Extraction, Purification, and Characterization of Lignin Fractions from Sugarcane Bagasse

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Three-step extraction of lignin fractions from ball-milled sugarcane bagasse (SCB) was studied with 96% dioxane, 50% dioxane, and 80% dioxane containing 1% NaOH at boiling temperature followed by purification to remove hemicelluloses. The total yields of hemicelluloses and lignin were 15.8% and 7.2% based on dried SCB, respectively. In the first step, 5.1% lignin (70.8% of the total extracted lignin) was isolated with 96% dioxane, which is higher than the traditional MWL procedure (3.3% lignin). In the second step, 10.4% hemicelluloses (65.8% of the total extracted hemicelluloses) were obtained with 50% dioxane. The obtained lignin fractions were characterized with FT-IR, ^{31}P-NMR, and 2D ^{1}H-^{13}C HSQC. The results suggested that the 'core' of the lignin structure did not change dramatically during the sequential neutral and alkaline dioxane treatment processes. The contents of phenolic hydroxyl groups in the three lignin fractions based on ^{31}P-NMR analysis were 1.66, 4.46, and 9.42 mmol/g lignin, respectively, higher than those from wood. The results also indicated that the lignin fractions obtained from SCB contained some amount of *p*-coumaric acid and ferulic acid, significantly different from those extracted from softwood and hardwood.

Keywords: Sugarcane bagasse; Lignin; Isolation; Dioxane; Characterization; 2D ^{1}H-^{13}C NMR

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INTRODUCTION

The lignin from herbaceous plants is known to be different from those from softwoods and hardwoods. It is composed of guaiacyl, syringyl, and *p*-hydroxyphenyl monomeric units, while wood lignins are composed of only guaiacyl units and/or syringyl units, or guaiacyl and *p*-hydroxyphenyl units (compression wood). Moreover, herbaceous cell walls are typified by the presence of considerable amounts of ferulic acid (FA) and *p*-coumaric acids (*p*-CA), linked to polysaccharides and/or lignins (Jacquet *et al.* 1995). It is considered that lignin plays an important role in the fractionation and utilization of lignocellulosic biomass. As one of the three main components of plant cell walls and a potentially useful renewable resource, lignin has attracted increasing interest in recent years. The detailed chemical and structural characteristics of lignin is becoming an important scientific approach as part of efforts to improve the utilization of lignocellulosic biomass.

A major problem in the elucidation of the chemical structure of native lignin is the isolation of lignin to as great an extent as possible while minimizing the extent of

chemical modification. Due to the complex nature of herbaceous lignin, the elucidation of its structure has been found to be more difficult than wood lignin. There are a series of standard preparation methods traditionally used for wood lignin studies (Bartuska *et al.* 1980; Maciel *et al.* 1981; Kolodziejski *et al.* 1982; Lindberg *et al.* 1988), among which the most common is based on extensive grinding of plant materials in a non-swelling liquid followed by dioxane extraction (Björkman 1956). The lignin preparations, milled wood lignin (MWL), obtained by this method have been considered as the basic standard material to perform most chemical and biological studies (Jung and Himmelsbach 1989; Ben-Ghedalia and Yosef 1994; Terrón *et al.* 1996). However, when the same approach is applied to non-wood material, the results are not as successful as expected (Himmelsbach and Barton 1980).

In China, sugarcane bagasse (SCB) is the second most commonly used non-wood fiber material after cereal straws for pulp and paper production (Bajpai *et al.* 2004). MWL from SCB was investigated in our group, and the yield was only 3.3% based on dried SCB. It is necessary to develop a method for the isolation of lignin with high yield for structure analysis. In our previous study (Zhang *et al.* 2010b), a three-step sequential extraction of lignin with 96% dioxane, 50% dioxane, and 80% dioxane containing 1% NaOH was developed for fractional isolation of lignin from wood. About 37% of the original lignin was released from eucalyptus, whereas only 13.5% could be isolated with traditional MWL method. Therefore, in the present study, a three-step sequential method was investigated for isolation of lignin from SCB with neutral and alkaline dioxane extraction, and the physico-chemical properties of lignin fractions obtained were characterized with FT-IR, ^{31}P-NMR, and 2D ^{1}H-^{13}C NMR (HSQC).

EXPERIMENTAL

Materials

SCB was obtained from a local sugar factory (Guangdong, China). The bagasse was depithed, air-dried, ground, and screened to prepare 20 to 40 mesh size particles. The ground bagasse was dried in a cabinet oven with air circulation for 16 h at 50^{o}C. It was then extracted in a Soxhlet extractor with toluene and ethanol (v/v, 2:1) for 7 h. The extractive-free bagasse was air-dried and further pulverized with a vibratory ball mill for 72 h in a stainless steel jar. The yield of lignin from SCB through the MWL procedure was only 3.3% based on dried SCB, and that of Klason lignin was 17.2%.

All chemicals were of analytical reagent grade and obtained from Guangzhou Chemical Reagent Factory, China.

Fractional Isolation of Lignin from SCB

The ball-milled bagasse was suspended in 96% aqueous dioxane (dioxane/water 96:4, v/v) with a solid-to-liquid ratio of 1:20 (g/mL) and refluxed for 2 h. The resulting suspension was filtered and washed with 96% aqueous dioxane until the filtrate was clear. The solid residues were dried in a cabinet oven with air circulation for 16 h at 50^{o}C to obtain the 96% dioxane-insoluble residues R1. The combined filtrates were concentrated with a rotary evaporator under reduced pressure to about 50 mL and then transferred into 3 volumes of 95% ethanol with agitation. The precipitates were filtered out, washed with 70% ethanol, and freeze-dried to obtain the 96% dioxane-soluble hemicellulosic fraction H1. The filtrates were combined and concentrated to remove ethanol and the remaining

dioxane. The resulted concentrate was transferred into 10 volumes of H_2O (pH 1.5 to 2.0, adjusted with 6 M HCl) with agitation. The precipitates were filtered out, washed with acidified water (pH 2.0), and freeze-dried to obtain the 96% dioxane-soluble lignin fraction L1.

The residues R1 obtained after 96% aqueous dioxane extraction were successively extracted with 50% aqueous dioxane (dioxane/water 50:50, v/v) with a solid-to-liquid ratio of 1:20 (g/mL) at boiling temperature for 2 h. The resulting suspension was filtered and washed with 50% aqueous dioxane until the filtrate was clear. The solid residues were dried in a cabinet oven with air circulation for 16 h at 50°C to obtain the 50% dioxane-insoluble residues R2. The filtrates were combined and treated with the same method described above to obtain the 50% dioxane-soluble hemicelluloses (H2) and lignin (L2) fractions.

The residues R2 obtained after 50% aqueous dioxane extraction were refluxed with 80% aqueous dioxane (dioxane/water 80:20, v/v) containing 1% NaOH with a solid-to-liquid ratio of 1:20 (g/mL) for 2 h. The resulting suspension was filtered and washed with 80% aqueous dioxane. The solid residues were dried in a cabinet oven with air circulation for 16 h at 50°C to obtain the alkaline 80% dioxane-insoluble residues R3. The combined filtrates were adjusted to pH 5.5-6.0 and treated with the same method described above to obtain the alkaline 80% dioxane-soluble hemicelluloses (H3) and lignin (L3) fractions.

To reduce errors and confirm the results, each extraction procedure was performed at least three times under the same conditions to make sure the standard deviation was lower than 5.0%, and the yields of hemicelluloses and lignin fractions represent the average value.

Characterization of Lignin and Hemicellulosic Fractions with FT-IR

FT-IR spectra of the lignin and hemicellulosic fractions were recorded on an FT-IR spectrophotometer (Nicolet 510) using a KBr disc containing 1% finely ground samples. Thirty-two scans were taken for each sample with a resolution of 2 cm^{-1} in transmittance mode in the range 4000 to 400 cm^{-1}.

Quantitative Determination of Hydroxyls in Lignin Fractions using ^{31}P-NMR

Quantitative ^{31}P-NMR spectra of the three lignin fractions were recorded on a Bruker DRX-400 spectrometer according to published procedures (Granata and Argyropoulos 1995; Akim *et al.* 2001). A solvent mixture composed of pyridine and deuterated chloroform (1.6:1, v/v) was prepared. An internal standard solution was prepared with cyclohexanol (400 mg) and chromium (III) acetylacetonate (40 mg) dissolved in 10 mL above solvent. Relaxation reagent was prepared with chromium (III) acetylacetonate (27.9 mg) dissolved in 5 mL of the above solvent. Dried lignin (40 mg) and internal standard solution (100 µL) were added into 800 µL of pyridine and deuterated chloroform (1.6:1, v/v). The resulting suspension was left at room temperature until the lignin was totally dissolved before 5 mL relaxation reagent was added. 2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholate (130 µL) was added and the mixture was transferred into a NMR tube for analysis.

Characterization of Lignin Fractions with ^{1}H-^{13}C Correlation NMR (HSQC)

^{1}H-^{13}C correlation 2D NMR (HSQC) spectra were recorded with a standard program at 300 K on a Bruker Avance-360 spectrometer (Bruker, Germany). Lignin

samples (66 mg) were dissolved in 0.5 mL DMSO-d_6, and the solvent signals (δ_H/δ_C 2.50/39.51 ppm) were used as a standard.

RESULTS AND DISCUSSION

Isolation of Lignin and Hemicellulosic Fractions

It is well known that lignin is covalently linked to polysaccharides, forming a lignin-hemicelluloses network made up of benzyl-ether, benzyl-ester, and phenyl-glycoside bonds (Guerra *et al.* 2006). Milled wood lignin is considered to be the most standard method for isolation of native lignin from wood. However, when it is applied to cereal straws and grass materials, the isolated lignin preparations contain great amounts of associated polysaccharides, which hampers the determination of lignin structure. In order to obtain relatively pure lignin with high yield, in present study, sequential isolation of lignin from SCB with 96% dioxane, 50% dioxane, and 80% dioxane containing 1% NaOH at boiling temperature was studied. Prior to precipitation of lignin, dioxane-soluble hemicelluloses were firstly precipitated with 70% ethanol to obtain relatively pure lignin in each step.

96% dioxane is generally considered as the standard lignin solvent, and 3.3% lignin based on dried materials could be obtained from wood when using the traditional MWL procedure. In the first step, most of extractable lignin was isolated with 96% dioxane from SCB, and the yields of hemicelluloses and lignin were 5.4% and 5.1%, respectively, higher than those from wood. In the second step, 10.4% hemicelluloses and 1.4% lignin were solubilized with 50% dioxane, which indicated that the solubility of hemicelluloses in 50% dioxane was higher than in 96% dioxane. Jung and Himmelsbach (1989) compared the 50% dioxane-soluble lignin and 96% dioxane-soluble lignin extracted from wheat straw and found that lignin extracted with 50% dioxane would be just as a representative of native lignin as that extracted with 96% dioxane. It was also reported that about 60 to 70% of the lignin from wheat straw was extracted with dilute alkali at temperatures lower than 100°C and that such mild alkaline treatment did not cause much chemical modification except the saponification of ester bonds between *p*-coumaric acid (*p*-CA) and lignin or ferulic acid (FA) and polysaccharides (Lu and Ralph 2010). In the third step, alkaline dioxane was used to isolate the hemicelluloses and lignin linked with ester bonds. However, only trace amounts of hemicelluloses were obtained with 80% dioxane containing 1% NaOH, which was probably from the alkaline cleavage of ester linkages between lignin and hemicelluloses with alkaline dioxane treatment (Sun *et al.* 2003). Simultaneously, 0.8% lignin was obtained from the ester linkage cleavage. The total yields of hemicelluloses and lignin with the three-step extraction were 15.8% and 7.2%, respectively, where 70.8% of the total extracted lignin was isolated in the first step with 96% dioxane, and 65.8% of the total extracted hemicelluloses were obtained in the second step with 50% dioxane.

FT-IR Spectra

In the present study, Fourier transform infrared (FT-IR) spectroscopy was used to characterize the structure of the isolated hemicelluloses and lignin. FT-IR spectra of hemicellulosic fractions H1 and H2 isolated with 96% dioxane and 50% dioxane, respectively, are illustrated in Fig. 1. The broad band at 3421 cm^{-1} is related to the stretching of hydroxyl groups. The absorption at 2925 cm^{-1} arises from C-H stretching. The band at

1736 cm^{-1} is originated from the acetyl and uronic ester groups of the hemicelluloses. The signal at 1632 cm^{-1} can be attributed to the absorbed water (Kacurakova *et al.* 1998). The small absorbances at 1605, 1515, 1464, and 1425 cm^{-1} correspond to the aromatic skeletal vibrations and ring breathing with C-O stretching in small amounts of associated lignin in hemicelluloses. The bands at 1383, 1250, and 1168 cm^{-1} are attributed to the C-H bending, the symmetric stretching of C-O, and the antisymmetric stretching of C-O in ester. The strong peak at 1044 cm^{-1} arises from the glycosidic bond stretching (C-O-C). A small sharp band at 897 cm^{-1} is characteristic of β-glycosidic linkages between the sugar units (Sun *et al.* 1996). Two low-intensity shoulders at 987 and 1083 cm^{-1} are assigned to arabinose substitution at C-3 of the xylose residues, a characteristic typical of substituted arabinoxylans (Blakeney *et al.* 1983). The intensities of the bands in these two spectra were rather similar, indicating the similar structures of the hemicellulosic fractions.

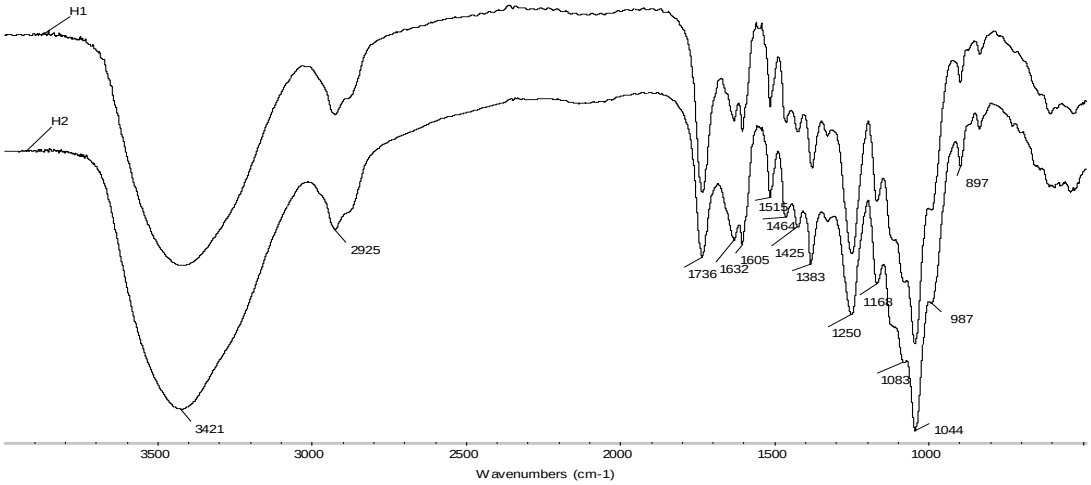

Fig. 1. FT-IR spectra of hemicellulosic fractions with 96% dioxane (H1) and 50% dioxane (H2)

The FT-IR spectra of the three lignin fractions are illustrated in Fig. 2. There were only minor changes in the absorbance intensities, which suggests that the 'core' of the lignin structure did not change dramatically during the sequential treatment processes (Zhang *et al.* 2010a; Chatterjee *et al.* 2012). The band at 1698 cm^{-1} arises from the stretching of carbonyl group in ferulate (Jung and Himmelsbach 1989). The intensity of this absorbance decreased from L1 to L3 and to L2, indicating that ferulates bonded to lignin could easily be extracted with 96% dioxane, and those crosslinked to lignin and hemicelluloses could be extracted after the cleavage of ester bond between FA and hemicelluloses under alkaline conditions. The very small signal at 1657 cm^{-1} is assigned to the carbonyl stretching in trace amount of *p*-CA. This signal was present with increased intensity in L2 compared to L1, indicating that most *p*-CA bonded to lignin could be extracted with 50% dioxane with increased solubility; however, the signal disappeared in L3, indicating that *p*-CA was not crosslinked to lignin and hemicelluloses as a bridge like FA. The absorbances at 1224 and 1125 cm^{-1} for the symmetric and antisymmetric C-O stretching connected to C=O also indicated the present of FA and *p*-CA in lignin fractions. The bands at 1597, 1509, 1463, and 1422 cm^{-1} are related to the aromatic skeletal vibrations and ring breathing with C-O stretching of lignin. The band at 1032 cm^{-1} probably derives from the associated polysaccharides (Lawther *et al.* 1996). The relatively high intensities of the absorbances at 1032 and 3422 cm^{-1} in the spectrum

of L2 indicate that the 50% dioxane-soluble lignin fraction L2 contained a much higher amount of carbohydrates than other two lignin fractions.

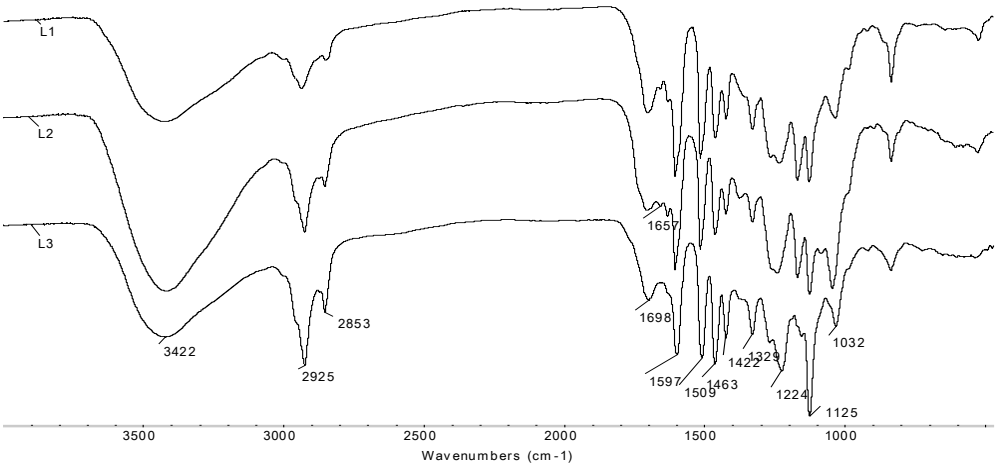

Fig. 2. FT-IR spectra of lignin fractions extracted with 96% dioxane (L1), 50% dioxane (L2), and alkaline 80% dioxane (L3)

^{31}P-NMR Spectra

The considerable proportion of free phenolic and aliphatic hydroxyl groups present in grass lignins plays an important role in the solubility properties of Gramineae lignins (Lapierre *et al.* 1989). The free hydroxyl groups of lignin also determines its reactivity during the fractionation and utilization of lignocelluloses (Akim *et al.* 2001). In order to elucidate the hydroxyl groups in lignin, the isolated lignin fractions (L1, L2, and L3) phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane were analyzed with ^{31}P-NMR according to the published method (Argyropoulos 1994), and the ^{31}P-NMR spectra are illustrated in Fig. 3.

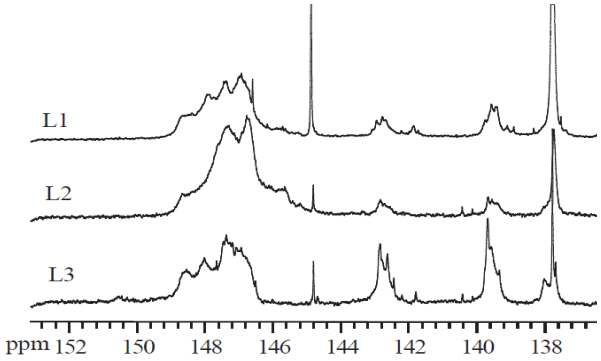

Fig. 3. ^{31}P-NMR spectra of three lignin fractions from SCB

All signals in the range of 149.2 to 146.0 and 144.5 to 137.2 ppm are associated with aliphatic and phenolic hydroxyl groups, respectively. In the phenolic hydroxyl region, the signals in the range of 140.0 to 138.8, 143.1 to 142.4, and 138.2 to 137.4 ppm are assigned to hydroxyl groups in non-condensed guaiacyl, syringyl, and *p*-hydroxy-phenyl units, respectively, and those in the range of 142.4 to 141.5 ppm are attributed to condensed guaiacyl unit. The contents of various hydroxyl groups in lignin fractions obtained from ^{31}P-NMR spectra (mmol/g lignin) are listed in Table 1. The presence of

signals of hydroxyl groups in guaiacyl, syringyl, and *p*-hydroxyphenyl units indicated that the isolated lignin fractions were all GSH-type lignin, suggesting that the core of the lignin structure did not change significantly during the sequential neutral and alkaline dioxane extraction processes, which is in agreement with the results obtained from FT-IR. As shown in Table 1, the contents of phenolic hydroxyl groups in the three lignin fractions were 1.66, 4.46, and 9.42 mmol/g lignin, respectively, higher than those from wood (Zhang *et al.* 2010a,b), which was in agreement with the herbaceous lignin characteristics. L3 contained much more phenolic hydroxyls than L1 and L2. In addition, relatively higher amount of aliphatic hydroxyls were found in L2 than in L1 and L3, which was probably due to more amount of associated carbohydrates present in L2, corresponding to the results from FT-IR analysis.

Table 1. Contents of Various Hydroxyl Groups in Lignin Fractions Obtained from ^{31}P-NMR Spectra (mmol/g lignin)

Lignin fractions	Aliphatic	Phenolic					
		Condensed phenolic	Non-condensed phenolic				Total
			H	G	S	Total	
L1	2.40	0.047	1.08	0.35	0.18	1.61	1.66
L2	20.92	0	2.18	1.34	0.94	4.46	4.46
L3	13.75	0	2.56	3.83	3.03	9.42	9.42

2D ^{1}H-^{13}C NMR Spectra

2D ^{1}H–^{13}C HSQC is a powerful tool for qualitative and quantitative analysis of lignin structures. It provides resolution of signals overlapping in the ^{1}H and ^{13}C NMR spectra and reveals both the aromatic units and the different inter-unit linkages present in lignin. In the present study the lignin fractions were characterized with 2D HSQC spectroscopy to better understand the structure of lignin. L1 and L3 could be easily dissolved in DMSO-d_6, while L2 could not be completely dissolved in DMSO-d_6 due to the relatively high content of associated carbohydrates. It swelled and produced a gel after ultrasound irradiation. Figure 4 lists the HSQC spectra of L1 and L3 as well as the predominant lignin structure with same color.

In HSQC spectra, the side chain aliphatic ^{1}H–^{13}C correlations (δ_H/δ_C 2.8–6.0/50–110 ppm region) and the aromatic ^{1}H–^{13}C correlations (δ_H/δ_C 6.0–8.0/100–150 ppm region) provide information on side-chain inter-unit linkages and lignin aromatic units, respectively. The signals were defined according to earlier publications (Kim *et al.* 2008; Zhang *et al.* 2010b). In the aliphatic regions, the major lignin correlating signals are from the typical lignin linkages β-O-4 (substructure A, blue color) and β-5 (B, green color). Linkage β-β (C, red color) could also be easily observed in L3, but could not be found in L1 due to the relatively low ratio of this type of substructure compared with other inter-unit linkages and associated carbohydrates. Obviously, in comparison with L3 isolated with alkaline dioxane, L1 isolated with neutral dioxane contained much more associated carbohydrates, which was probably due to the alkaline cleavage of the cross-linkages between lignin and carbohydrates via ferulate as a bridge under alkaline conditions. Similar results were also reported on the cleavage of the ester linkages between lignin and hemicelluloses during alkaline treatment (Spencer and Akin 1980). The results also suggested that the remained carbohydrates in L3 were probably linked to lignin through alkaline resistant linkages such as ether or C-C linkages. The residual hemicelluloses 2-acetylated xylan (2-O-Ac-D-Xylp) and 3-acetylated xylan (3-O-Ac-D-Xylp) were found

in L1. In addition, the reducing end of glucopyranoside (β-D-GlcpR) and some other polysaccharides were also detected (not assigned color, in black) (Yelle *et al.* 2008).

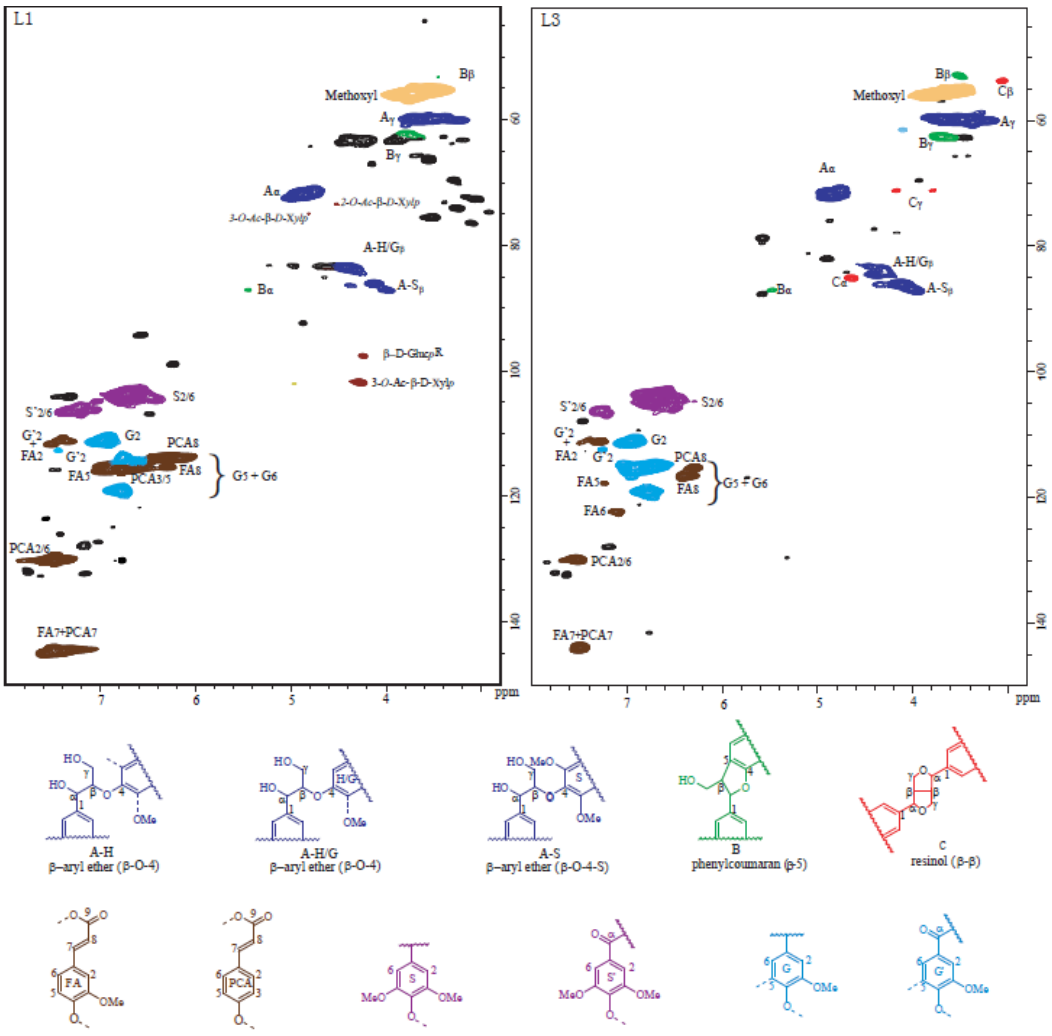

Fig. 4. HSQC spectra of lignin fractions L1 and L3

In the aromatic region, L1 and L3 exhibited similar basic structural units (S/G ratio) to lignin except much higher amounts of *p*-CA and FA in L1. It was reported that grass cell wall contains a small amount of ester-linked hydroxycinnamic acid derivatives, such as *p*-CA and FA phenolic constituents (Hartley and Ford 1989). A considerable amount of these hydroxycinnamates remained in lignin fractions extracted with neutral dioxane from bagasse, as shown in the aromatic region with brown color in Fig. 4, which is in agreement with the results from FT-IR and ^{31}P-NMR analyses. Clearly, the content of *p*-CA in L1 is much more than in L3, but that of FA is almost same in L1 and L3, indicating the different linkages of lignin with *p*-CA and with FA. FA is always present as a bridge to crosslink lignin and carbohydrates, and it is always present in lignin fractions whether ester bonds between FA and lignin are cleaved or not, while *p*-CA is just linked to the lignin side chain, resulting in the absence of some amount of *p*-CA in alkaline dioxane treatment. These results were in parallel to those from FT-IR analysis.

CONCLUSIONS

1. The total yields of hemicelluloses and lignin are 15.8% and 7.2% based on dried sugarcane bagasse (SCB), respectively, after three-step extraction of lignin fractions from ball-milled SCB with 96% dioxane, 50% dioxane, and 80% dioxane containing 1% NaOH at boiling temperature.
2. 70.8% of the total extracted lignin was isolated in the first step with 96% dioxane, and 65.8% of the total extracted hemicelluloses were obtained in the second step with 50% dioxane.
3. The 'core' of the lignin structure did not change dramatically during the sequential neutral and alkaline dioxane treatment processes.
4. The contents of phenolic hydroxyl groups in the three lignin fractions based on ^{31}P-NMR analysis were 1.66, 4.46, and 9.42 mmol/g lignin, respectively, which indicates higher levels than those present in wood.
5. The lignin fractions contained some amount of *p*-coumaric acid and ferulic acid, which is significantly different from those extracted from softwood and hardwood.

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