The hot stainless bomb was cooled down immediately by cold water, and 1 mL of 5-iodovanillin (80 mg dissolved in 5 mL acetone) was added as internal standard. The mixture was extracted with CH_2Cl_2 (20 mL) three times and organic phase (CH_2Cl_2) was discarded. The remaining water phase (alkali solution) was acidified with 2N HCl to pH 3~4. The acidified solution was further extracted again with CH_2Cl_2 three times, and collected the organic phase (CH_2Cl_2). The organic phase was dried by Na_2SO_4(s) and the volume was adjusted to 100 mL. 1 mL of this solution was dried by rotavapor at 30 ˚C. The dried product was dissolved in 50 μL

of pyridine, and 50 µL of N-methyl-N-(trimethylsilyl) trifluoraacetamide (MSTFA) was added. The derivatized mixture was then directly injected (2 µL) into the GC. Quantitative GC analysis was carried out on a HP6890 GC equipped with a flame ionization detector and HP-1 column (30 m×0.32 mm×0.25 µm). The injection temperature was 200 ˚C, the detector temperature was 270 ˚C, and the column flow rate was 2 mL of helium per min. The column was held for 3 min at 120 ˚C, raised at 5 ˚C per min to 200 ˚C, followed by 10 ˚C per min to 260 ˚C, and kept isothermal at 260˚C for 5 min.

Isolation of Cellulolytic Enzyme Lignin

The cellulolytic enzyme lignin (CEL) (Bjorkman 1956; Chang *et al*. 1975) was acquired with the following procedure. The extractive-free and alkaline-treated samples were subjected to 4 to 12 h of milling at 600 rpm using ZrO_2 bowls and 17 ZrO_2 balls in a planetary ball milling apparatus (Pulverisette 7, Fritsch, Germany). Then, the meal was treated with cellulase (from Trichoderma viride, 4.7 U/mg solid, Sigma; loading: 500 U/g sample) in an acetate buffer solution (pH 4.5) at 50 °C for 48 h. For the extractive-free samples, the enzyme-treated residues were washed with H_2O and then air dried. Then, they were extracted by 1, 4-dioxane (96% v/v) for 24 h at 25 °C three times to obtain CEL (Capanema *et al*. 2004). For the alkaline-treated samples, enzymatic hydrolysis was carried out. Then, the enzymatically hydrolyzed residue was washed with H_2O and freeze-dried. The final vacuum-dried sample was used as CEL in this study.

^{1}H-^{13}C HSQC NMR Acquisition

The CEL (about 60 mg) was dissolved in 200 µL of DMSO-d_6 and then characterized (Capanema *et al.* 2004). ^{1}H-^{13}C HSQC NMR was performed on a Bruker AVANCE 500-MHz spectrometer equipped with a 5-mm BBI probe. The acquisition parameters used were 160 transients (scans per block) acquired using 1,000 data points in the F2 (^{1}H) dimension with an acquisition time of 151 ms and 256 data points in the F1 (^{13}C) dimension with an acquisition time of 7.68 ms. A coupling constant ^{1}J C–H of 147 Hz was used. The 2D data set was processed with 1,000 and 91,000 data points using the Qsine function in both dimensions.

RESULTS AND DISCUSSION

Chemical Composition of Samples

The chemical composition of the samples (stem, cob, and leaf) is summarized in Tables 1 and 2.

Table 1. Composition of the Extractive-free Sample (Average ± SD)

No.	Glucan	Xylan	TS*	TL*	Ash
Stem	36.1 ± 0.36	20.8 ± 0.12	59.7 ± 0.60	22.3 ± 0.24	7.8 ± 0.05
Cob	32.4 ± 0.66	28.9 ± 0.51	65.2 ± 1.24	17.4 ± 1.86	2.0 ± 0.01
Leaf	36.4 ± 0.46	23.9 ± 1.18	66.2 ± 1.68	20.6 ± 0.16	5.4 ± 0.02

Note: Composition is expressed as % (w/w) of the extractive-free sample. TS: Total sugars. TL: Total lignin, including acid-insoluble lignin and acid-soluble lignin.

Table 2. Composition of the Alkaline-treated Sample (Average ± SD)

No.	Glucan	Xylan	TS*	TL*
Stem	34.6 ± 0.02	6.3 ± 0.01	42.1 ± 0.02	5.3 ± 0.01
Cob	30.2 ± 0.39	9.8 ± 0.23	41.6 ± 0.09	3.4 ± 0.01
Leaf	33.0 ± 0.24	6.4 ± 0.01	41.1 ± 0.25	4.6 ± 0.12

Note: Composition is expressed as % (w/w) of the extractive-free sample. TS: Total sugars. TL: Total lignin, including acid-insoluble lignin and acid-soluble lignin.

For the extractive-free samples, the stem had the lowest proportion of carbohydrates (about 60%) and the highest lignin (22.3%). Cob and leaf had similar levels of carbohydrates (around 65%), and lower lignin content (17.4% and 20.6%), respectively. With the mild alkaline treatment, both lignin and carbohydrates in stem, cob, and leaf decreased to similar levels, although the delignification and the removal of carbohydrates varied among the samples. Compared to woody samples (data not shown), an unexpected delignification (76% to 82%) of corn stover by the mild alkaline treatment indicated that its lignin was different from that in woody samples.

Quantitation of *p*-Coumarate and Ferulate

A significant amount of *p*-coumarate esters and ferulate esters involved in herbaceous samples has been reported (Buranov and Mazza 2008; Ralph *et al*. 1994). For example, around 8% *p*-coumaric acid was liberated and determined from maize stem through mild alkaline extraction (Sun *et al*. 2002). In this study, 3 to 9% *p*-coumaric acid and ferulic acid hydrolyzed from samples were identified and quantified in the alkaline treatment liquor by HPLC (Table 3). It is known that *p*-coumarate esters and ferulate esters can be oxidized into *p*-hydroxybenzaldehyde and vanillin, respectively, by alkaline nitrobenzene oxidation. Thus, it was essential to remove *p*-coumarate esters and ferulate esters to minimize their interruptions on the yield of aldehydes, S/V, and H/V of samples. Based on our study, mild alkaline treatment removed *p*-coumarate esters and ferulate esters from samples effectively.

Table 3. Content of *p*-Coumarate and Ferulate of Samples

Sample	% Based on lignin		
	p-coumarate	Ferulate	Total
Stem	7.12±0.24	1.15±0.05	8.27±0.32
Cob	6.33±0.15	2.60±0.10	8.93±0.54
Leaf	2.06±0.08	0.86±0.04	2.92±0.12

Characterization of S/V and H/V of Samples

Alkaline nitrobenzene oxidation is still the most important and commonly used degradation method for characterizing lignin structure in lignocellulosic materials. In nitrobenzene oxidation, three constitutive lignin building units, *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) moieties, are degraded into aldehydes: *p*-hydroxybenzaldehyde (H), vanillin (V) and syringaldehyde (S), and a small amount of their corresponding acids: *p*-hydroxybenzoic acid, vanillic acid, and syringic acid, respectively. The ratios of syringaldehyde to vanillin (S/V) and *p*-hydroxybenzaldehyde to vanillin (H/V) have long been identified as significant lignin characteristics of biomass. The yield of aldehydes, S/V, and H/V of the extractive-free samples and

alkaline-treated samples is summarized in Table 4. The variations of the yield of aldehydes, S/V, and H/V indicated the structural variations of lignin in the extractive-free samples. For instance, lignin in the stem was the least condensed, but lignin in the leaf was the most condensed based on the yield of aldehydes. Meanwhile, nitrobenzene oxidation demonstrated a substantial change of lignin induced by the mild alkaline treatment (Table 4). A significant decrease in the yield of aldehydes, S/V, and H/V indicated that lignin became more condensed in the alkaline-treated sample. Two reasons were proposed to explain the decrease in the yield of aldehydes, S/V, and H/V with the mild alkaline treatment. The first reason was the removal of *p*-coumarate esters and ferulate esters from samples. Another reason was related to the removal of parts of non-condensed lignin, which were theoretically composed of S units. Thus, it is reasonable to apply the alkaline nitrobenzene oxidation to the extractive-free sample if *p*-coumarate esters and ferulate esters were thought of as the lignin-composing units. Otherwise, it is essential to remove *p*-coumarate esters and ferulate esters to acquire structural information for lignin.

Table 4. Lignin Composition from Alkaline Nitrobenzene Oxidation (Average ± SD)

Sample		Yield	S/V	H/V
Extractive-free	Stem	35.6±1.20	1.35±0.11	0.80±0.02
	Cob	27.0±0.24	1.71±0.21	0.72±0.01
	Leaf	19.4±0.63	0.98±0.06	0.41±0.01
Alkaline-treated	Stem	25.5±1.31	1.29±0.14	0.15±0.05
	Cob	17.3±0.85	1.27±0.09	0.23±0.02
	Leaf	13.1±0.26	0.78±0.04	0.43±0.04

Note: % yield of aldehydes was based on lignin of sample; S/V and H/V were molar ratios.

Isolation of Cellulolytic Enzyme Lignin (CEL)

Several methods have been discussed and applied to isolate lignin from woody samples (Capanema *et al*. 2004; Min *et al*. 2013). Although ball-milling is known to induce minimal bond cleavage, methods that involve ball-milling are the basis of several cell wall characterization procedures that are well established and accepted by the scientific community. The bond cleavage was not sufficiently intense to significantly change the native structure of the lignin. Because of the high yield of the isolated lignin and minimal structure change, cellulolytic enzyme lignin was used for the structural characterization of native lignin, intensively. In this research, with 4 h of ball milling, the CELs of the extractive-free stem and cob obtained yields of 53% and 77% lignin, respectively. However, the yield of CEL of the extractive-free leaf increased to 45.9% with 12 h of ball-milling, indicating that its lignin was more difficult to be isolated. CELs of the alkaline-treated samples were achieved as the residues of enzymatic hydrolysis without further 1, 4-dioxane extraction.

NMR Spectroscopy

^{1}H-^{13}C HSQC NMR had been widely applied to characterize lignin and lignin-carbohydrate complexes (Balakshin *et al*. 2011; Yelle *et al*. 2008). The major lignin moieties in corn stover are shown in Fig. 1. Lignin in corn stover is more complicated than the counterpart in woody samples because it involves a significant amount of *p*-coumarate esters and ferulate esters, in addition to *p*-hydrobenzaldehyde. (Buranov and

Mazza 2008). As a result, the occurrence of *p*-coumarate esters and ferulate esters interfere with the quantitative analysis of lignin by ^{1}H-^{13}C HSQC NMR. Meanwhile, the interference of carbohydrates on the characterization of lignin was also reported (Yelle *et al*. 2008; Balakshin *et al*. 2011). Therefore, CEL was applied for lignin characterization because of a low carbohydrate content in this study.

Four main inter-unit linkages in lignin are shown in Fig. 2. Because of a low carbohydrate content, CEL rather than MWLc (crude milled wood lignin) was applied to characterize the structure of lignin.

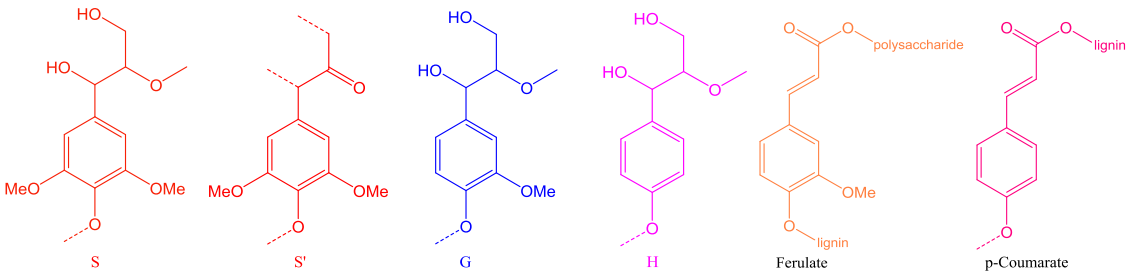

Fig. 1. Major moieties of corn stover lignin

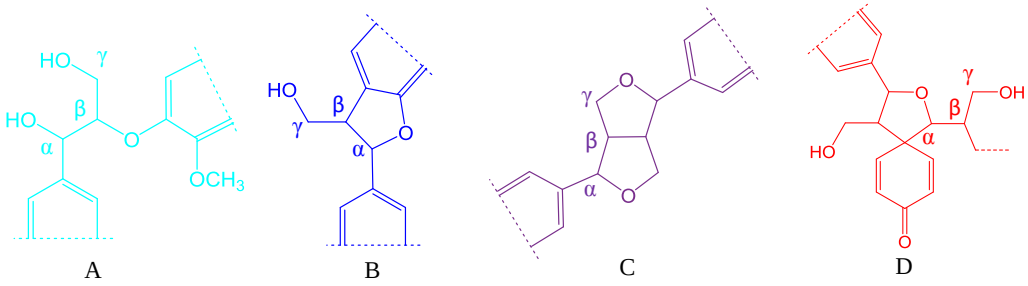

Fig. 2. Main inter-unit linkages in lignin; A: β-O-4′; B: β-5′; C: β-β′ and D: Spirodienone

The contents of major units and inter-linkages in lignin are summarized in Table 5. The intensive contours of the acylation of γ-OH of the samples are revealed in the aliphatic oxygenated region (Fig. 3a). First of all, 25% to 47% of γ-OH in lignin were acylated in the extractive-free samples. It was proposed that γ-OH was exclusively actylated by *p*-coumaric acid (Buranov and Mazza 2008). However, with the mild alkaline treatment was carried out on samples, the actylation of γ-OH of samples decreased dramatically. For example, the actylation of γ-OH of stem decreased from 43% to 2% after the alkaline treatment.

Meanwhile, the intensive contours of *p*-coumarate and ferulate of samples were also identified in the aromatic region (Fig. 3b). Furthermore, the significant removal of *p*-coumarate esters and ferulate esters by the mild alkaline treatment was confirmed in the aromatic region by the comparison with Fig. 3b and Fig. 3d. With the removal of *p*-coumarate and ferulate esters, the accurate S/G and H/G of the sample was acquired from the alkali-treated samples. The contents of *p*-coumarate and ferulate esters also could be quantified through ^{1}H-^{13}C HSQC NMR. However, it was essential to identify a reference for such quantitative analysis.

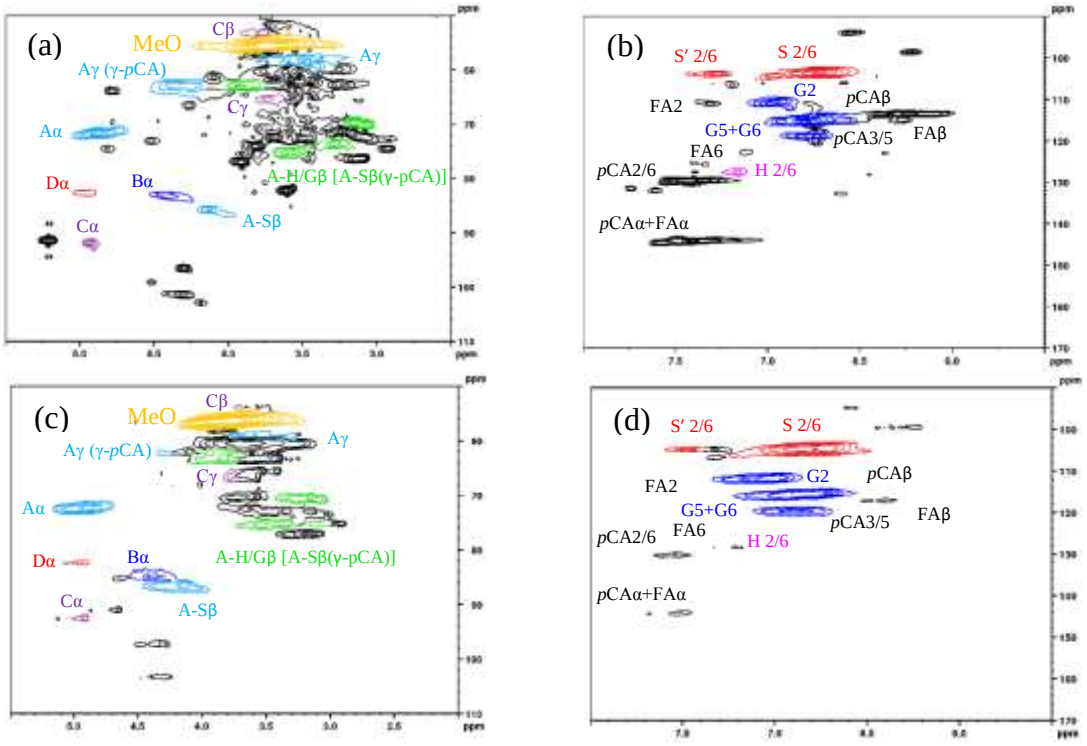

Fig. 3. ^{1}H-^{13}C HSQC spectra: (a) aliphatic oxygenated region of extractive-free stem; (b) aromatic region of extractive-free stem; (c) aliphatic oxygenated region of alkaline extracted stem; (d) aromatic region of alkaline extracted stem

Secondly, the four major inter-linkages of lignin were characterized as well (Table 5). Generally, the β-O-4 linkage ranged from 40% to 60%, as the most abundant inter-unit linkages in all samples. However, the C-O linkage *e.g.* β-O-4 referring to the non-condensed linkage in lignin decreased dramatically (about 30%) with the mild alkaline treatment. In the other hand, the C-C linkages *e.g.* β-5 referring to the condensed linkage in lignin increased by around 50%, relatively. The alkaline pre-extraction of wood resulted in the loss of a part of lignin enriched in H- and G- units and phenolic OH as compared to the bulk lignin, significant differences in the amounts of inter-unit linkages were also observed (Balakshin *et al.* 2001a,b).

Lastly, the variations of the ratios of composing units were quantified with measurement of the contour volume integrals in ^{1}H-^{13}C HSQC NMR. It was demonstrated that the contours of aromatic parts of composing units (G and H units) were overlapped with the contours of aromatic parts of *p*-coumarate and ferulate in Fig. 3b and d. However, the values of syringyl to guaiacyl ratio (S/G) and *p*-hydroxyphenyl to guaiacyl ratio (H/G) were still calculated by using the reported assignments of the aromatic contour of each composing unit (Kim *et al.* 2008). The measurement of volume integrals on the assigned contours was using the processing software (Bruker Topspin 3.0). The $S_{2/6}$, G_2 and $H_{2/6}$ contours were used assuming the C-H pairs had similar environments and G_2 integral was logically doubled. It had been convincingly shown that NMR-based S/G values are accurate (Marita *et al.* 2003; Vanholme *et al.* 2010; Ralph *et al.* 2012). The same result could be inferred for minor H units. But the H unit often corresponds to end-groups and thus may be overrepresented (Mansfield *et al.* 2012).

Eventually, the values of the ratios of composing units of the samples were calculated and summarized in Table 5. The decrease of S/G could be explained by the removal of parts of non-condensed lignin during the alkaline treatment. Technically, non-condensed lignin was mainly composed by S units. Thus, lignin in the alkaline-treated samples was more condensed compared to lignin in the extractive-free samples. Compared to alkaline nitrobenzene oxidation, NMR does profile the "entire" lignin (the isolated lignin), in principle including the condensed and the non-condensed parts of lignin. However, the isolation process induced some level of degradation and there are errors associated the yield recovery, both of which could bias the information derived from the NMR spectra.

Table 5. Inter-unit Linkages and Main Units in Lignin

Unit	Extractive-free			Alkaline treated		
	Stem	Cob	Leaf	Stem	Cob	Leaf
β-O-4'	60	56	53	44	42	40
β-5'	27	28	29	43	40	43
β-β'	10	11	11	9	11	9
β-1'	3	5	7	4	7	8
γ-Acyl.	43	47	25	2	2	3
S/G	1.3	1.5	0.7	1.1	1.3	0.6
H/G	0.2	0.5	0.3	0.2	0.4	0.3

Note: assuming the sum of β-O-4', β-1', β-β', and β-5' was 100% linkages in lignin;
γ-Acyl.: γ-acylation

CONCLUSIONS

1. A remarkable amount of lignin was removed from corn stover by mild alkaline treatment. Significant amounts of *p*-coumaric acid and ferulic acid were identified and recovered in the extraction liquors, although the quantifications of them varied among stem, cob, and leaf.
2. The structural changes of lignin induced by the mild alkaline treatment were first elucidated by alkaline nitrobenzene oxidation. Because of the removal of parts of non-condensed lignin, *p*-coumarate, and ferulate esters, the lignin of the alkaline-treated samples gave lower yield of aldehydes, S/V, and H/V. It was demonstrated that the lignin of alkaline-treated samples was more condensed.
3. ^{1}H-^{13}C HSQC NMR is a sophisticated technique that can be used to characterize lignin intensively. Therefore, the occurrence of *p*-coumarate esters and ferulate esters in corn stover was confirmed; the variations of lignin of stem, cob, and leaf were characterized; and the structural changes in lignin induced by mild alkaline treatment were elucidated in this study. The increase of β-5' and the decrease of β-O-4' indicated lignin became more condensed with the alkaline treatment. The acylation degree of the γ-OH of lignin also decreased dramatically due to the saponification of *p*-coumarate esters induced by the alkaline treatment.

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