Structural Changes of Lignin in the Soda-AQ Pulping Process Studied Using the Carbon-13 Tracer Method

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To reveal the structural changes of lignin during the soda–AQ cooking process and chemical bonds between lignin and xylan of wheat straw, ^{13}C isotope-labeled technology was used in this study. First, ^{13}C isotope-labeled xylose was injected into the living wheat straw. The success of ^{13}C isotope-labeling was confirmed by the results of ^{13}C abundance determination. Then, milled wood lignin-^{13}C was extracted from wheat straw. The wheat straw, which had already been labeled by xylose-^{13}C, was cooked by the soda-AQ process. Soda-AQ Lignin-^{13}C and Residual Lignin-^{13}C were extracted from black liquor and residual pulp. FT-IR, ^{13}C-NMR, and 2D HSQC NMR analyses indicated that all lignin preparations were HGS-type lignin. The main lignin linkages were *β-O-4'* units, *β-β'* units, *β-5'* units, and *β-1* units, with the highest content of *β-O-4'*. Furthermore, 82.8% of *β-O-4'* units, 77.2% of *β-β'* units, 75.4% of *β-5'* units, and 75.4% of *β-1* units were degraded during the cooking process. LC bonds between lignin and xylan were at C_2 and C_5 positions of xylan. It was found that the C-2 position of xylan in wheat straw could be mainly connected to lignin by γ-ester bonds, and C-5 position of xylan in wheat straw was possibly linked with lignin by benzyl ether bonds.

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

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INTRODUCTION

Numerous studies have been carried out on the structure of lignin and lignin-carbohydrate complexes (LCCs). Using the traditional analytical methods to study the structure of lignin and LCC will inevitably cause some structural changes during the separation process. Furthermore, the separated products cannot be representative of the precise structure of the plant itself or the residual lignin and LCCs in the pulp. Common chemical degradation methods, such as oxidation (Watanabe and Koshijima 1988), acid-catalyzed hydrolysis (Eriksson *et al.* 1980), and alkali hydrolysis (Takakashi and Koshijima 1988), are unable to cleave all of the bonds connecting lignin and carbohydrates. In addition, it is likely that some unknown side reactions occur in the process of strong chemical decomposition. Therefore, traditional analytical methods cannot effectively determine the structural characteristics of lignin and LCC.

With the development of science and technology, more experimental technologies have been successfully used to study the structure of lignin and LCCs. Karlsson *et al.* (2001) applied molecular exclusion chromatography to isolate components from pine sulfate pulp after treatment with enzyme, and showed that it had characteristic absorption of lignin and carbohydrates in ultraviolet spectra, thus proving that chemical bonds

existed between lignin and sugar units. Then, Lawoko *et al.* (2003) adopted enzymolysis with chemical treatment methods to systematically fractionate unbleached softwood kraft pulp (fiber); the lignin and sugar content of the degraded products were also detected. The coexistence of lignin and sugar in some fractions indirectly showed the presence of bonds between lignin and carbohydrates in softwood kraft pulp. Although researchers have determined there are covalent bonds connecting lignin and carbohydrates, it is still unknown what types of bonds bind these components together.

In the 1980s, ^{13}C-NMR technology began to be widely used in the study of the bonds connecting lignin and lignin-carbohydrates complexes. However, lignin macro-molecules and carbohydrate compounds are extremely complicated. It is impossible to distinguish the bonds connecting lignin and carbohydrates from the ordinary ^{13}C-NMR spectra without the additional use of special analytical methods, such as ^{13}C isotope labeling techniques. The use of isotope labeling technology can overcome the chemical destruction of the native structure of LCCs in plants during isolation, making the results more accurate and representative. Some researchers (Su *et al*. 2002; Xie *et al*. 2003; Wang *et al*. 2006; Gu *et al*. 2002a,b) have used isotope-labeled coniferin as a lignin precursor to bio-synthesize dehydrogenated polymers (DHPs) with xylan, holo-cellulose, cellulose, or pectin. It was found that lignin and glycan can form LCCs, which were connected by ether, ester, and acetal bonds, as detected by ^{13}C-NMR. On this basis, various investigators (Yang *et al.* 2004; Gu *et al.* 2002a,b; Xie and Terashima 1991; Xie *et al.* 2000) have successfully used the ^{13}C isotope tracer technique combined with ^{13}C-NMR to analyze lignin and LCC bonds in plants such as straw and ginkgo. Recently, 2D-HSQC has been commonly used to analyze the structure of lignin and LCC, which can overcome the overlapping problem from lignin and carbohydrate. But, this approach evaluates only relative proportions of various signals and is unable to provide with the absolute values. A combination of HSQC and ^{13}C-NMR, as suggested by Zhang and Gellerstedt (2007), is a method to obtain the absolute values of various signals. This method is very effective to analyze the content of various lignin structures and has been widely applied (Balakshin *et al*, 2011; Wen *et al*, 2013a,2013b,2013c; Yuan *et al*. 2011).

In this study, ^{13}C isotope-labeled xylose was efficiently injected into living wheat straw, according to the results of ^{13}C abundance detection. The structural characteristics of ML extracted from wheat straw were studied using the ^{13}C-NMR spectra technique. Then, ^{13}C isotope-labeled wheat straw was pulped using the soda-AQ method. Finally, the changes in lignin before and after cooking were analyzed by FT-IR, ^{13}C-NMR and 2D-HSQC.

EXPERIMENTAL

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

A conventional variety of wheat straw, *Triticum aestivum* E-mai 352, was planted in the field in March 2012. A mixed 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 2nd, 3rd, and 4th sections of the internodes (starting from the root) of the wheat straw within 30 days. After the injection, the wheat straw was allowed to grow for 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, then ground and sieved to obtain an 80- to 100-mesh fraction. This fraction was subjected to extraction with benzene/ethanol (2:1, v/v) in a Soxhlet apparatus for 6 h and hot water successively. Finally, the extractive-free wheat straw powder was freeze-dried.

Preparation of ML (^{13}C Isotope-Labeled Xylose Wheat Straw and Natural Wheat Straw)

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 prepare the ML using the procedure of Björkman (1956).

Preparation of Soda-AQ Lignin and Residual Lignin

Soda-AQ pulping was performed under the following conditions: 15% NaOH, 0.05% AQ, 5:1 liquor-to-wheat straw ratio. Wheat straw black liquor lignin was obtained by acid precipitation (Yuan *et al*. 2009). Residual lignin was prepared and purified according to the procedures of Kapareju and Wu (Kaparaju and Felby 2010; Wu and Argyropoulos 2003).

^{13}C Abundance Determination

Sample (0.1 mg) stable isotopic values ($\delta^{13}C$) were analyzed in duplicate, using a continuous flow system isotope ratio mass spectrometry (Isoprime 100) coupled with an elemental analyzer (Vario Pyro Cube), after being wrapped in a tin boat. The average standard deviations of the measurements were ±0.15‰ for $\delta^{13}C$. Values of $\delta^{13}C$ are expressed in standard delta notation relative to the Pee Dee Belemnite (PDB) standard. The sample $\delta^{13}C$ was corrected using the two-point correction method according to the international standards IAEA-CH3 and IAEA-601. The ^{13}C abundances (%) of samples were calculated using the $\delta^{13}C$ values and the $^{13}C/^{12}C$ ratio of the PDB standard ($^{13}C/^{12}C = 11237.2\pm90*10^{-6}$).

^{13}C-NMR Spectroscopy

All NMR spectra were recorded on a Bruker AVIII 400 MHz spectrometer operated at 25 °C utilizing DMSO-d_6 as the solvent. For quantitative ^{13}C NMR, 125 mg of the lignin was dissolved in 0.5 mL of DMSO-d_6. The quantitative ^{13}C NMR spectra were recorded in the FT mode at 100.6 MHz. The inverse-gated decoupling sequence, which allows quantitative analysis and comparison of the signal intensities, was used with the following parameters: 30° pulse angle; 1.4-s acquisition time; 2-s relaxation delay; 64,000 data points; and 30,000 scans. Chromium (III) acetylacetonate (0.01 M) was added to the lignin solution to provide complete relaxation of all nuclei.

2D-HSQC Spectroscopy

Lignin (60 mg) was dissolved in 0.5 mL of DMSO-d_6. 2D HSQC NMR spectra were recorded in the HSQC experiments. 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.

RESULTS AND DISCUSSION

$^{13}C/^{12}C$ Isotopic Ratio

The ^{13}C content (Vienna Pee Dee Belemnite (VPDB) $\delta^{13}C$ scale) of the xylose-$^{13}C_5$ (all five carbons in xylose were isotope-labeled) enriched wheat straw was significantly higher than the control (Table 1). These results showed that with the AOPP and coniferin, the xylose-$^{13}C_5$ was successfully introduced into the wheat straw and took part in the plant's normal metabolism.

Table 1. ^{13}C Abundance of ^{13}C Isotope-Labeled Wheat Straw

Sample	^{13}C scale (VPDB)	^{13}C%
^{13}C-X5 enriched wheat straw	43.73	1.1503
control	0.12	1.1087

FT-IR Determination of Lignin Samples

The FT-IR spectra of the lignin samples are shown in Fig. 1. The spectrum of the ML-^{13}C was quite similar to that of ML, soda-AQ lignin, and residual lignin. The signals at 1600 cm^{-1}, 1510 cm^{-1}, and 1420 cm^{-1}, corresponding to the aromatic ring (Yang *et al.* 2013), were obvious in the spectra, which indicated that the basic lignin structure was not changed during the cooking process.

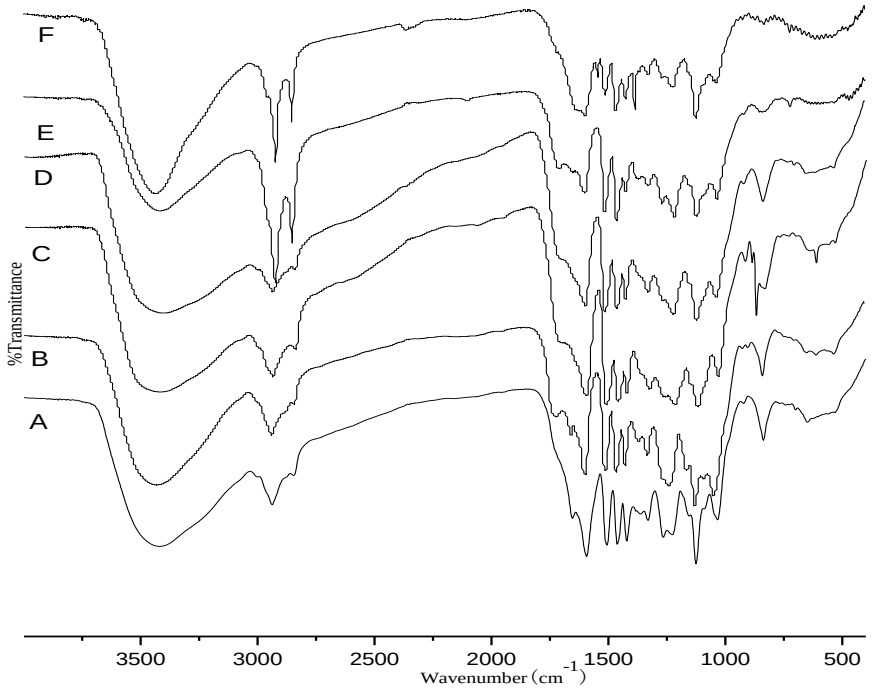

Fig. 1. FT-IR spectra of lignin samples (A-ML; B-ML-^{13}C; C-Soda-AQ Lignin; D-Soda-AQ Lignin-^{13}C; E-Residual Lignin ; F-Residual Lignin-^{13}C; ML was extracted from wheat straw; Soda-AQ lignin was extracted from black liquor; Residual lignin was extracted from pulp.)

The signal at 1740 cm^{-1} was from non-conjugated carbonyl groups (Faix 1991). In lignin samples, that was mainly from the carboxyl or ester linkage of Cγ of lignin side chain. The intensity of this signal in ML-^{13}C was stronger than that of ML. It may be due to the length of time of ball milling during ML extraction, resulting in oxidation of partial structures. Furthermore, by comparing the six spectra, it was found that residual lignin had the lowest content of non-conjugated carbonyl groups, indicating the dissolution of most of this structure in black liquor.

The FT-IR spectra of ML and ML-^{13}C were almost identical. Thus, it was concluded that the injection of xylose-$^{13}C_5$ did not interfere with the normal metabolism of wheat straw. Compared with soda-AQ lignin, the signals at 2920 and 2850 cm^{-1} from the C-H stretching vibration of methyl and methylene units were intensified in the residual lignin, indicating the breakdown and rearrangement of chemical bonds between lignin monomer units. The signal from H-type lignin was at 834 cm^{-1}. The intensity of this signal was also reduced in residual lignin, compared with that of ML and soda-AQ lignin, indicating the dissolving of H-type lignin during the cooking process.

^{13}C NMR Determination of Lignin Samples

^{13}C-NMR spectra of lignin samples are shown in Fig. 2. The region between 160 and 103 ppm was used as a reference to compare other signal shifts.

In the ^{13}C-NMR spectra of lignin samples, it was easy to find the signals from *p*-coumaric acid (Scalbert *et al*. 1985) at 167.1 ppm (C_9), 160.6 ppm (C_4), 130.9 ppm (C_2/C_6), 125.6 ppm (C_1), and 116.5ppm (C_3/C_5). The weak signal between 90 and 102 ppm indicated the low carbohydrate content in lignin samples.

The signals assigned to guaiacyl (Sun *et al*. 2005) were at 149.7 ppm (C_3, etherified), 148.1 ppm (C_3), 145.9 ppm (C_4, etherified), 119.9 ppm (C_6), 115.3 ppm (C_5), and 111.6 ppm (C_2). The strong signals from syringyl (Kim and Ralph 2010) at 152.8 ppm (C_3/C_5, etherified), 138.7 ppm (C_4, etherified), 134.8 ppm (C_1, etherified), 133.6 ppm (C_1, non-etherified), 106.9 ppm (C_2/C_6, S with α-CO), and 104.8 ppm (C_2/C_6) could also be seen. The signal from C_2/C_6 of *p*-hydroxyphenyl (H) was at 128.6 ppm (Wen *et al*. 2013a). Based on these signals, it could be concluded that the lignin of wheat straw was HGS type.

In the 90 to 50 ppm region, the main signals were from methoxyl (-OCH_3) at 56.5 ppm and *β*-O-4', *β*-*β*', and *β*-5' units. The signals assigned to *β*-O-4' (Nimz *et al*. 1981) were at 85.7 ppm (C_β in S type *β*-O-4' units) and 84.2 ppm (C_β in G type *β*-O-4' units), 72.8 ppm (C_α, *β*-O-4'), 63.8 ppm (C_γ in G type *β*-O-4' units with α-C=O), and 60.7 ppm (C_γ, *β*-O-4'). The signals at 84.3 ppm and 71.1ppm were from C_α and C_γ of *β*-*β*' (Sun *et al.* 1996). And the signal of C_α from *β*-5' was at 86.7 ppm.

The intensities of some signals in ML-^{13}C were stronger than those of ML. Examples include the signals at 101.8 ppm (C_1 of xylan), 75.4 ppm (C_4 of xylan), 74.0 ppm (C_3 of xylan), 73.4 ppm (C_2 of xylan), and 62.5 ppm (C_5 of xylan). Compared with soda-AQ lignin, there were differences of signals from C_1-C_5 of xylan at 101.4 ppm (C_1), 75.1 ppm (C_4), 73.7 ppm (C_3), 72.5 ppm (C_2), and 62.9 ppm (C_5) for soda-AQ lignin-^{13}C. The increased signals indicated that xylose-$^{13}C_5$ had been successfully introduced into wheat straw and synthesized to xylan. It was also found that the locations of signals of C_2, C_5 from xylan in ML-^{13}C, and C_5 of xylan in soda-AQ lignin-^{13}C were different from the purified xylan (Cheng 2011). This indicated that C_2 and C_5 of xylan were possibly linked with lignin by benzyl ether bonds or γ-ester bonds in wheat straw. As is well known, γ-

ester linkages are unstable in the process of alkali treatment. Therefore, it was suggested that C_2 position of xylan in wheat straw could be mainly connected to lignin by γ-ester bonds, and the C_5 position of xylan in wheat straw was possibly linked with lignin by benzyl ether bonds.

In comparison to ML, some signals of the soda-AQ lignin and residual lignin were weakened. The signals at 72.8, 85.7, and 60.7 ppm from α, *β*, γ of *β*-O-4' units were all weakened (Fig. 2). The chemical shifts between 103.6 and 163 ppm were set as 600, the corresponding values of α, *β*, γ of *β*-O-4' units for ML, soda-AQ lignin, and residual lignin were then obtained as 17.08, 2.99, 29.30 (ML), 11.37, 0.34, 13.07 (soda-AQ lignin), and 6.04, 0.48, 3.71 (residual lignin), respectively. These data indicated that the degradation of *β*-O-4' units were the main reaction during soda-AQ cooking process, with most of *β*-O-4' units dissolving in black liquor, minor left in residual pulp. Meanwhile, the intentions of signals from C_α of *β*-*β*' units and *β*-5' units were also decreased from 1.85, 16.61 (ML) to 0.44, 2.05 (soda-AQ lignin) and 0.50, 2.82 (residual lignin), indicating of the degradation of the two structures in the process of pulping.

The intensities of the signals at 10 to 34 ppm from the aliphatic side chain of the phenylpropane units were the strongest in the residual lignin. Aliphatic carbon signals may come from the degradation of *β*-aryl ether linkages or from the condensation reaction of methyl and methylene units. This result was in accordance with the analysis of FT-IR spectra.

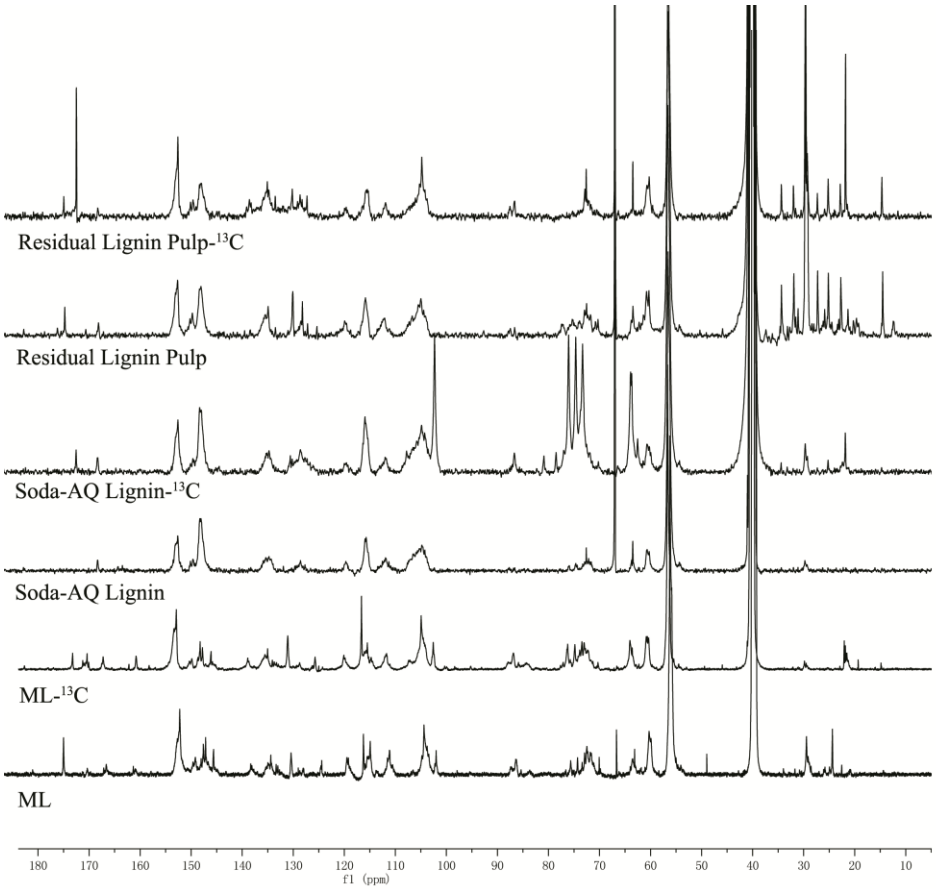

Fig. 2. ^{13}C-NMR spectra of lignin samples

Analysis of 2D-HSQC Spectra of Lignin Samples

The 2D-HSQC spectra of lignin samples were shown in Fig. 3. Main substructures of lignins of wheat straw are shown in Fig. 4. The assignments of various signals are shown in Table 2. The main signals of the aliphatic side chains (δC/δH 50-90 /2.5-6.0) were from -OCH_3 (δC/δH 55.6 /3.69) and *β-O*-4' unit. The signal from C_γ-H_γ of the *β-O*-4' structure of the lignin samples was approximately at δC/δH 60.6/3.41 (Rencoret *et al.* 2009). Acylated C*γ* from *β-O*-4' structures of ML and of residual lignin was at δC/δH 63.8/4.14. However, this signal was not observed in soda-AQ lignins. The C_α-H_α signal from *β-O*-4' structures was at δC/δH 72.3/4.81 (Del *et al.* 2012a,) for all lignin, and the signals from C_β-H_β were at δC/δH 86.1/4.08 (*β-O*-4' connected with a S unit) and δC/δH 84.3/4.29 (*β-O*-4'connected with a G unit). The C_α-H_α signal from *β-β*' structures (Martinez *et al*. 2008) was at approximately δC/δH 84.3/4.64, while the signals of C_γ-H_γ were at δC/δH 71.4/4.17 and δC/δH 71.8/3.80. The signals from *β*-5' structures were obvious to see in all lignin samples' spectra. C_α-H_α of *β*-5' was at δC/δH 87.1/5.45 (Yuan *et al*. 2013). The C_γ-H_γ signal (δC/δH 62.5/3.73) of *β*-5' structures was overlapped with the signals of C_5-H_5 from xylan. The C_α-H_α signals from *β*-1' structures were at δC/δH 81.8 /5.05 (Del *et al*. 2012b; Wen *et al*. 2013c) in the residual lignin-^{13}C. Furthermore, the signals at δC/δH 10-30/0.5-1.5 of the residual lignin were from methyl and methylene groups. It can also be seen clearly that there were signals from C_1-C_5 of xylan in all lignins, which were located at δC/δH 101.8/4.28, δC/δH 72.8/3.06, δC/δH 74.0/3.27, δC/δH 75.4/3.52, δC/δH 62.7/3.41, respectively.

In the 2D-HSQC spectra of the lignin samples, the $C_{2/6}$-$H_{2/6}$ signal from S units (Yuan *et al.* 2011; Wen *et al.*, 2013c) was at δC/δH 104.3/6.66, and the $C_{2/6}$-$H_{2/6}$ signal of C_α-oxidized S units was at δC/δH 106.4/7.22. C_2-H_2, C_5-H_5, and C_6-H_6 signals from G units were at δC/δH 111.4/6.94, δC/δH 116.0/6.73, and δC/δH 118.6/6.75, respectively (Del *et al*. 2012b). In the 2D-HSQC spectra of ML and soda-AQ lignin, the signal from C_2-H_2 of (FA_2) (Wen *et al.* 2013c) was found at δC/δH 111.7/7.31. This result was consistent with the results of FI-IR that the ferulic acid structure from wheat straw was dissolved in the black liquor in the process of pulping. The $C_{2,6}$-$H_{2,6}$ signal from H units was at δC/δH 127.5/7.02 and $C_{3,5}$-$H_{3,5}$ signal from H units was overlapped with the C_5-H_5 signal from G units. The relative content of H-type lignin in ML, soda-AQ lignin, and residual lignin were 15.79%, 10.42%, 5.81% respectively, indicating the degradation of H-type lignin during cooking process. Besides, the signals from *p*-coumaric acid ($PCA_{2,6}$), end groups of cinnamyl alcohol and end groups of cinnamaldehyde (J) were also found.

Conclusions from the 2D-HSQC spectra analysis results are as follows: the main substructures of lignin were H, G, and S units; and that the main lignin linkages were *β-O*-4' units, *β-β*' units, *β*-5' units, and *β*-1' units.

From the quantitative analysis of the ^{13}C-NMR combination with 2D-HSQC, the absolute contents of β-O-4' units, β-β' units, β-5' units and β-1' units were obtained (Wen *et al*. 2013b). The four substructures contents for ML, soda-AQ lignin, and residual lignin were shown in Table 3. The results showed that the highest content substructure was β-O-4' units, then was β-β' units, with minor content of β-5' units and β-1' units. Furthermore, it was found that 82.8% of *β-O*-4' units, 77.2% of *β-β*' units, 75.4% of *β*-5' units, and 75.4% of *β*-1' units were degraded during the cooking process, and 15.8% of *β-O*-4' units, 17.3% of *β-β*' units, 22.8% of β-5' units, and 18.0% of β-1' units were left in the residual pulp.

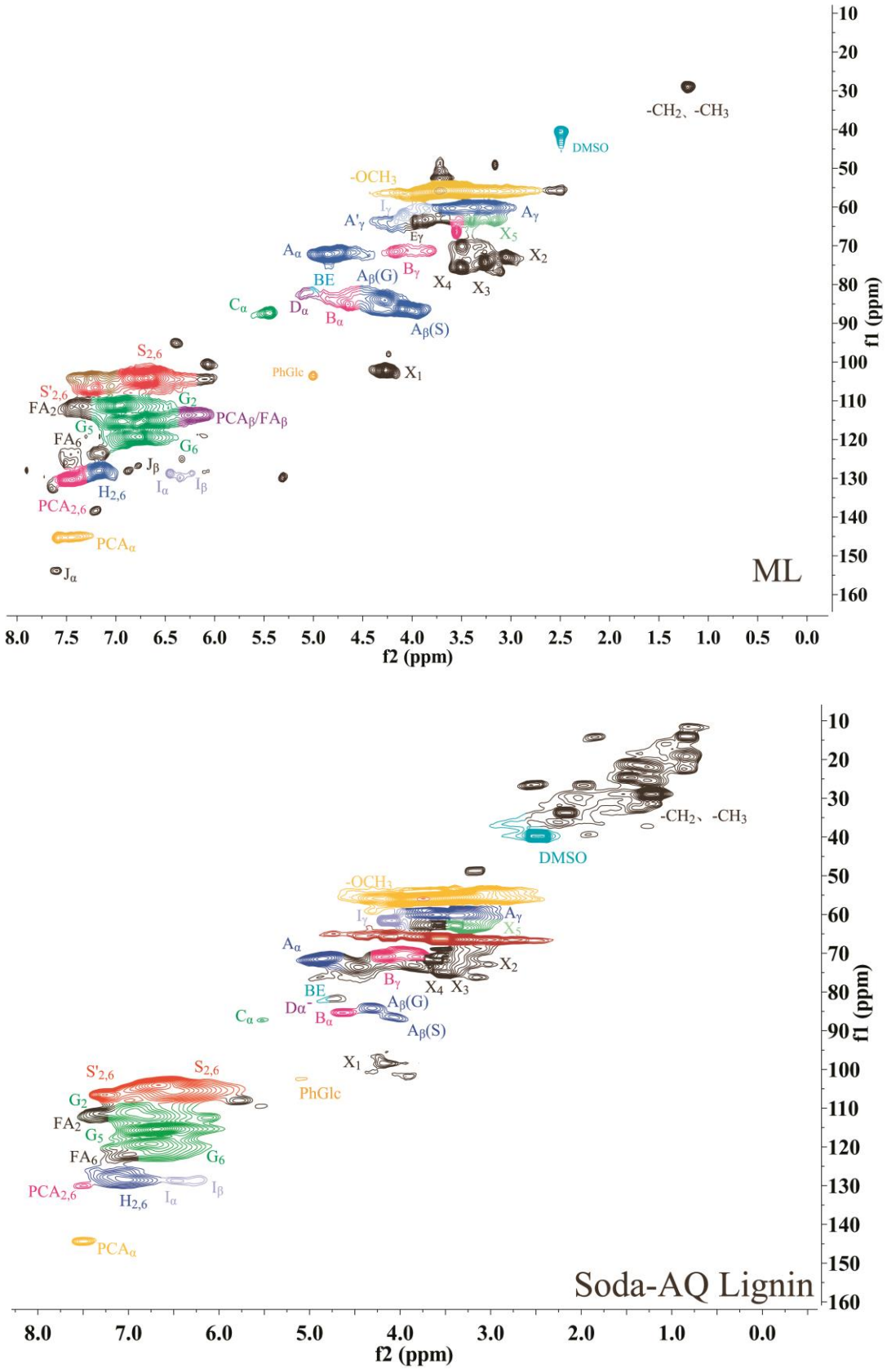

Fig. 3 (top two). 2D HSQC NMR spectra of lignin samples (ML, Soda-AQ lignin, Residual lignin)

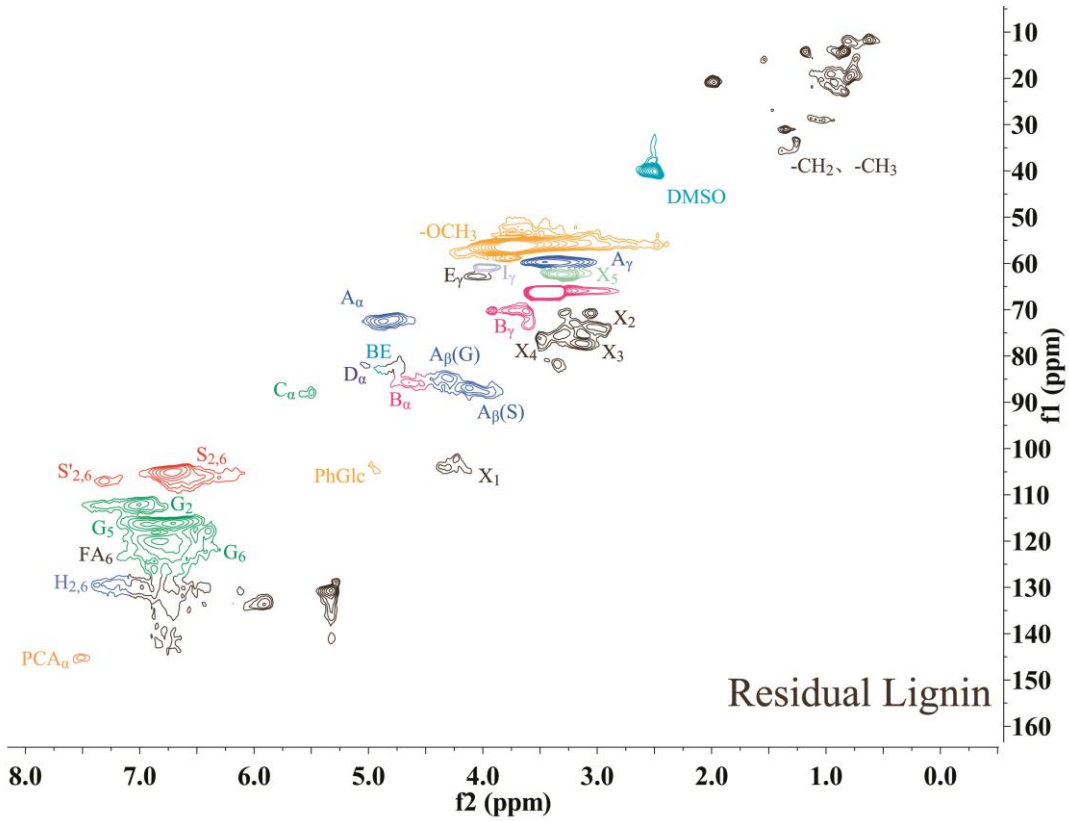

Fig. 3. 2D HSQC NMR spectra of lignin samples (ML, Soda-AQ lignin, Residual lignin)

From Table 3, it was found that the S/G ratio of the lignin samples were 1.28 (ML), 1.42 (Soda-AQ lignin), and 1.41 (Residual lignin).

The other method of calculating the S/G ratio was from analysis of ^{13}C-NMR (Wen *et al*. 2013c). The chemical shifts between 103 and 160 ppm were set as 600, while the corresponding values of methoxyl (55-57 ppm) for the lignin samples were 457.29/600 (ML), 519.63/600 (soda-AQ lignin), and 407.23/600 (residual lignin), respectively.

The results from the two methods were coincident. The S/G ratio of soda-AQ lignin and residual lignin were higher than that of ML, indicating that it was easier to dissolve G. That was due to more methoxyl groups of the S-type lignin than G-type lignin, resulting in more stereospecific blockade.

Table 2. Assignments of the Lignin ^{13}C-^{1}H Correlation Peaks in the 2D HSQC NMR Spectra of lignin samples (ML, Soda-AQ lignin, Residual lignin)

Label	δC/δH(ppm)	Assignment
-OCH_3	55.6/3.69	C-H in methoxyls
Aγ	60.6/3.41	Cγ-Hγ in γ-hydroxylated *β*-O-4' substructures
Iγ	61.9/4.09	Cγ-Hγ in cinnamyl alcohol end-groups
A'γ	63.8/4.14	Cγ-Hγ in γ-acylated *β*-O-4' substructures
Aα	72.3/4.81	Cα-Hα in *β*-O-4' substructures linked to a G(S) unit
Bγ	71.4/4.17 71.8/3.80	Cγ-Hγ in *β*-*β*' resinol substructures
Bα	84.3/4.64	Cα -Hα in *β*-*β*' resinol substructures
Cα	87.1/5.45	Cα-Hα in phenylcoumaran substructures
Dα	81.8/5.05	Cα-Hα in spirodienone substructures
A_{β}(G)	84.3/4.29	C_{β}-H_{β} in *β*-O-4' substructures linked to a G unit
A_{β}(S)	86.1/4.08	C_{β}-H_{β} in *β*-O-4' substructures linked to a S unit
X_1	101.8/4.28	C_1-H_1 in *β*-D-xylopyranoside
X_2	72.8/3.06	C_2-H_2 in *β*-D-xylopyranoside
X_3	74.0/3.27	C_3-H_3 in *β*-D-xylopyranoside
X_4	75.4/3.52	C_4-H_4 in *β*-D-xylopyranoside
X_5	62.7/3.41	C_5-H_5 in *β*-D-xylopyranoside
$S_{2,6}$	104.3/6.66	C_2-H_2 and C_6-H_6 in etherified syringyl units
$S'_{2,6}$	106.4/7.22	C_2-H_2 and C_6-H_6 in oxidized(CαOOH) syringyl units
G_2	111.4/6.94	C_2-H_2 in guaiacyl units
G_5	116.0/6.73	C_5-H_5 in guaiacyl units
G_6	118.6/6.75	C_6-H_6 in guaiacyl units
FA_2	111.7/7.31	C_2-H_2 in ferulate
PCA_{β}/FA_{β}	113.6/6.28	C_{β}-H_{β} in *p*-coumarate and ferulate
FA_6	125.3/7.14	C_6-H_6 in ferulate
$H_{2,6}$	127.5/7.02	$C_{2,6}$-$H_{2,6}$ in p-hydroxyphenyl units
$PCA_{2,6}$	130.8/7.45	C_2-H_2 and C_6-H_6 in p-coumarate
I_{β}	129.7/6.35	C_{β}-H_{β} in cinnamy alcohol end-groups
Iα	128.7/6.46	Cα-Hαin cinnamy alcohol end-groups
J_{β}	126.6/6.78	C_{β}-H_{β} in cinnamyl aldehyde end-groups
Jα	153.7/7.61	Cα-Hα in cinnamyl aldehyde end-groups
PCAα	145.3/7.59	Cα-Hα in *p*-coumarate
BE	80.6/5.08	Benzyl ether LCC linkages
Eγ	63.6/4.07	γ-Ester LCC linkages
PhGlc	102.2/5.03	Phenyl glycoside linkages

Table 3. Quantitative Characteristics of the Lignin Preparations from Quantitative NMR Method

Sample	*β*-*O*-4' (%)	*β*-*β*' (%)	*β*-5'(%)	*β*-1' (%)	S/G
ML	24.24	2.72	1.67	0.61	1.28
Soda-AQ lignin	0.36	0.15	0.03	0.04	1.42
Residual lignin	3.82	0.47	0.38	0.11	1.41

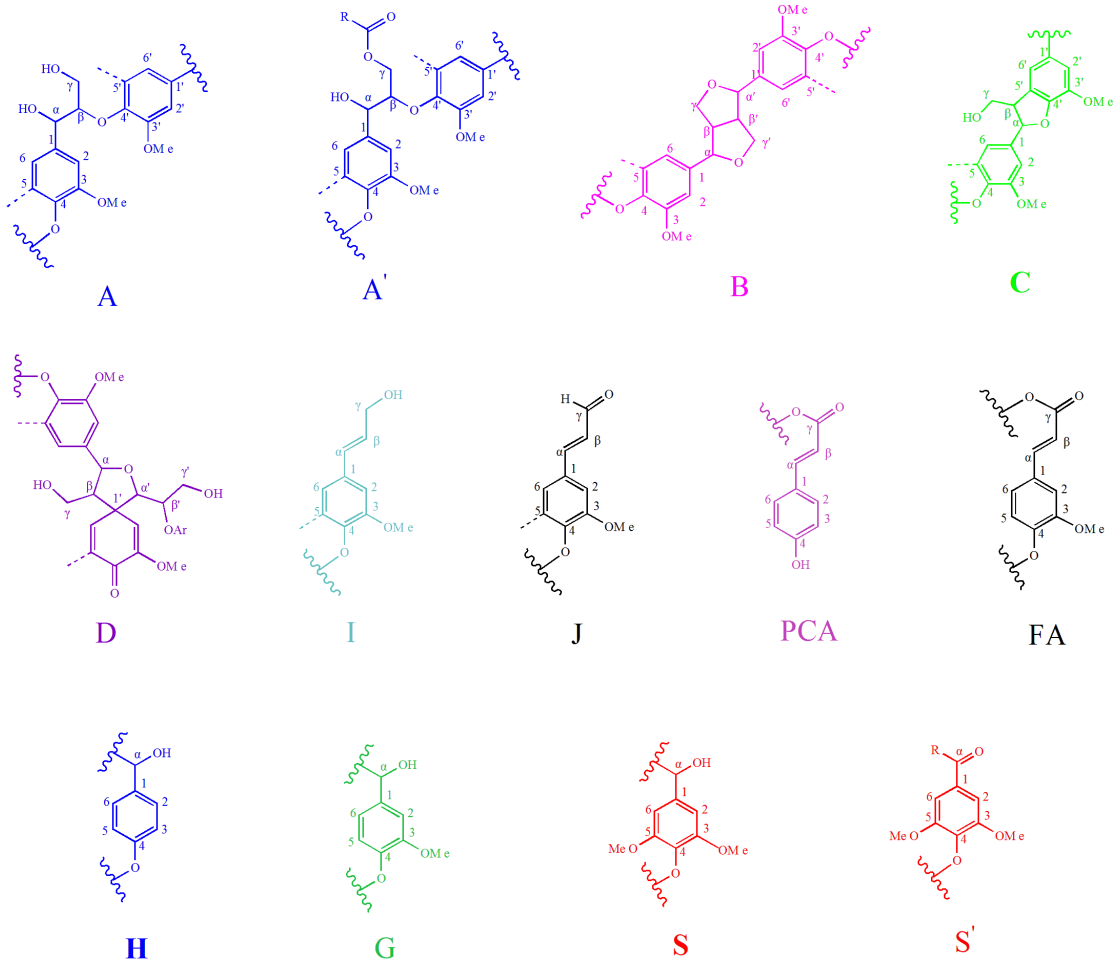

Fig. 4. Main structures present in the lignins of wheat straw: (A) *β*-O-4' alkyl-aryl ethers; (A') *β*-O-4' alkyl-aryl ethers with acylated γ-OH; (B) resinols; (C) phenylcoumarane; (D) spirodienones; (I)cinnamyl alcohol end-groups; (J) cinnamyl aldehyde end-groups; (PCA)p-coumarates; (FA) ferulates;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

CONCLUSIONS

1. ^{13}C abundance detection showed that ^{13}C isotope-labeled xylose was incorporated into the wheat straw *in vivo* and took part in the plant's normal metabolism.
2. The analysis results from FT-IR showed the lowest content of p-hydroxyphenyl units in residual lignin, indicating that it was the easiest for p-hydroxyphenyl units to dissolve in black liquor during cooking process.
3. By analyzing the ^{13}C-NMR spectra of ML-^{13}C and Soda-AQ lignin-^{13}C, it was found that the C_2 position of xylan in wheat straw could be mainly connected to lignin by γ-ester bonds, and C_5 position of xylan in wheat straw was possibly linked with lignin by benzyl ether bonds.
4. From the analysis results from ^{13}C-NMR combination with 2D-HSQC, it was concluded that the main sub-structures of lignin were H, G, and S units and that the main lignin linkages were *β-O*-4', *β-β*', *β*-5', and *β*-1, with the highest content of β-O-4'. Furthermore, it was found that most of *β-O*-4', *β-β*', *β*-5', and *β*-1 were dissolved in black liquor during cooking process, with minor left in residual pulp. From the results of S/G ration, it indicated of easier dissolving of G than S during cooking process.

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REFERENCES CITED

Balakshin, M., Capanema, E., Gracz, H., Chang, H., and Jameel, H. (2011). "Quantification of lignin–carbohydrate linkages with high-resolution NMR spectroscopy," *Planta* 233(6), 1097-1110.

Björkman, A. (1956). "Studies on finely divided wood. Part 1. - Extraction of lignin with neutral solvent," *Svensk Papperstidn.* 59(13), 477-485.

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

Del Río, J. C., Prinsen, P., Rencoret, J., Nieto, L., Barbero, J. J., Ralph, J., Martínez, A. T., Ralph, J., 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, 3169-3634.

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(14), 5922-5935.

Eriksson, Ö., Goring, D. A. I., and Lindgren, B. O. (1980). "Structural studies on the chemical bonds between lignin and carbohydrates in spruce," *Wood Sci. Technol.* 14(4), 267-279.

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

Gu, R. J., Xie, Y.-M., Wu, H., and Lai, Y.-M. (2002a). "Carbon-13 enrichment of dehydrogenation polymers of monolignols traced by solid-state ^{13}C nuclear magnetic resonance," *Chem. Ind. Forest Prod.* 22(1), 1-6. (Chinese)

Gu, R. J., Xie, Y.-M., Zeng, S. Q., Wu, H., and City, Y. (2002b). "Carbon-13 enrichment of rice stalk lignin traced by solid state ^{13}C NMR spectroscopy," *Chem. J. Chinese U.* 23(6), 1073-1076. (Chinese)

Kaparaju, P., and Felby, C. (2010). "Characterization of lignin during oxidative and hydrothermal pre-treatment processes of wheat straw and corn stover," *Bioresour. Technol.* 101(9), 3175-3181.

Kaparaju, P., and Felby, C. (2010). "Characterization of lignin during oxidative and hydrothermal pre-treatment processes of wheat straw and corn stover," *Bioresour. Technol.* 101(9), 3175-3181.

Karlsson, O., Pettersson, B., and Westermark, U. (2001). "The use of cellulases and hemicellulases to study lignin-cellulose as well as lignin-hemicellulose bonds in kraft pulps," *J. Pulp Pap. Sci.* 27(6), 196-201.

Kim, H., and Ralph, J. (2010). "Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d_6/pyridine-d_5," *Org. Biomol. Chem.* 8(3), 576-591.

Lawoko, M., Henriksson, G., and Gellerstedt, G. (2003). "New method for quantitative preparation of lignin-carbohydrate complex from unbleached softwood kraft pulp: Lignin-polysaccharidenn," *Holzforschung* 57(1), 69-74.

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.

Nimz, H. H., Robert, D., Faix, O., and Nemr, M. (1981). "Carbon-13 NMR spectra of lignins. 8: Structural differences between lignins of hardwood, softwoods, grasses and compression wood," *Holzforschung* 35(1), 16-26.

Rencoret, J., Marques, G., Gutiérrez, A., Nieto, L., Santos, J. I., Jiménez-Barbero, J., Martínez, Á. T., and del Río, 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.

Scalbert, A., Monties, B., Lallemand, J. Y., Guittet, E., and Rolando, C. (1985). "Ether linkage between phenolic acids and lignin fractions from wheat straw," *Phytochemistry* 24(6), 1359-1362.

Su, Q., Xie, Y. M., Gu, R. J., Hu, Z., Wu, H., and Yang, H. (2002). "Study on lignin-carbohydrate complexes from monolygnol (II) – Synthesis of DHP from micro crystalline cellulose and coniferin," *Paper Sci. Technol.* 21(5), 9-11. (Chinese)

Sun, R. C., Lawther, J. M., and Banks, W. B. (1996). "Effects of extraction time and different alkalis on the composition of alkali-soluble wheat straw lignins," *J. Agric. Food Chem.* 44(12), 3965-3970.

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.

Takakashi, N., and Koshijima, T. (1988). "Ester linkages between lignin and glucuronoxylan in lignin-carbohydrate complex from beech (*Fagus crenata*) wood," *Wood Sci. Technol.* 22(3), 231-241.

Wang, P., Xie, Y. M., and Fan, J. Y. (2006). "Study on pectin-lignin complexes from monolignols by carbon-13 isotope tracer method," *J. Shanxi U. Sci. Technol.* 24, 17-23 (Chinese).

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

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

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.

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, 332-343.

Wu, S., and Argyropoulos, D .S. (2003). "An improved method for isolating lignin in high yield and purity," *J. Pulp Pap. Sci.* 29(7), 235-240.

Xie, Y. M., Gu, R. J., and Watanabe, T. (2003). "Studies on the linkages between dehydrogenation polymer and cell wall polysaccharide by carbon-13 isotopic tracer method," *12th International Symposium on Wood and Pulping Chemistry*, Madison, WI, USA, pp. 207-210.

Xie, Y. M., and Terashima, N. (1991). "Selective carbon-13 enrichment of side carbons of ginkgo lignin traced by carbon-13 nuclear magnetic resonance," *Mokuzai Gakkaishi* 37(10), 935-941.

Xie, Y. M., Yasuda, S., Wu, H., and Liu, H. (2000). "Analysis of the structure of lignin-carbohydrate complexes by the specific ^{13}C tracer method," *J. Wood Sci.* 46(2), 130-136.

Yang, H. T., Xie, Y. M., and Yang, Y. S. (2004). "The structure of rice stalk lignin analyzed by tracer isotope carbon-13 and carbon-13 nuclear magnetic resonance," *Paper Sci. Technol.* 23(1), 1-5. (Chinese)

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

Yuan, T.-Q., He, J., and Sun, R.-C. (2009). "Fractionation and physico-chemical analysis of degraded lignins from the black liquor of *Eucalyptus pellita* KP-AQ pulping," *Polym. Degrad. Stab.* 94(7), 1142-1150.

Yuan, T. Q., Sun, S.-N., Xu, F., Gu, R., 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.

Yuan, T. Q., You, T. T., Wang, W., and Sun, R. C. (2013). "Synergistic benefits of ionic liquid and alkaline pretreatments of poplar wood. Part 2: Characterization of lignin and hemicelluloses," *Bioresour. Technol.* 136(5), 345-350.

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

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