PHYSICO-CHEMICAL CHARACTERIZATION OF DIFFERENT ALCOHOL-SOLUBLE LIGNINS FROM RICE STRAW

D. SHE, X. N. NIE, F. XU,[*] Z. C. GENG, H. T. JIA,[**] G. L. JONES[***] and M. S. BAIRD[****]

College of Resources and Environment, Northwest Agriculture and Forest University, Yangling 712100, China
[*]*Institute of Biomass Chemistry and Technology, Beijing Forestry University, Beijing 10083, China*
[**]*College of Pratacultural and Environmental Sciences, Xinjiang Agricultural University, Urumqi 830052, China*
[***]*The BioComposites Centre, University of Wales, Bangor LL57 2UW, United Kingdom*
[****]*Department of Chemistry, University of Wales, Bangor LL57 2UW, United Kingdom*

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The treatment of dewaxed rice straw with 60% methanol, 60% ethanol, 60% *n*-propanol, 60% *n*-butanol, 60% ethanol containing 0.01 M HCl and 60% ethanol containing 0.25 M NaOH at 75 ºC for 3 h resulted in the solubilization of 14.6, 13.0, 16.3, 12.2, 13.0 and 75.6% of the original lignin, respectively. All alcohol-soluble lignin fractions showed spectral features of GSH-type lignin. The yield and purity of the acid-insoluble lignin extracted with 60% ethanol containing 0.25 M NaOH was much higher than of those prepared with other alcohols. The purity of lignin prepared with different alcohols followed the order: methanol > *n*-propanol > *n*-butanol > ethanol. Furthermore, the treatment of 60% ethanol under alkali catalyst produced purer lignin than the 60% ethanol treatments with or without acid catalyst. The six alcohol-soluble lignin fractions were comparatively characterized by both destructive methods, such as alkaline nitrobenzene oxidation, and non-destructive techniques, such as ultraviolet (UV), Fourier Transform Infrared (FTIR), ^{13}C and two-dimensional Heteronuclear Single Quantum Correlation Nuclear Magnetic Resonance (^{13}C and 2D-HSQC NMR) Spectroscopy, and Gel Permeation Chromatography (GPC). The 2D-HSQC NMR spectrum showed that the prominent linkage of the lignin fraction obtained with 60% ethanol containing 0.25 M NaOH was between the *β-O*-4 ether substructure and the *β*-5′ and *β*-*β*′ ones.

Keywords: rice straw, lignin, alcohol, 2D-HSQC, FT-IR, ^{13}C NMR

INTRODUCTION

Rice is one of the most important agricultural crops in China. Associated to rice production is a corresponding annual production of nearly 200 million tons of rice straw. Rice straw has been traditionally used as animal feed, feedstock for paper industry and organic fertilizer.[1,2] However, modern views of animal breeding practices and the growing interest in environmental problems tend to limit these uses, therefore negatively affecting its demand.[3] To develop a new route of its uses and to increase its added value, the conversion of rice straw into ethanol and other chemicals has been extensively studied.[1,4] Chemical composition and structural characteristics are key factors that affect the efficiency of its utilization and the nutritive quality of rice straw during the conversion process. Therefore, a thorough study of the isolation and structural characterization of the hemicelluloses, cellulose and lignin from rice straw is necessary.

Lignin, one of the main components of rice straw, is expected to play an important role in the near future as a raw material for the production of bio-products and chemicals. Unlike other natural polymers, such as cellulose and proteins, which have only one type of linkage between units, lignin is a complex three-dimensional polymer formed by dehydrogenative polymerization of *p*-hydroxycinnamyl, coniferyl and sinapyl alcohols.[5]

These three lignin precursors give rise to the so-called H (*p*-hydroxyphenyl), G (guaiacyl) and S (syringyl) phenylpropanoid units, which can be acylated, showing different abundancy, as depending on their origin.[6] Furthermore, lignin is an amorphous three-dimensional polymer of the phenylpropanoid units linked[7] through ether and carbon-carbon bonds, such as *β*-*O*-4′, 4-*O*-5′, *β*-*β*′, *β*-1′, *β*-5′ and 5-5′. Lignin is covalently linked to polysaccharides, forming a lignin-hemicelluloses network made up of benzyl-ether,[8-10] benzyl-ester[11,12] and phenyl-glycoside bonds.[13] It provides mechanical support for plants, facilitates the transport of nutrients, and defends against the attack of microogranisms.[14] However, the tight physical binding and chemical linkages between lignin and cell-wall polysaccharides prevent its clean isolation in an unaltered form.[15]

Therefore, rice straw has to be pre-treated to get the free lignin. Among the various pretreatment methods currently studied for the production of pulp and/or ethanol, the organosolv process seems to be very promising.[16] Organosolv lignins are usually recovered from spent organosolv liquor by precipitation in water, after evaporating most of the organic solvents. The most frequently used solvents for the organosolv process are primary alcohols with a low boiling point, such as ethanol and methanol, although other solvents, namely acetic and formic acids, have also been used. Recently, Pan *et al*.[17-19] have successfully developed this pretreatment technology for hybrid poplar and lodgepole pine destroyed by the mountain pine beetle, producing substrates with very good enzymatic digestibility. This procedure also produces a large amount of high-quality lignin, which is relatively pure, primarily unaltered and less condensed than other lignin products. It is soluble in many organic solvents and could find applications in the fields of adhesives,[20] fibres, films[21] and biodegradable polymers.[22] Decisions on the suitability of these lignin fractions for potential applications require prior investigation of their structural and chemical characteristics. However, the available literature reports on the structural characterization of rice straw lignin are scarce. A previous research of ours was dedicated to the physico-chemical characterization of rice straw lignin obtained by hydrogen peroxide treatment.[23] The present paper investigates the structure and composition of different alcohol-soluble lignins from rice straw. To this end, six lignin fractions of rice straw were isolated by treatments with 60% methanol, 60% ethanol, 60% *n*-propanol, 60% *n*-butanol, 60% ethanol containing 0.01M HCl and 60% ethanol containing 0.25 M NaOH at 75 ºC for 3 h, respectively. The solubilized lignin fractions were analyzed to characterize their chemical composition, physico-chemical properties and linkages between units by means of degradation methods, such as alkaline nitrobenzene oxidation and thermal analysis, and non-destructive techniques, *e.g*. ultraviolet (UV), Fourier Transform Infrared (FT-IR), carbon-13 nuclear and two-dimensional Heteronuclear Single Quantum Correlation Nuclear Magnetic Resonance Spectroscopy (^{13}C NMR and 2D HSQC NMR) and Gel Permeation Chromatography (GPC).

EXPERIMENTAL

Materials

Rice straw, obtained from the experimental farm of the Northwest Agricultural and Forestry University (Yangling, China), was dried in sunlight and cut into small pieces. After oven-drying at 60 ºC for 16 h, the sample was ground to pass a 0.8 mm size screen, and the powder was further dried in a cabinet oven with air circulation at 60 ºC for 16 h and stored in a desiccator. The composition (w/w) of the rice straw used was the following: cellulose – 36.5%, hemicelluloses – 33.8%, chlorite lignin – 12.3%, wax – 3.8% and ash – 13.3% (70.8% silica). Prior to the treatment, rice straw was first dewaxed with toluene-ethanol (2:1, v/v) in a Soxhlet extractor for 6 h.

Fractional isolation of lignin

Different alcohol treatments for rice straw were carried out according to the scheme plotted in Figure 1. The dewaxed rice straw was extracted with 60% methanol, 60% ethanol, 60% *n*-propanol, 60% *n*-butanol, 60% ethanol containing 0.01 M HCl and 60% ethanol containing 0.25 M NaOH as a catalyst, at 75 ºC for 3 h, at a solid-to-liquor ratio of 1:25 (g/mL), under stirring. The solubilized filtrations were recovered, neutralized at pH 5.5-6.0 with 6 M HCl, concentrated to about 50-60 mL, and then precipitated by pouring the concentrated supernatant fluid into 3 volumes of 95% ethanol. After filtration, the isolated lignins were obtained from the corresponding supernatants by concentration to about 20 mL, adjustment to pH 2.0, then centrifugation, the preparations obtained being labelled as lignins L_1, L_2, L_3, L_4, L_5 and L_6, respectively. The yields of the residual hemicelluloses and lignins are given on a dry weight basis, related to the initial dewaxed rice straw.

Characterization of lignin fractions

The neutral sugars in the lignin fractions were liberated by hydrolysis with 6% H_2SO_4 for 2.5 h at 105

ºC. After hydrolysis, the sample was diluted 30 times, filtered and injected into a high-performance anion exchange chromatography (HPAEC) system (Dionex ISC 3000) equipped with an amperometric detector, an AS50 autosampler and a Carbopac PA1 column (4×250 mm, Dionex). Neutral sugars were separated in 18 mM NaOH (carbonate-free and purged with nitrogen) with post-column addition of 0.3 M NaOH, at a rate of 0.5 mL/min. Run time was 45 min, followed by a 10 min elution with 18 mM NaOH, to re-equilibrate the column. The uronic acid was eluted with 0.4 M NaOH for 20 min at a 1 mL/min rate, with post-column addition of 0.3 M NaOH, at a 0.5 mL/min rate. Calibration was performed with standard solutions of L-arabinose, D-glucose, D-xylose, D-mannose, D-galactose, glucuronic and galacturonic acids.

The molecular weights of the lignin fractions were determined by gel permeation chromatography (GPC, Agilent 1200, USA), with a refraction index detector (RID) on a PL-gel 10 um Mixed-B 7.5 mm ID column, calibrated with polystyrene standards (peak average molecular weights of 1320, 9200, 66000, 435500, Polymer Laboratories Ltd.). A 4 mg sample was dissolved in 2 mL tetrahydrofuran, and a 20 µL sample in solution was injected. The column was operated at ambient temperature and eluted with tetrahydrofuran at a flow rate of 1 mL/min. The chemical composition of the phenolic acids and aldehydes liberated from alkaline nitrobenzene oxidation of lignin was determined by high-performance liquid chromatography (HPLC, Agilent 1200, USA), on a 250×4.6 mm ZORBAX Eclipse XDB-C18 column with a diode-array detector (DAD). Separations were obtained using a linear gradient of two solvent systems: solvent A (water-methanol-acetic acid – 89:10:1) and solvent B (methanol-water-acetic acid – 90:9:1). A linear gradient was run over 42 min from 0 to 32.5%, at a flow rate of 1 mL/min. The identification of the individual compounds was made at 280 nm by computer comparison of the retention times and peak areas with the authentic phenolic acids and aldehydes. Measurements were conducted in two parallel series, and the reproducibility of the values was kept within the 6.0% range.

The UV spectra were recorded on an ultraviolet/visible spectrophotometer (Techcomp, UV2300). A lignin sample (5 mg) was dissolved in a 95% (v/v) dioxane aqueous solution (10 mL). A 1 mL aliquot was diluted to 10 mL with a 50% (v/v) dioxane aqueous solution and the absorbance between 260 and 400 nm was measured. The FT-IR spectrophotometer (Nicolet, 750) was operated with KBr discs containing 1% finely ground lignin fractions (32 scans, 4 cm^{-1} resolution). The ^{13}C-NMR spectrum was obtained on a Bruker spectrometer at 100 MHz. The sample (100 mg) was dissolved in 0.5 mL of DMSO-d_6, and the spectrum was recorded at 25 ºC after 30000 scans. A 30° pulse flipping angle, a 9.2 µs pulse width, 1.89 s delay time and 1.36 s acquired time between scans were used. The spectral widths for the HSQC (Heteronuclear Single Quantum Correlation) (semi-quantitative mode) were of 5000 and 2000 Hz for the ^{1}H and ^{13}C dimensions, respectively. The number of collected complex points was of 1024 for the ^{1}H dimension with a recycle delay (d_1) of 5 s, while the number of transients for the HSQC spectra was 128, the 256 time increments being always recorded in the ^{13}C dimension. The $^{1}J_{C\text{-}H}$ used was of 146 Hz. Prior to Fourier transformation, the data matrices were zero-filled to 1024 points in the ^{13}C dimension. Data processing was performed using standard Bruker Topspin-NMR software.

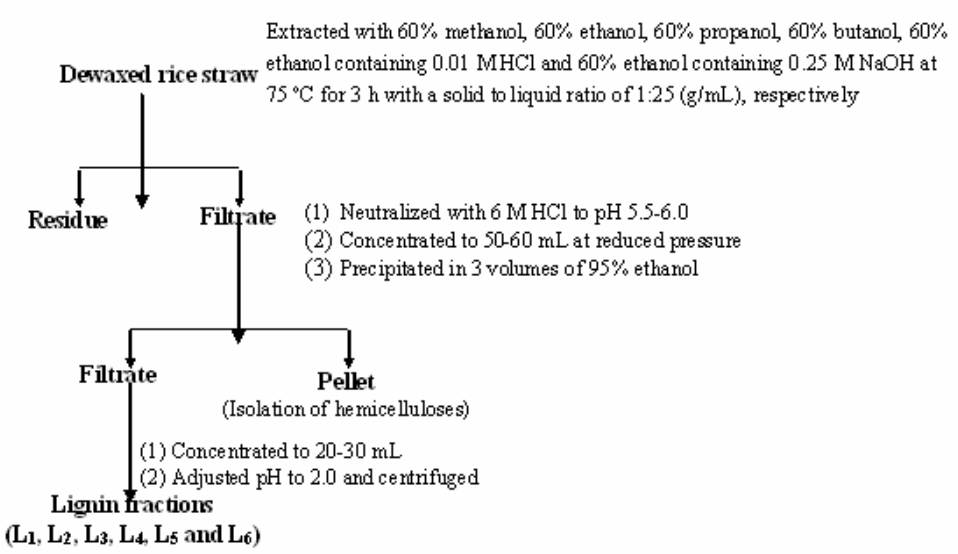

Figure 1: Scheme for isolation of lignins from rice straw

RESULTS AND DISCUSSION

Fractional yield and purity of lignin

Organosolv degradation processes have attracted increasing attention as alternatives to conventional degradation processes, because of their lower environmental impact and lower energy consumption. By-products, such as degraded lignin and hemicelluloses, can be utilized for many purposes.[24] The yield (% initial dewaxed rice straw, w/w) of the alcohol-soluble lignin fractions attained in this experiment is shown in Table 1. As one can see, the extractions of the dewaxed rice straw with 60% methanol, 60% ethanol, 60% propanol, 60% butanol, 60% ethanol containing 0.01 M HCl and 60% ethanol containing 0.25 M NaOH at 75 ºC for 3 h, yielded 1.8, 1.6, 2.0, 1.5, 1.6 and 9.3% lignin, corresponding to 14.6, 13.0, 16.3, 12.2, 13.0 and 75.6% of the original lignin, respectively. It should be noted that the yield of acid-insoluble lignin of the 60% ethanol containing 0.25 M NaOH treatment was much higher than those resulting from other treatments. This indicated that a significant degradation of the lignin macromolecules occurred during the treatments with alkaline ethanol. Interestingly, the lignin yields of 60% ethanol and 60% ethanol containing 0.01 M HCl treatments were the same, indicating that a low amount of acid catalyst had no significant effect on the ethanol degradation of lignin macromolecules. Furthermore, the data in Table 1 also show that the treatment of 60% ethanol containing 0.25 M NaOH from rice straw dewaxed at 75 ºC for 3 h led to a release of 55.1% of the original hemicelluloses and of 75.5%, respectively, of the original lignin. Meanwhile, the acidic treatment of 60% ethanol with 0.01 M HCl under the same conditions led to a release of only 7.4% of the original hemicelluloses and of 13.0%, respectively, of the original lignin. This indicated that some alkali-labile linkages between lignin monomers, or between lignin and polysaccharides, might have broken down during the alkali treatment.

UV spectroscopy has been used to determine the purity of lignin and to monitor its distribution among various tissues of gymnosperms and dicotyledonous angiosperms with respect to concentration. In the present study, UV-Vis absorption measurements of the acid-insoluble lignin fractions were carried out with a dioxane/water mixture, which solubilized the lignins, even if limited to wavelengths above 240 nm. Figure 2 illustrates the UV spectra of the acid-insoluble fractions L_1, L_2, L_3, L_4 (Fig. 2a), and L_2, L_5, L_6 (Fig. 2b). As shown in the spectra, the 6 lignin fractions exhibit basic UV spectra typical of lignins, which have a first maximum absorption at 274 nm and a second maximum absorption at 318 nm.[25] The maximum absorption at 274 nm originates from the non-conjugated phenolic groups present in lignin, such as sinapyl alcohol, coniferyl alcohol and a small amount of *p*-coumaryl alcohol. Generally, the three structural moieties of lignin give different absorption maxima and extinction coefficients. The extinction coefficient of the guaiacyl units at 280 nm is 3.5 times higher than that of the syringyl units, while the extinction coefficient of the *p*-coumaryl units is lower than that of the guaiacyl ones, yet higher than that of the syringyl units.[26] The second maximum absorption – around 315 nm – is mainly due to the esters of hydroxycinnamic acids, such as ferulic and *p*-coumaric acids.[25,27]

Figure 2a shows that the maximum absorption of the 4 different alcohol-soluble lignin preparations occurs in the order: $L_1 > L_3 > L_4 > L_2$, suggesting that the lignin fractions solubilized in methanol had a higher purity than those solubilized in propanol and butanol, while the ethanol-soluble lignin fractions had a lower purity.

Table 1
Yield (% initial dry rice straw, w/w) of degraded hemicelluloses, lignin and cellulose-rich residue

	Yield					
	F_1[a]	F_2[a]	F_3[a]	F_4[a]	F_5[a]	F_6[a]
Hemicelluloses	3.5	3.5	2.1	2.2	2.5	18.6
Lignin	1.8	1.6	2.0	1.5	1.6	9.3
Residue	89.0	89.4	91.2	86.9	86.3	56.6

[a] F_1, F_2, F_3, F_4, F_5 and F_6 – degraded polymeric preparations of hemicelluloses, lignin and residue obtained by treatment of dewaxed rice straw with 60% methanol, 60% ethanol, 60% *n*-propanol, 60% *n*-butanol, 60% ethanol containing 0.01 M HCl, and 60% ethanol containing 0.25 M NaOH at 75 ºC for 3 h, respectively

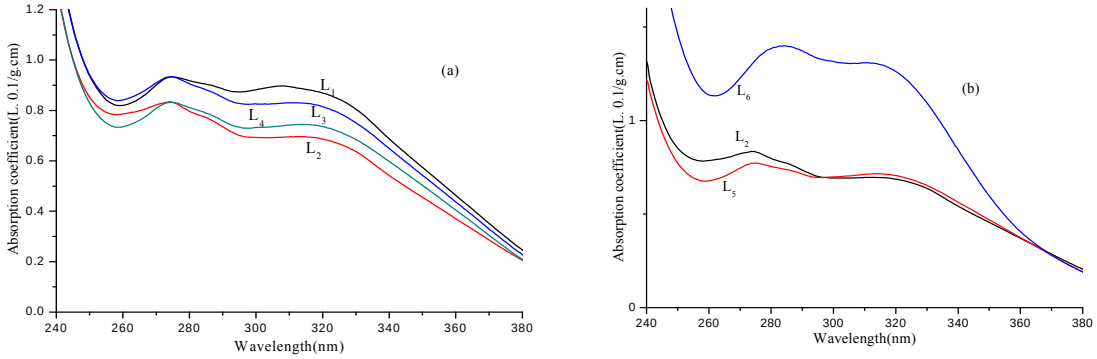

Figure 2: UV spectra of acid-insoluble lignin fractions: (a) L_1, L_2, L_3, L_4, and (b) L_2, L_5, L_6

Clearly, as shown in Figure 2b, lignin fraction L_5 had a similar purity with that of lignin fraction L_2, lignin fraction L_6 being much purer than both of them, indicating that the treatment with 60% ethanol under alkali catalyst produced purer lignin than those with 60% ethanol and with 60% ethanol under acid catalysis.

Data on the content and neutral sugar composition of the bound hemicelluloses in the 6 acid-insoluble lignin fractions are listed in Table 2. As one can see, glucose was identified as the predominant sugar in the 6 alcohol-soluble lignin fractions, containing 57.3-70.9% of the associated hemicelluloses. Xylose, galactose and arabinose were identified in noticeable amounts, while rhamnose occurred only as traces in the six lignin fractions. Obviously, lignin fraction L_6, which was isolated by 60% ethanol containing 0.25 M NaOH, was much purer than the other 5 lignin fractions (the total sugar content being of only 0.79%). This relative freedom *versus* the associated polysaccharides in lignin fraction L_6 is probably explained by the fact that the alkali treatment cleaved a large amount of *α*-ether bonds between lignin and hemicelluloses, and saponified the hydroxycinnamic esters between *p*-coumaric acid and lignin or between ferulic acid and hemicelluloses.[28] The highest purity of lignin fraction L_6 agreed with the results of the UV analysis. A comparison between data on the bound polysaccharides of alcohol-soluble lignin fractions (5.37-8.07%) and our data on H_2O_2-soluble lignin fractions (0.57-1.12%) showed[23] that the purity of alcohol-soluble lignin fractions is not only lower than that of the alkaline alcohol-soluble ones, but also lower than that of the alkaline H_2O_2-soluble lignin preparations.

Composition of phenolic acids and aldehydes

To elucidate the structural differences among the lignins obtained from dewaxed rice straw, the 6 alcohol-soluble lignin fractions were submitted to alkaline nitrobenzene oxidation, which has been widely used for assaying and identifying the structural units of lignin, because, during the oxidation process, the three constitutive monomeric lignin units (*p*-hydroxyphenyl, guaiacyl and syringyl) produce the corresponding *p*-hydroxybenzaldehyde, vanillin and syringaldehyde. The amounts and relative distribution of the degradation products can be subsequently used to derive information on the composition of the lignin fractions.[29]

Table 3 lists the results on the characterization of phenolic acids and aldehydes from the 6 acid-insoluble lignin fractions, obtained from dewaxed rice straw by different alcohol treatments. As shown in Table 3, the molar ratios of G (relative total moles of vanillin, vanillic acid and acetovanillin), of S (relative total moles of syringaldehyde, syringic acid and acetosyringone) and of H (relative total moles of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid) were found to be 0.6:0.7:1 in L_1, 0.8:0.5:1 in L_2, 1.1:0.8:1 in L_3, 1.1:0.8:1 in L_4, 0.5:0.6:1 in L_5, and 0.6:1.2:1 in L_6, respectively. The occurrence of large amounts of non-condensed guaiacyl, syringyl and *p*-hydroxyphenyl units implied that the 6 lignin preparations from rice straw can be considered as GSH lignin.

Table 2
Content of neutral sugars (% of lignin sample, w/w) in the isolated lignin fractions

Neutral sugars	Lignin fractions[a]					
	L_1	L_2	L_3	L_4	L_5	L_6
Rhamnose	0.10	0.08	0.06	0.05	0.04	nd[b]
Arabinose	0.42	0.51	1.18	1.18	0.62	0.06
Galactose	0.47	0.55	1.14	1.13	0.58	0.03
Glucose	3.81	4.14	4.47	4.75	4.20	0.55
Xylose	0.57	0.64	0.95	0.97	0.64	0.15
Total	5.37	5.92	7.80	8.07	6.07	0.79

[a]L_1, L_2, L_3, L_4, L_5, and L_6 represent the degraded lignin preparations obtained by treatment of dewaxed rice straw with 60% methanol, 60% ethanol, 60% *n*-propanol, 60% *n*-butanol, 60% ethanol containing 0.01M HCl, 60% ethanol containing 0.25 M NaOH at 75 ºC for 3 h, respectively; [b]nd: not detected

Table 3
Yield of phenolic acids and aldehydes (% lignin sample, w/w) obtained by alkaline nitrobenzene oxidation of isolated acid-insoluble lignin preparations

	L_1	L_2	L_3	L_4	L_5	L_6
p-Hydroxybenzoic acid	1.62	1.81	1.10	1.08	1.99	1.65
Syringaldehyde	0.84	0.63	0.59	0.50	0.59	1.98
p-Hydroxybenzaldehyde	1.53	0.81	0.76	0.67	0.50	1.05
Vanillic acid	0.49	1.57	1.69	1.60	0.46	0.57
Syringic acid	2.42	1.38	1.45	1.37	1.64	2.57
Vanillin	1.67	0.71	0.72	0.68	0.67	1.28
Acetovanillone	0.09	0.16	0.16	0.15	0.22	0.16
p-Coumaric acid	0.24	0.53	0.59	0.55	0.34	0.58
Acetosyringone	T	T	0.15	0.13	0.08	T
Ferulic acid	0.42	1.82	1.38	1.56	1.13	T
Total	9.31	9.41	8.59	8.29	7.63	9.84
Molar ratio (G:S:H)	0.6:0.7:1	0.8:0.5:1	1.1:0.8:1	1.1:0.8:1	0.5:0.6:1	0.6:1.2:1

[a] Corresponding to lignin fractions in Table 2
[b] T – trace
[c] G – the sum of total moles of vanillin, vanillic acid and acetovanillone; S – the sum of total moles of syringaldehyde and syringic acid; H – the sum of total moles of *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde

Table 4
Weight-average ($\bar{M}_w$) and number-average ($\bar{\bar{M}}_n$) molecular weights and polydispersity ($\bar{M}_w/\bar{M}_n$) of lignin fractions

	Lignin fractions[a]					
	L_1	L_2	L_3	L_4	L_5	L_6
$\bar{M}_w$	1370	1420	1350	1250	1340	1460
$\bar{\bar{M}}_n$	1230	1380	1320	1230	1300	1420
$\bar{M}_w/\bar{M}_n$	1.12	1.03	1.03	1.02	1.03	1.03

[a] Corresponding to lignin fractions in Table 2

Clearly, lignin fraction L_6, obtained by a treatment with 60% ethanol containing 0.25 M NaOH, yielded a relatively lower monomeric ratio of vanillin-to-syringaldedyde than that of the other 5 alcohol-soluble lignin fractions. This implied that the non-condensed syringyl units were more easily degradable than the non-condensed guaiacyl ones from the lignin fractions,

during the treatment with 60% ethanol under alkali catalysis. Generally, a large ratio of ferulic acid was quantitatively oxidized to vanillin, and most of the *p*-coumaric acids were quantitatively oxidized to *p*-hydroxybenzaldehydes during alkaline nitrobenzene oxidation. As shown in Table 3, the *p*-coumaric and ferulic acids from the cell walls of rice straw were present only in minimal amounts (0.24-0.59% and 0.42-1.82%, respectively) in the mixture of nitrobenzene oxidation. This suggested that these two hydroxycinnamic acids are strongly linked to the lignin from the cell walls of rice straw, which agrees with the conclusions of some previous studies of ours.[30,31]

Molecular weight

Table 4 lists the weight-average ($\bar{M}_w$), number-average ($\bar{M}_n$) molecular weights and polydispersity ($\bar{M}_w/\bar{M}_n$) of the acid-insoluble lignin fractions L_1-L_6 degraded during different alcohol treatments. Special mention should be made of the fact that the molecular weights discussed throughout this work were obtained from a calibration performed with monodisperse polystyrene standards. Therefore, these values should be considered only as relative and not absolute molecular weights. The acid-insoluble lignin fractions were shown to have $\bar{M}_w$ between 1248 and 1462 g mol^{-1}, with a polydispersity of 1.02-1.12, while the 6 lignin fractions showed no significant difference in their molecular average weights. A comparison with the $\bar{M}_w$ (3890 g mol^{-1}) of the alkali-soluble lignin fraction from rice straw showed[25] that these lower molecular weights imply that all alcohol treatments, performed under the given conditions, led to a significant cleavage of the *β-O*-4′ ether and ester bonds in these lignin fractions, with less opportunity for repolymerisation, which again agrees with the result of Sannigrahi *et al*.,[31] who indicated that, in contrast to the lignin produced by other technical processes, organosolv lignin gave a low molecular weight product of high purity.[32] In addition, according to the data shown in Table 4, $\bar{M}_w$ increased from 1340 g mol^{-1} (L_5) in the acidic-ethanol treatment, to 1416 g mol^{-1} (L_2) in the neutral-ethanol treatment, and to 1462 g mol^{-1} (L_6), respectively, in the alkali-ethanol treatment, which indicates that the alkali conditions caused an increment of lignin repolymerisation, while the acidic treatment favoured the dissolution of low molecular-size lignins.

FT-IR spectra

Figure 3 shows the FTIR spectra of rice straw lignin extracted with 60% methanol (spectrum 1), 60% ethanol (spectrum 2) and 60% propanol (spectrum 3) at 75 °C for 3 h. The absence of the band at 1745 cm^{-1} revealed that the labile ester bonds were completely cleaved during alcohol treatment. A small band at 1721 cm^{-1} is assigned to unconjugated ketone and carboxyl group stretching. The relative intensities of the bands for aromatic skeleton vibrations in the lignin fractions are assigned to 1603, 1511 and 1424 cm^{-1}, confirming that the "core" of the lignin structure did not change significantly during the alcohol treatments under the given conditions. The band at 1655 cm^{-1} in the spectra is attributed to the conjugated carbonyl groups (*α*-carbonyl groups) stretching occurring in lignin. It can be also caused by the carbonyl groups of some residual aliphatic esters from hydroxycinnamic acids. The absorption at 1603 cm^{-1} might originate from an enol structure present in lignin, while the absorption at 1460 cm^{-1} might be affected by the absorption of the C-H deformations and aromatic ring vibrations. The intensive bands at 1369 and 1265 cm^{-1} in the spectra were assigned to syringyl and guaiacyl ring breathing with C=O stretching, respectively. The relative band at 1170 cm^{-1} showed aromatic ether ring breathing and aromatic C-H deformation of the syringyl and guaiacyl units. The band at 1127 cm^{-1} indicated aromatic C-H in-plane deformation, suggesting that a great similarity of the aromatic ring skeleton existed in the 6 alcohol-soluble lignin fractions. In addition, aromatic C-H out-of-plane bending appears at 840 cm^{-1}.

The FTIR spectra of rice straw lignin extracted with 60% butanol (spectrum 1), 60% ethanol containing 0.01 M HCl (spectrum 2) and 60% ethanol containing 0.25 M NaOH (spectrum 3), at 75 °C for 3 h, are shown in Figure 4. Comparatively with spectrum 3 of the corresponding lignin fraction (Fig. 4), the absence of a band at 1035 cm^{-1} in spectra 1-2 (Fig. 4) and in spectra 1-3 (Fig. 3) is attributed to the C-O stretching vibration in the first-order aliphatic C-OH and ether linkages (C-O-C). Besides, the band absence at 1224 cm^{-1} in spectra 1-2 of Figures 3 and 4 is assigned to C-O and C-C stretching vibrations, C-O deformation in secondary alcohols, or to aromatic C-H in-plane deformation.

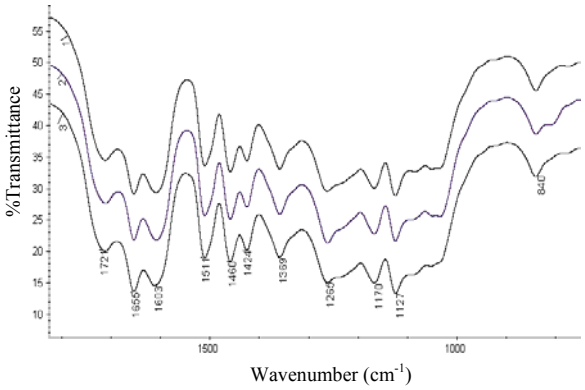

Figure 3: FT-IR spectra of rice straw lignin fractions isolated with 60% methanol (spectrum 1), 60% ethanol (spectrum 2) and 60% propanol (spectrum 3) at 75 ºC for 3 h

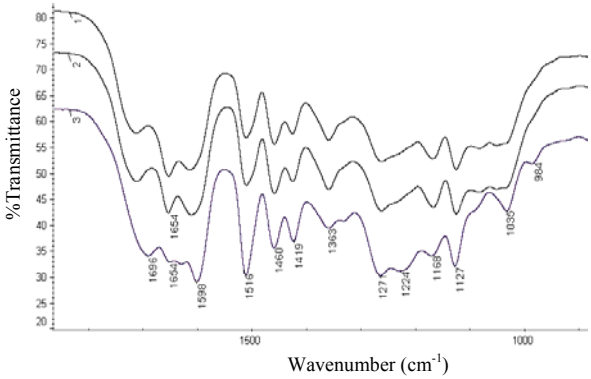

Figure 4: FT-IR spectra of rice straw lignin fractions isolated with 60% butanol (spectrum 1), 60% ethanol containing 0.01 M HCl (spectrum 2) and 60% ethanol containing 0.25 M NaOH (spectrum 3) at 75 ºC for 3 h

In summary, the spectra for the treatments with 60% methanol (spectrum 1), 60% ethanol (spectrum 2) and 60% propanol (spectrum 3) in Figure 3, and 60% butanol (spectrum 1) and 60% ethanol containing 0.01 M HCl (spectrum 2) in Figure 4 preserved the same structure, being different from the spectrum of the 60% ethanol containing 0.25 M NaOH (spectrum 3, Fig. 4) treatment. This phenomenon implies that the alkaline treatment led to several changes in the internal structure of lignin and to a relative increase in carbonyl group stretching. All lignin fractions in Figures 3 and 4 showed[33] the spectral features of GSH-type lignin with the characteristic bands at 1127 and 840 cm^{-1} (data not shown in Fig. 4) and a small band at 1168 cm^{-1} in Figure 4 and at 1170 cm^{-1} in Figure 3.

^{13}C NMR spectra

The ^{13}C-NMR spectrum of lignin fraction L_6 isolated by 60% ethanol containing 0.25 M NaOH is illustrated in Figure 5, while the chemical shifts (δ, ppm), intensity and assignment are listed in Table 5. Most of the observed signals have been previously assigned to straw and wood lignin spectra.[24,34] The lignin fraction evidences no typical polysaccharide signals between 57 and 103 ppm, indicating that the lignin preparation contained only traces of associated polysaccharides, which corresponded to the data obtained from sugar analysis (Table 2).

Other characteristic signals observed in the spectrum are 7 strong resonances (168.1, 159.8, 144.3, 130.1, 125.3, 115.9, 115.3 ppm), corresponding to C-γ, C-4, C-*α*, C-2/C-6, C-1, C-3/C-5, C-β in *p*-coumaric ester (PC ester), respectively.[35] In addition, the ether-linked ferulic acid gives signals at 168.1 (C-γ) and 122.1 ppm (C-6, data not shown), whereas the esterified ferulic acid exhibits a signal at 122.9 ppm (C-6). These signals indicate that the *p*-coumaric acid is linked to lignin by ester bonds, whereas the ferulic acid is linked to lignin by both ether and ester bonds. These signals also imply that the lignin fraction contained noticeable amounts of esterified *p*-coumaric acid and traces of etherified ferulic acid. These results agree with the data on alkaline nitrobenzene oxidation (Table 3).

Information on the aromatic region of lignin fraction L_6 is presented between 104.3 and 168.1 ppm in the ^{13}C-NMR spectrum. The syringyl (S) residues were detected by signals at 152.4 (C-3/C-5, S etherified), 147.5 (C-3/C-5, S non-etherified), 138.6 (C-4, S etherified, data not shown), 134.7 (C-1, S etherified), 104.4 and 103.9 ppm (C-2/C-6, S). Guaiacyl (G) residues were verified by signals at 149.2 (C-3, G etherified), 147.5 (C-4, G etherified), 145.6 (C-4, G non-etherified), 134.7 (C-1, G etherified), 119.5 (C-6, G) and 111.2 ppm (C-2, G). The *p*-hydroxyphenyl (H) residues were identified at 128.3 ppm (C-2/C-6, H). These signals revealed that the sample could be verified as a GSH-type lignin.

One of the most important reactions for lignin degradation in alkaline media is the cleavage of the *β-O*-aryl ether structures. In Figure 5, the occurrence of the *β-O*-aryl ether structure in lignin preparation L_6 isolated with 60% ethanol containing 0.25 M NaOH is identified with 3 resonances – at 86.2, 71.8 and 60.1 ppm – related to the C-*β*, C-*α* and C-*γ* in *β-O*-4′ structures, respectively. These signals indicate that the *β-O*-

4′ units were not completely cleaved under the alkaline conditions applied. Furthermore, the two distinct signals at 55.8 ppm arise from the $–OCH_3$ syringyl and $–OCH_3$ guaiacyl groups. The signals for the *γ*–methyl, *α*- and *β*-methylene groups in the n-propyl side chains of the lignin fraction appear in the spectrum between 14.0 and 25.6-29.1 ppm, respectively.

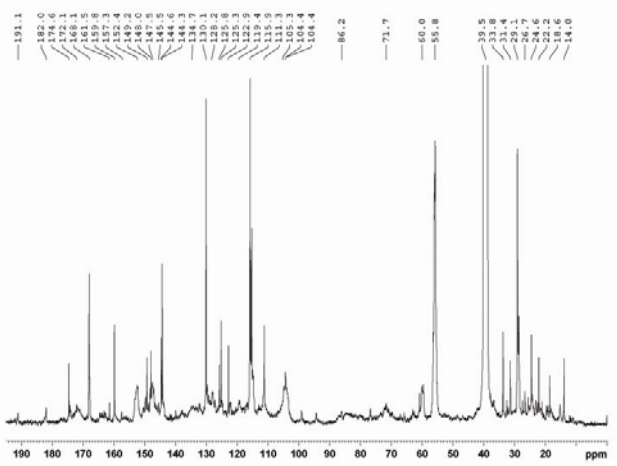

Figure 5: ^{13}C-NMR spectrum of lignin fraction L_6 isolated with 60% ethanol containing 0.25 M NaOH at 75 ºC for 3 h

Table 5
Chemical shift values (δ, ppm), intensity and signal assignment of lignin fraction L_6

PPM	Intensity	Assignment	PPM	Intensity	Assignment
191.1	vw	*α*-CHO in cinnamaldehyde	119.5	vw	C-6, G unit
168.1	s	C-*γ*, PC ester; C-*γ*, FE ether	115.9	s	C-3/C-5, PC ester
159.8	s	C-4, PC ester	115.3	s	C-*β*, PC ester
152.4	w	C-3/C-5, S units	111.2	w	C-2, G unit
149.7	w	C-3, G etherified	104.4	w	C-2/C-6, S etherified
148.0	w	C-3, G units	86.2	vw	C-*β*, *β-O*-4
147.5	w	C-3/C-5, S units	71.8	vw	C-*α*, *β-O*-4
145.6	vw	C-4, G non-etherified	60.1	w	C-*γ*, *β-O*-4
144.3	s	C-*α*, FE ether	55.8	s	OCH_3, G and S unit
138.6	vw	C-4, S etherified	39.5	s	DMSO
134.7	vw	C-1, G etherified	33.8	w	CH_3 in ketones (conj) or in aliphatic
130.1	s	C-2/C-6, PC ester	31.4	w	CH_3 in ketones (conj) or in aliphatic
128.3	vw	C-2/C-6, H unit	29.1	s	CH_2 in aliphatic side chain
125.8	w	C-1, PC ester	25.6	w	CH_3 or CH_2 group in saturated side chains
125.3	s	C-1, PC ester	22.2	w	CH_3 or CH_2 group in saturated side chains
122.9	s	C-6, FE ester	14.0	w	*γ*-CH_3, in n-propyl side chain

Notes: Intensity abbreviations: w – weak; s – strong; vw – very weak; PC ester – *p*-coumaric ester; FE ether – etherified ferulic acid

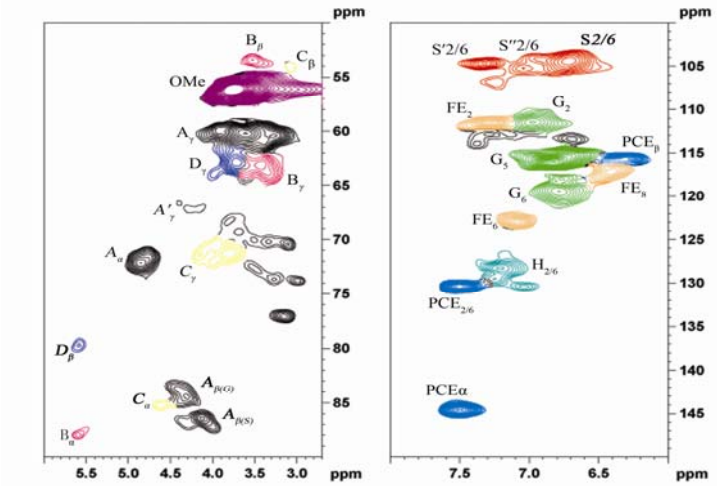

Figure 6: 2D-HSQC spectrum of lignin fraction L_6 isolated with 60% ethanol containing 0.25 M NaOH at 75 ºC for 3 h

2D HSQC NMR spectra

2D HSQC NMR is a powerful tool for qualitative and quantitative analysis of lignin structures, due to its well-resolved signals from various structural environments in a complex molecule and more detailed diagnostic information on the structure.[36] It also provides the resolution of the 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 the lignin fractions. To acquire further information on the structural characterization of rice straw, the alcohol-soluble lignin fraction isolated by 60% ethanol containing 0.25 M NaOH was subjected to 2D-HSQC NMR analysis.

The HSQC NMR spectrum of lignin fraction L_6 exhibits three regions corresponding to aliphatic (δ_C/δ_H 10-40/0.5-2.5 ppm), side chain (δ_C/δ_H 50-90/2.5-6.0 ppm) and aromatic (δ_C/δ_H 100-150/6.0-8.0 ppm) ^{13}C-^{1}H correlations.[37] The aliphatic region showed signals without significant structural information on lignin fraction L_6, so that only the side chain and the aromatic regions of the spectrum are shown in Figure 6. As one can see, the side chain or aromatic region of the HSQC NMR spectrum provided useful information on the various linkages among the structural units present in lignin fraction L_6. The prominent correlating signals observed in the spectrum were *β-O*-4′ ether linkages (substructure A). The correlations at δ_C/δ_H 71.7/4.84 ppm, δ_C/δ_H 83.8-85.9/4.09-4.31 ppm and δ_C/δ_H 60.0-64.2/3.13-4.03 ppm belong to the C_α-H_α, C_β-H_β and C_γ-H_γ correlations of the *β-O*-4′ ether substructures. Besides the *β-O*-4′ ether substructures, other linkages observed were *β*-5′ (phenylcoumaran, B) and *β-β*′ (resinol, C) linkages. Phenylcoumaran substructures B were found at a relatively low level, as shown by the signals at δ_C/δ_H 87.5/5.59, 53.0/3.51 and 62.6/3.41 ppm, corresponding to their C_α-H_α, C_β-H_β and C_γ-H_γ correlations, respectively. The very weak signals for resinol substructures C were observed in the spectrum, as shown by their C_α-H_α, C_β-H_β and C_γ-H_γ correlations at δ_C/δ_H 84.8/4.61, 53.5/3.06 and 70.8/3.79 and 70.8/4.12 ppm, respectively. This indicated that inter-unit linkages *β-β*′ existed in the alkaline ethanol-soluble lignin fraction. However, the relatively lower amount of this type of substructures was probably explained by the fact that these inter-unit linkages were unstable under alkaline conditions.

The main signals in the aromatic region (δ_C/δ_H 100-150/6.0-8.0 ppm) of the HSQC NMR spectrum (Fig. 6) corresponded to the aromatic ring-based lignin units. The primary correlation at δ_C/δ_H 103.8/6.69 ppm is for the 2/6 positions of the S units, while the G units give different correlations for C_2-H_2 (δ_C/δ_H 110.8/6.96 ppm), C_5-H_5 (δ_C/δ_H 115.5/6.84 ppm) and C_6-H_6 (δ_C/δ_H 119.0/6.77 ppm). Signals corresponding to the $C_{2/6}$-$H_{2/6}$ correlations in oxidized (C_α=O) phenolic syringyl units (S′ and S″) were observed at δ_C/δ_H 104.5/7.32 and 104.5/7.03 ppm, respectively. Signals of the *p*-hydroxylphenyl units (H-type lignin) were also detected in the HSQC spectrum at δ_C/δ_H 129.7/7.02 ppm. All signals in the aromatic region revealed that the lignin fraction

obtained from rice straw could be verified as a typical GSH grass lignin.

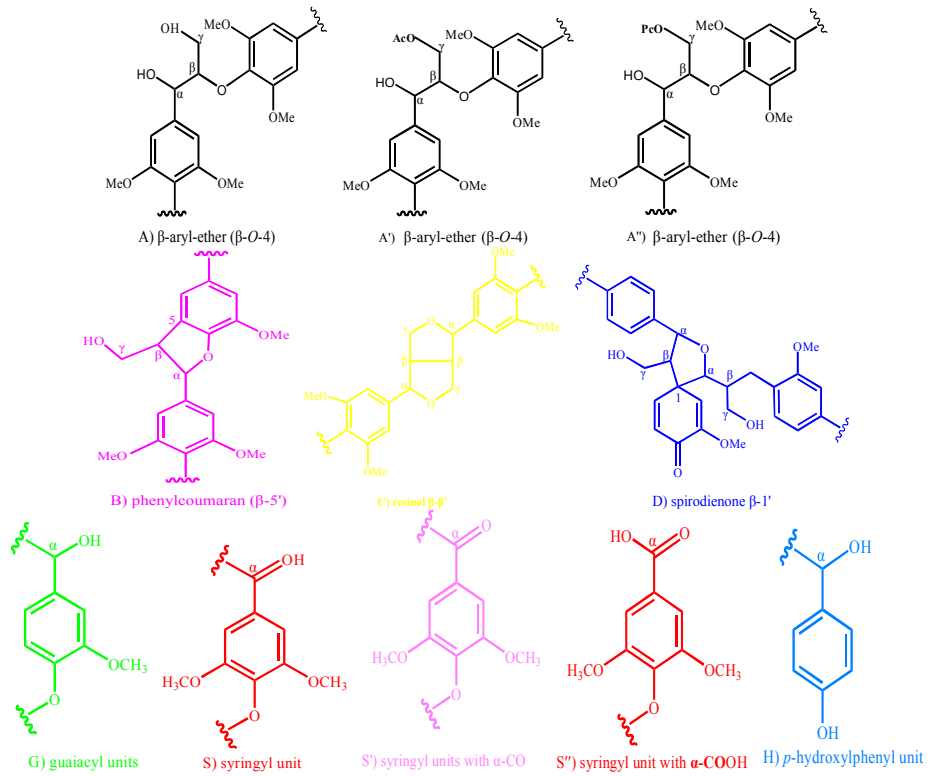

Figure 7: Main structures present in alkaline rice straw lignin (A) *β–O*-4′ linkages; (A′) *γ*-acetylated *β–O*-4′ substructures; (A″) *γ*-p-coumaroylated *β–O*-4′ linkages; (B) phenylcoumarane structures formed by *β*–5′/*α-O*-4′ linkages; (C) resinol structures formed by *β–β*′/*α-O-γ*′/ *γ-O-α*′ linkages; (D) spirodienone structures formed by *β*–1′ linkages; (F) *p*-hydroxycinnamyl alcohol end groups; (G) guaiacyl unit; (S) syringyl unit; (S′) oxidized syringyl unit linked with a carbonyl group at C_α (phenolic); (S″) oxidized syringyl unit linked with a carboxyl group at C_α

Table 6
Assignments of ^{13}C-^{1}H correlation signals in HSQC spectrum of lignin fraction L_6

Label	δ_C/δ_H(ppm)	Assignment
C_β	53.5/3.06	C_β-H_β in phenylcoumaran substructures (C)
B_β	53.0/3.51	C_β-H_β in resinol substructures (B)
OMe	55.65/3.74	C-H in methoxyls
A_γ	60.0/3.13 and 3.60	$C_\gamma H_\gamma$ in *β*–O-4′ substructures (A)
D_β	79.2/5.58	C_β-H_β in spirodienone substructures (D)
D_γ	62.4/3.70	C_γ-H_γ in spirodienone substructures (D)
B_γ	62.6/3.41	C_γ-H_γ in *β-β* resinol substructures (B)
A'_γ	64.2/4.03	C_γ-H_γ in *γ*-acetylated *β*–O-4′ substructures (A′ and A″)
C_γ	70.8/3.79 and 4.12	C_γ-H_γ in phenylcoumaran substructures (C)
B_γ	62.59/3.41	$C_\gamma H_\gamma$ in resinol substructures (B)
A_α	71.7/4.84	C_α-H_α in *β*–O-4′ substructures linked to an S unit (A)
$A_{\beta(G)}$	83.8/4.31	C_β-H_β in *β*–O-4′ substructures linked to a G unit (A)
B_α	87.5/5.59	C_α-H_α in resinol substructures (B)
$A_{\beta(S)}$	85.9/4.09	in *β*–O-4′ substructures linked to an S unit (A)
C_α	84.8/4.61	C_α-H_α in phenylcoumaran substructures (C)
$S_{2/6}$	103.8/6.69	$C_{2,6}$-$H_{2,6}$ in etherified syringyl units (S)
$S'_{2/6}$	104.5/7.32	$C_{2,6}$-$H_{2,6}$ in oxidized (C_α=O) phenolic syringyl units (S′)

$S''_{2/6}$	104.5/7.03	$C_{2,6}$-$H_{2,6}$ in oxidized (C_αOOH) syringyl units (S″)
G_2	110.8/6.96	C_2-H_2 in guaiacyl units (G)
FE_2	110.8/7.30	C_2-H_2 in ferulic ester (FE)
FE_8	116.7/6.39	C_8-H_8 in ferulic ester (FE)
G_5	115.5/6.84	C_5-H_5 in etherified guaiacyl units (G)
G_6	119.0/6.77	C_6-H_6 in guaiacyl units (G)
FE_6	122.4/7.09	C_6-H_6 in ferulic ester (FE)
$H_{2/6}$	129.7/7.02	$C_{2,6}$-$H_{2,6}$ in *p*-hydroxylphenyl unit (H)
pCE$_{2/6}$	129.82/7.48	$C_{2,6}$-$H_{2,6}$ in *p*-coumaroylated substructures (A″)
pCE$_\alpha$	144.1/7.47	C_α-H_α in *p*-coumaroylated substructures (A″)
pCE$_\beta$	115.3/6.32	C_β-H_β in *p*-coumaroylated substructures (A″)

The most interesting features are the clearly revealed intense *p*CE and FE peaks. Ferulates, largely acylating arabinoxylans, exist in grasses.[36] Ferulates are also involved in lignification, cross-coupling with lignin monomers, oligomers possibly resulting from lignin-polysaccharide cross-linking, which is an important feature of grass cell walls.[38] The peak at 110.8/7.30 ppm belongs to 2-position, 122.4/7.09 ppm belongs to 6-position and 116.7/6.39 ppm belongs to 8-position of ferulates (FE), respectively. The FE_7 correlation coincides with that of pCE$_\alpha$ at 144.1/7.47 ppm. The FE_5 correlation overlaps with the G_5 correlations. As observed above, ferulates acylate arabinoxylans, their presence in the lignin fraction suggesting the association between lignins and polysaccharides mediated by ferulates.[36]

p-Coumarates also acylate arabinoxylans, to a lesser degree than ferulates, yet mainly acylating the lignin sidechains in grasses.[39] Easy to identify were also the correlations of the esterified *p*-coumarates structures in the HSQC NMR spectrum, as due to their very prominent signals (Fig. 6). The *p*CE2/6 correlations occur at 129.8/7.48 ppm, while the 3,5-correlations at 115.5/6.84 ppm overlap those of the guaiacyl units. pCE$_\alpha$ and pCE$_\beta$ correlations (144.1/7.47 and 115.3/6.32) were also observed in the spectrum. The assignments of δ_C/δ_H are listed in Table 6.

CONCLUSIONS

Organosolv alcohol solvents were used to isolate lignin from rice straw. The results showed that the different alcohol treatments resulted in low molecular weight lignin fractions. The order of lignin purity of different alcohol treatments was the following: methanol > propanol > butanol > ethanol. Furthermore, the treatment of ethanol under alkali catalyst produced purer lignin than the ethanol treatments with or without acid catalyst. Possibly, the alcohol treatments significantly cleaved the *β-O*-4′ ether and ester bonds in the lignin fractions. The minimal amounts of *p*-coumaric and ferulic acids present in the extracted lignin fractions confirmed that these two hydroxycinnamic acids were strongly linked to lignin in the cell walls of rice straw. 2D-HSQC NMR spectra showed a prominent linkage of the lignin fraction obtained by ethanol containing 0.25 M NaOH was *β-O*-4′ ether, together with the *β*-5′ and *β*-*β*′ ones.

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