**Structural elucidation of *Eucalyptus* lignin and its dynamic changes
in the cell walls during an integrated process of ionic liquids and
successive alkali treatments**

Han-Yin Li ^a, Chen-Zhou Wang ^a, Xue Chen ^a, Xue-Fei Cao ^{a,b}, Shao-Ni Sun ^{a,*},
Run-Cang Sun ^a

^a *Beijing Key Laboratory of Lignocellulosic Chemistry, Beijing Forestry University,
Beijing, 100083, China.*

^b *State Key Laboratory of Pulp and Paper Engineering, South China University of
Technology, Guangzhou, 510640, China.*

*Corresponding authors. Address:

Beijing Key Laboratory of Lignocellulosic Chemistry, Beijing, 100083, China.

Tel: +86 10 62336972; fax: +86 10 62336972.

E-mail address: rcsun@bjfu.edu.cn (R.C. Sun); sunshaoni@126.com (S.N. Sun)

22

23 **A B S T R A C T**

24 An integrated process based on ionic liquids ([Bmim]Cl and [Bmim]OAc)
25 pretreatment and successive alkali post-treatments (0.5, 2.0, and 4.0% NaOH at 90 °C
26 for 2 h) was performed to isolate lignins from *Eucalyptus*. The structural features and
27 spatial distribution of lignin in the *Eucalyptus* cell wall were investigated thoroughly.
28 Results revealed that the ionic liquids pretreatment promoted the isolation of alkaline
29 lignin from the pretreated samples without obvious structural changes. Additionally,
30 the integrated process **resulted in** syringyl-rich lignin macromolecules with more
31 β -O-4' linkages and less phenolic hydroxyl groups. Confocal Raman microscopy
32 analysis showed that the dissolution behavior of lignin was varied in the
33 morphologically distinct regions during the successive alkali treatments, and lignin
34 dissolved was mainly stemmed from the secondary wall regions. These results
35 provided some useful information for understanding the mechanisms of
36 delignification during the integrated process and enhancing the potential utilizations
37 of lignin in future biorefineries.

38

39 Keywords: *Eucalyptus*; ionic liquids pretreatment; successive alkali treatments; lignin;
40 confocal Raman microscopy

41

42

43

44 **1. Introduction**

45 Lignin, one of the major components in the plant cell walls, is a structurally
46 complex polyphenolic biomacromolecule, mainly consisting of three basic structural
47 phenylpropane units: syringyl (S), guaiacyl (G), and *p*-hydroxyphenyl (H) (Zeng et al.,
48 2013). The interaction of lignin with cellulose and hemicelluloses as well as its
49 inherent complexity and heterogeneity make lignin a major contribution to biomass
50 recalcitrance (Perera et al., 2012). Generally, lignin is considered as an undesirable
51 component in the pulp and paper making industry. However, it has shown potential in
52 certain areas such as polymeric materials and chemicals in recent years (Wang et al.,
53 2016). Therefore, it is not surprising that an economical and conducive isolation
54 method for extracting lignin is the prerequisite for high-value applications of lignin.

55

56 Ionic liquids (ILs) as the promising “green solvents” for the pretreatment of
57 lignocellulosic biomass have attracted a significant attention. The ILs pretreatment
58 can enhance the delignification process and reduce the cellulose crystallinity, thus
59 increasing the accessibility of cellulase to the biomass (Qiu et al., 2012; Wen et al.,
60 2014). In addition, the physical and chemical properties of ILs can be tailored by
61 designing their cations and anions to meet the application requirements. Particularly,
62 the role of anion is predominant in the cellulose dissolution process (Lu et al., 2014).
63 It has been reported that cellulose from a variety of sources could be efficiently

dissolved in 1-butyl-3-methylimidazolium chloride ([Bmim]Cl) with no degradation of the solutes (Remsing et al., 2006; Swatloski et al., 2002), and the 1-butyl-3-methylimidazolium acetate ([Bmim]OAc) was identified as the most effective swelling and dissolution agent for biomass (Brandt et al., 2010). Therefore, in this work, [Bmim]Cl and [Bmim]OAc with the same cation but different anions were selected to pretreat *Eucalyptus*. The present refinery cycle based on ILs pretreatment focused on cellulose as the major active participant, while less attention has been paid to lignin. As is known that the residual lignin in the ILs pretreated biomass significantly affected the enzymatic hydrolysis. At the same time, considering the potential applications of lignin, alkali post-treatment as a cost-effective and widespread method was used to further isolate lignin from the pretreated biomass, since it could make some part of alkali-labile linkages between lignin units or between lignin and hemicelluloses broken, without degrading carbohydrates (Kim et al., 2016). The combined process based on ILs and alkali treatment was viewed as a promising method to promote the fractionation of lignin and efficient utilization of lignocellulosic biomass. During the process, the structure of lignin may be changed, which could be used to illustrate the deconstruction mechanism of this integrated method on the plant cell walls. In addition, the structural information of lignin is conducive to evaluate the utilization of lignin. Therefore, it is necessary to clarify the structural features of lignin.

85 Recently, many studies about ILs pretreatment and alkali treatment acting on
86 biomass have been conducted (Geng & Henderson, 2012; Kim et al., 2016; Qiu et al.,
87 2012). However, the detailed information of lignin changes in the cell walls at
88 subcellular level during the treatment process is lacking. Confocal Raman microscopy
89 (CRM) as a label-free and non-destructive microscopic technique has been widely
90 used in the elucidation of cell walls composition and structure in situ with a high
91 spatial resolution and chemical specificity (Gierlinger et al., 2012; Zeng et al., 2015).
92 Therefore, the utilization of this in situ technique is greatly beneficial for the selection
93 and development of pretreatment conditions to develop a cost effective deconstruction
94 strategy.

95
96 In the present study, the structural characteristics and chemical reactivity of the
97 *Eucalyptus* lignin fractions obtained by the ILs pretreatment and successive alkali
98 treatments were thoroughly characterized by high-performance anion-exchange
99 chromatography (HPAEC), gel permeation chromatography (GPC), Fourier transform
100 infrared (FT-IR), quantitative two-dimensional heteronuclear single-quantum
101 coherence (2D-HSQC) spectroscopy, and ^{31}P NMR spectroscopy. Additionally, in
102 order to monitor the course of delignification visually, the spatial distribution changes
103 of lignin in the *Eucalyptus* cell walls during this integrated process were investigated
104 by CRM.

2. Materials and methods

2.1. Materials

Eucalyptus grandis × *E. urophylla* wood (5 years old) was harvested from Guangxi province, China. The air dried raw material was cut into small pieces and then ground. The particles obtained (80-100 mesh) were extracted with toluene-ethanol (2:1, v/v) in a Soxhlet instrument for 6 h, and then dried for 16 h in an oven at 60 °C for further use. For Raman detection, 8 µm thickness cross sections were cut from the raw material by a sliding microtome. Then, the sections were used for ILs pretreatment and successive alkali treatments. The main composition of the dried dewaxed material was cellulose 41.6%, hemicelluloses 19.6%, acid-insoluble lignin 25.4%, and acid-soluble lignin 4.0%, which was determined by National Renewable Energy laboratory's (NREL) standard analytical procedure (Sluiter et al., 2008). The used ILs ([Bmim]Cl and [Bmim]OAc) were purchased from Lanzhou Institute of Chemical Physics, Lanzhou, China. All chemicals purchased were of analytical or reagent grade and used without further purification.

2.2. Lignin fractionation by a combination of ILs and successive alkali treatments

The scheme for the isolation of lignin is presented in Fig. 1. The dewaxed *Eucalyptus* (5 g) were pretreated with [Bmim]Cl and [Bmim]OAc at a biomass loading of 5 wt% in an oil bath at 120 °C for 30 min with magnetic stirring,

respectively. At the end of the designated time, quintuple deionized water as an antisolvent was slowly added into the slurries with constant stirring for 30 min at room temperature. Subsequently, the insoluble residues were collected by filtration, and then washed repeatedly with deionized water for removing any remaining ILs from the residues until the wash solution appeared colorless. Finally, the pretreated samples were dried at 60 °C for 24 h.

In order to extract the lignin fractions, the *Eucalyptus* pretreated by ILs was successively treated with 0.5, 2.0, and 4.0% NaOH at 90 °C for 2 h under a solid to liquid ratio of 1:20 (g/mL). After filtration, the residues in each step were washed thoroughly with distilled water and then oven-dried. The combined filtrates in each step were adjusted to pH 5.5 with HCl and concentrated to about 30 mL. Subsequently, the concentrated filtrate was poured into 3 volumes of 95% ethanol to precipitate hemicelluloses. After that, the filtrate was concentrated to about 30 mL, and the pH was adjusted to 1.5–2.0 with HCl. The lignin regenerated was added into a large amount of acid water with pH 2.0 to precipitate lignin. The precipitate was centrifuged and then freeze-dried to obtain lignin fractions. The lignin fractions obtained from the *Eucalyptus* pretreated by [Bmim]Cl and [Bmim]OAc were labeled as L_{BCl-0.5}, L_{BCl-2.0}, L_{BCl-4.0}, and L_{BAC-0.5}, L_{BAC-2.0}, L_{BAC-4.0}, respectively, corresponding to the successive alkali treatments with 0.5, 2.0, and 4.0% NaOH at 90 °C for 2 h. In comparison, the lignin fractions obtained by using the same alkali solutions without

ILs pretreatment were labeled as L_{C-0.5}, L_{C-2.0}, and L_{C-4.0}.

2.3. Analysis procedures

The associated polysaccharides in the lignin fractions obtained were determined by HPAEC, and the molecular weights were detected by GPC, which were based on the procedures described in a previous literature (Li et al., 2015). The SEM images of the untreated and pretreated *Eucalyptus* were recorded with a Hitachi 3400 N scanning electron microscopy (SEM) (S-3400N, HITACHI, Japan) at acceleration voltages of 5 kV. All samples were sputtered with gold in a sputter coater (E-1010, HITACHI, Japan) prior to observation. FT-IR spectra of the lignin fractions were recorded on a thermo scientific nicolet iN10 FT-IR microscope (Thermo Nicolet Corporation, Madison, WI, USA) in the range of 4000–600 cm⁻¹ at 4 cm⁻¹ resolution with 128 scans per sample. The HSQC NMR spectra of the lignin fractions were acquired on a Bruker AVIII 400MHz spectrometer at 25 °C using 30 mg of lignin in 0.5 mL of DMSO-d₆. ³¹P NMR spectra were used to detect the functional groups (phenolic hydroxyl, aliphatic hydroxyl, and carboxyl groups) of the lignin (Wen et al., 2013b). Raman spectra and images of untreated and treated cross sections of *Eucalyptus* cell walls were obtained on a LabRam Xplora confocal Raman microscope (Horiba Jobin Yvon, Longjumeau, France) according to the procedure described in a recent paper (Li et al., 2015). All experiments in this study were performed in duplicate, and the data listed in tables were the average values. The reproducibility of the values was found

less than 5%.

3. Results and discussion

3.1. Yield of the isolated lignin and content of associated carbohydrates

The yields and associated polysaccharides contents of the lignin fractions obtained from *Eucalyptus* are shown in Table 1. It was found that the yields of lignin increased after the ILs pretreatment, which was probably due to that the ILs pretreatment could disrupt the inter- and intra-molecular hydrogen bonding between crystalline cellulose, thus causing the swelling of plant cell walls, which is important for transporting lignin fragments out of the cell walls, as well as for the dissolution of cellulose and hemicelluloses (Sun et al., 2013; Tadesse & Luque, 2011). Additionally, SEM images (Fig. S1b) showed that after the ILs pretreatment, the rigid and compact morphological structure of the raw material was disrupted, as the structure became rough and loose, thus promoting the penetration of alkali into the ILs pretreated samples. Therefore, the dissolution of lignin in the following alkali extraction processes was increased slightly by the ILs pretreatment. The highest yield (10.65%) was achieved under the condition of [Bmim]OAc pretreatment. As can be seen from Fig. S1a, the cell walls were swollen significantly by the [Bmim]OAc pretreatment, while there was no obvious swelling occurred in the cell walls by the [Bmim]Cl pretreatment. Furthermore, many cracks and holes were formed on the surface morphology of the sample pretreated with [Bmim]OAc (Fig. S1b), indicating more

release of reactive sites and accessible area. Therefore, the pretreatment with [Bmim]OAc had a better performance in swelling and disruption of cell wall than **that** **with** [Bmim]Cl, which could promote more dissolution of lignin in the following alkali extraction, thus resulting in the higher yield of lignin fraction. The reason for the different effects of the two ILs on the samples may be that the acetate anion had a **stronger** hydrogen bond basicity as compared with the chloride anion, which can effectively weaken the hydrogen-bonding interaction in the polymer chains (Brandt et al., 2010).

The contents of the associated carbohydrates determined by HPAEC were used to evaluate the purity of the lignin fractions. As shown in Table 1, all the lignin fractions contained small amounts of associated carbohydrates (0.25–1.98%), suggesting that the alkali treatment significantly cleaved the linkages between lignin and hemicelluloses in the cell walls of *Eucalyptus* (Kim et al., 2016). Some differences about the carbohydrate contents were observed in the lignin fractions isolated from ILs pretreated *Eucalyptus*, which may be due to the discrepancy of ILs properties. The relatively pure lignins obtained are beneficial for the subsequent structure analysis.

3.2. Molecular weights analysis

The weight-average (M_w) and number-average (M_n) molecular weights, and polydispersity (M_w/M_n) of all the lignin fractions obtained were analyzed by GPC, and

the results are given in Table 2. The M_w value of lignin extracted with 0.5% NaOH was decreased slightly after the ILs pretreatment. This phenomenon suggested that dissociation or depolymerization of lignin possibly occurred during the ILs pretreatment processes, resulting in the decrease of lignin size (Wen et al., 2014). As the concentrations of NaOH increased from 0.5% to 4.0%, the M_w of the lignin fractions raised slightly, which may be related to the increasing content of associated carbohydrates in the lignin fractions according to the results of sugar analysis. It has been reported that the carbohydrates linked to lignin could increase the molar mass of lignin when measured by GPC (Jääskeläinen et al., 2003). The low M_w values (2870–3540 g/mol) were observed in all the lignin fractions in this study, which may be caused by the cleavage of some sensitive bonds in the lignin macromolecule under the alkaline conditions given. Furthermore, the polydispersity of all the lignin fractions showed relatively low values (1.46–1.75), implying the relatively narrow molecular weight distributions of these lignin fractions. The low polydispersity index of lignin is likely related to the mild treatment conditions and the two-step precipitation method, in which the lignin was firstly dissolved in ethanol and then precipitated in acidic water in this study. In short, the lignins isolated from the samples pretreated with ILs following by aqueous alkali had relatively uniform fragment sizes, which is conducive to the further utilization of lignin.

3.3. FT-IR spectra analysis

The FT-IR spectra of all the lignin fractions obtained from *Eucalyptus* are shown in Fig. S2. The characteristic bands at 1593, 1504, and 1423 cm^{-1} are assigned to aromatic skeletal vibrations, and the C–H deformation combined with aromatic ring vibration was observed at 1462 cm^{-1} , indicating a primary structure of lignin. The similarity of the spectra suggested that the ILs pretreatment and/or the successive alkali treatments with the increasing concentrations had little influence on the basic lignin structure. The S and condensed G absorptions were observed at 1327 cm^{-1} , and the G ring breathing with C=O stretching gives a signal at 1265 cm^{-1} . The strong band at 1122 cm^{-1} is attributed to the aromatic C–H in-plane bending deformation (typical for S units), and the aromatic C–H out-of-plane deformation appeared at 918 cm^{-1} (G) and 833 cm^{-1} (S) (Villaverde et al., 2009). Obviously, the **intensity** of S units was relatively high than that of G units in these lignin fractions, suggesting that the S units were more easily released under the alkali treatment conditions.

3.4. 2D-HSQC spectra

To investigate the detailed information about the fine chemical structures of the lignins, the lignin samples ($\text{L}_{\text{C-0.5}}$, $\text{L}_{\text{BCl-0.5}}$ and $\text{L}_{\text{BAC-0.5}}$) were analyzed by the 2D-HSQC NMR technique. The information about the inter-unit bonding patterns in the *Eucalyptus* lignin can be found in the side-chain region, and the signals from lignin units with a good resolution can be well separated in the aromatic regions. The side-chain regions ($\delta_{\text{C}}/\delta_{\text{H}}$ 50.0–90.0/2.70–6.00) and aromatic regions ($\delta_{\text{C}}/\delta_{\text{H}}$

100.0–130.0/6.20–7.50) of 2D-HSQC spectra are shown in Fig. S3, and the main substructures are depicted in Fig. S4. The assignments of the main lignin cross signals are shown in Table S1 according to the previous literatures (Rencoret et al., 2010; Rencoret et al., 2008; Wen et al., 2013a).

In the side-chain regions, the inter-unit linkages and substructures such as β -aryl ether (β -O-4', A), resinol (β - β' , B), phenylcoumaran (β -5', C), and phydroxycinnamyl alcohol end groups (I) were present, in which β -O-4' was the most abundant linkages in the *Eucalyptus* lignins. In the aromatic regions, the signals of S and G lignin were observed clearly, and small amounts of C $_{\alpha}$ -oxidized S units (S') were also detected at δ_C/δ_H 106.1/7.30 corresponding to C_{2,6}-H_{2,6} correlations.

The relative abundances of the basic substructures and the main linkages in the *Eucalyptus* lignin were calculated by the 2D-HSQC NMR quantitative method (Wen et al., 2013a). As shown in Table 3, the S/G ratios of L_{C-0.5}, L_{BCL-0.5} and L_{BAC-0.5} were 1.16, 1.54 and 1.47, respectively, suggesting that S units were easily released from the plant cell walls under the alkaline condition with a low concentration. In addition, the lignin fractions L_{BCL-0.5} and L_{BAC-0.5} had higher S/G ratios than L_{C-0.5}. The reason for the higher ratios was that the G lignin was more easily degraded than the S-type lignin during the ILs pretreatment at 120 °C, resulting in a relatively high content of S lignin in the pretreated substrates (Kim et al., 2011; Varanasi et al., 2012). Therefore, the

dissolution of S lignin could be improved in the following alkali treatment, and the lignins with higher S/G ratios were obtained. As compared with L_{BAC-0.5}, L_{BCl-0.5} showed a relatively high S/G ratio, which may be related to the higher acidity of [Bmim]Cl than [Bmim]OAc, since the acidity of the ILs could influence the lignin depolymerization (Eldridge & Ellison; Gräsvik et al., 2014). Furthermore, the β -O-4' aryl ether units (A) were the predominant substructures in all the lignin fractions (60.4–65.3%), followed by β - β' resinol-type linkages (B, 10.7–13.4%), and the lowest amounts of β -5' phenylcoumaran (C, 1.9–2.9%). The contents of the β -O-4' linkages in L_{BCl-0.5} and L_{BAC-0.5} were higher than that in L_{C-0.5}, which was probably attributed to the existence of abundant S units in L_{BCl-0.5} and L_{BAC-0.5}. Basically, the only single pathway available for monolignol addition to an S unit is β -O-4' coupling (Kim & Ralph, 2010). However, the contents of β -5' structures in L_{BCl-0.5} and L_{BAC-0.5} decreased as compared with L_{C-0.5}, probably resulting from the reduction of G lignin in the two lignin fractions. That is, β -5' structures can only be obtained from the coupling of a monolignol with G or *p*-hydroxyphenyl (H) unit (Kim & Ralph, 2010).

3.5. ³¹P NMR spectral analysis

For further evaluating the structural changes of lignin obtained from the ILs combined with alkali extraction processes, the kinds and amounts of hydroxyl groups in lignin were detected by the quantitative ³¹P NMR technique. The spectra of L_{C-0.5}, L_{BCl-0.5} and L_{BAC-0.5} are shown in Fig. S5 and the corresponding results are shown in

Table 4. It was observed that the contents of aliphatic OH in $L_{\text{BCl-0.5}}$ (3.89 mmol/g) and $L_{\text{BAc-0.5}}$ (3.43 mmol/g) were lower than that in $L_{\text{C-0.5}}$ (4.25 mmol/g), which may be owing to the occurrence of dehydration reaction in the side-chain of lignin. The content of COOH was relatively stable or slightly decreased in the lignin extracted from the ILs pretreated samples, probably owing to no obvious oxidation reaction under the conditions given. This further confirmed that the dehydration reaction of aliphatic OH occurred rather than oxidation reaction during the ILs pretreatment (Wen et al., 2014). The S-type OH content was less than the corresponding G-type OH content in all the three lignin fractions, suggesting that the majority of S-type lignin was involved in forming the β -O-4' linkages, and only a small amount of S-type OH could be detected by the ^{31}P NMR technique. The lower contents of phenolic OH in $L_{\text{BCl-0.5}}$ (0.64 mmol/g) and $L_{\text{BAc-0.5}}$ (0.68 mmol/g) than that in $L_{\text{C-0.5}}$ (0.82 mmol/g) indicated that the lignin extracted from the ILs pretreated samples may have more etherified lignin units owing to the formation of β -O-4' structures and phenyl glycoside linkages (Granata & Argyropoulos, 1995). This was in line with the results of 2D-HSQC NMR, in which more amount of β -O-4' linkages were observed in $L_{\text{BCl-0.5}}$ and $L_{\text{BAc-0.5}}$ than in $L_{\text{C-0.5}}$.

3.6. Confocal Raman microscopy analysis

CRM as a non-destructive method was used to in-situ monitor the changes in the spatial distribution of lignin from the *Eucalyptus* cell walls during the ILs

316 pretreatment and the alkali post-treatments. Raman images were acquired by
317 integrating the intensity of the main lignin Raman bands over the range from 1560 to
318 1690 cm^{-1} (Fig. 2). As can be seen, the raw material had a heterogeneous distribution
319 of lignin, as evidenced by the various intensities within morphologically distinct
320 regions, which reflected the different concentrations of lignin. The highest
321 concentration of lignin was observed in the cell corner middle lamella (CCML)
322 regions, followed by compound middle lamella (CML), and the lowest in the
323 secondary wall (S) regions. By comparing with the raw material, there was no
324 obvious changes appeared in the [Bmim]Cl pretreated sample, while a substantial
325 decrease of lignin concentration occurred after the [Bmim]OAc pretreatment. It has
326 been reported that the π - π interactions of the IL cation with lignin assisted in the
327 dissolution of lignin during the ILs pretreatment (Shill et al., 2011). Moreover, the
328 solubility of lignin in ILs was also influenced by the nature of the anions in which the
329 chloride anion was not as effective as the acetate anion for dissolving lignin (Pu et al.,
330 2007; Shill et al., 2011). After the successive alkali treatments, the lignin
331 concentrations showed decreasing trends in the raw material and ILs pretreated
332 samples. The different intensity changes of lignin among morphologically distinct
333 regions were generally related to the varied rates of delignification. Obviously, the
334 concentration of lignin decreased in the S regions with the 0.5% NaOH treatment, but
335 unobvious in the CCML regions. A significant reduction of lignin in the whole
336 cell wall regions was observed in the samples treated with 2.0% NaOH. As the NaOH

concentration further increased to 4.0%, the intensities of lignin became weaker in the S regions.

To investigate the detailed dynamic changes of lignin in the distinct morphological regions, the average Raman spectra (Fig. S6) were acquired from the S and CCML regions of the successive alkali treated samples with or without [Bmim]OAc pretreatment. The Raman signal intensity of lignin from the [Bmim]OAc pretreated sample showed a substantial reduction both in the S and CCML regions as compared with the raw material. After the successive alkali treatments, the band intensities decreased gradually in the S and CCML regions with different patterns. A significant decrease of the lignin intensity was observed in the S regions during each alkali treatment process, indicating the extensive removal of lignin, while minor changes of the band intensity occurred in the CCML regions. The different dissolution behaviors of lignin in the S and CCML regions may be related to the reactivities of varied lignin units in the morphologically distinct regions. It has been reported that the S regions occupied the highest content of lignin in the raw material because of the largest portion of cell walls (Boerjan et al., 2003). Additionally, in view of the high degree of delignification in the S regions, the lignin dissolved was mainly originated from the S layers. The phenomenon was in line with the previous report that the removal of lignin first occurred in the S layers during the alkaline treatment (Ferraz et al., 2014). Owing to the relatively high content of S units in the lignin obtained as evidenced by

the FT-IR and NMR analyses, the S units were predominantly existed in the S regions.

The higher reactivity of S units was attributed to the easier cleavage of β -O-4'

linkages in S lignin under the alkali conditions, resulting in the relatively high

delignification rate in the S regions (Studer et al., 2011).

4. Conclusions

The lignin fractions with high purities extracted from *Eucalyptus* by the ILs

pretreatment combined with successive alkali treatments were investigated. The ILs

pretreatment process slightly increased the yield of the alkali-soluble lignin fractions

and it had a minor effect on their M_w . The lignin fractions isolated from the ILs

pretreated samples had relatively high S/G ratios, abundant β -O-4' linkages, and small

amounts of phenolic hydroxyl groups. Additionally, the delignification process in the

Eucalyptus cell walls was monitored visually by CRM, and the degree of

delignification in the S regions was higher than that in the CCML regions.

Acknowledgements

This work was supported by the Fundamental Research Funds for the Central

Universities (2015ZCQ-CL-02), the National Science Foundation of China for Key

Project (31430092), Program of International Science & Technology Cooperation of

China (2015DFG31860), and the Fundamental Research Funds for the Central

Universities (BLYJ201621).

379

380 References

- 381 Boerjan, W., Ralph, J., Baucher, M., 2003. Lignin biosynthesis. *Annu. Rev. Plant Biol.*
382 54, 519-546.
- 383 Brandt, A., Hallett, J.P., Leak, D.J., Murphy, R.J., Welton, T., 2010. The effect of the
384 ionic liquid anion in the pretreatment of pine wood chips. *Green Chem.* 12,
385 672-679.
- 386 Eldridge, R.B., Ellison, C.J., 2012. Depolymerization of lignin for biomass processing
387 in ionic liquids.
- 388 Ferraz, A., Costa, T.H., Siqueira, G., Milagres, A.M. 2014. Mapping of cell wall
389 components in lignified biomass as a tool to understand recalcitrance. in:
390 *Biofuels in Brazil*, Springer, pp. 173-202.
- 391 Geng, X., Henderson, W.A., 2012. Pretreatment of corn stover by combining ionic
392 liquid dissolution with alkali extraction. *Biotechnol. Bioeng.* 109, 84-91.
- 393 Gierlinger, N., Keplinger, T., Harrington, M., 2012. Imaging of plant cell walls by
394 confocal Raman microscopy. *Nat. Protoc.* 7, 1694-1708.
- 395 Gräsvik, J., Winstrand, S., Normark, M., Jönsson, L.J., Mikkola, J.P., 2014.
396 Evaluation of four ionic liquids for pretreatment of lignocellulosic biomass.
397 *BMC Biotechnol.* 14, 1.
- 398 Granata, A., Argyropoulos, D.S., 1995. 2-Chloro-4, 4, 5, 5-tetramethyl-1, 3,
399 2-dioxaphospholane, a reagent for the accurate determination of the
400 uncondensed and condensed phenolic moieties in lignins. *J. Agric. Food Chem.*
401 43, 1538-1544.
- 402 Jääskeläinen, A., Sun, Y., Argyropoulos, D., Tamminen, T., Hortling, B., 2003. The
403 effect of isolation method on the chemical structure of residual lignin. *Wood*
404 *Sci. Technol.* 37, 91-102.
- 405 Kim, H., Ralph, J., 2010. Solution-state 2D NMR of ball-milled plant cell wall gels in
406 DMSO-d₆/pyridine-d₅. *Org. Biomol. Chem.* 8, 576-591.
- 407 Kim, J.Y., Shin, E.J., Eom, I.Y., Won, K., Kim, Y.H., Choi, D., Choi, I.G., Choi, J.W.,
408 2011. Structural features of lignin macromolecules extracted with ionic liquid
409 from poplar wood. *Bioresour. Technol.* 102, 9020-9025.
- 410 Kim, J.S., Lee, Y., Kim, T.H., 2016. A review on alkaline pretreatment technology for
411 bioconversion of lignocellulosic biomass. *Bioresour. Technol.* 199, 42-48.
- 412 Li, H.Y., Sun, S.N., Wang, C.Z., Sun, R.C., 2015. Structural and dynamic changes of
413 lignin in *Eucalyptus* cell walls during successive alkaline ethanol treatments.
414 *Ind. Crop. Prod.* 74, 200-208.
- 415 Lu, B., Xu, A., Wang, J., 2014. Cation does matter: how cationic structure affects the
416 dissolution of cellulose in ionic liquids. *Green Chem.* 16, 1326-1335.
- 417 Perera, P.N., Schmidt, M., Chiang, V.L., Schuck, P.J., Adams, P.D., 2012.
418 Raman-spectroscopy-based noninvasive microanalysis of native lignin

- structure. Anal. Bioanal. Chem. 402, 983-987.
- Pu, Y., Jiang, N., Ragauskas, A.J., 2007. Ionic liquid as a green solvent for lignin. J. Wood Chem. Technol. 27, 23-33.
- Qiu, Z., Aita, G.M., Walker, M.S., 2012. Effect of ionic liquid pretreatment on the chemical composition, structure and enzymatic hydrolysis of energy cane bagasse. Bioresour. Technol. 117, 251-256.
- Remsing, R.C., Swatloski, R.P., Rogers, R.D., Moyna, G., 2006. Mechanism of cellulose dissolution in the ionic liquid 1-n-butyl-3-methylimidazolium chloride: a ^{13}C and $^{35/37}\text{Cl}$ NMR relaxation study on model systems. Chem. Commun., 1271-1273.
- Rencoret, J., Gutiérrez, A., Nieto, L., Jiménez-Barbero, J., Faulds, C.B., Kim, H., Ralph, J., Martínez, Á.T., José, C., 2010. Lignin composition and structure in young versus adult *Eucalyptus globulus* plants. Plant Physiol., 110.
- Rencoret, J., Marques, G., Gutierrez, A., Ibarra, D., Li, J., Gellerstedt, G., Santos, J.I., Jimenez-Barbero, J., Martinez, A.T., del Rio, J.C., 2008. Structural characterization of milled wood lignins from different eucalypt species. Holzforschung 62, 514-526.
- Shill, K., Padmanabhan, S., Xin, Q., Prausnitz, J.M., Clark, D.S., Blanch, H.W., 2011. Ionic liquid pretreatment of cellulosic biomass: enzymatic hydrolysis and ionic liquid recycle. Biotechnol. Bioeng. 108, 511-520.
- Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008. Laboratory Analytical Procedure (LAP): Determination of Structural Carbohydrates and Lignin in Biomass. Technical Report NREL/TP-510-42618. National Renewable Energy Laboratory Golden Co., USA.
- Studer, M.H., DeMartini, J.D., Davis, M.F., Sykes, R.W., Davison, B., Keller, M., Tuskan, G.A., Wyman, C.E., 2011. Lignin content in natural *Populus* variants affects sugar release. P. Natl. Acad. Sci. 108, 6300-6305.
- Sun, L., Li, C., Xue, Z., Simmons, B.A., Singh, S., 2013. Unveiling high-resolution, tissue specific dynamic changes in corn stover during ionic liquid pretreatment. Rsc Adv. 3, 2017-2027.
- Swatloski, R.P., Spear, S.K., Holbrey, J.D., Rogers, R.D., 2002. Dissolution of cellulose with ionic liquids. J. Am. Chem. Soc. 124, 4974-4975.
- Tadesse, H., Luque, R., 2011. Advances on biomass pretreatment using ionic liquids: an overview. Energy Environ. Sci. 4, 3913-3929.
- Varanasi, P., Singh, P., Arora, R., Adams, P.D., Auer, M., Simmons, B.A., Singh, S., 2012. Understanding changes in lignin of *Panicum virgatum* and *Eucalyptus globulus* as a function of ionic liquid pretreatment. Bioresour. Technol. 126, 156-161.
- Villaverde, J.J., Li, J., Ek, M., Ligeró, P., de Vega, A., 2009. Native lignin structure of *Miscanthus x giganteus* and its changes during acetic and formic acid fractionation. J. Agric. Food Chem. 57, 6262-6270.
- Wang, C., Kelley, S.S., Venditti, R.A., 2016. Lignin-Based Thermoplastic Materials.

461 ChemSusChem.

462 Wen, J.L., Sun, S.L., Xue, B.L., Sun, R.C., 2013a. Recent advances in

463 characterization of lignin polymer by solution-state nuclear magnetic

464 resonance (NMR) methodology. *Materials* 6, 359-391.

465 Wen, J.L., Yuan, T.Q., Sun, S.L., Xu, F., Sun, R.C., 2014. Understanding the chemical

466 transformations of lignin during ionic liquid pretreatment. *Green Chem.* 16,

467 181-190.

468 Wen, J.L., Sun, S.L., Yuan, T.Q., Xu, F., Sun, R.C., 2013b. Structural Elucidation of

469 Lignin Polymers of *Eucalyptus* Chips during Organosolv Pretreatment and

470 Extended Delignification. *J. Agric. Food Chem.* 61, 11067-11075.

471 Zeng, J., Helms, G.L., Gao, X., Chen, S., 2013. Quantification of wheat straw lignin

472 structure by comprehensive NMR analysis. *J. Agric. Food Chem.* 61,

473 10848-10857.

474 Zeng, Y., Zhao, S., Wei, H., Tucker, M.P., Himmel, M.E., Mosier, N.S., Meilan, R.,

475 Ding, S.Y., 2015. In situ micro-spectroscopic investigation of lignin in poplar

476 cell walls pretreated by maleic acid. *Biotechnol. Biofuels* 8, 1.

477

Figure captions

Fig. 1. Scheme for ILs combined with successive alkali extractions of lignin fractions from *Eucalyptus*.

Fig. 2. Raman images of the lignin distribution in the *Eucalyptus* cell walls before and after ILs pretreatment and each step of successive alkali post-treatments by integrating from 1560 to 1690 cm^{-1} . Intensity scale locates at the right. Bright white/yellow regions suggest a high concentration of lignin; dark blue/black locations indicate very low concentration.

Fig. 1.

Fig. 2.

Table 1

Yields and associated carbohydrate contents of lignin fractions obtained from *Eucalyptus* extracted by the combination of ILs and sequential alkali solutions at different alkali concentrations.

Lignin fractions ^a	Yield ^b	Sugars ^c						
		Ara	Gal	Glc	Xly	Man	Uro	Total ^d
L _C -0.5	7.27	0.07	0.06	0.08	0.05	0.09	ND ^e	0.35
L _C -2.0	5.26	0.04	0.05	0.08	0.05	0.03	ND	0.25
L _C -4.0	4.01	0.03	0.03	0.05	0.52	ND	ND	0.62
L _{BCl} -0.5	7.86	0.19	0.15	0.06	0.15	0.08	ND	0.63
L _{BCl} -2.0	6.78	0.08	0.07	0.08	0.43	0.04	0.06	0.75
L _{BCl} -4.0	4.24	0.09	0.13	0.10	0.84	0.04	0.16	1.36
L _{BAC} -0.5	10.65	0.07	0.08	0.05	0.15	0.06	ND	0.41
L _{BAC} -2.0	8.23	0.12	0.15	0.07	1.25	0.06	0.11	1.75
L _{BAC} -4.0	3.77	0.18	0.11	0.13	1.36	ND	0.20	1.98

^a The lignin fractions L_C-0.5, L_C-2.0 and L_C-4.0 were extracted sequentially by alkali solutions with 0.5, 2.0, and 4.0% NaOH from *Eucalyptus* without ILs preparation.

The lignin fractions L_{BCl}-0.5, L_{BCl}-2.0 and L_{BCl}-4.0, and L_{BAC}-0.5, L_{BAC}-2.0 and L_{BAC}-4.0 isolated successively by alkali solutions with 0.5, 2.0, and 4.0% NaOH from the [Bmim]Cl and [Bmim]OAc pretreated *Eucalyptus*, respectively.

^b Based on the acid-insoluble lignin content in the raw material (% , w/w).

^c Ara, arabinose; Gal, galactose; Glc, glucose; Xyl, xylose; Man, mannose; Uro, uronic acid.

^d Represents the contents of total associated carbohydrates in the lignin fractions.

^e ND, not detectable.

Table 2

Weight-average (M_w) and number-average (M_n) molecular weights and polydispersity

(M_w/M_n) of the lignin fractions obtained from the integrated treatment.

	Lignin fractions ^a								
	L _C -0.5	L _C -2.0	L _C -4.0	L _{BCl} -0.5	L _{BCl} -2.0	L _{BCl} -4.0	L _{BAC} -0.5	L _{BAC} -2.0	L _{BAC} -4.0
M_w	3200	3180	3430	2870	3180	3540	2900	2960	3350
M_n	2120	2040	2350	1850	2080	2070	1910	1790	1910
M_w/M_n	1.51	1.56	1.46	1.55	1.53	1.71	1.52	1.65	1.75

^a Corresponding to the lignin fractions in Table 1.

Table 3

Quantification of the lignin fractions $L_{C-0.5}$, $L_{BCl-0.5}$ and $L_{BAc-0.5}$ by quantitative 2D-HSQC NMR.

	Percentages of linkages between structural units			
	$\beta-O-4'$	$\beta-\beta'$	$\beta-5'$	S/G
$L_{C-0.5}$	60.4	13.2	2.9	1.16
$L_{BCl-0.5}$	65.3	13.4	1.9	1.54
$L_{BAc-0.5}$	61.9	10.7	2.5	1.47

Table 4

Quantification of the functional groups (mmol/g) in the alkali lignins obtained from the raw materials and ILs pretreated samples under the various pretreatment conditions by quantitative ^{31}P -NMR method.

Lignin fractions	Aliphatic OH	Syringyl OH	Guaiacyl OH	Carboxylic group	Total phenolic OH
L _C -0.5	4.25	0.24	0.58	0.26	0.82
L _{BCI} -0.5	3.89	0.18	0.46	0.23	0.64
L _{BAC} -0.5	3.43	0.25	0.43	0.27	0.68