

Chemical Elucidation of Structurally Diverse Willow Lignins

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A new fast-growing wood raw material, willow (*Salix matsudana* cv. Zhuliu), was subjected to pulping to identify the structure of its lignin. Thus, the black liquor lignin (AL) and enzymatic mild acidolysis lignin (EMAL) were prepared, and their molecular structure and molecular weight of the isolated lignin polymers were comprehensively investigated by Fourier transform infrared spectroscopy (FT-IR), two-dimensional nuclear magnetic resonance (2D-NMR HSQC), ¹³C nuclear magnetic resonance (¹³C-NMR), and gel permeation chromatography (GPC). The NMR results showed that syringyl (S) unit was the predominant structural monomeric unit in willow lignin, as opposed to guaiacyl (G) and *p*-hydroxyphenyl (H) units. The S/G ratio for the EMAL was found to be 2.02, whereas that for the AL was 0.94. The lignin in the black liquor (AL) fraction was modified during pulping, as shown by its reduced molecular weight. The two isolated lignin polymers, EMAL and AL showed low weight-average molecular weight: 4127 g/mol and 3522.5 g/mol, and in addition they exhibited low polydispersity index (Mw/Mn < 2.0).

Keywords: Lignin; FT-IR; ¹³C-NMR; 2D-NMR; GPC

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INTRODUCTION

Recently, willow has been identified as a potential wood substrate because of the global shortage of various forest resources (Klašnja *et al.* 2005; Barimani *et al.* 2014). Willow not only has a good potential for the pulp and paper industry because it is fast-growing, but it also has great potential for biomass refining (Krzyżaniak *et al.* 2014). Indeed, more focus had been directed to the “biorefinery” based on fast-growing hardwood (Ayrilmis and Kaymakci 2014). A wider application for it in the future therefore demands a deeper understanding of its lignin structure and its ensuing chemistry reaction (Wu *et al.* 2015; Wen *et al.* 2015).

Lignin is the second most abundant natural biological macromolecule in the biosphere, and is isolated as a byproduct of wood pulping. It is an aromatic polymer widely present in plants and amorphous in nature, containing oxo-benzene-propanol or its derivatives as structural units (Boerjan *et al.* 2003). The main structural unit in lignin can be divided into three distinct units: syringyl units (S-lignin); guaiacyl units (G-lignin); and *p*-hydroxyphenyl units (H-lignin). Different preparation methods yield lignin with different chemical compositions and structures (Rencoret *et al.* 2009).

In today’s pulp industry, black liquor is being burned to obtain energy to run the pulp mill. However, more and more researchers are considering other applications for conversion of lignin to energy; for example, lignin has been considered as a replacement

for phenol in phenolformaldehyde resins (Tejado *et al.* 2007, 2008), vanillin production (Araújo *et al.* 2010; Pinto *et al.* 2010), synthetic tanning agents production (Suparno *et al.* 2005), a biosorbent for heavy metals (Yun *et al.* 2008; Hengky *et al.* 2009), and dyes (Suteu *et al.* 2009; Saad *et al.* 2012).

FitzPatrick *et al.* (2010) pointed out that biomass has a complex composition, similar to petroleum, and its primary fractionation can yield a wide range of products. Nuclear magnetic resonance (NMR) spectrometry is one of the available techniques that offer a deeper knowledge of lignin structure. In particular, 2D-NMR provides higher resolution and the ability to distinguish between different structural units (Ämmälahti *et al.* 1998). The 2D-NMR technique has been employed widely to characterize residual lignins (Balakshin *et al.* 2003; Rencoret *et al.* 2009; Rutkowska *et al.* 2009; Yuan *et al.* 2011) and to a lesser extent to characterize dissolved lignin from black liquors (Capanema *et al.* 2001; Liitiä *et al.* 2003). Additionally, the lignin molecular weight is an important physical property. Araújo *et al.* (2010) pointed out that lignin with low molecular weight preferred to obtain a better vanillin yield than high-molecular weight lignin. So understanding molecular weight of lignin can contribute to its more effective utilization. Gel permeation chromatography (GPC) has been used to determine the molecular weight distributions of lignin in black liquor and wood, and this will help to understand the law of lignin dissolved during pulping.

In the current study, two isolated lignins from willow were compared by their NMR spectra in addition to a variety of other techniques to characterize the lignins in the development of high-value added products.

EXPERIMENTAL

Materials

The willow (*Salix matsudana* cv. *Zhuliu*) was provided by Shandong Shengshi industrial development group, China. The tree age is three years. Lignin content 24.3 %. The commercial cellulase was supplied by Sinopharm Chemical Reagent Co., Ltd., Beijing, China. The enzyme activity of cellulase was measured as 15000 μg at a pH value of 4.5.

Preparation of EMAL

Willow wood was crushed and screened through a 60-mesh sieve. It was then acetone extracted for 48 h and dried in a vacuum drying box at 25 °C. Approximately 12 g of wood was subjected to planetary ball milling (PM200, Retsch, Germany) for 72 h at a rotation frequency of 500 rpm.

The enzymatic mild acidic hydrolysis was done in two separate stages. In the first stage, cellulase and hemicellulase were added to the wood flour slurry in the buffer medium. The slurry was agitated in a rocking incubator (HZQ-F100, Shanghai FUMA Equipment Co., Ltd., China) for 72 h at 40 °C at 5% consistency. When the enzyme treatment was finished, the slurry was centrifuged at 3000 rpm for 15 min with a high-speed centrifuge (GL-20G-II, Shanghai Anting Scientific Instruments, China). The solid obtained was washed three times using acidified water (pH = 2) then freeze-dried (DZF-6020, Shanghai Jing Hong Experimental Equipment Co., Ltd) for approximately 72 h to obtain a crude lignin sample.

In the second stage, the crude lignin was added to a dioxane-water solution (dioxane:water, 81:15 v/v). Under a nitrogen atmosphere, the mixture was heated at 80 °C for about 2 h. The product was then filtered and washed 3 times using acidified water (pH = 2). Then the mixture was neutralized with sodium bicarbonate and concentrated by rotary evaporator (RE-52A, Gongyi Yuhua Instrument Co., Ltd, China) under reduced pressure at 45 °C. The concentrated mixture was added to acidified deionized water (pH = 2) in order to isolate the lignin by letting it stand for 8 h, followed by centrifugation and freeze-drying. Lastly, the lignin was washed with hexane to obtain a purified sample. The yield was 24.7 % for total lignin content.

Preparation of AL

The pulping conditions were as follows: alkali charge (Na₂O) 16%, sulfidity 25%, liquor ratio 1:5, temperature raising period 1.5 h, at the max temperature 170 °C for 2 h. After pulping, a Buchner funnel was used for filtration of liquor.

Approximately 100 mL of black liquor was diluted with 900 mL of deionized water and left under agitation for 2 h. Then, pH was lowered to 2 by the slow addition of sulfuric acid (volume fraction = 22 %) and stirred for 30 min, followed by standing for 12 h. The precipitated lignin was filtered through a 1.2-mm cellulose nitrate filter. After filtration, the precipitate was washed 2 times with acidified water (pH = 2), and freeze-dried for 72 h. Finally, the solid obtained was powdered in an agate mortar and stored in an amber glass bottle. The yield was 5 g for 100 mL of black liquor.

Acetylation of samples

Approximately 100 mg of lignin were placed in a dry vial, and 0.5 mL of pyridine was added. Then, the vial was sealed and constantly stirred at 37 °C for 1 h or until the lignin was completely dissolved. Afterwards, 1 mL of the acetylation agent, acetic anhydride, was added. The reaction was carried out with constant agitation at 37 °C for 72 h. Finally, the vial was opened and 0.44 mL of methanol was added to remove the excess of pyridine and acetic anhydride. After 2 h of stirring, the contents of the vial were dried and milled with an agate mortar and pestle.

FT-IR Analysis

The FT-IR spectra were obtained from an IRPrestige-21 (Shimadzu Corporation, Japan) spectrometer using the KBr technique. Their spectra were recorded in the range from 4,000 to 500 cm⁻¹ at 4 cm⁻¹ resolution and 64 scans per sample. The fingerprint region was baseline corrected between 1,900 and 700 cm⁻¹. Before data collection, a background scanning was performed for background correction.

¹³C-NMR Spectroscopy

Approximately 150 mg of acetylated lignin was mixed with 500 μL of dimethyl sulphoxide-d₆ (DMSO-d₆) and stirred to fully dissolve. Then analyzed by an Advance II 400 MHz spectrometer (BRUKER, Germany) for 16 h, adopt inverse gate decoupling sequence (C13IG sequence) from Bruker Standard Pulse Library.

2D-NMR Spectroscopy

About 80 mg of non-acetylated lignin and 500 μL of dimethyl sulphoxide-d₆ (DMSO-d₆) were stirred fully to obtain a solution. The solution was then analyzed using

an Advance II 400 MHz spectrometer (BRUKER, Germany) for 12 h, adopting the pulse sequence “hsqcetgpsisp.2” from Bruker Standard Pulse Library.

Molecular Weight Distribution

The column used was a PL-gel 10 mm mixed-B 7.5 mm i.d. column, which was calibrated with PL polystyrene standards. The molecular weight distributions of the non-acetylated lignin samples were obtained using liquid chromatography 1200 (Agilent Technologies Inc., America). Approximately 10 mg of oven-dried non-acetylated lignin sample was fully dissolved in 10 mL of tetrahydrofuran (THF) and 20 μ L solutions were injected into the column. The temperature was maintained at 40 $^{\circ}$ C and the flow rate was kept at 1.0 mL/min.

RESULTS AND DISCUSSION

FT-IR Spectra of Lignin

Both EMAL and AL lignins exhibited some lignin common characteristic FT-IR absorption peaks (Faix 1991; Lin *et al.* 1992). Specific signal attributions can be seen in Table 1. For black liquor lignin the characteristic absorption peak intensity was lower compared to EMAL. The signals at 1500, 1510, and 1600 cm^{-1} represented the stretching vibrations of the benzene ring skeleton.

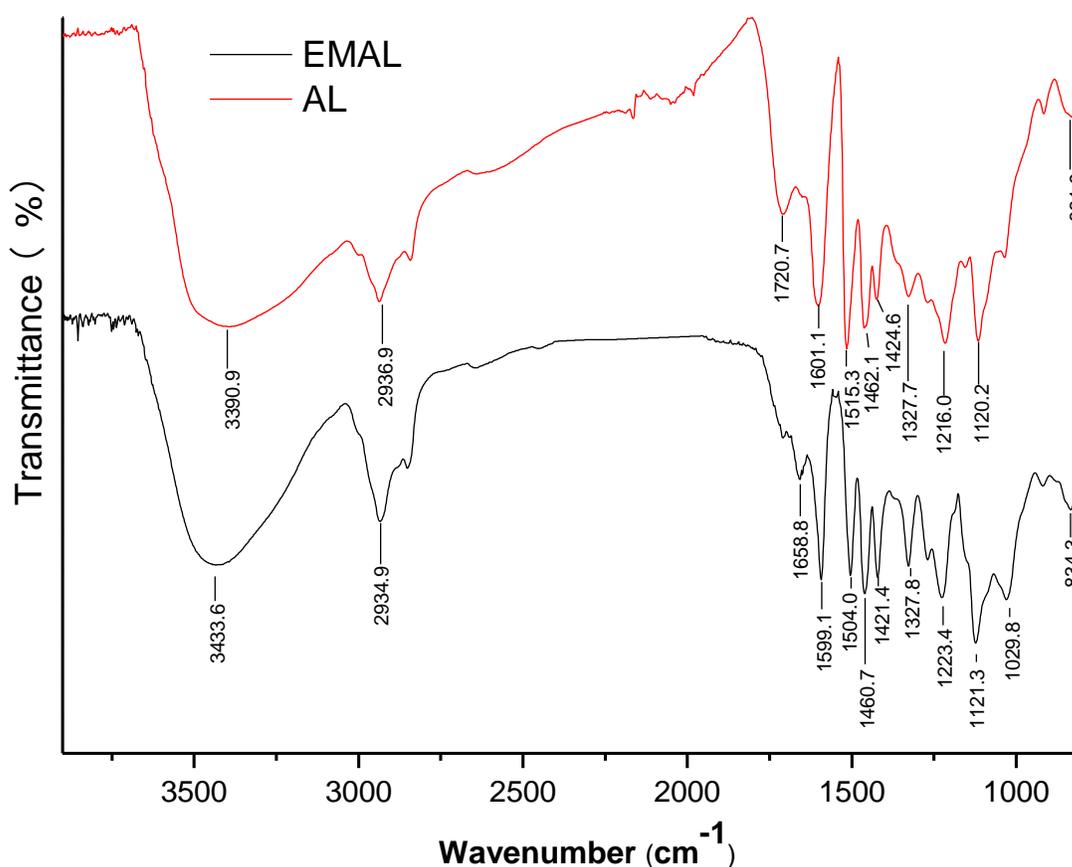


Fig. 1. FT-IR spectra of two kinds of prepared lignins

The signals from 1730 to 1000 cm^{-1} represent the carbonyl, benzene, and ether linkages in lignin. During the pulping process, the quinoids in lignin was destroyed at high temperature and give rise to compounds with carbonyl functional groups. Compared to the EMAL, the signal intensity at 1720.7 cm^{-1} of AL increased in the FT-IR spectrum, and accordingly the absorption peak of EMAL at 1658.8 cm^{-1} disappeared in the AL. The absorption peak at 1327.7 cm^{-1} represents the guaiacyl and condensed syringyl lignin vibration in black liquor and appeared to be noticeably attenuated in relation to the EMAL at 1327.8 cm^{-1} , since AL affected in the separation process, resulting in a change of its content. Due to the presence of HS^- in the cooking liquor, and bearing in mind the nucleophilic character of HS^- , one can expect a reaction of lignin and removal of hydroxyl groups out of the primary alcohols. Thus the absorption peak at 1029.8 cm^{-1} was present for EMAL but it disappeared in the AL.

Table 1. EMAL and AL Infrared Absorption Peaks and Bands Assignments

Wavenumber (cm^{-1})		Functional group
EMAL	AL	
3433.6	3390.9	O-H stretch
2934.9	2936.9	C-H stretch in methyl and methylene groups
	1720.7	C=O stretch in unconjugated ketones, carbonyls and in ester groups
1658.8		C=O stretch; in unconjugated p-subst. aryl ketones
1599.1	1601.1	Aromatic skeletal vibrations plus C=O stretch
1504.0	1515.3	Aromatic skeletal vibrations
1460.7	1462.1	C-H deformations; asym in $-\text{CH}_3$ and $-\text{CH}_2-$
1421.4	1424.6	Aromatic skeletal vibrations combined with C-H in plane deform
1327.8	1327.7	S ring plus G ring condensed
1223.4	1216.0	C-C plus C-O plus C=O stretch
1121.3	1120.2	Aromatic C-H in-plane deformation; secondary alcohols plus C=O stretch
1029.8		Aromatic C-H in plane deformation; C-O deform in primary alcohols; C=O stretch
834.3	831.9	C-H out-of-plane in position 2 and 6 of S, and in all positions of H units

¹³C-NMR Spectroscopy of Lignin

The ¹³C-NMR spectra of acetylated EMAL and acetylated AL samples are presented in Fig. 2. Recent literature reports can provide the assignments of typical substructures (Fernández-Costas *et al.* 2014). The comparison between the ¹³C-NMR spectrum of native wood lignin and the dissolved lignin from kraft liquor showed that the latter has a more altered structure and a higher amount of carbohydrates contamination. When lignin samples were acetylated, the ¹³C-NMR can differentiate amongst phenolic hydroxyl groups and primary and secondary aliphatic hydroxyl (Fasching *et al.* 2008) groups in lignin structure. In ¹³C-NMR spectra of EMAL, there appeared a weak phenolic hydroxyl (169.01 ppm) and strong primary aliphatic hydroxyls (170.48 ppm) absorption peaks and secondary aliphatic hydroxyls (169.93 ppm) absorption peaks. In AL ¹³C-NMR spectra, there appeared strong phenolic hydroxyl groups (168.66 ppm) and weak primary (170.50 ppm) aliphatic hydroxyls absorption peaks and a secondary aliphatic hydroxyls absorption peak.

In the aromatic region (156 to 100 ppm) of EMAL, the strongest signals corresponded to the C-2/C-6 carbon units (broad signal centered at 104.12 ppm), the C-3/C-5 carbon units (152.91 ppm), and C-1 carbon unit (134.90 ppm) of S-lignin structure.

In the aromatic region (153 to 104 ppm) of AL, the strongest signals corresponded to the C-2/C-6 carbon units (broad signal centered at 105.25 ppm) and the C-3/C-5 carbon units (152.16 ppm) of S-lignin. These signals reveal the relative abundance of the syringyl structures in the lignin polymer. Signals related to G-units were weak: EMAL 111.51 ppm (C-2), 122.73 ppm (C-5), 134.9 ppm (C-1); AL 148.22 (C-3). Due to the noise and other overlapping signals, it is often difficult to distinguish other carbon signals such as C-1 and C-4 between 148 and 138 ppm, although they appeared. Between 131 and 139 ppm, there were three less intense signals that were not yet assigned. Signals at 139.52, 136.01, and 123.79 ppm were attributed to carbons in residual pyridine which was used in acetylation reaction.

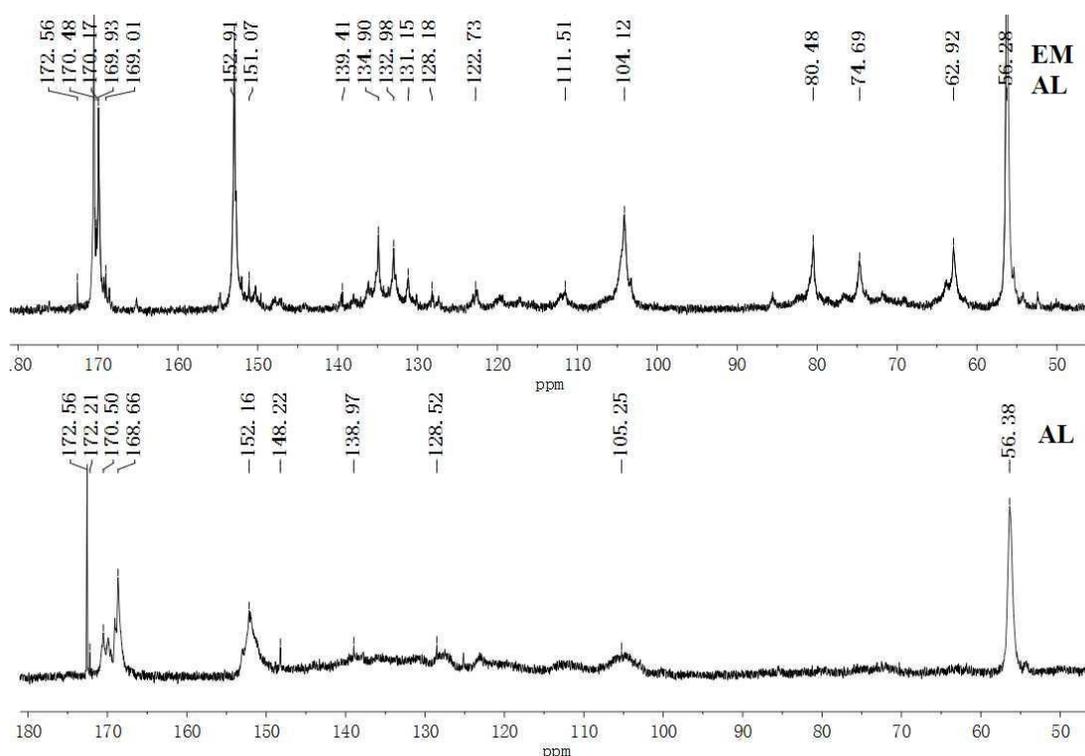


Fig. 2. The ^{13}C -NMR spectra of two kinds of lignins

The region from 86 to 50 ppm in the ^{13}C -NMR spectra contained signals that represented oxygenated and non-oxygenated inter-unit linkages in lignin. The most intense signal belonged to O-CH₃ in S and G units. In Fig. 2, one EMAL absorption peak appears at 56.28 ppm, whereas the AL absorption peak appears at 56.38 ppm. The spectrum also exhibits signals at 80.48 and 74.69 ppm corresponding to α and β carbons, respectively, in resinol substructures. The two absorption peaks indicate that the guaiacyl resinol substructure is one of the main structural compounds in EMAL, although its concentration was found to be less in AL. Indeed, the β -O-4 structure content in AL was reduced, although it should abound in native lignin (EMAL). However, kraft pulping led to the degradation of the lignin structure. Condensation reactions, conjugated double bonds, and saturated hydrocarbon structures may be introduced into the side chains of the lignin polymer. Duarte *et al.* (2001) showed that both kraft cooking and isolation of lignin by acid hydrolysis would change the structure of lignin and reduce the number of characteristic functional groups.

2D- NMR Spectra of Lignin

The 2D-HSQC NMR method provides important information on the structural transformations of lignin. Substructural information could be obtained by assigning the various signals and estimating the S/G ratio according to the literature (Wen *et al.* 2012; Wen *et al.* 2013; Fernández-Costas *et al.* 2014).

Raw lignin could be defined as an H/G/S lignin according to the 2D-HSQC spectra. The substructures in EMAL, the C_{α} - H_{α} correlations in classical β -O-4 substructures, were observed at $\delta C/\delta H$ 72.0/4.88 ppm. The C_{β} - H_{β} correlations were at $\delta C/\delta H$ 86.0/4.13 ppm, and the C_{γ} - H_{γ} correlations were at $\delta C/\delta H$ 59.6/3.72 (3.40) ppm for the β -O-4 substructures in EMAL. Additionally the C_{α} - H_{α} , C_{β} - H_{β} , and C_{γ} - H_{γ} correlations in β - β substructure were found at $\delta C/\delta H$ 85.0/4.67, $\delta C/\delta H$ 53.2/3.11, and $\delta C/\delta H$ 71.2/3.83 (4.19) ppm, respectively. The main cross-signals in the aromatic region of the 2D-HSQC spectra corresponded to the phenyl rings of the different lignin units. The S-units showed prominent resonances for $C_{2,6}$ - $H_{2,6}$ correlations at $\delta C/\delta H$ 104.2/6.71 ppm. The G-lignin units showed three varying correlations for C_2 - H_2 , C_5 - H_5 , and C_6 - H_6 , and the correlations were also found at $\delta C/\delta H$ 111.1/7.00, $\delta C/\delta H$ 115.1/6.78, and $\delta C/\delta H$ 119.0/6.85 ppm, respectively. As compared to raw lignin, the spectra of AL have only weak signals for β -O-4 linkages and resinol (β - β) substructures. The β -O-4 aryl ether (A), resinol (β - β , B) were identified by the cross peaks at $\delta C/\delta H$ 59.5/3.67 (A_{γ}), 71.1/4.17 (B_{γ}), and 53.6/3.06 (B_{β}) ppm, respectively. This observation could be attributed to the cleavage of β -O-4 aryl ether linkages in lignin during pulping. The S-units showed a prominent resonance for $C_{2,6}$ - $H_{2,6}$ correlations at $\delta C/\delta H$ 103.5/6.60 ppm. The different correlations of G-lignin units for C_2 - H_2 , C_5 - H_5 , and C_6 - H_6 were found at $\delta C/\delta H$ 111.3/6.86, $\delta C/\delta H$ 115.4/6.75, and $\delta C/\delta H$ 119.2/6.94 ppm, respectively.

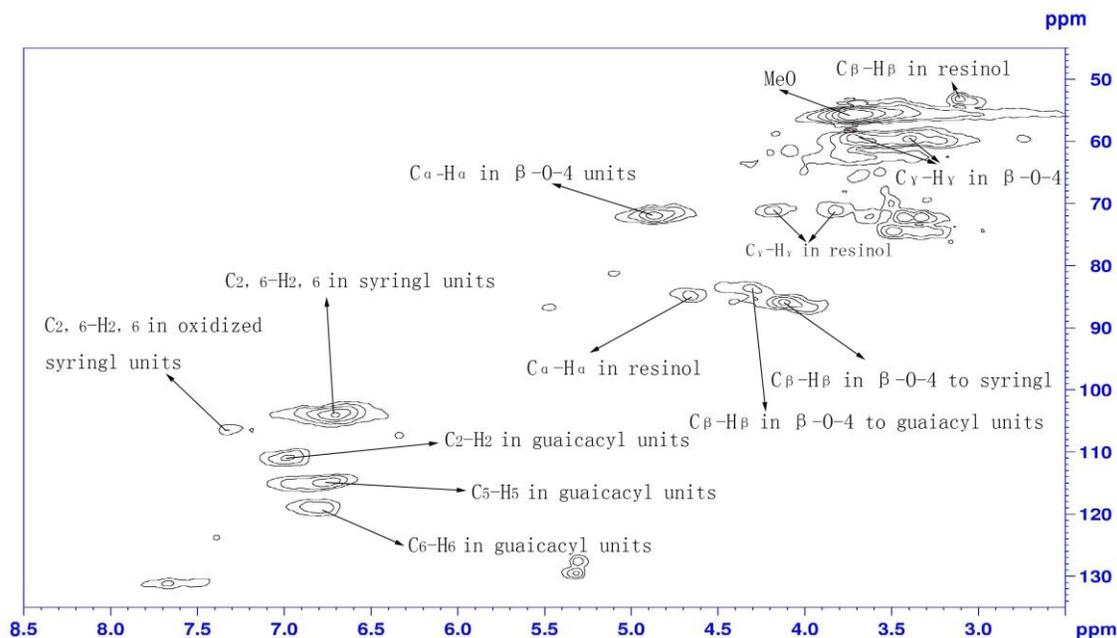


Fig. 3. The 2D-HSQC NMR spectra of EMAL

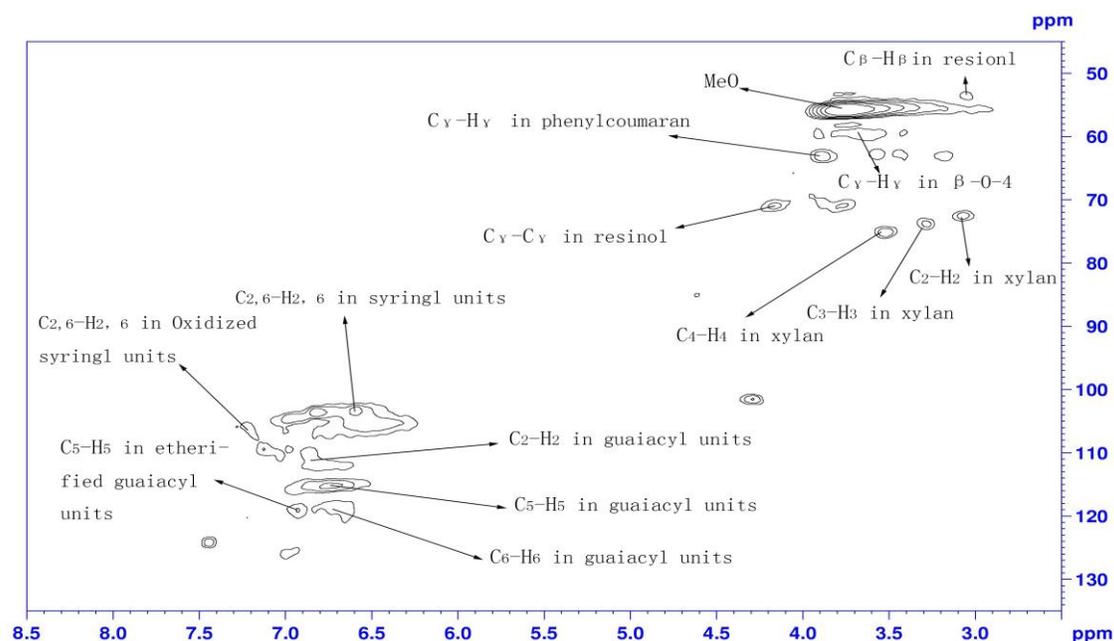


Fig. 4. The 2D-HSQC NMR spectra of AL

The S/G ratio of EMAL was 2.02, whereas the S/G ratio of AL was 0.94. In 2D-HSQC NMR of EMAL, there are a number of $C_{\alpha}-H_{\alpha}$, $C_{\beta}-H_{\beta}$, and $C_{\gamma}-H_{\gamma}$ linkages associated with β -O-4 structure and resinol structure. As can be seen by the NMR detection, after cooking (temperature = 170 °C), ether bonds and carbon-carbon bonds were drastically reduced, and primary and secondary aliphatic hydroxyl groups were removed, which is helpful for lignin pyrolysis or other conversion. In addition, as cooking progresses, most of the lignin structure was destroyed and removed. Only a small amount of lignin remain in the pulp, which is a very beneficial to bleaching and papermaking.

Average Molecular Weight Changes of Lignin

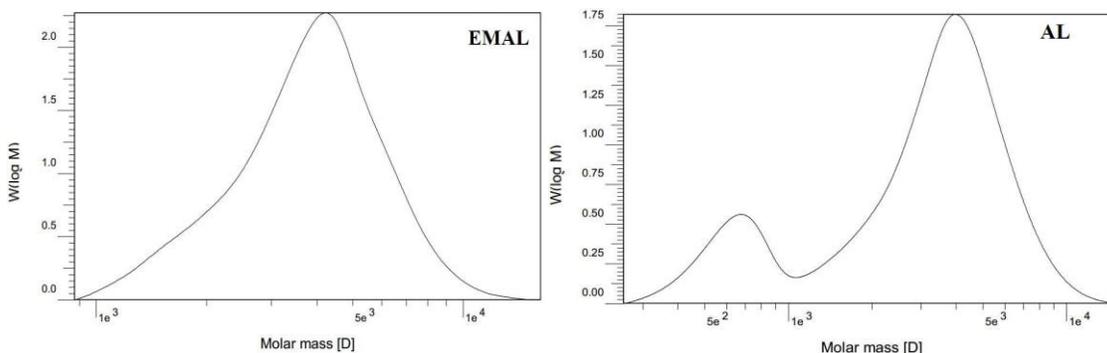


Fig. 5. The average molecular weight distribution curves of the two lignin samples from GPC-analysis

The values of the weight average (M_w) and number-average (M_n) molecular weights from the GPC curves (relative values to polystyrene), and the polydispersity index (PDI, M_w/M_n) of MWL and AL are shown in Table 2. The two lignin fractions had

similar molecular weight distributions. However, after high temperature and ensuing reactions, the β -O-4 aryl ether, β - β substructure in the original lignin was hydrolyzed. The AL became fragmented, and the molecular weight became smaller. In addition, the M_w of the willow EMAL was smaller than that of poplar, cotton, and reeds (Kondo *et al.* 1992; Liu *et al.* 2012; Wu *et al.* 2015). It was found that the PDIs (Table 1) of the two lignin polymers were narrow ($M_w/M_n < 2.0$). Also, the two lignins exhibited high polydispersity, but it was less than that of the lignins from the other three raw materials mentioned above. It was found that the isolation process was effective to obtain more homogeneous lignin polymers.

Table 2. Average Molecular Weight and Polydispersity of Lignin

Lignin	M_w (g/mol)	M_n (g/mol)	M_z (g/mol)	M_w/M_n
EMAL	4127.0	3324.9	4966.7	1.2412
AL	3522.5	1871.2	4831.8	1.8825

*Values are an average of n samples for each group. M_n : number-average molecular weight which can be averaged according to the number of molecules; M_w : weight-average molecular weight which can be averaged according to the weight of molecules; M_z : Z-average molecular weight; M_w/M_n : polydispersity.

CONCLUSIONS

1. As shown by FT-IR, the two willow lignin sample (AL and EMAL) had typical infrared absorption characteristics of lignin. The difference is that the quinoid structure was destroyed and gave rise to compounds with carbonyl functional groups. Also, guaiacyl and syringyl structures were destroyed, and hydroxyls were removed out of the primary alcohols, such that the AL sample (black liquor lignin) characteristic absorption peaks were changed.
2. The sequential extraction method proposed yielded in-homogeneous lignin polymers for demonstrating the characteristics of the native lignin of willow. It was found that the lignins are composed of *p*-hydroxyphenyl, guaiacyl, and syringyl monomeric units. From ^{13}C -NMR and 2D NMR results it was apparent that syringyl (S) was the predominant unit in willow lignin over guaiacyl (G) and *p*-hydroxyphenyl (H) units. The S/G ratio of EMAL was 2.02, whereas the S/G ratio of AL was 0.94.
3. After pulping, linkages of lignin were destroyed. Whereas the original lignin had a larger molecular weight (4127 g/mol), after pulping it became smaller (3522.5 g/mol). The PDIs of the two lignin polymers were found to be narrow ($M_w/M_n < 2.0$). The two lignins exhibited high polydispersity, but it was less than the other raw materials viz., poplar, cotton, and reeds.
4. Lignin after pulping, the quinoid structure was transformed to carbonyl functional groups, ether bonds in the β -O-4 structure of syringyl and guaiacyl groups was broken, and carbon-carbon bonds also exhibited rupture. The HS^- ion in cooking liquor was able to react with lignin with the removal of hydroxyl functions from the primary and secondary alcohol. In addition, methoxyl groups were also removal out

of the lignin basic unit. Thereby the molecules weight of lignin broken into small and lignin structure became incomplete. Finally, lignin byproducts were dissolved into the black liquor from the wood.

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