




# Selective precipitation and characterization of lignin–carbohydrate complexes (LCCs) from *Eucalyptus*

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## Abstract

**Main conclusion** Six types of lignin–carbohydrate complex (LCC) fractions were isolated from *Eucalyptus*. The acidic dioxane treatment applied significantly improved the yield of LCCs. The extraction conditions had a limited impact on the LCC structures and linkages.

Characterization of the lignin–carbohydrate complex (LCC) structures and linkages promises to offer insight on plant cell wall chemistry. In this case, *Eucalyptus* LCCs were extracted by aqueous dioxane, and then precipitated sequentially by 70% ethanol, 100% ethanol, and acidic water (pH = 2). The composition and structure of the six LCC fractions obtained by selective precipitation were investigated by sugar analysis, molecular weight determination, and 2D HSQC NMR. It was found that the acidic (0.05-M HCl) dioxane treatment significantly improved the yield of LCCs (66.4% based on Klason lignin), which was higher than the neutral aqueous dioxane extraction, and the extraction condition showed limited impact on the LCC structures and linkages. In the fractionation process, the low-molecular-weight LCCs containing a high content of carbohydrates (60.3–63.2%) were first precipitated by 70% ethanol from the extractable solution. The phenyl glycoside (PhGlc) bonds (13.0–17.0 per 100Ar) and highly acetylated xylans were observed in the fractions recovered by the precipitation with 100% ethanol. On the other hand, such xylan-rich LCCs exhibited the highest frequency of  $\beta$ -O-4 linkages. The benzyl ether (BE) bonds were only detected in the fractions obtained by acidic water precipitation.

**Keywords** *Eucalyptus* · Lignin–carbohydrate complexes (LCCs) · Selective precipitation · Structure · Linkage · 2D HSQC NMR

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## Introduction

*Eucalyptus* is widely planted in the tropics and subtropics, not only in subtropical China but also throughout the world. It is one of the most important fast growing trees for pulp & paper as well as biorefinery industries, and can produce many value-added fermentation products such as ethanol and xylitol (Romaní et al. 2012; Chen and Fu 2013). The earliest plantings of *Eucalyptus* in China can be dated back to 120 years ago, and *Eucalyptus* plantations in China have been covered 4.6 million hectares in 2014 (Li et al. 2015). Yet, there are only a few reports about lignin–carbohydrate complexes (LCCs) in *Eucalyptus* cell walls (Li et al. 2011; Miyagawa et al. 2012). Therefore, a clear understanding of the LCC structures and linkages in *Eucalyptus* will facilitate the optimization of the biorefinery process and explore the potential utilization of *Eucalyptus* biomass.

Lignocellulose, an extremely complex natural material, is made up of three main biopolymers: cellulose, hemicelluloses, and lignin (Isikgor and Becer 2015). Unlike cellulose, hemicelluloses are branched heterogeneous polysaccharides that are composed of hexose and pentose sugar units, such as xylose, mannose, arabinose, glucose, and uronic acid (Gírio et al. 2010). Lignin, accounting for 15–30% dry weight of terrestrial plants and approximately 30% of the organic carbon in the biosphere (Goldstein 1981; Yue et al. 2016), is mainly composed of three different phenyl propane monomers such as guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) units (Ragauskas et al. 2014). In addition, lignins have complex and heterogeneous structures that are neither absolutely definable nor determinable (Yue et al. 2017). Separation of lignin, cellulose, and hemicelluloses had been found to be difficult, since the hemicelluloses act as glue holding cellulose and lignin together, and the lignin and polysaccharides (mainly hemicelluloses) are linked by covalent bonds, forming lignin–carbohydrate complexes (Balakshin et al. 2008). It is generally accepted that there are three main types of LCC linkages: phenyl glycoside (PhGlc), benzyl ethers (BE), and  $\gamma$ -esters (Fengel and Wegener 1983; Balakshin et al. 2011). Besides, the presence of LCCs impedes the effective separation of the wood components and the efficiency of enzymatic hydrolysis of carbohydrates in biorefining processes (Balan et al. 2009; Balakshin et al. 2014). Although the complex structure of LCCs has been investigated extensively, it has not been clearly elucidated (Salanti et al. 2012). Therefore, how to isolate LCCs with minimal chemical modification from plant cell walls is prerequisite for an extensive investigation on the structures and linkages of LCCs.

In the past several decades, various methods have been developed to isolate LCCs from plant cell walls. The first directly extracted LCCs from poplar wood with hot water (140 °C) were obtained by Traynard in (1953). Afterwards, (Bjorkman 1957) introduced lignin and LCCs isolation procedures from ball-milled wood with aqueous dioxane. Lawoko et al. (2005) reported the quantitative fractionation method of LCCs followed with selective alkaline dissolution steps by cellulase treatment. However, this protocol (Lawoko et al. 2005) was only applicable to softwood, and such isolation procedure was rather complicated. Recently, another fractionation method was reported that the ball-milled wood was dissolved in tetrabutylammonium hydroxide (TBAH) and dimethyl sulfoxide (DMSO) solution for the high yield fractionation of LCCs from both hardwood and softwood (Li et al. 2011; Du et al. 2013). However, this protocol induced the deacetylation of the partially acetylated hydroxyl groups in hemicelluloses (Giummarella and Lawoko 2016). Giummarella and Lawoko (2016) applied a mild protocol for quantitative fractionation of LCCs and unveiled insights into the role of molecular structure of xylan on forming the type of

lignin–carbohydrate (LC) bonds. Wang et al. developed a solvent system, containing lithium chloride and dimethyl sulfoxide (LiCl/DMSO), capable of directly dissolving ball-milled wood to study LCCs (Wang et al. 2013). Although this protocol is mild, the detailed structural features of individual LCC fraction were lost (Giummarella and Lawoko 2016). Balakshin (Balakshin et al. 2011) and (Yang et al. 2015) introduced different precipitation procedures to isolate the LCCs from different woods by aqueous dioxane extraction. In these two previous works, although the LCC structures and linkages were clearly observed by two-dimensional nuclear magnetic resonance (2D NMR), the yield of the isolated LCCs was relatively low (7.7–22.0% based on Klason lignin).

In the present study, to identify and quantify the LCC structures and linkages, 70% ethanol, 100% ethanol, and acidic water (pH = 2) were applied to fractionate LCCs by selectively precipitation from the neutral aqueous dioxane and acidic aqueous dioxane, respectively. Six fractions of LCC were obtained in mild conditions and fully elucidated by composition analysis, 2D HSQC NMR spectroscopies, and gel permeation chromatography (GPC).

## Materials and methods

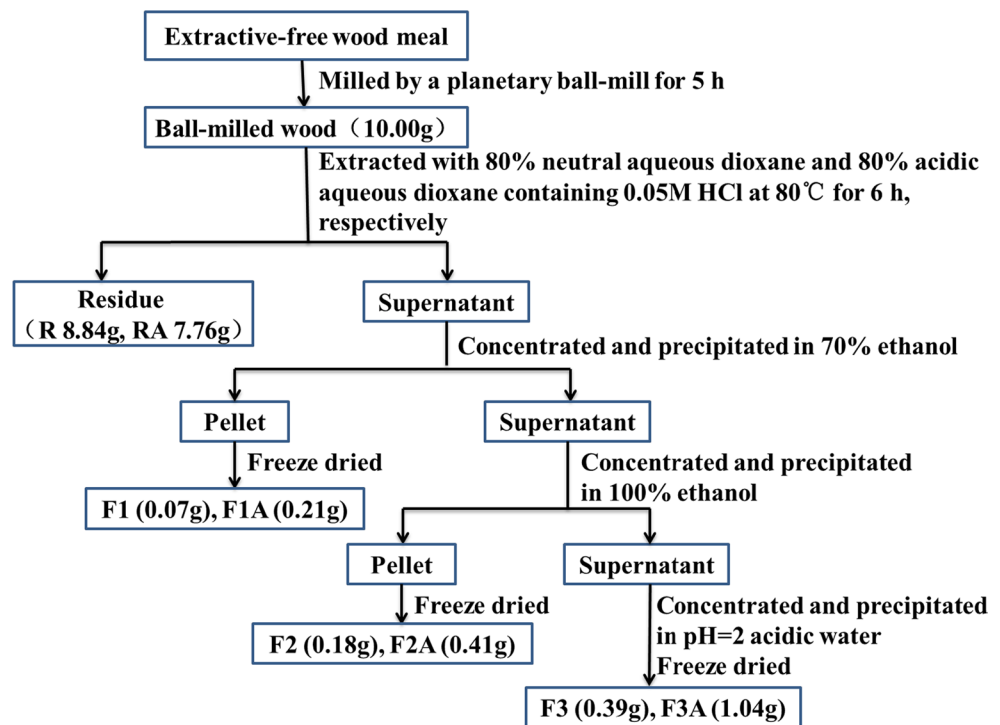
### Materials

Wood sample was obtained from the *Eucalyptus grandis* × *E. urophylla*, 4-year-old, which was collected from Guangxi province, China. The sample was dried in an oven at 60 °C before ground. The 40–60 mesh wood powders were extracted with toluene/ethanol (2:1, v/v) in a Soxhlet extractor for 12 h to remove wax. The extractive-free *Eucalyptus* contained 40.4% cellulose, 17.5% hemicelluloses, and 24.4% Klason lignin, determined according to the methods given by National Renewable Energy Laboratory (NREL) (Sluiter et al. 2008). The dewaxed sample was milled in a planetary ball mill (Fritsch, Germany) equipped with a 500-mL ZrO<sub>2</sub> bowl containing mixed balls (10 balls of 2-cm diameter and 25 balls of 1-cm diameter). The milling was conducted for 5 h (a 10-min lull after every 10 min of milling) under a nitrogen atmosphere at 450 rpm. All chemicals were analytical or reagent grade without further purification.

### Selective precipitation of LCCs

Six LCC fractions were isolated according to the scheme in Fig. 1. The procedures were as follows: The 10-g ball-milled wood sample was stirred in 80% neutral aqueous dioxane and 80% acidic dioxane containing 0.05-M HCl with a solid to liquid ratio of 1:20 (g/mL) at 80 °C for 6 h, respectively. The extraction procedure was repeated twice and the solid residue was washed with fresh dioxane until the filtrate was

**Fig. 1** Selective precipitation scheme of lignin–carbohydrate complexes (LCCs) from *Eucalyptus*



clear. The combined supernatants were first concentrated to 80 mL at reduced pressure and then precipitated in three volumes of 70% ethanol. The precipitates were collected through filtration and freeze-dried to obtain F1 and F1A fractions, based on 80% neutral aqueous dioxane and 80% acidic aqueous dioxane extraction, respectively. After that, the supernatant was concentrated to 60 mL and then precipitated in three volumes 100% ethanol. A pellet rich in lignin was recovered as F2 and F2A fractions, respectively. Subsequently, the corresponding supernatant was concentrated to 50 mL with a rotary evaporator under reduced pressure, and then precipitated in 10 volumes acidic water (pH = 2) to obtain F3 and F3A fractions, respectively.

### Characterization of LCCs

The analysis of the carbohydrate moieties associated with the six LCC fractions was conducted by hydrolysis with dilute sulfuric acid according to the previous literature (Yuan et al. 2011). 50 mg of LCC fraction was completely dissolved in DMSO/*N*-methylimidazole (2:1, v/v, 3 mL); after continually shaking for 24 h at room temperature in the dark, the acetic anhydride (1 mL) was added in the mixture and maintained for 2 h. At the end of the designated time, the solution was dropped slowly into acid water (pH = 2, 100 mL) to precipitate and then freeze-dried to obtain the acetylated LCC sample. The weight-average ( $M_w$ ) and number-average ( $M_n$ ) molecular weights of the acetylated LCC preparations were detected by gel permeation chromatography (GPC)

with an ultraviolet (UV) detector on a PL-gel 10- $\mu$ m mixed-B 7.5 mm i.d. column according to the previous literature (Wen et al. 2013b; Zhao et al. 2016). The 2D HSQC and  $^{31}\text{P}$  NMR spectra were recorded on a Bruker AVIII 400 MHz spectrometer at 25 °C. For the 2D HSQC NMR, about 40 mg of sample was dissolved in 0.5 mL of DMSO- $d_6$ . For the  $^{31}\text{P}$  NMR, sample (20 mg) was dissolved in 500- $\mu$ L anhydrous  $\text{CDCl}_3$ /pyridine (1:1.6, v/v, liquid A). 100- $\mu$ L cyclohexanol solution (10.85 mg/mL, in liquid A) and 100- $\mu$ L chromium (III) acetylacetonate solution (5 mg/mL, in liquid A) were added. 100- $\mu$ L phosphorylating agents (2-chloro-4, 4, 5, 5-tetramethyl-1, 3, 2-dioxaphospholane, TMDP) was added into the above solution and the mixture was kept for 10 min. The final phosphatized sample was transferred into a 5-mm NMR tube for subsequent determination. A semi-quantitative method based on 2D HSQC spectra was used to calculate the relative amount of lignin–lignin and LCC linkages (Wen et al. 2013a; Zhao et al. 2016).

## Results and discussion

### Yield and carbohydrate composition

Wu and Argyropoulos (Wu and Argyropoulos 2003) introduced the enzymatic mild acidolysis method to isolate the high yield of lignin and minimizing damage on the lignin structure from plant cell walls. To improve the yield of LCCs, the acidic dioxane containing 0.05 M HCl was

developed to extract the LCCs from *Eucalyptus* based on the above-mentioned enlightenments. For comparison purpose, the neutral aqueous dioxane was also applied to extract the LCCs as the control. As shown in Table 1, the treatment of the ball-milled *Eucalyptus* with neutral aqueous dioxane at 80 °C extracted 26.1% LCCs based on Klason lignin. By contrast, the LCCs yield increased to 68.4% based on Klason lignin with acidic dioxane extraction. Moreover, the sugar content of the LCC fractions obtained from acidic aqueous dioxane treatment was generally higher than those fractions of neutral aqueous dioxane extraction. These results indicated that the acidic aqueous dioxane was able to isolate more LCCs than the neutral aqueous dioxane from the plant cell walls at the same temperature. It should be noted that the other 31.6% LCCs for the acidic dioxane treatment, which still retained in the residue and the final supernatant after precipitation of F3A, were not analyzed in the present study.

The six LCC fractions in this study were obtained by a selective precipitation process from neutral aqueous dioxane and acidic dioxane extracts, respectively. Different ethanol concentrations have been used to grade precipitate hemicelluloses (Peng et al. 2009; Bian et al. 2010). The distribution of branches along the xylan backbone was different among the hemicellulosic fractions isolated at the various ethanol concentrations. Therefore, 70% ethanol and 100% ethanol were developed to precipitate LCCs in the present study. As shown in Table 1, the sugar contents of the F1, F2, and F3 fractions were 60.3, 30.5, and 5.4%, respectively. Most of the carbohydrates in the neutral aqueous dioxane extracts were precipitated as F1 and F2 fractions by 70 and 100% ethanol, respectively. However, due to the covalent linkages between lignin and carbohydrates (Björkman 1954), a part of the lignin was also incorporated in the F1 and F2 fractions. In addition, the sugar content of the F3 fraction was the lowest among the three fractions, suggesting that most of the dissolved lignin (15.2% without sugar) was precipitated in F3 fraction by pH = 2 acidic water. Besides, the yield of

the F1 and F2 fractions was only 2.7% and 7.3%, respectively, based on the Klason lignin of wood, much lower than that of the F3 fraction (16.1%). Similarly, F1A fraction possessed the highest sugar content (63.2%) and lowest yield of lignin (8.8% based on Klason) among the three fractions precipitated by acid aqueous dioxane extraction. It had been reported that fractionation of LCC can be classified as “carbohydrate-rich LCC”, and “lignin-rich LCC” (Lawoko et al. 2005). Therefore, the F1, F2, F1A, and F2A fractions can be categorized as carbohydrate-rich LCC, and the F3 and F3A fractions can be regarded as lignin-rich LCC preparations.

It has been reported that xylans are the predominant hemicellulosic components in the hardwood cell walls, and most of the hemicelluloses are located in secondary wall (Sun et al. 2001). This could explain the reason that xylose was the predominant sugar constituent in all the obtained six LCC fractions. In addition, other sugars, such as glucose, galactose, arabinose, and uronic acid, were also observed in these LCC fractions. Interestingly, the contents of glucose and xylose reduced significantly in the F1A fraction as compared to F1, and the impact of acid hydrolysis with acidic aqueous dioxane was milder on F2A and F3A as compared to F2 and F3, respectively. On the other hand, rhamnose was not detected in the F1A, F2A, and F3A fractions, and the content of uronic acid in the F1A fraction was significantly higher than that of the F1 fraction. This suggested that the acidic aqueous dioxane extraction favored the isolation of the hemicelluloses enriched in uronic acids. This result was well in line with the previous literature (Sun et al. 2005).

### Molecular weight distributions

It has been reported that the molecular weight of LCC preparation depends on the isolated methods (Sakagami et al. 2010). As shown in Table 2, the molecular weight of the six LCC fractions ranged from 1380 to 14580 g/mol. The lowest molecular weight of the LCC was found in the F1

**Table 1** Yields and carbohydrate contents of the precipitated LCC fractions

Samples	Yield (%)		Sugar content <sup>d</sup>	Carbohydrate content <sup>a</sup> (%)						
	With sugar <sup>b</sup>	Without sugar <sup>c</sup>		Rha	Ara	Gal	Glc	Xyl	Man	Uro
F1	2.7 ± 0.1	1.1	60.3 ± 2.3	1.3	1.7	4.9	33.6	45.4	3.1	10.1
F2	7.3 ± 0.3	5.1	30.5 ± 1.0	2.4	2.6	7.3	2.73	67.9	1.7	15.4
F3	16.1 ± 0.6	15.2	5.4 ± 0.2	3.7	6.7	8.8	6.54	57.8	3.9	5.4
F1A	8.8 ± 0.4	3.2	63.2 ± 2.5	N.D. <sup>e</sup>	6.3	5.6	25.4	19.2	1.3	42.3
F2A	16.8 ± 0.7	11.4	32.1 ± 1.2	N.D.	4.2	10.0	3.5	55.1	2.0	25.2
F3A	42.8 ± 1.6	40.0	6.6 ± 0.3	N.D.	1.3	4.0	3.7	68.2	2.3	20.5

<sup>a</sup>Rha rhamnose, Ara arabinose, Gal galactose, Glc glucose, Man mannose, Xyl xylose, Uro uronic acid

<sup>b</sup>Yield of LCC fraction (based on Klason lignin of wood)

<sup>c</sup>Yield of LCC fraction subtracted the sugar content (based on Klason lignin of wood)

<sup>d</sup>Yield of the associated carbohydrate (based on LCC fraction)

<sup>e</sup>Not detected

**Table 2** Weight-average ( $M_w$ ) and number-average ( $M_n$ ) and  $M_w/M_n$  of the precipitated LCC fractions

Samples	$M_w$	$M_n$	$M_w/M_n$
F1	1380 ± 20	1370 ± 30	1.01
F2	14580 ± 150	11060 ± 150	1.32
F3	4590 ± 60	2870 ± 40	1.60
F1A	1400 ± 40	1380 ± 20	1.01
F2A	9940 ± 100	8620 ± 100	1.15
F3A	4780 ± 80	3060 ± 50	1.56

fraction with a very high carbohydrate content (60.3%), suggesting that some of the lignin and most of the carbohydrates were first coprecipitated in 70% ethanol. In addition, the F2 fraction exhibited the highest molecular weight of 14580 g/mol due to the associated polysaccharose linked with lignin in LCCs. The carbohydrate chains linked to lignin can increase the hydrodynamic volume of lignin, thus increasing the apparent molar mass of lignin in GPC measurements (Jääskeläinen et al. 2003). Furthermore, 2D HSQC analysis revealed covalent linkages (mainly PhGlc bonds) between lignin and hemicelluloses in the F2 fraction. The molecular weight of the F3 fraction which possesses the lowest sugar content was 4590 g/mol, close to the value of milled wood lignin (MWL) in our previous study (Zhao et al. 2016).

The average molecular weights of the F1A, F2A, and F3A fractions were similar to the value of the F1, F2, and F3 fractions, respectively. However, the molecular weight of F2A fraction was significantly lower (32%) than that of F2 fraction owing to the cleavage of some LCC linkages during the acidic aqueous dioxane treatment. This was in line with the results of semi-quantitative integration of the 2D NMR spectra, as shown in Table 3. The content of the PhGlc linkages decreased from 17.0 per 100Ar (monomeric lignin unit) in the F2 fraction to 13.0 per 100Ar in the F2A fraction. Besides, all the six LCC fractions exhibited relatively narrow-molecular-weight distributions ranging from 1.01 to 1.60.

**Table 3** Semi-quantitative HSQC analysis of lignin substructures and LCC linkages in the precipitated fractions (the values were presented on the basis of per 100 aromatic units)

Samples	$\beta$ -O-4	$\beta$ - $\beta$	$\beta$ -5	$\beta$ -1	BE	PhGlc	S/G
F2	86.1	14.8	N.D.	N.D.	N.D.	17.0	2.96
F3	58.8	13.2	1.8	0.2	1.7	N.D.	2.21
F2A	83.0	6.8	N.D.	N.D.	N.D.	13.0	3.13
F3A	59.6	11.5	2.3	N.D.	1.6	N.D.	2.37

N.D. = not detected

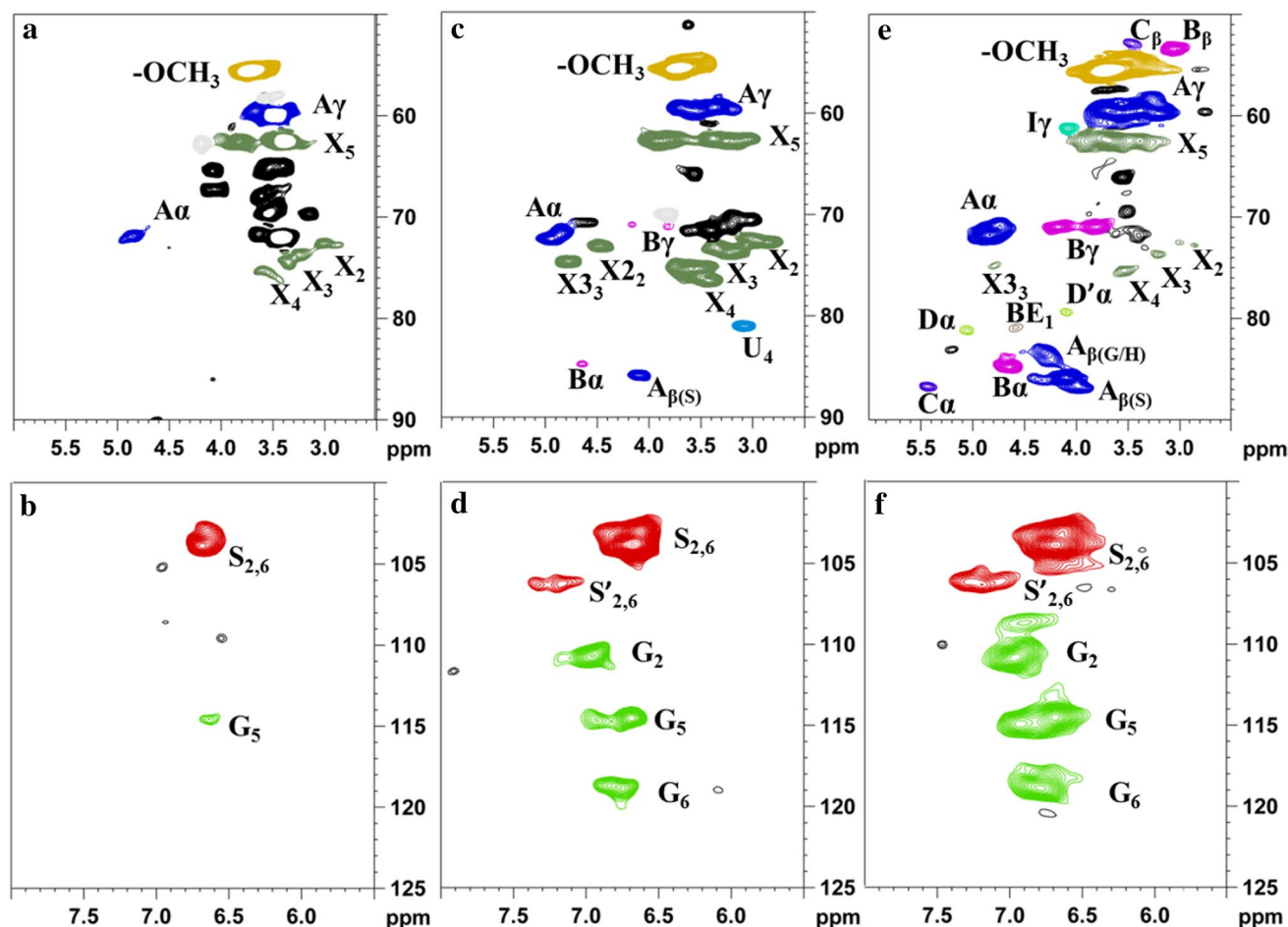
## 2D HSQC NMR analysis

2D HSQC NMR can provide important compositional and structural information of the LCCs and lignin (Du et al. 2014). In addition, the application of 2D HSQC NMR can also provide a direct evidence of the structural characteristics and the linkages of LCCs (Balakshin et al. 2014). The HSQC spectra of the LCC fractions isolated by neutral aqueous dioxane and acidic aqueous dioxane treatment are shown in Figs. 2 and 3, respectively. The anomeric correlations ( $\delta_C/\delta_H$  90–110/3.5–6.0) of the associated carbohydrates in the six LCC fractions are displayed in Fig. 4. The main LCC linkages and substructures of lignin are depicted in Fig. 5. The HSQC crossing signals of lignin and the associated carbohydrates are assigned by the published literature (Kim and Ralph 2010; Mansfield et al. 2012). The assignments of the main lignin and associated carbohydrate cross signals in the HSQC spectra are listed in Table S1.

### Lignin side-chain and aromatic regions

As shown in Figs. 2 and 3, all the side-chain regions ( $\delta_C/\delta_H$  50–90/2.5–6.0) of the 2D HSQC spectra showed in prominent signals corresponding to methoxy groups and  $\beta$ -O-4 aryl ether linkages. The  $C_\alpha$ - $H_\alpha$  and  $C_\gamma$ - $H_\gamma$  correlations in  $\beta$ -O-4 substructures A were observed in the spectra of the F1 and F1A fractions. However, other substructures, such as  $\beta$ - $\beta$  (resinol, B),  $\beta$ -5 (phenylcoumaran, C), and  $\beta$ -1 (spirodienone, D), were not found at the same contour levels in the side chain of the spectra of the F1 and F1A fractions. In addition, the signals of the  $C_{2,6}$ - $H_{2,6}$  correlation in S-type lignin units ( $S_{2,6}$ ) and  $C_5$ - $H_5$  correlation in G lignin units ( $G_5$ ) were observed in the aromatic regions ( $\delta_C/\delta_H$  100–125/5.5–8.0) of the spectra in the F1 and F1A fractions. This suggested that the low molecular weight of LCCs (mainly S-type lignin units) and most of the carbohydrates were coprecipitated at the relatively lower concentration of ethanol (70% ethanol). Moreover, the most frequent inter-unit linkages of  $\beta$ -O-4 were linked by S units of lignin. The  $C_\beta$ - $H_\beta$  correlations in substructures A linked to G/H lignin units and the  $C_\alpha$ - $H_\alpha$  correlations in the substructures C and D were not detected in the F2 and F2A fractions. However, the  $\beta$ -5 and  $\beta$ -1 subunits were observed in the F3 fraction. This showed that more





**Fig. 2** Side-chain ( $\delta_C/\delta_H$  50–90/2.5–6.0; **a**, **c**, and **e**) and aromatic regions ( $\delta_C/\delta_H$  100–125/5.5–8.0; **b**, **d**, and **f**) in the 2D HSQC spectra of F1, F2, and F3 fractions. F1 fraction (**a**, **b**), F2 fraction (**c**, **d**), and F3 fraction (**e**, **f**) were isolated by neutral aqueous dioxane (DMSO- $d_6$  as solvent). Specific lignin–carbohydrate and lignin substructures are

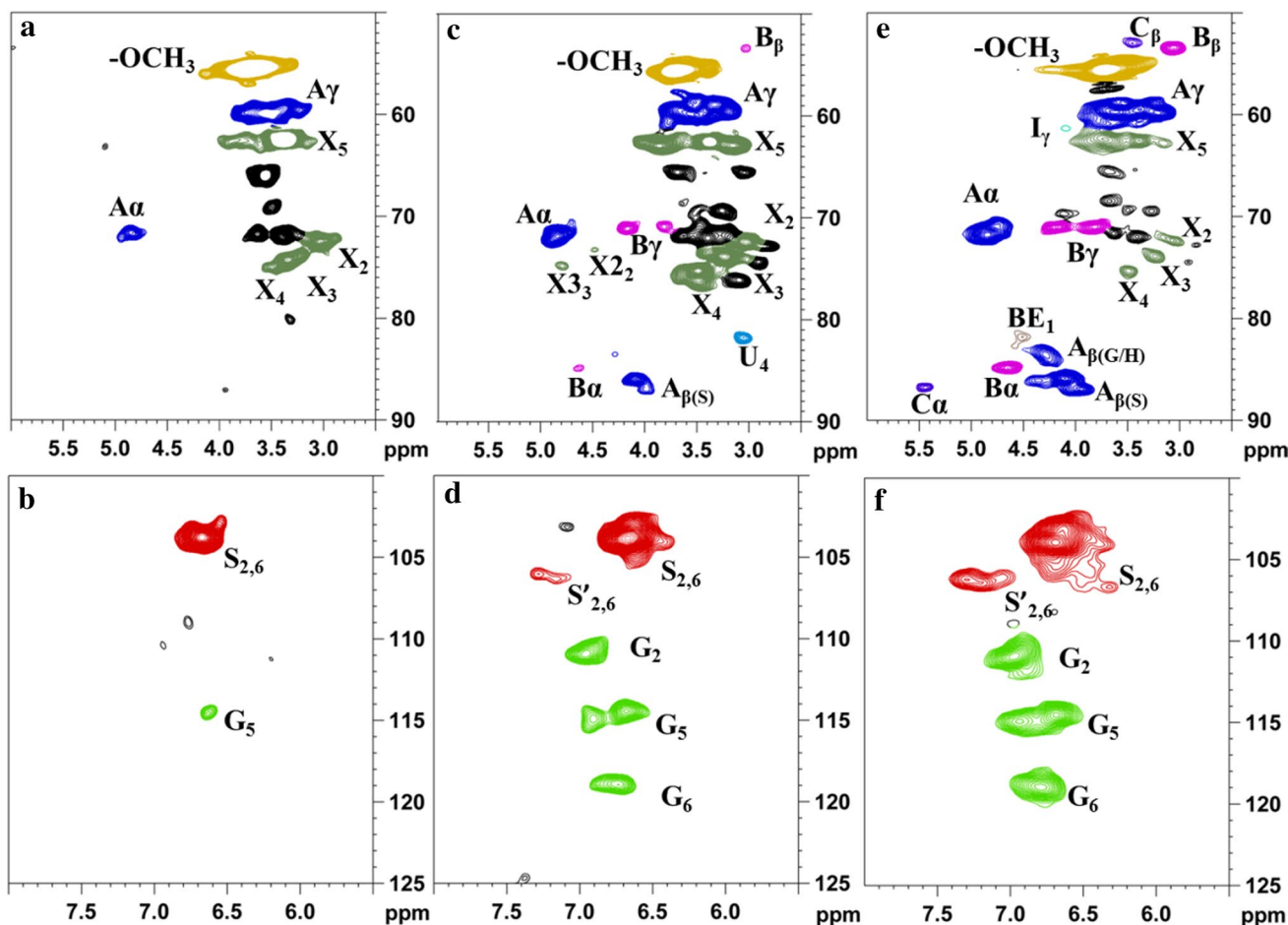
labeled according to Fig. 5. Carbohydrate signals were labeled as follows: U and X are  $\alpha$ -D-GlcUA and  $\beta$ -D-xylopyranoside units, respectively. X<sub>2</sub> and X<sub>3</sub> are  $\beta$ -D-xylopyranoside units acetylated at C-2 and C-3 positions, respectively

resistant ( $\beta$ - $\beta$ ,  $\beta$ -5, and  $\beta$ -1) linkages were connected by G lignin units according to the previous literature (Boerjan et al. 2003). Interestingly, the C $_{\alpha}$ -H $_{\alpha}$  and C $_{\alpha'}$ -H $_{\alpha'}$  correlations in spirodienone substructures D were observed in the F3 fraction, but not detected in the F3A fraction. This suggested that spirodienones are more vulnerable to cleavage than other substructures during the acidic aqueous dioxane treatment.

### Associated carbohydrates

Various signals from the associated carbohydrates could be found in the 2D HSQC spectra of the isolated LCC fractions (Figs. 2, 3, and 4). The most important carbohydrate signals corresponded to C<sub>2</sub>-H<sub>2</sub>, C<sub>3</sub>-H<sub>3</sub>, C<sub>4</sub>-H<sub>4</sub>, and C<sub>5</sub>-H<sub>5</sub> correlations in xylans (X<sub>2</sub>, X<sub>3</sub>, X<sub>4</sub>, and X<sub>5</sub>) were observed in the six LCC fractions. The signals of the C<sub>2</sub>-H<sub>2</sub> correlations from 2-*O*-acetyl- $\beta$ -D-xylopyranoside units (X<sub>2</sub>) and

C<sub>3</sub>-H<sub>3</sub> correlations from 3-*O*-acetyl- $\beta$ -D-xylopyranoside units (X<sub>3</sub>) were observed in the HSQC spectra of the F2 and F2A fractions. However, these signals could not be found at the same contour levels in the spectra of the F1, F1A, and F3A fractions, and only a part of signals of X<sub>3</sub> were found in the spectra of the F3 fraction (Fig. 4). This phenomenon suggested that *O*-2 and *O*-3 acetyl groups on the xylan chain were precipitated by 100% ethanol. A high substitution by acetylation would reduce the probability of formation of BE on xylan, since BE bonds were not detectable in the F2 and F2A fractions according to the previous literature (Giummarella and Lawoko 2016). In addition, the C<sub>4</sub>-H<sub>4</sub> correlations from 4-*O*-methyl- $\alpha$ -D-GlcUA (U<sub>4</sub>) were observed at  $\delta_C/\delta_H$  81.1/3.10 in the spectra of the F2 and F2A fractions, corresponding to the results obtained by sugar analysis. These results confirmed that the acetylated 4-*O*-methyl-glucuronoxylan is the major carbohydrate associated with the lignin macromolecules (Bian et al. 2010; Yuan et al. 2011).

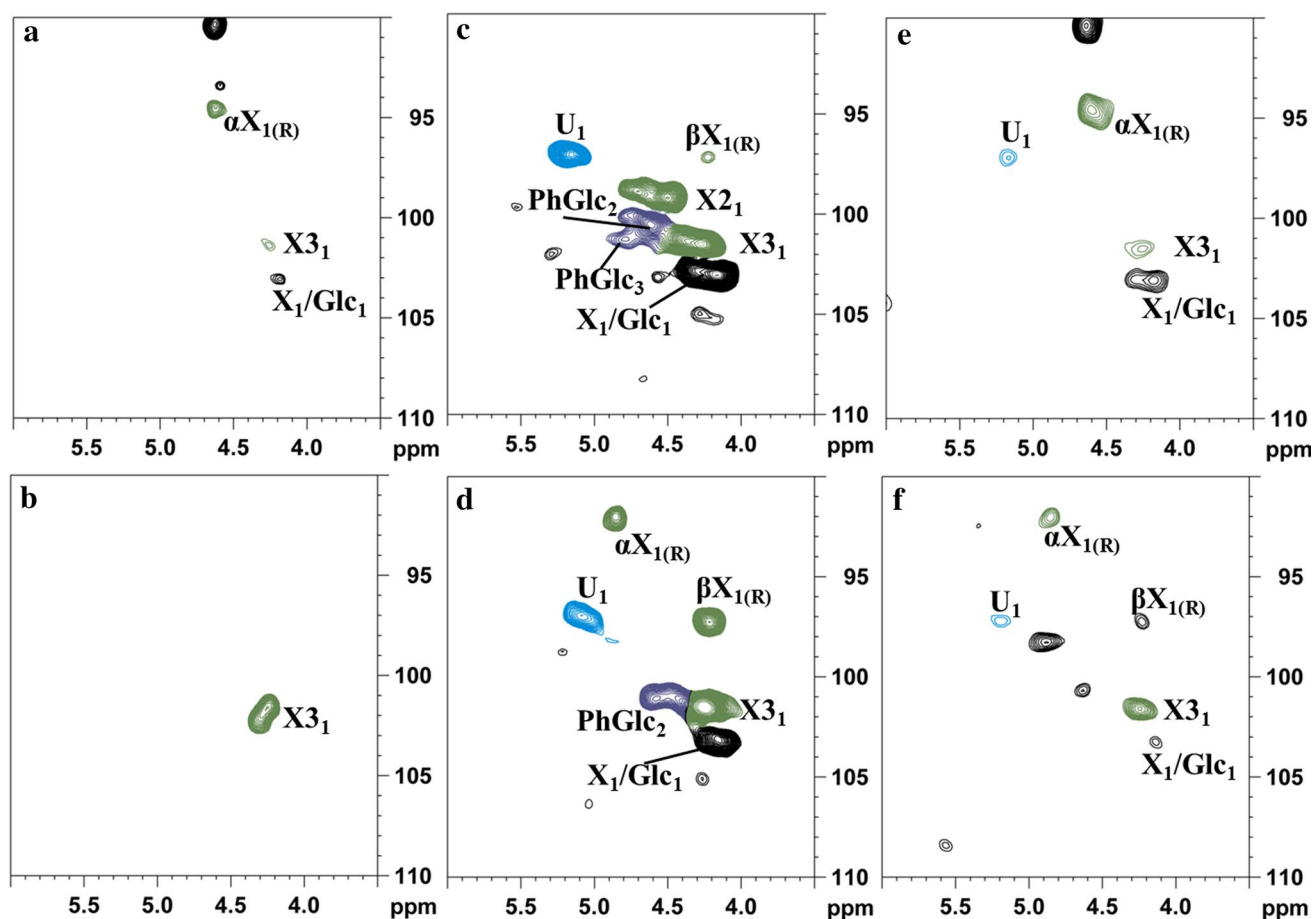


**Fig. 3** Side-chain ( $\delta_C/\delta_H$  50–90/2.5–6.0; **a**, **c**, and **e**) and aromatic regions ( $\delta_C/\delta_H$  100–125/5.5–8.0; **b**, **d**, and **f**) in the 2D HSQC spectra of F1A, F2A, and F3A fractions. F1A fraction (**a**, **b**), F2A fraction (**c**, **d**), and F3A fraction (**e**, **f**) were isolated by acidic aqueous dioxane containing 0.05 M HCl (DMSO- $d_6$  as solvent). Specific lignin–carbohydrate and lignin substructures are labeled according to Fig. 5. Carbohydrate signals were labeled as follows: U and X are  $\alpha$ -D-

GlcUA and  $\beta$ -D-xylopyranoside units, respectively. X<sub>2</sub> and X<sub>3</sub> are  $\beta$ -D-xylopyranoside units acetylated at C-2 and C-3 positions, respectively. Specific lignin–carbohydrate and lignin structures are labeled according to Fig. 5. Carbohydrate signals were labeled as follows: U and X are  $\alpha$ -D-GlcUA and  $\beta$ -D-xylopyranoside units, respectively. X<sub>2</sub> and X<sub>3</sub> are  $\beta$ -D-xylopyranoside units acetylated at C-2 and C-3 positions, respectively

According to lignin–carbohydrate model compounds, benzyl ether LCC structures can be subdivided into two types: (a) BE<sub>1</sub> linkages between the  $\alpha$ -position of lignin and the primary OH groups of carbohydrates (at C-6 of Glc, Gal, and Man, and C-5 of Ara); and (b) BE<sub>2</sub> linkages between the  $\alpha$ -position of lignin and secondary OH groups of carbohydrates, mainly of lignin–xylan type (at C-2 or C-3 of Xyl). However, the signal of BE<sub>2</sub> overlaps with the correlations of C <sub>$\alpha$</sub> -H <sub>$\alpha$</sub>  in the spirodienone structure (D) at  $\delta_C/\delta_H$  81.3/5.07 (Balakshin et al. 2011). In the present study, the signals of BE<sub>1</sub> were only detected in the spectra of the F3 and F3A fractions by NMR analysis. Besides, the signals of  $\gamma$ -esters linkages at  $\delta_C/\delta_H$  65–62/4.0–4.5 were not observed (Balakshin et al. 2011). Except for BE and  $\gamma$ -esters linkages, the PhGlc linkages are also found in native LCCs in wood. According to the previous literature (Yuan et al. 2011;

Miyagawa et al. 2014), the cross signals at  $\delta_C/\delta_H$  100.6/4.65 and  $\delta_C/\delta_H$  101.5/4.79 were labeled as PhGlc<sub>2</sub> and PhGlc<sub>3</sub>, respectively. As shown in Fig. 4, the signals of PhGlc<sub>2</sub> and PhGlc<sub>3</sub> were detected in the spectra of the F2 fraction, implying that the involvement of two different types of sugar units forms glycosidic-type linkages with phenolic hydroxyls in lignin. However, the signals of PhGlc<sub>3</sub> were not observed in the spectra of the F2A fraction, suggesting that PhGlc<sub>3</sub> linkages are more vulnerable to cleavage than PhGlc<sub>2</sub> linkages during the acidic aqueous dioxane treatment. According to the previous study (Miyagawa et al. 2014), PhGlc<sub>3</sub> ( $\delta_C/\delta_H$  101.5/4.79) should be linked with the xylose unit, whereas xylan was the predominant associated carbohydrate in both F2 and F2A fractions (Table 1). Therefore, further research was needed to confirm the different sugar components of the PhGlc linkages. In addition, the signals of PhGlc were



**Fig. 4** Anomeric regions ( $\delta_C/\delta_H$  90–110/3.5–6.0) in the 2D HSQC spectra of F1 (a), F1A (b), F2 (c), F2A (d), F3 (e), and F3A (f) fractions. The F1, F2, and F3 fractions were isolated by neutral aqueous dioxane, and the F1A, F2A, and F3A fractions were isolated by acidic

aqueous dioxane containing 0.05 M HCl (DMSO- $d_6$  as solvent). Specific lignin-carbohydrate and lignin structures are labeled according to Fig. 5. Carbohydrate signals were labeled as follows: U and X are  $\alpha$ -D-GlcUA and  $\beta$ -D-xylopyranoside units, respectively

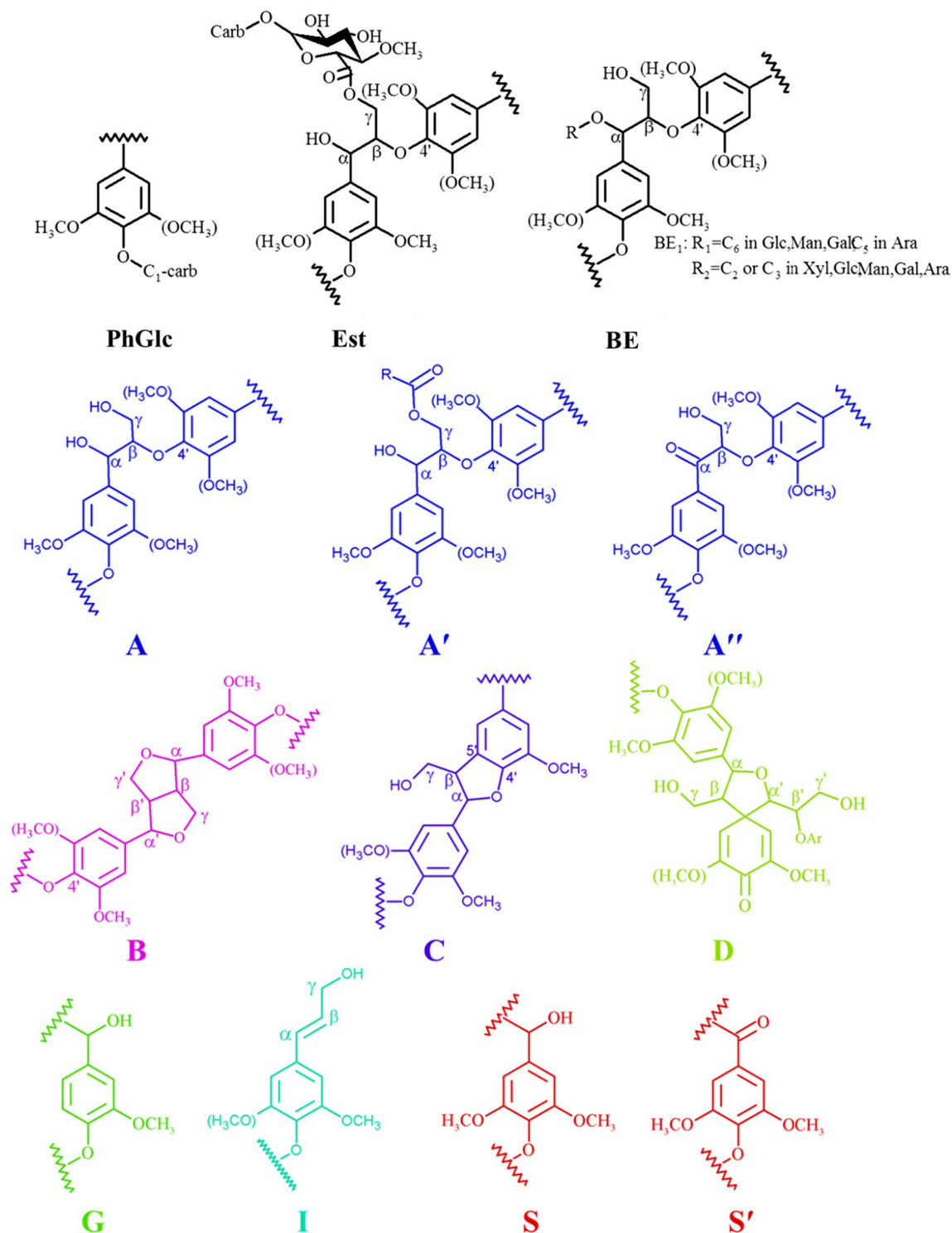
not found in the spectra of other fractions except the F2 and F2A fractions.

### Semi-quantification of lignin and LCC linkages

Table 3 displays the semi-quantitative HSQC results of the main lignin substructures and LCC linkages (referred to as per 100 aromatic units) in the F2, F3, F2A, and F3A fractions. Because of the low lignin content (less than 50%) and the missing of the  $C_2$ -H $_2$  correlations in G lignin units ( $G_2$ ) in the F1 and F1A fractions, the semi-quantitative integration of these two fractions were not conducted. Interestingly, the amount of the  $\beta$ -O-4 linkage in the F2 fraction was higher than that of F2A fraction, and the  $\beta$ - $\beta$  linkage content in the former was also higher than that of the latter. However, the  $\beta$ -O-4 linkage content in the F3 and F3A fractions was similar, and the amount of resinols and phenylcoumarans in these two fractions was also similar. This implied that acidic dioxane treatment might partially cleave the

structures of the lignin recovered by 100% ethanol precipitation. The  $\beta$ -O-4 linkage content in the F2 and F2A fractions was the relatively higher among the F2, F3, F2A, and F3A fractions. According to the previous study, the LCC fraction isolated by 95% ethanol precipitation also had the high  $\beta$ -O-4 linkage content (84.5 per 100Ar) (Yang et al. 2015). Furthermore, the S/G ratios in the F2 and F2A fractions were higher than their corresponding F3 and F3A fractions, and xylose contents in the formers were significantly higher than other monomer saccharides (Table 1). Therefore, it can be concluded that the xylan-rich LCC fractions, i.e., F2 and F2A, had less-branched lignin macromolecular configurations and tended to precipitate in 100% ethanol (Lawoko et al. 2005). The amount of BE linkages in the F3 fraction (1.7 per 100Ar) was similar with that of the F3A fraction (1.6 per 100Ar). However, the amount of PhGlc moieties in the F2A fraction (13.0 per 100Ar) was lower than that of the F2 fraction (17.0 per 100Ar). These results suggested that the acidic dioxane treatment could result in some cleavages





**Fig. 5** Main lignin–carbohydrate complex (LCC) linkages and substructures of lignin: (PhGlc) phenyl glycoside; (Est)  $\gamma$ -ester; (BE) benzyl ether; **a**  $\beta$ -O-4 linkages; **b** resinol substructures formed by  $\beta$ - $\beta$ , $\alpha$ -O- $\gamma$  and  $\gamma$ -O- $\alpha$  linkages; **c** phenylcoumaran structures formed

by  $\beta$ -5 and  $\alpha$ -O- $\alpha$  linkages; **d** spirodienone structures formed by  $\beta$ -1 and  $\alpha$ -O- $\alpha$  linkages; **i** *p*-hydroxycinnamyl alcohol end groups; **s** syringyl units; **s'** oxidized syringyl units; and **g** guaiacyl units

**Table 4** Quantification of the F3 and F3A fractions by quantitative  $^{31}\text{P}$ -NMR analysis (mmol/g lignin)

Samples	Aliphatic OH	Syringyl OH	Guaiacyl OH		Carboxylic group	Total phenolic OH
			C	NC		
F3	4.32	0.44	0.18	0.74	0.16	1.36
F3A	4.94	0.53	0.10	0.52	0.16	1.15

C condensed, NC noncondensed

of the PhGlc linkages, and BE bonds are more stabilized than PhGlc linkages from *Eucalyptus*.

### $^{31}\text{P}$ NMR analysis

Quantitative  $^{31}\text{P}$  NMR technique was applied to investigate the functional groups of the LCC fractions (Table 4, Fig. S1). Because of the high carbohydrate content, the LCC fractions like F1, F2, F1A, and F2A could not dissolve completely in the phosphating reagent. For the F3 and F3A fractions, the contents of S-OH groups were lower than those of G-OH groups, suggesting that most S-type lignin units are involved in the formation of  $\beta$ -O-4 linkages in these fractions and only a small amount of S-OH could be reacted with TMDP and detected by the  $^{31}\text{P}$  NMR technique. It was found that the amounts of the COOH and phenolic OH groups in the F3 fraction were similar with that of the F3A fraction. These indicated that the acidic aqueous dioxane extraction favored the isolation of the high yield of the LCCs and the lignin structure was not significantly modified from *Eucalyptus*.

### Conclusions

In summary, to investigate the LCC structures and linkages of *Eucalyptus*, a selective precipitation process was developed in this study to isolate LCCs in the neutral aqueous dioxane and acidic aqueous dioxane extractable solutions from plant cell walls, respectively. The results indicated that the acidic aqueous dioxane treatment favored the extraction of a high yield of LCCs, and the LCC linkages were not substantially cleaved. Moreover, both carbohydrates and lignin were first coprecipitated by 70% ethanol, and then, the polysaccharose linked with lignin through LCC linkages was selectively precipitated by 100% ethanol. Therefore, this selective precipitation protocol of LCCs introduced in this study could improve the understanding of the LCC structures and linkages in the plant cell walls, and help to develop more effective plant deconstruction or depolymerization strategies in biorefinery.

**Author contribution statement** RS and TY conceived and designed the research; BZ, JX, and BC conducted the

experiments; BZ, JX, and XC analyzed the data; BZ wrote the manuscript; SW and AC corrected the manuscript. All authors read and approved the manuscript.

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