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# Deep eutectic solvent pretreatment of biomass: Influence of hydrogen bond donor and temperature on lignin extraction with high $\beta$ -O-4 content

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## Abstract

Recovering lignin with high  $\beta$ -O-4 content is of prime interest for further high yield depolymerization in low molecular weight phenolic compounds. Pretreatment of lignocellulosic biomass with deep eutectic solvents (DES) was studied to extract this type of tailored lignin from softwood and brewer's spent grains. In this work, choline chloride (ChCl) based DES with two different hydrogen bond donors (HBD) (lactic acid (LA) and Glycerol (Gly)), were investigated at mild temperatures (60 and 80°C). The influence of DES pretreatment on extracted lignin molecular weight and structural characteristics was analysed. The acidity and density of DES were proved to affect lignin extraction yield and its features. The lignin characteristics (type of interunits, accessibility) were shown to impact their ability to be recovered. Acidic-DES ChCl:LA at 80°C with woody biomass

gave promising results with 78% of lignin extracted exclusively composed of G units with 61% of  $\beta$ -O-4 linkages with narrow molecular weights distribution.

**Keywords:**  $\beta$ -O-4 linkages, DES pretreatment, HBD, lignin, structural characteristics

## 1. Introduction

Lignocellulosic biomass is mainly composed of lignin, hemicelluloses and cellulose. In most of the current biorefineries, cellulose is considered to be the most valuable polymer due to its large range of applications in areas such as celluloses of speciality and bioethanol production after saccharification. Lignin is often considered as a low-value co-product and burned for energy. Today, the valorisation of lignin is part of the new industrial concept of biorefineries because it is the only aromatic biopolymer composing lignocellulosic biomass, as well as the second most abundant compound on Earth since it represents 30% of the world's organic carbon (Tan et al., 2019). Due to its aromaticity, lignin appears to be an alternative to petroleum derivatives and a precursor for the formation of active biomolecules and biomaterials. To extract lignin, the pretreatment step is crucial in the biorefinery process and its contribution to the total cost of the global biorefinery process was estimated to be 40 % (Halder and Purkait, 2021). Due to the complexity of lignocellulosic biomass with three main polymers linked with hydrogen and covalent bonds, the development of pretreatments of high efficiency represents a real challenge. Currently, the main industrial sourcing of lignin came from several processes used especially in the paper industry, such as kraft or bisulfite processes. However, these processes deteriorate the native lignin structure, use polluting solvents, and require high energy input. Recently, processes based on ionic liquids have emerged, they allow less degradation of lignin due to the use of milder

operating conditions than in the paper industry and are applicable to all types of lignocellulosic biomasses. However, the lack of toxicological data and the high cost of these products are limiting factors for their industrial development (Kumar et al., 2018). To extract native lignin with sustainable processes, so-called "green", in particular, solvents called deep eutectic solvents (DES) are currently being studied. They are composed of a hydrogen bond acceptor (HBA) which in many cases is choline chloride (ChCl) (Chen et al., 2020) and a hydrogen bond donor (HBD) which can be for example a polyol or a carboxylic acid. DES are easy to prepare, biodegradable, non-toxic, durable and low cost. They are solvents formed from a mixture of two or more compounds at a precise molar ratio and whose melting point is much lower than the melting temperature of each of their components. This property, specific to these solvents, makes it possible to work under less drastic conditions to extract lignin, whose structure is sensitive to chemical and physical attacks. Finally, their behaviour and extraction efficiencies depend on the type of compounds to be extracted as well as on the involvement of hydrogen and Van Der Waals bonds between the DES and the molecule to be extracted (Owczarek et al., 2016). Recent studies have been done on the extraction of lignin with DES on different types of solid biomass, *i.e.* straw, wood chips, and other biowastes (Chen et al., 2018; Tan et al., 2019; Wang and Lee, 2021). Preserving labile linkages from native lignin structure ( $\beta$ -O-4 bonds) guarantee high value-added applications for lignin (Zijlstra et al., 2019) and the high recovery of low molecular weight aromatic compounds by depolymerisation (Sun et al., 2018). According to Chen et al, (2020), to retain a majority of  $\beta$ -O-4 bonds in extracted lignin, HBD has to be of carboxylic acid such as lactic acid (LA) or polyol such as glycerol (Gly) type. Here, the

main objective was to study the influence of HBD (LA or Gly) on the lignin extraction efficiency for two different biomasses: sawmill chips and brewers' spent grains. Those biomasses were selected as they were composed mainly of guaiacyl units (G) for sawmill chips and a mixture of G, *p*-hydroxyphenyl (H) and syringyl units (S). Furthermore, in the literature, it is accepted that temperature affects significantly the  $\beta$ -O-4 bonds content of lignin (Hong et al., 2020). Shen et al, (2020) have reported a 69 % decrease in  $\beta$ -O-4 bonds content when the temperature during DES pretreatment raised from 80 to 130°C. Lignin extraction was tested in mild conditions of temperature at 60 and 80°C. In the various experimental conditions tested, to elucidate the structural changes related to DES pretreatment conditions, a complete characterization of extracted lignin was performed by Fourier Transform Infra-Red (FTIR) Spectrometry, size exclusion chromatography and NMR 2D-HSQC.

## **2. Materials and methods**

### **2.1. Materials**

The sawmill chips came from the Norske Skog paper mill (Golbey, France). A mix of softwoods composed of spruce and pine was used as raw material in the pulp mill. They have been ground and sieved to 0.2 mm to obtain a softwood chips powder (SCP) used for DES pretreatment. The brewer's spent grains (BSG) came from a microbrewery (Strasbourg, France) and have been washed with water, dried at 45°C until constant weight, then crushed and sieved to 0.2 mm. A hydrothermal treatment (60°C during 24h) using a solid to liquid ratio of 2.5% w/v was applied on both biomasses. After the hydrothermal experiment, extractives were removed according to the protocol

referenced in part 2.2. The extractive-free SCP and BSG were stored at -20°C until use for DES pretreatment.

## **2.2. Methods of characterisation, extraction and preparation**

To characterize both lignocellulosic biomasses, the extractives, the dry matter and the ashes contents were determined using NREL/TP-510-42619, NREL/TP-510-42621, NREL/TP-510-42622 methods respectively (Sluiter et al., 2008d, 2008a, 2008b). The inorganic elemental composition was measured using inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Variant 720 ES). The sample was previously digested using HNO<sub>3</sub> in a microwaves reactor (Anton Paar Multiwave Eco). Protein was estimated from the nitrogen content measured in the biomass sample. For nitrogen evaluation, the elemental composition (C, H, N) was assessed with an elemental analyzer (FLASH 2000 Thermofischer). Protein content was calculated by using the 6.25\*N ratio. The measurement of cellulose, hemicellulose and lignin was determined using NREL/TP-510-42618 method (Sluiter et al., 2008c) with slight modifications. In a 100 mL flask, 0.175g of lignocellulosic biomasses were weighed, previously dried overnight in an oven at 105°C. Then, 1.5 mL of H<sub>2</sub>SO<sub>4</sub> 72% was added. Each flask was incubated in a thermostatic bath for 1h at 30°C. Every 10 minutes, flasks were shaken to dissolve all the biomass in the acid. Then, flasks were cooled to room temperature and 42 mL of distilled water were added to obtain a 4% acid solution and flasks were put in an oven for 1h at 121°C. The acid-insoluble residues (AIR) were gravimetrically determined after filtration through a glass crucible. The liquid fractions containing acid-soluble lignin (ASL) were quantified by a Shimadzu UV-2401PC dual focus spectrophotometer at 240 nm with an extinction coefficient of 12 L.g<sup>-1</sup>.cm<sup>-1</sup>. The acid-

insoluble lignin (AIL) content was defined as the amount of ash and protein-free Klason lignin. The monomeric reducing sugars of the previous liquid fractions were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD ICS-3000 Dionex) with a Dionex CarboPac PA-20 analytical column (3x150 mm). Monosaccharides were eluted at 35°C, with an eluent flow rate of 0.4 mL.min<sup>-1</sup> and with the following composition: 99.2% pure water/250 mM NaOH 0.8%: 0-20 min; 75% pure water/250 mM NaOH 20%/ NaOAc (1 M) NaOH (20 mM) 5%: 20-37 min ; 40% pure water/250 mM NaOH 20%/NaOAc (1 M) NaOH (20 mM) 40%: 37-41 min. Each elution was followed by a wash and equilibration time. Standards for the external sugars and uronic acids used for analyses were: fucose, glucose, xylose, galactose, mannose, rhamnose, arabinose, and galacturonic acid. All measurements were performed in triplicate. Table 1 presents the chemical characterization of both crude biomasses.

### **2.3. DES preparation and conditions for biomasses pretreatment**

Two deep eutectic solvents were used. They were formulated with the same hydrogen bond acceptor (HBA): chloride choline (ChCl). Lactic acid (LA) or glycerol (Gly) were tested as hydrogen bond donors (HBD) in DES synthesis. Each DES was synthesized at a specific molar ratio (ChCl:LA 1:10 ; ChCl:Gly 1:2) according to Shen et al, (2020) and Chen et al, (2018). Each DES was stirred at 80°C for 2h. Biomasses were mixed with magnetic stirring in the mass ratio biomass/DES of 1:20 at 60 or 80°C in an oil bath for 24 hours. Those conditions are detailed in table 2. Extraction of lignin from extractive-free BSG

and SCP were carried out in a single-step DES pretreatment. The pretreatment protocol is described in figure 1.

After the DES pretreatment, the pretreated liquid and solid were separated by filtration on a sintered-glass filter. The solid was washed with 95% ethanol until the washing liquid appeared clear. The ethanol washing liquid was mixed with the pretreated liquid fraction and this liquid fraction was treated with ethanol/water mixture at a ratio of 9:1 (v/v) as antisolvent, then evaporated in the rotary evaporator to precipitate lignin. Due to lignin precipitation, two fractions were obtained. They were separated by a sintered-glass filtration: a residual liquid fraction (LF) and a solid with lignin precipitate. Finally, the fraction containing lignin precipitate was washed three times with ultrapure water to remove residual hemicelluloses and solvent impurities. The lignin precipitate was dried at 45°C for a minimum of 24 hours (Chen et al., 2018; Tan et al., 2019) and was named DES-extracted lignin (DEEL).

## **2.4. Characterization of solid fraction and DES-extracted lignin**

### **2.4.1 FTIR Spectroscopy**

FTIR spectra were acquired by attenuated total reflectance (ATR) through a diamond cell on a Nicolet 6700. All spectra were recorded at a spectral resolution of 4  $\text{cm}^{-1}$ , and 16 scans were performed per sample in the 4000 – 650  $\text{cm}^{-1}$  range. Omnic 5.2 (Thermo Scientific) software was used for acquisition, while Opus Viewer (Bruker) version 6.5 was used for data processing.

### **2.4.2 Size-Exclusion Chromatography**

The weight-average molecular weight ( $M_w$ ), number-average molecular weight ( $M_n$ ) and polydispersity index (PDI) of lignin were measured by Size-Exclusion



Chromatography (SEC). Analyses were performed using a Shimadzu Prominence system equipped with a UV detector at 254 nm on a column sequence consisting of a Shodex GPC KF806L followed by a Phenogel 00H-0442-K0 column. The separation was performed at 30°C and eluted with tetrahydrofuran (THF) at a flow rate of 0.7 mL.min<sup>-1</sup>. The lignin was dissolved in unstabilised THF (1 mg.mL<sup>-1</sup>), filtered through a 0.45 µm PTFE filter and 20 µL of the solution was injected. Standard polystyrene samples were used for the calibration curve. Measurements were carried out in triplicate.

### 2.4.3 NMR experiments

For all NMR analyses, approximately 70 mg of samples were dissolved in 0.5 mL of dimethylsulfoxide (DMSO-d<sub>6</sub>). Spectra were recorded on a Bruker AVANCE III 500 MHz spectrometer equipped with a BBFO cryoscope. The 2D HSQC NMR spectra were acquired with the following parameters: 128 transients, 16 dummy scans, a relaxation time of 2.5 seconds, 1024 data points in the F2 dimension (<sup>1</sup>H) and 256 data points in the F1 dimension (<sup>13</sup>C). A <sup>1</sup>J<sub>C-H</sub> coupling constant of 145 Hz was used. The 2D data set was processed with MestRenova using a 90° shifted square sinusoidal acquisition window. Data matrices were filled from zero to 1024 points in both dimensions, baseline and phase corrections were applied in both dimensions. The central solvent (DMSO-d<sub>6</sub>) was used as the internal chemical shift reference point (δ<sub>C</sub>/δ<sub>H</sub> 40.1/2.51). To quantify, the percentage of subunits and the type of bonds composing the lignin of BSG and SCP, the following Equations were used based on Zijlstra et al, (2019):

$$\text{Total Aromatics} = (((S_{2/6} + S'_{2/6})/2) + S_{\text{condensed}}) + ((G_2 + G_5 + G_6 - H_{2/6})/3) + (H_{2/6}/2) \quad (1)$$

$$\text{Ratio G} = (((G_2 + G_5 + G_6 + H_{2/6})/3) / \text{Total Aromatics}) \times 100 \quad (2)$$

$$\text{Ratio S} = (((S_{2/6} + S'_{2/6})/2) + S_{\text{condensed}}) / \text{Total Aromatics} \times 100 \quad (3)$$

$$\text{Ratio H} = (H_{2/6}/2) / \text{Total Aromatics} \times 100 \quad (4)$$

$$\beta\text{-O-4 content} = (\beta\text{-O-4}\alpha / \text{Total Aromatics}) \times 100 \quad (5)$$

$$\beta\text{-}\beta \text{ content} = (\beta\text{-}\beta_{\beta} / \text{Total Aromatics}) \times 100 \quad (6)$$

$$\beta\text{-5 content} = (\beta\text{-5}_{\beta} / \text{Total Aromatics}) \times 100 \quad (7)$$

The terms in the equations for quantifying the percentage of subunits in lignin are represented by the  $^1\text{H}$  NMR chemical shifts of the aromatic protons ( $S_{2/6}$ ,  $S'_{2/6}$ ,  $G_2$ ,  $G_5$ ,  $G_6$  and  $H_{2/6}$ ). For the calculation of the ratio of inter-unit bonds in lignin, the alpha proton shift in  $^1\text{H}$  NMR is used for  $\beta\text{-O-4}$  and the beta proton shift for  $\beta\text{-}\beta$  and  $\beta\text{-5}$  quantification.

### 3. Results and Discussion

#### 3.1. Mass yields of DEEL

##### 3.1.1. Influence of temperature and deep eutectic solvent

Figure 2 presents the lignin extraction yields obtained for pretreated BSG and SCP at 60 and 80°C with ChCl-DES composed of lactic acid or glycerol as HBD. For ChCl:LA pretreatment, the lignin extraction yield for both biomasses increased with a temperature rise from 60°C to 80°C. This result is in agreement with the literature data (Alvarez-Vasco et al., 2016; Hong et al., 2020) and could be explained with the decrease of DES viscosity with an elevation of temperature allowing the improvement of heat and mass transfers and therefore the performances of the DES pretreatment. Augmentations with the temperature of 5 and 20 % of lignin extraction rate were assessed for BSG and SCP respectively. Whatever the temperature with ChCl:LA, lignin extraction rates of SCP (56.7-77.7%) were higher than the ones obtained with BSG (34.5-39.3%). Those extraction rates are close to those reported previously. As an example,

the lignin extraction yield of cereal biomass (*i.e.* corncob) extracted with ChCl:LA (molar ratio 1:2) at 80°C during 24h was 31.1% (Chen et al., 2019; Zhang et al., 2016). According to Chen et al, (2019), it was found with softwood biomass (poplar) that lignin extraction yield is around 60% during 6h of ChCl: LA (molar ratio 1:9) at 100°C. According to Stevanovic, (2016), lignocellulosic biomass from softwoods had a higher theoretical percentage of lignin (27-35%) than lignocellulosic biomass from cereals (17-24%). Furthermore, lignin from cereals possessed a more complex structure than lignin from softwoods as it can be composed of the three subunits G-H and S whereas softwood lignin contains mainly G subunits. Lou et al, (2019) mentioned that chemical composition, as well as lignin properties, have an impact on lignin extraction with DES. They also specified that fiber morphological structures could influence those extraction yields. As an example, those authors announced that lignin extraction from herbaceous biomass (*i.e.* wheat straw) was facilitated compared to lignin from woody biomass as herbaceous biomass has a lower density with higher porosity than wood, and its vascular tissue cells present a thinner cell wall and shorter cell length.

The lignin extraction rate from BSG and SCP were compared at 80°C for DES ChCl:LA and ChCl:Gly. ChCl:LA pointed out higher lignin extraction yields with 39.3% for BSG and 77.7% for SCP, whereas ChCl:Gly exhibited a lignin yield of 14.3% and 27.0% for BSG and SCP respectively. This result is in agreement with the literature data (Xu et al., 2020) which proved that acidic DES with carboxylic acid used as HBD ( $pK_a = 3.08$  for LA) showed a higher lignin extraction rate than other types of HBD (*i.e.* polyol HBD ;  $pK_a = 14.15$  for Gly).

As reported by several studies (Chen et al., 2019; Procentese et al., 2015), ChCl:Gly could be a promising candidate for lignin extraction (60-90%). However, it often needs drastic pretreatment conditions which are not compatible with the preservation of  $\beta$ -O-4 bonds. For example, 59% of lignin could be recovered from corn cob with a DES pretreatment during 15h at 150°C (Procentese et al., 2015). To improve pretreatment efficiency toward lignin extraction with ChCl:Gly, Chen et al, (2018) used this DES at 120°C during 1h acidified with 1% of H<sub>2</sub>SO<sub>4</sub> and with a water content of 20%. This tuning DES was able to extract at least 50% of lignin from switchgrass with partial preservation of  $\beta$ -O-4 inter-unit links.

### **3.2. Characterisation of DEEL**

#### **3.2.1. FTIR spectra**

The FTIR spectra of the lignin precipitates (DEEL) extracted with ChCl:LA and ChCl:Gly were studied and compared with the FTIR spectra of SCP and BSG initial biomasses and the corresponding solid fractions remaining after DES treatment (Figure 3). The assignment of the different FTIR signals is presented in Table 3. The presence of cellulose and hemicellulose in the SF and the initial biomasses was demonstrated by the presence of the broad OH band at 3390 cm<sup>-1</sup> (a). The specific bands of the lignin aromatic skeleton vibration were defined at 1590 and 1510 cm<sup>-1</sup> (d and e). In addition, the band at 1455 cm<sup>-1</sup> (f) represented the asymmetric deformation of CH<sub>3</sub> and CH<sub>2</sub>. Finally, the band at 1250 cm<sup>-1</sup> (g) represented the specific C-O vibration of the guaiacyl (G) subunit, which is the lignin subunit mainly present in softwoods. The specific C-O vibration band of the syringyl (S) subunit present in cereal or herbaceous biomasses was at 1140 cm<sup>-1</sup> (h), and the band at 1030 cm<sup>-1</sup> (i) was representative of the methoxy groups present on

the G and S subunits. In the spectra of the SF obtained after ChCl:LA at 60 and 80°C, the presence of an additional band at 1758 cm<sup>-1</sup> (c) was detected (Steinmetz et al., 2019). This band corresponds to the carbonyl groups of the phenyl esters. It was not present in the initial biomasses nor in the DEEL. It was assumed that the presence of band (c) was due to esterification reactions that occurred during the pretreatment and extraction processes between lignin and DES ChCl:LA. Here, DES ChCl:LA had several actions, on the one hand, it was able to extract lignin from lignocellulosic biomass but on the other hand, it made parasitic reactions by forming esters by binding through the phenolic OH of lignin subunits by grafting. Furthermore, in the area of the G and S subunits, extraction with ChCl:LA allowed better distinction of the specific bands of the G and S subunits compared to extraction with ChCl:Gly, which proved that acid DES were much more efficient than polyol DES for the extraction of lignin from lignocellulosic biomass. In contrast, the spectra of DEEL from SCP and BSG extracted with ChCl:LA and ChCl:Gly were not as resolved as those of the SF. Firstly, the absence of cellulose and hemicellulose in the lignin precipitates was demonstrated by the reduction of the broad OH band at 3390 cm<sup>-1</sup>. Secondly, the vibration of aliphatic CH<sub>3</sub> and CH<sub>2</sub> was represented by the band at 2940 cm<sup>-1</sup> (b). In addition, part of the specific vibration and deformation bands of the aromatic skeleton of lignin were present with the band at 1590 cm<sup>-1</sup> and the band at 1455 cm<sup>-1</sup>. In contrast, in SCP as in BSG, the formation of a bounce at 1250 cm<sup>-1</sup> did not allow to distinguish the peak of the specific C-O vibration of the G subunit. However, the presence of the methoxy groups specific to this subunit with the band at 1030 cm<sup>-1</sup> was clearly visible (Oh et al., 2020; Shen et al., 2019; Steinmetz et al., 2019). In the case of BSG, the significant band of the C-O vibration of the S subunit disappeared

in the broad band comprising the methoxy groups specific to the G and S subunits at  $1030\text{ cm}^{-1}$  (Oh et al, 2020; Shen et al, 2019; Steinmetz et al, 2019).

### 3.2.2. Molecular weight characterization of DEEL

The evolution of DEEL molecular weights can indirectly monitor the state of depolymerization and recondensation reactions in lignin during DES extraction. The chromatographic data (*i.e.* Mn, Mw and PDI) for SCP and BSG samples are summarised in Table 4 and molecular weight distributions are visible in Figure 4. Chromatographic profiles of DEEL from both biomasses extracted with ChCl:Gly were nearly similar. Peaks of low intensity in the same range of molecular weights were visible for SCP lignin with Mn at 1,410, 493 and 252 Da and with Mn at 1,275 and 431 Da for BSG lignin. The DEEL extracted from both biomasses with ChCl:LA at 60 and 80°C exhibited peaks of higher intensities compared to peaks of DEEL obtained with ChCl:Gly. For DEEL from SCP at 80°C and BSG at both temperatures, two peaks in the Mn range 735-857 and 259-277 Da were noticed. The elevation of temperature from 60 to 80°C did not influence the molecular weight distribution of DEEL from BSG. However, for SCP DEEL, a unique peak with a Mn of 504 Da and a Mw of 770 Da was detected at 60°C whereas two populations with Mn at 857 and 259 Da and Mw at 1,124 and 274 Da were visible at 80°C. Degradation and recondensation reactions could explain this molecular weight evolution with temperature increase. The unique peak for lignin extracted by ChCl:LA at 60°C presented the broadest distribution among all the samples with a PDI at 1.53. For all the other SEC chromatograms, peaks presented a narrow distribution with PDI between 1.03 and 1.31. This implied, as already referred to the literature that the DES pretreatment could result in homogeneous lignin fragments recovery (Shen et al., 2020). The DES ChCl:Gly seems to be able to extract lignin fragments of BSG and SCP with larger sizes than ChCl:LA but with low extraction yield in the tested conditions as referred in results of part 3.1. The temperature used for the extraction with DES ChCl:LA seems to present various effects on molecular weight

distribution depending on biomass type. The molecular weights  $M_n$  and  $M_w$  obtained in the present study were slightly lower than the ones observed in the literature. As an example, Hong et al, (2020) measured a  $M_w$  at 2,230 and a  $M_n$  at 1,900 Da for their alkaline lignin from woody biomass (poplar) pretreated with ChCl:Formic acid at 80°C for 6h. Their PDI of 1.17 was comparable to the ones determined in the present study. Shen et al, (2020) isolated lignin from eucalyptus wood with a double enzymatic treatment which presented a  $M_n$  of 7,630 Da, a  $M_w$  of 15,770 Da and a PDI of 2.07. DES extraction with ChCl:LA at 60 and 80°C during 6h was proceeded on this lignin. At 60°C,  $M_n$ ,  $M_w$  and PDI of respectively 6,840, 12,930 Da and 1.89 were assessed and those data decreased to 4,950, 8,670 Da and 1.75 at 80°C. Authors affirmed that degradation reaction (in opposition to recondensation reactions) became the primary reaction during the DES pretreatment as lignin fragments of lower sizes were obtained with an increase of temperature from 60 to 80°C. Therefore, lignin sourcing and DES treatment (*i.e.* DES type and operating conditions such as temperature, contact time) were revealed to have various influences on lignin molecular weights parameters.

### 3.2.3. DEEL structural characterization by NMR

From the NMR analysis of lignin from BSG and SCP extracted by ChCl:LA and ChCl:Gly at 60 and 80°C, the G/H/S ratio and the amounts of  $\beta$ -O-4,  $\beta$ - $\beta$  and  $\beta$ -5 bonds were determined (Table 5). The main visible subunits (*i.e.* G and H) of lignin as well as the inter-unit linkages were represented in Figure 5. In Figure 6.A, in the region between 50 and 90 ppm of the spectrum of the DEEL extracted from SCP by ChCl:LA at 80°C,  $\beta$ -O-4  $\gamma$ -acetylated ( $A'\gamma$ ) bonds were attributed to the structure of lactic acid-acetylated lignin after ChCl:LA pretreatment (Oh et al, 2020). In addition, in this region, there were also the presence of  $\beta$ -O-4 ( $\delta_C$  70.44/  $\delta_H$



4.91(A<sub>α</sub>), β-β (δ<sub>C</sub> 53.56/ δ<sub>H</sub> 3.05 (C<sub>β</sub>)) and β-5 (δ<sub>C</sub> 87.9/ δ<sub>H</sub> 5.45 (B<sub>α</sub>)) bonds. The SCP came from a softwood which was composed predominantly of G subunits, according to Zhu and Theliander, (2015). In the aromatic region between 115 and 125 ppm, Figure 6.A showed the presence of spots representing exclusively the protons G2, G5 and G6 specific to this subunit and thus proved that the extracted lignin was composed of mostly G subunits. Then thanks to the chemical shifts of the G subunit proton, the ratio of G subunits was calculated for the DEEL from SCP and a rate near 100% of G subunits was found. This value corresponded well with the one determined by Zijlstra et al, (2019) as they also found G ratio equal to 99% for the lignin of pine. Furthermore, after quantification of β-O-4, β-β and β-5 bonds, the DEEL from SCP extracted at 80°C with ChCl:LA presented a 61% β-O-4 ratio, a 15% β-5 ratio and a 3% β-β ratio (Table 5). Those data were closed to the ones obtained by Zijlstra et al, (2019) for lignin from pine extracted under mild conditions with ethanol, as they found 59% of β-O-4, 14% of β-5 and 0% of β-β. Lowering the temperature to 60°C for DEEL extraction from SCP with ChCl:LA had consequences on lignin inter-unit characteristics as β-O-4, β-5 and β-β respectively decreased to 41, 6 and 2%. Those ratios were again reduced to 12, 3 and 1% when ChCl:Gly was applied at 80°C.

BSG, as grass or herbaceous biomasses were composed of the G, H and S subunits. Here, the determined G/H/S ratios of BSG were 35/36/29 (the S subunit ratio was estimated based on G and H ratios measured by NMR 2D-HSQC). In the NMR spectra, Figure 6.B showed spots in the aromatic region corresponding to the G subunits between 115 and 125 ppm and the H subunit at 130 ppm (G2, G5, G6 and H2,6). However, depending on the DES and temperature, the spots were larger or smaller and represented a greater number of protons characteristic of the G and H subunits. Despite all the integrated analysis spectra for the different lignin samples extracted from BSG, there was no presence of the S subunit either in native or

condensed form between 100 and 110 ppm. This result is in agreement with that of Alvarez-Vasco et al, (2016) who observed that G units were more prone to the collection than the S units during DES pretreatment of lignocellulosic biomass between 80-140°C. In contrast to lignin extracted from SCP, the spectra of lignin from BSG showed few spots corresponding to the  $\alpha$ ,  $\beta$  and  $\gamma$  protons of inter-unit bonds. Regarding the rates of  $\beta$ -O-4 bonds, lignin from BSG extracted by ChCl:LA at 80°C showed a rate of 53% while it decreased to 45% when a temperature of 60°C was applied. This ratio also dropped to 16% when ChCl:Gly was used at 80°C. The ratio of  $\beta$ -5 and  $\beta$ - $\beta$  were quite stable and comprised respectively between 3-6% and 3-11%. Among the different conditions tested and for both biomasses, extraction of lignin with high ratio of  $\beta$ -O-4 aryl-ether bonds was realized with acidic DES ChCl:LA at 80°C.

#### 4. Conclusion

ChCl-based DES pretreatment with Lactic acid or Glycerol as HBD were investigated to recover lignin from softwood or cereal biomass with high content of  $\beta$ -O-4 at mild temperature (60 and 80°C). Acidic-DES ChCl:LA at 80°C with woody biomass gave promising results as around 78% of lignin was extracted exclusively composed of G units with 61% of  $\beta$ -O-4 linkages. In the same conditions, the extraction of lignin from cereal biomass was lower (40%) probably due to the complexity (chemical composition, lignin properties) of this lignin sourcing. DES with glycerol was not relevant in the tested conditions and further studies are needed to improve the lignin recovery rate of this type of DES.

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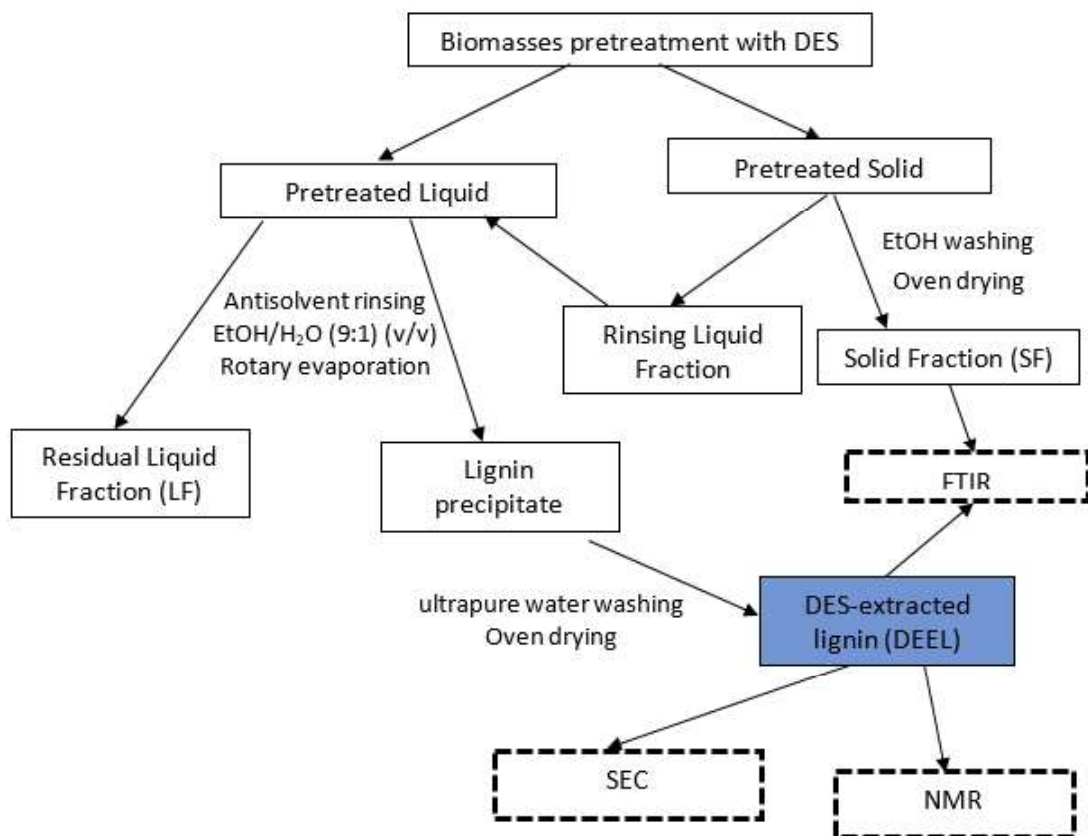
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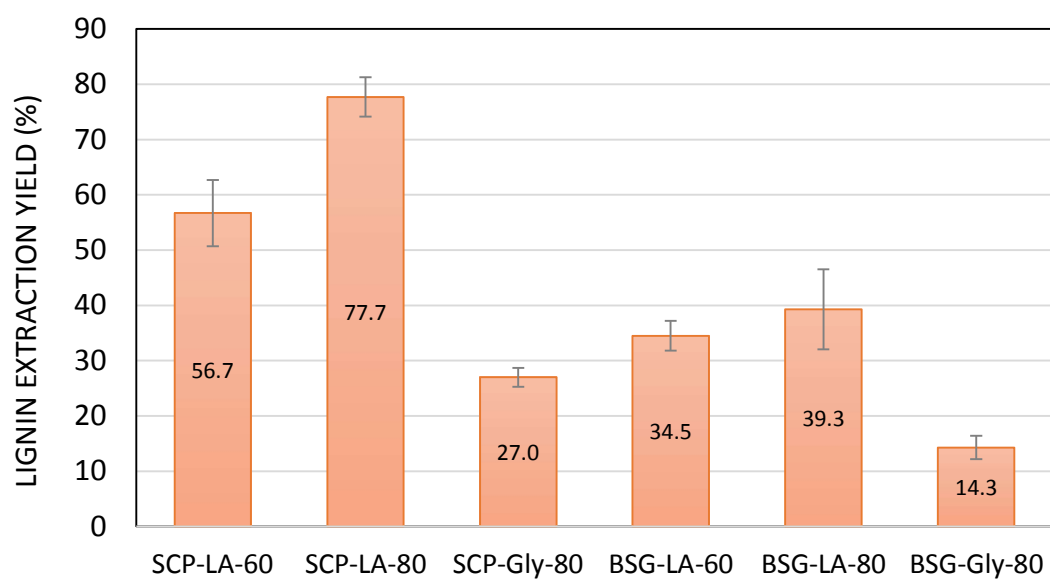
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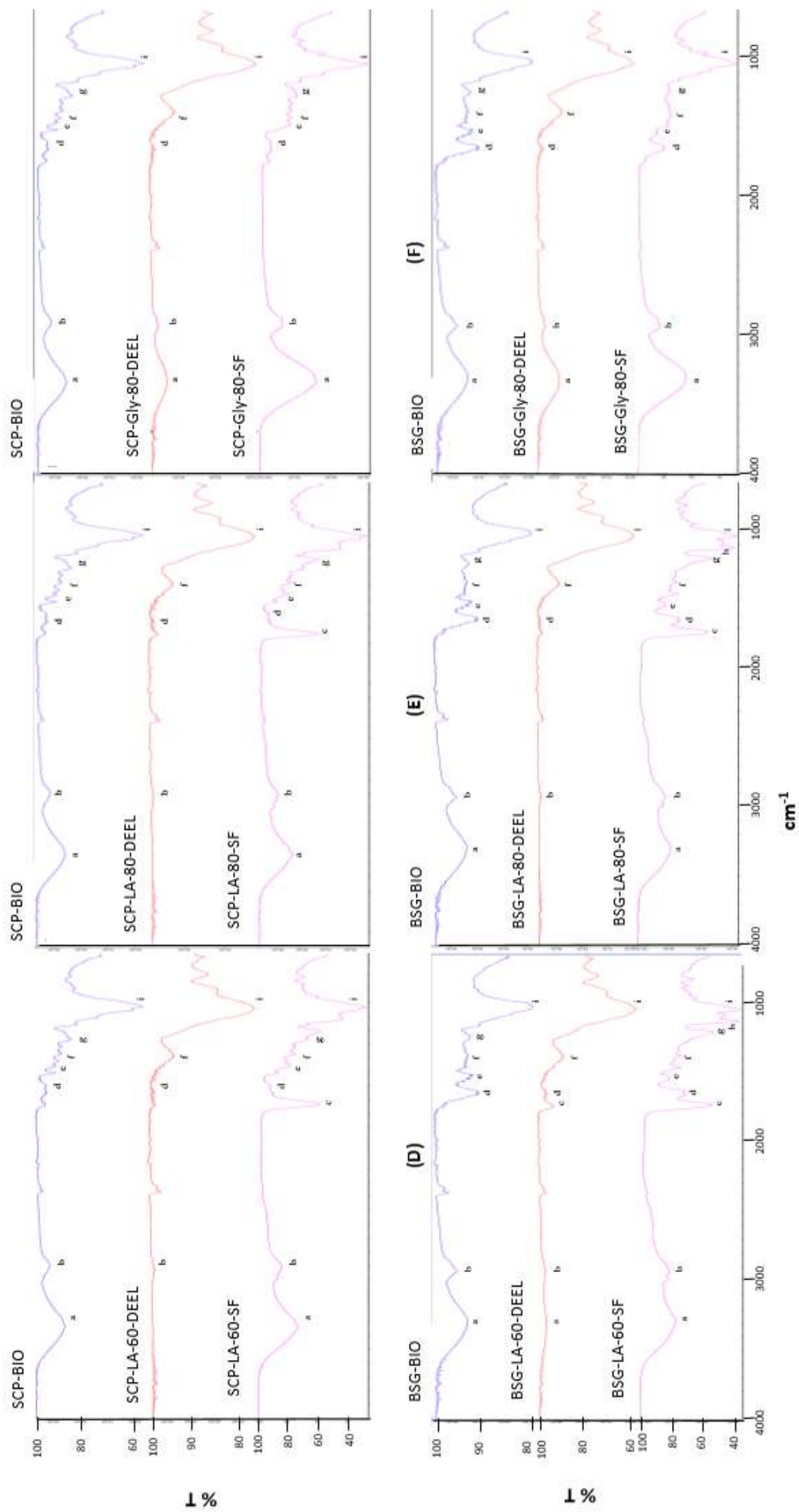


**Figure 1:** Protocol for lignin extraction by DES (color online only)

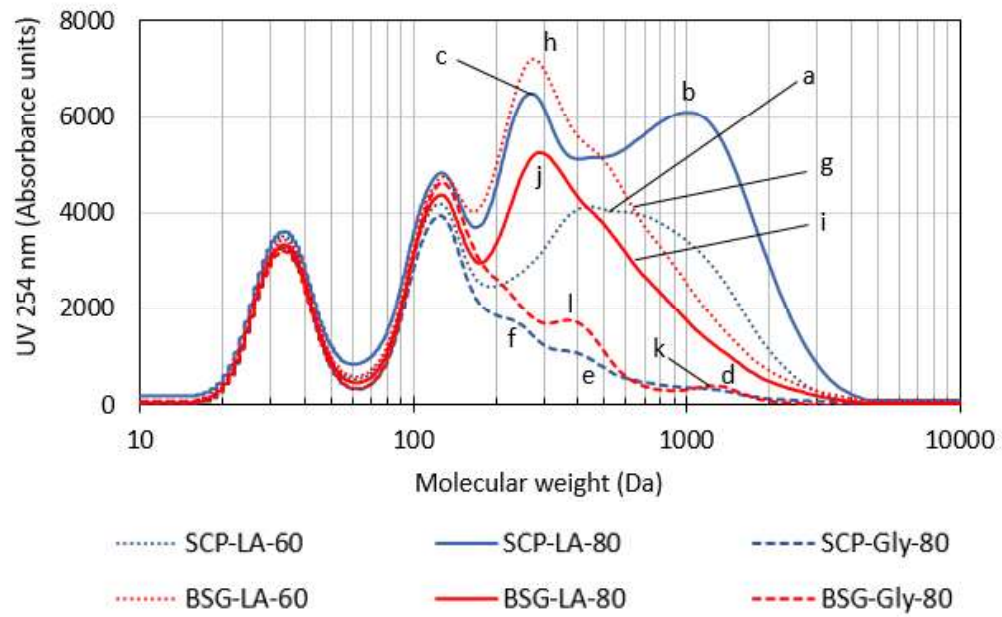


**Figure 2:** Influence of temperature (60 or 80°C) and DES HBD type (LA or Gly) on lignin extraction yield of SCP and BSG (**color online only**)

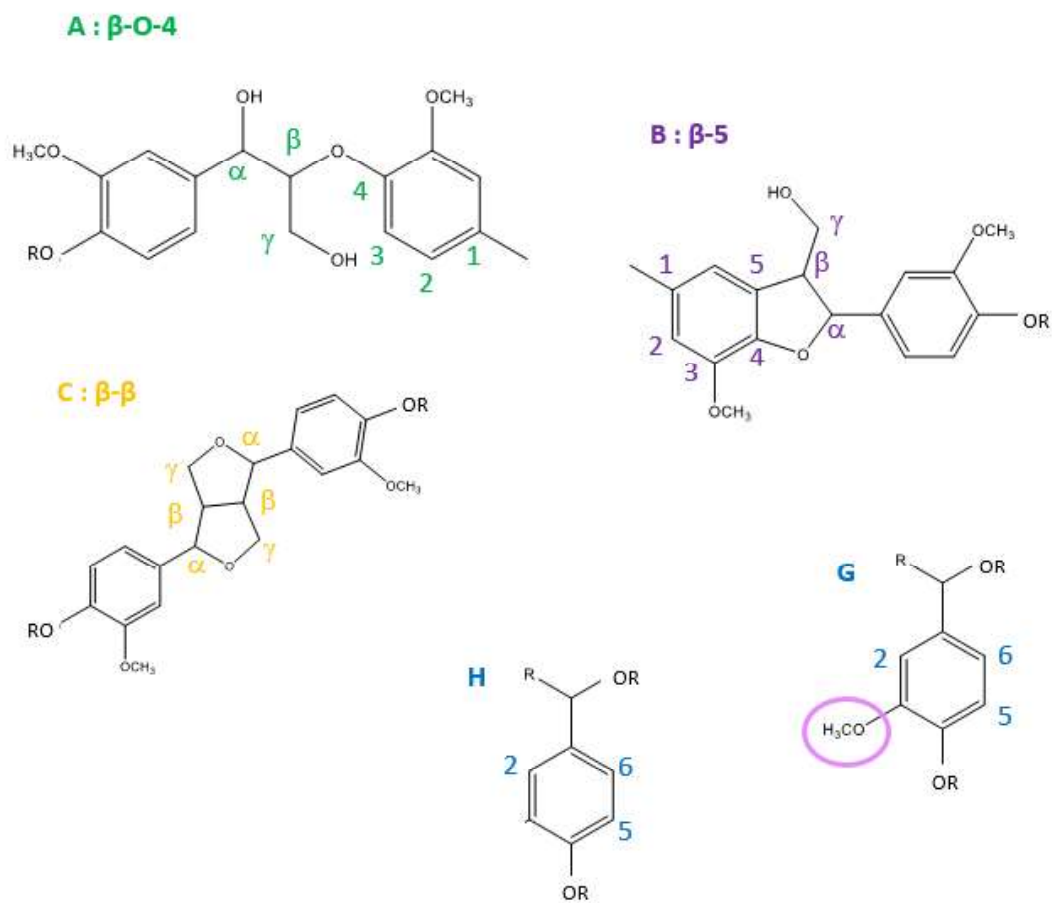




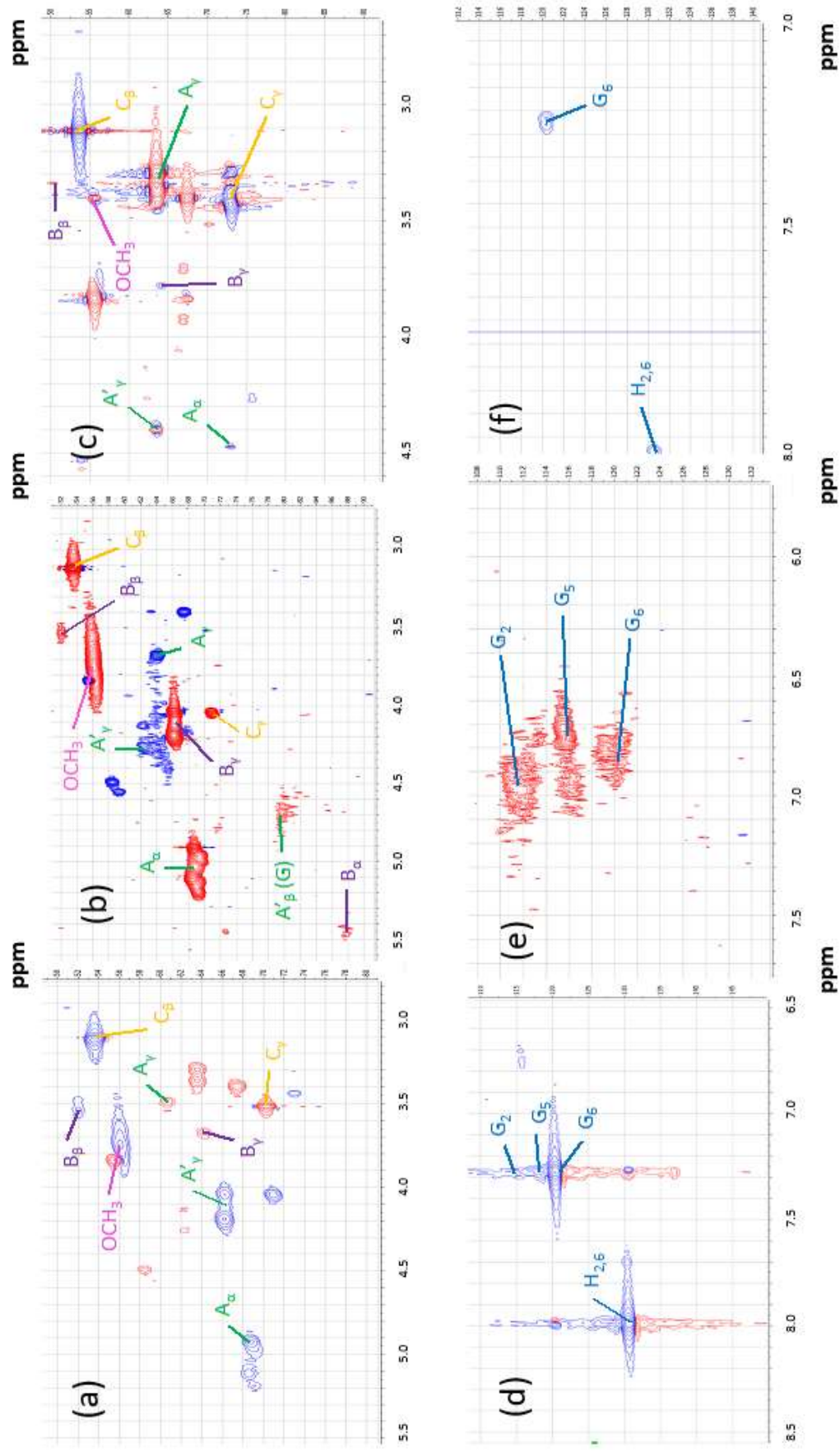
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(color online only)



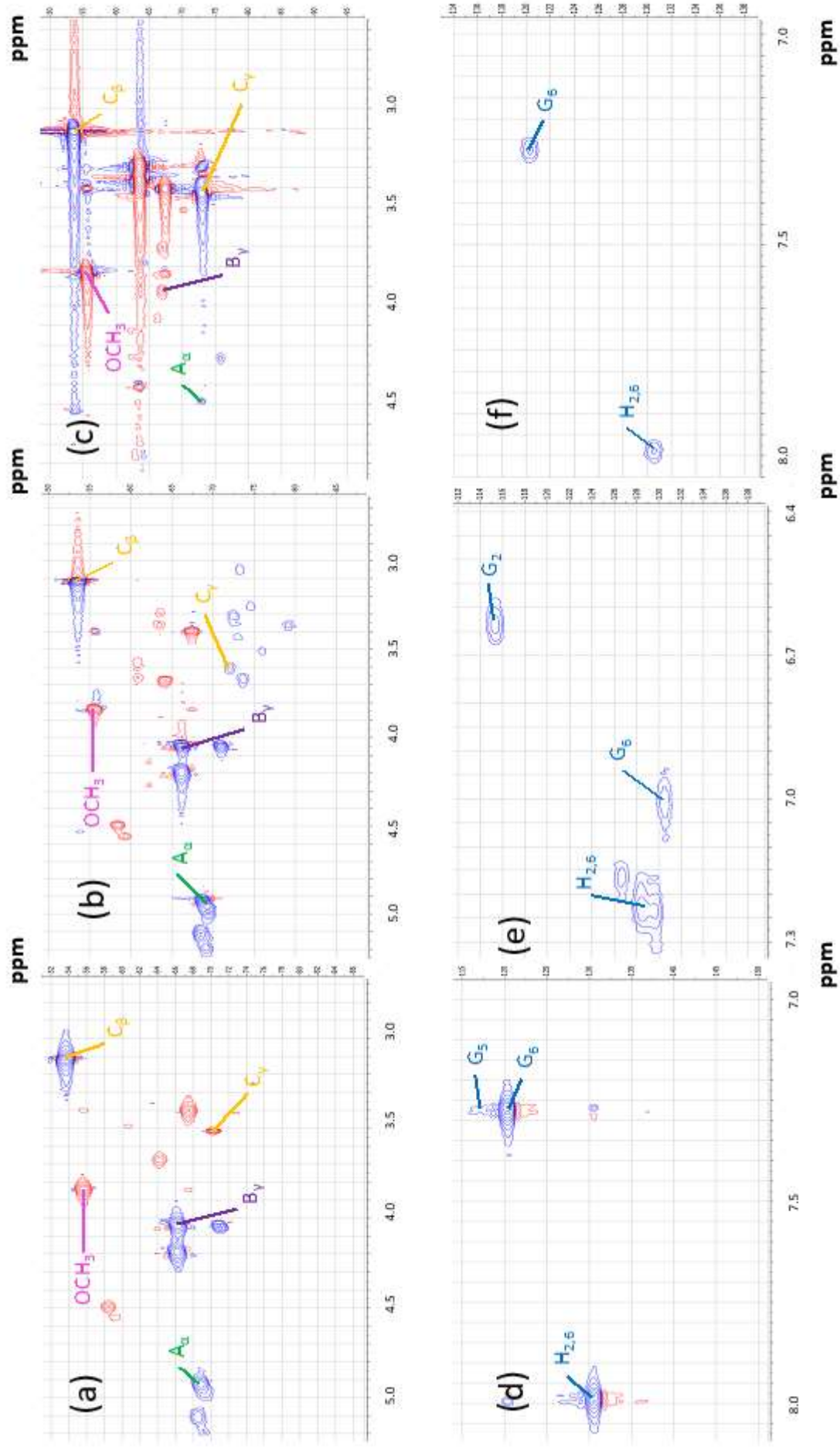
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**Figure 6.B:** NMR 2D HSQC spectra of the aliphatic and aromatic parts of BSG lignin extracted by ChCl:LA at 60°C (a and d respectively), 80°C (b and e) and by ChCl:Gly at 80°C (c and f (**color online only**))

**Table 1:** Chemical composition of crude BSG and SCP used

<b>Component</b>	<b>Composition (g/100g dry weight)</b>	
	<b>BSG</b>	<b>SCP</b>
<b>Carbohydrates</b>	40.21 ± 2.44	46.86 ± 3.69
<b>Cellulose (as glucose)</b>	16.47 ± 1.39	27.01 ± 2.06
<b>Hemicelluloses</b>	23.74 ± 1.05	19.85 ± 1.63
<b>as Arabinose</b>	10.39 ± 0.41	1.58 ± 0.11
<b>as Xylose</b>	11.45 ± 0.49	5.18 ± 0.31
<b>as Mannose</b>	0.70 ± 0.08	11.37 ± 1.10
<b>as Galactose</b>	1.19 ± 0.07	1.72 ± 0.12
<b>Lignin</b>	19.4 ± 0.70	28.5 ± 2.60
<b>as AIL</b>	12.9 ± 0.50	25.5 ± 2.05
<b>as ASL</b>	6.5 ± 0.20	3.0 ± 0.55
<b>Ashes</b>	4.1 ± 0.06	0.6 ± 0.20
<b>Extractives</b>	10.5 ± 2.22	4.7 ± 0.93
<b>Proteins</b>	24.9 ± 1.60	nd

**Table 2:** Preparation and pretreatment of lignocellulosic biomasses by DES

DES	HBA	HBD	HBD pKa	Molar ratio	Ratio biomass/DES	Experimental conditions
ChCl : LA	Choline chloride  (T <sub>m</sub> *= 302°C)	Lactic acid (T <sub>m</sub> = 17°C)	3.08	1 : 10	1 : 20	60 or 80°C, oil bath, magnetic
ChCl : Gly		Glycerol (T <sub>m</sub> = 17.8°C)	14.15	1 : 2	1 : 20	stirring, 24 hours

\* T<sub>m</sub> corresponds to the melting temperature of compound.

**Table 3:** Assignment of FTIR signals from SCP and BSG

Peak Name	Wave Number	Assignment
<i>a</i>	3390 cm <sup>-1</sup>	Phenolic or aliphatic OH
<i>b</i>	2940 cm <sup>-1</sup>	Aliphatic CH <sub>3</sub> and CH <sub>2</sub>
<i>c</i>	1758 cm <sup>-1</sup>	Phenyl ester C=O
<i>d</i>	1590 cm <sup>-1</sup>	Aromatic C-C
<i>e</i>	1510 cm <sup>-1</sup>	Aromatic C-C
<i>f</i>	1455 cm <sup>-1</sup>	Asymmetric CH <sub>3</sub> and CH <sub>2</sub>
<i>g</i>	1250 cm <sup>-1</sup>	C-O (Subunits G)
<i>h</i>	1140 cm <sup>-1</sup>	C-O (Subunits S)
<i>i</i>	1030 cm <sup>-1</sup>	OCH <sub>3</sub>



**Table 4:** Molecular weights Mn and Mw and Polydispersity Index PDI of SCP and BSG DEEL at 254 nm

<b>Samples</b>	<b>Peak name</b>	<b>Mn (Da)</b>	<b>Mw (Da)</b>	<b>PDI</b>
<b>SCP-LA-60</b>	a	504	770	1.53
<b>SCP-LA-80</b>	b	857	1,124	1.31
	c	259	274	1.06
<b>SCP-Gly-80</b>	d	1,410	1,725	1.22
	e	493	526	1.07
	f	252	259	1.03
<b>BSG-LA-60</b>	g	735	948	1.29
	h	265	284	1.07
<b>BSG-LA-80</b>	i	738	919	1.25
	j	277	296	1.07
<b>BSG-Gly-80</b>	k	1,275	1,361	1.07
	l	431	460	1.07

**Table 5:** Quantification of subunits and inter-unit bonds of lignin from SCP and BSG by ChCl:LA and ChCl:Gly at 60 and 80°C

Samples	Ratio G/H/S (%)	$\beta$ -O-4 (%)	$\beta$ -5 (%)	$\beta$ - $\beta$ (%)
SCP-LA-60	99/1/0	41	6	2
SCP-LA-80	100/0/0	61	15	3
SCP-Gly-80	99/1/0	12	3	1
BSG-LA-60	35/36/29 <sup>a</sup>	45	6	5
BSG-LA-80	35/36/29 <sup>a</sup>	53	3	3
BSG-Gly-80	35/36/29 <sup>a</sup>	16	3	11

<sup>a</sup>S subunit ratio was estimated based on G and H ratios measured by NMR 2D-HSQC