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ARTICLE

Quantitative Extraction of *p*-Coumaric Acid and Ferulic Acid in Different Gramineous Materials and Structural Changes of Residual Alkali Lignin

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ABSTRACT

Ferulic acid (FA) and *p*-coumaric acid (*p*CA) in bagasse, wheat straw, corn straw, and corncob were extracted by alkaline hydrolysis and characterized by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). It was found that the FA and most of the *p*CA in gramineous biomass could be dissociated and released after being treated with 1 M NaOH at 100°C for 4 h. The yields of *p*CA/FA in bagasse, wheat straw, corn straw, and corncob determined by GC-FID are 39.8/11.5, 13.7/11.0, 28.0/11.0, and 35.1/14.5 mg/g, respectively. The raw materials and the treated solid residues were characterized by gel-state 2D Heteronuclear Single Quantum Coherence Nuclear Magnetic Resonance (2D HSQC NMR). It was found that only a small amount of lignin was detected in the residue after alkali treatment, indicating that the alkali treatment conditions can effectively cleave the FA and *p*CA. Additionally, the lignin in the alkali solution was recovered and characterized by 2D HSQC NMR. The FA was not able to be detected by NMR, whereas a small amount of *p*CA remained in the alkali lignin. This study reveals the structural change of residual lignins during the quantitative isolation of FA and *p*CA, which is essential for the selective isolation of *p*CA/FA and valorization of residual alkali lignin.

KEYWORDS

Gramineae; ferulic acid; *p*-coumaric acid; alkali lignin; alkaline pretreatment

1 Introduction

Ferulic acid (FA) and *p*-coumaric acid (*p*CA) are typical hydroxycinnamic acid structures that widely exist in the cell wall of gramineous plants. Normally, they are both involved in the lignification process during plant cell wall development. As reported, FA and *p*CA are incorporated into lignin by ester linkages via the dehydrogenated free-radical coupling, which produces the cross-linking between lignin and polysaccharides [1,2]. The presence of FA and *p*CA in gramineous lignin significantly impacts its structure and application properties. Nowadays, producing lignin-derived platform chemicals by catalytic degradation is a promising way for lignin application. The ester bonds between FA (or *p*CA) and carbohydrates in grass lignins are easily broken or chemically changed during the degradation process, increasing the diversity and the monomeric products [3,4]. In particular, FA has excellent antioxidant and free radical scavenging capacity, and *p*CA exhibits antibacterial and lipid-lowering functions [5]. FA and *p*CA have promising applications in food, drug, cosmetics, etc. Therefore, efficient extraction of FA

and *p*CA from gramineous raw materials is a crucial way to get FA and *p*CA for a future application while promoting the application of agricultural and forestry wastes.

At present, FA and *p*CA are mainly obtained from gramineous plants through enzymatic hydrolysis, organic solvent extraction, and chemical hydrolysis [6]. FA can be selectively recovered from plant cell walls through the synergistic action of xylanase/pectinase and FA esterase. However, it is a time-consuming and costly method [7]. Organic solvent extraction usually involves high solvent consumption. Compared to the enzymatic hydrolysis and organic solvent extraction, chemical hydrolysis is more efficient in isolating FA and *p*CA from plant raw materials. The most commonly used chemical hydrolysis are acid, alkali, and acid/base two-step hydrolysis. It is noted that hydrolysis under acidic conditions can destroy the glycosidic bonds between polysaccharides [8,9], and the soluble mono- and oligosaccharides complicate the purification process [10]. In addition, the condensation reaction of lignin more easily happens under acid conditions [11]. Considering the residual lignin reactivity, alkaline hydrolysis is more suitable for FA and *p*CA isolation [12].

The hydrolysis efficiency of FA and *p*CA in the alkaline treatment is essential for isolating and quantifying these two hydroxycinnamic acids. At the same time, the complete release of FA and *p*CA is of great significance for the evaluation of active ingredients and the chemical composition analysis of raw materials. For example, a series of chemical treatment methods are used to delignify biomass in the pulp and paper industry. And the ester bond structures from FA and *p*CA in the raw lignin will increase the reagent consumption. In the subsequent bleaching stage, the residual FA and *p*CA containing unsaturated double bonds that existed in pulp and paper are significant incentives for the cost of bleaching agents and cause the brightness reversion of paper [13,14]. Besides, lignin degradation and condensation occur during the alkaline treatment for the quantitative isolation of FA and *p*CA. However, the corresponding structural changes of lignin are still not clear.

Herein, we reported the quantitative isolation of FA and *p*CA from four different samples and analyzed them by GC-MS and GC-FID. The solid residual and isolated alkali lignin after alkaline treatment were characterized by 2D HSQC NMR to reveal the structural change of lignin during the extraction of FA and *p*CA, Fig. 1.

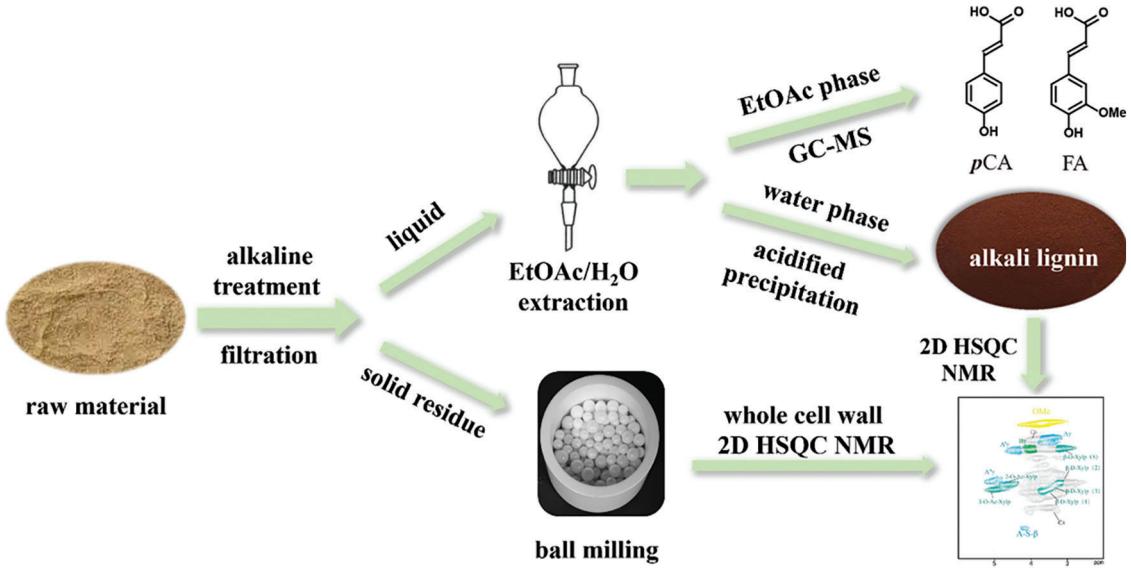

Figure 1: Schematic of extraction and characterization of *p*CA, FA, and residual lignin

2 Materials and Methods

2.1 Materials

Ferulic acid standard (99%) was purchased from Shanghai Shifeng Biochemical Co., Ltd. (China). *p*-Coumaric acid (99%), sodium hydroxide (99%), ethanol (95%), dichloromethane (99%), ethyl acetate (EtOAc) (99%), pyridine (99%), bis(trimethylsilyl) trifluoroacetamide (BSTFA) (98%) were purchased from Shanghai Macklin Biochemical Co., Ltd. (China). Sugarcane bagasse, wheat straw, and corn stove/corncob used in this study were provided by Nanen sugar Paper Co., Ltd. (Xinping, Yunnan Province, China), Jiaxiang County (Jining, Shandong Province, China), and Taobao (online retail), respectively. All raw materials were cut into pieces, grounded, and sieved to collect the 40–60 mesh. Then the grounded powders were dewaxed by ethanol aqueous solution (80%) extraction at 80°C for 8 h (twice) and dichloromethane extraction at 30°C for 15 h.

2.2 Lignin Contents

The lignin contents of four different samples were determined by the L-cysteine dissolution method according to the previous study [15]. The lignin contents of bagasse, wheat straw, corn stover, and corncob were 20.5%, 16.5%, 15.8%, and 12.3%, respectively.

2.3 Alkali Hydrolysis of Gramineous Raw Materials

1 g of dry raw material was put into a sealed glass vial with 20 mL of 1 M NaOH solution and kept stirring for 1–8 h at 100°C. After the reaction, the vial was cooled down and filtered to collect the filtrate by 200 mesh screen and the hydrolysis residue. The residue was washed with deionized water 3 times, combined with the filtrates, and then adjusted the pH to 3 with 1 M HCl. The filtrate was extracted with ethyl acetate. The supernatant was collected by centrifugation 3 times at 10000 rpm. The upper organic phase was filtered with a 0.22 μm membrane and evaporated under reduced pressure. The extracted hydrolysis product was silylated in pyridine using bis (trimethylsilyl) trifluoroacetamide (BSTFA) at 50°C for 40 min. The silylated product was characterized by GC-FID and GC-MS. In addition, the alkali lignin obtained by acidic precipitation was collected and freeze-dried for NMR characterization.

2.4 GC-MS Analysis

The structure of the extracted hydrolysis products, including FA and *p*CA, was identified by the GC-MS with Shimadzu SH-Rxi Sil column (GCMS-TQ8040, SHIMADZU). The procedure was as follows: the gasification temperature was set as 250°C. 1 μL of the sample was injected with split mode (split ratio 20:1). The oven temperature was held at 80°C for 5 min and then was programmed to 280°C (heating rate 15 °C/min) and kept for 10 min. The ion source temperature was 250°C. The MS scanning range was 50–800 m/z.

2.5 GC-FID Analysis

The quantitative analysis of FA and *p*CA was carried out by gas chromatography (SHIMADZU GC-FID Nexis GC-2030) equipped with SH-Rxi-1ms column with an FID detector. The concentration-response value correction curve was measured by the detector response values of FA and *p*CA standard solutions at different concentration gradients. The same temperature program was used for GC-MS determination. High-purity nitrogen was used as the carrier gas. The hydrogen flow rate of the flame ion detector was 40 mL/min. The airflow rate was 200 mL/min. The detector temperature was 300°C.

GC-FID, combined with the concentration gradient calibration curve method, was used for a more accurate quantitative analysis of products with known structures. The calibration curve of the concentration detector response value was plotted. The concentration was calculated through the detector response value of the product to be tested.

$$\text{FA:Y} = 533.24\text{C} - 136855 \left(\text{R}^2 = 0.9985\right)$$

$$p\text{CA:Y} = 519.44\text{C} - 114570 \left(\text{R}^2 = 0.9998\right)$$

Y is the response value of the detector; C is the sample concentration (unit: ppm)

2.6 2D HSQC NMR Characterization

2D HSQC NMR for whole cell wall (WCW) and solid residue. 0.5 g of dry raw materials (solid residue) was ball-milled for 48 h (milling by a planetary ball mill at 300 rpm for 5 min with 5 min intervals). 100 mg of ball-milled sample was added into a 2 mL glass vial with 1 mL of mixed deuterated reagent (DMSO-d_6: pyridine-d_5 4:1, v/v). The sample was sonicated for 10 min and then transferred to an NMR tube. The HSQC spectrum was recorded on a Bruker 500 MHz NMR. The pulse program was hsqcetgpsi2 with NS as 64, TD2 as 1024, TD1 as 256, and D1 as 1.5 s. The obtained spectrum was analyzed and processed by topspin software, The signal of DMSO (δ_C/δ_H 39.5/2.49) was used as reference.

2D HSQC NMR for alkali lignin. 60 mg of lignin sample was dissolved in an NMR tube with 0.6 mL DMSO-d_6. The 2D HSQC NMR determination procedure was the same as WCW.

$$c(x) = \frac{\text{A}(x)}{\text{A}(G2) + 0.5\text{A}(S2/6) + 0.5\text{A}(H2/6)} \times 100\%$$

$c(x)$ is the content of a structure relative to the total aromatic ring in the lignin structure, and A is the characteristic signal area of a structure in the spectrum (calculated as one hydrogen unit).

2.7 Molecular Weight

5 mg of lignin sample was dissolved in 0.5 mL of acetic anhydride: pyridine (1:1, v/v). The mixture was reacted at room temperature for 24 h in the dark. After the reaction, the mixture was transferred to a 50 mL round bottom flask, quenched by ethanol, and evaporated under reduced pressure at 50°C to remove the solvents. The acetylated product was dissolved in chromatographic grade tetrahydrofuran solvent and filtered with a 0.22 μm membrane. The sample concentration was 2 mg/mL. Polystyrene standard samples (the minimum weight average molecular weight is 208 g/mol and the maximum is 49,000 g/mol) were used for molecular weight calibration. The molecular weight of the product was determined by tetrahydrofuran phase gel permeation chromatography (GPC, Agilent1260) [16].

3 Results and Discussion

3.1 Quantification of FA and pCA

Alkaline hydrolysis could cleave the ester bonds between lignin and FA (or *p*CA) to release the FA and *p*CA monomers. The alkaline hydrolysis products were extracted by EtOAc to recover the released FA and *p*CA for isolation and quantification [8]. The recovered hydrolysis products of bagasse were identified by GC-MS, as shown in Fig. 2. Figs. 2a and 2b show the total ion chromatography (TIC) and mass spectrometry (MS) fragment diagram of ethyl acetate extracts from bagasse after hydrolysis reaction at 100°C for 4 h. Combined with the retention time and mass spectrum fragments of FA and *p*CA standards, it was confirmed that FA and *p*CA were the main products extracted by EtOAc.

During alkaline hydrolysis, the reaction time, temperature, and alkaline concentration are essential factors that affect the cleavage efficiency of FA and *p*CA. Considering the reactivity of lignin and FA/*p*CA under alkaline treatment, the isolation yields of FA and *p*CA by alkaline hydrolysis were investigated at 100°C and 1 M NaOH for varied reaction times [17]. As shown in Table 1, the yields of FA and *p*CA monomers increased by prolonging the hydrolysis time from 1 to 4 h. As the alkaline treatment time was prolonged to 4 h, *p*CA and FA monomer yielded 39.8 and 11.5 mg/g, respectively.

The total mass of these two monomers accounts for 25.03% based on lignin content. By comparison, the yields of *p*CA and FA at 4 h are 34% and 18.5% higher than those of 1 h. However, as the reaction time was extended to 8 h, the yields of *p*CA and FA were slightly decreased.

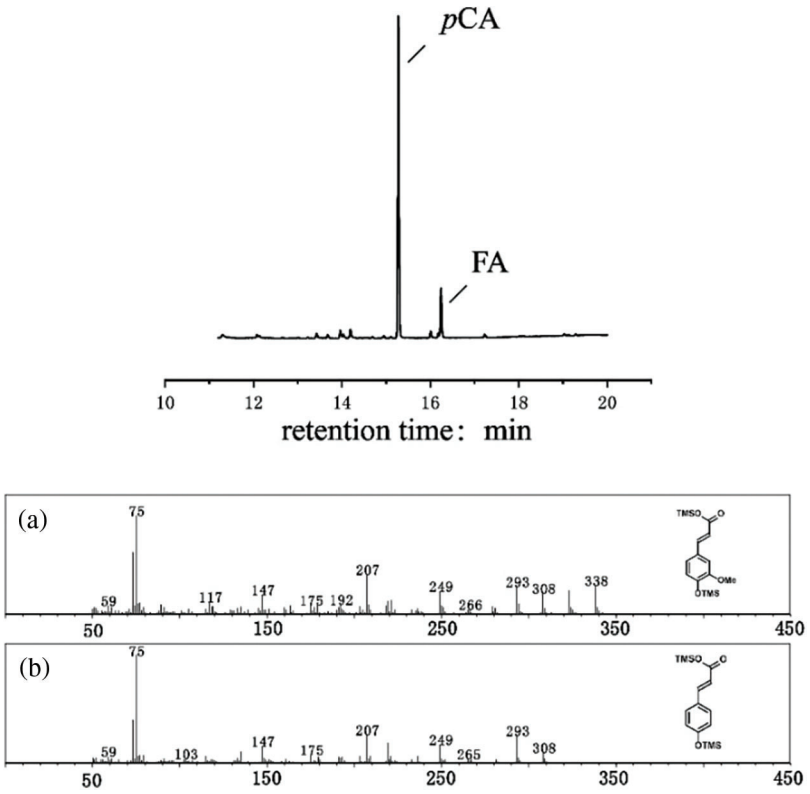

Figure 2: TIC and MS fragments of bagasse alkali hydrolysate (a) Silylated FA (b) Silylated *p*CA

Table 1: The yields of FA and *p*CA from bagasse by alkaline hydrolysis for different reaction times

Reaction time (h)	Yield of *p*CA (mg/g)	Yield of FA (mg/g)
1	29.7	9.7
2	36.3	10.3
4	39.8	11.5
8	37.6	10.5

Note: The isolation yields are based on the dry weight of raw materials.

Table 2 shows the isolation yield of FA and *p*CA from bagasse, wheat straw, corn stover, and corncob. Among these four raw materials, bagasse shows the highest yield of *p*CA, followed by corncob, corn stover, and wheat straw, which are 39.8, 35.1, 28.0, and 13.7 mg/g, respectively. The yield of FA in corncob hydrolysate is the highest among four different biomass, being 14.5 mg/g (11.79% based on lignin). Considering the lignin content of raw materials, the total yields of FA and *p*CA in bagasse, wheat straw, corn stover, and corncob are accounted for 25%, 15%, 25%, and 40%, respectively. It is evident that the FA and *p*CA are important components in Gramineae lignins. These results suggested that bagasse, corncob, and corn stover are all suitable raw materials for the source of FA and *p*CA.

Table 2: The yields of FA and *p*CA hydrolyzed by different raw materials at 100°C for 4 h

	Yield of *p*CA (mg/g)	Yield of FA (mg/g)
Bagasse	39.8	11.5
Wheat straw	13.7	11.0
Corn stover	28.0	11.0
Corncob	35.1	14.5

Note: The isolation yields are based on the dry weight of raw materials.

3.2 2D HSQC NMR Characterization of Raw Materials and Solid Residues

The HSQC spectra of the whole cell wall gel-state samples are shown in Figs. 3 and 4. The characteristic peaks G2, G5, and G6 of G-type units were observed in the aromatic region in Fig. 3 at δ_C/δ_H 111.3/6.95, 115/6.77, and 118.8/6.76 ppm. The characteristic signal of S-type unit S2/6 was located at δ_C/δ_H 103.9/6.67 ppm. The signals of FA and *p*CA were evident in the aromatic region of the four samples. The signal of *p*CA2/6 structure is located in δ_C/δ_H 130.2/7.47 ppm. The signal of *p*CA8 was at δ_C/δ_H 113.8/6.26 ppm. The crosslinking signals of *p*CA7 and FA7 were observed at δ_C/δ_H 144.6/7.44 ppm. The signals of FA2, FA6, and FA8 were at δ_C/δ_H 111.1/7.35, 123.4/7.13, and 115.3/6.40 ppm, respectively [18–20]. The NMR spectra showed that the four gramineous lignins are characteristic S/G/H type lignin but with small amount of H units. Meanwhile, the correlations of S/G/H units linked by β aryl ether linkage could be clearly observed in the aliphatic side-chain region (Fig. 4). By comparison, it indicates that some of the H-type β aryl ether linkages could be contributed by the cross-linkage between *p*CA and lignin. The characteristic signal of β-D-xylan (5,4,3,2) was also observed at δ_C/δ_H 63.15/3.27, 75.34/3.65, 74.1/3.42, and 72.62/3.22 ppm. Based on the integrals of S2/6 and G2, the S/G ratios of bagasse, wheat straw, corn straw, and corncob were calculated as 3.1, 0.8, 1.4, and 0.8, respectively.

The signal intensity of *p*CA in the four grass samples was significantly stronger than that of FA. The content of *p*CA is about 1.5–7.0 times higher than that of FA, according to the contour integration. Among these samples, the content of *p*CA in bagasse is the highest by integration. In contrast, the content of *p*CA in wheat straw is significantly lower than that of the other three samples, consistent with the result from GC quantitation.

For a better understanding of the removal efficiency of FA and *p*CA under alkaline condition, the hydrolysis solid residue was also characterized by 2D HSQC NMR (Fig. 5). Neither the FA nor *p*CA could be observed in the aromatic area, suggesting the complete (or nearly complete) removal of FA and *p*CA under the alkaline treatment in 1 M NaOH at 100°C for 4 h. A small amount of G/S-type units, including G2, G5, G6, and S2/6 correlations in the aromatic area and β aryl ether linkages in the aliphatic region, were observed in the solid residue of wheat straw. By comparison, the correlations of methoxyl group are observed in the solid residues of bagasse, wheat straw, and corn stover. In contrast, the correlations of the methoxyl group completely disappeared in the solid residue of the corncob. However, no characteristic lignin peak is observed in bagasse, corn stover, and corncob residues, indicating that the lignin has been effectively removed and dissolved in the alkaline liquid (filtrate) under the alkaline hydrolysis treatment, and the low levels of residual lignin in the solid residue beyond the 2D HSQC NMR's detection limit.

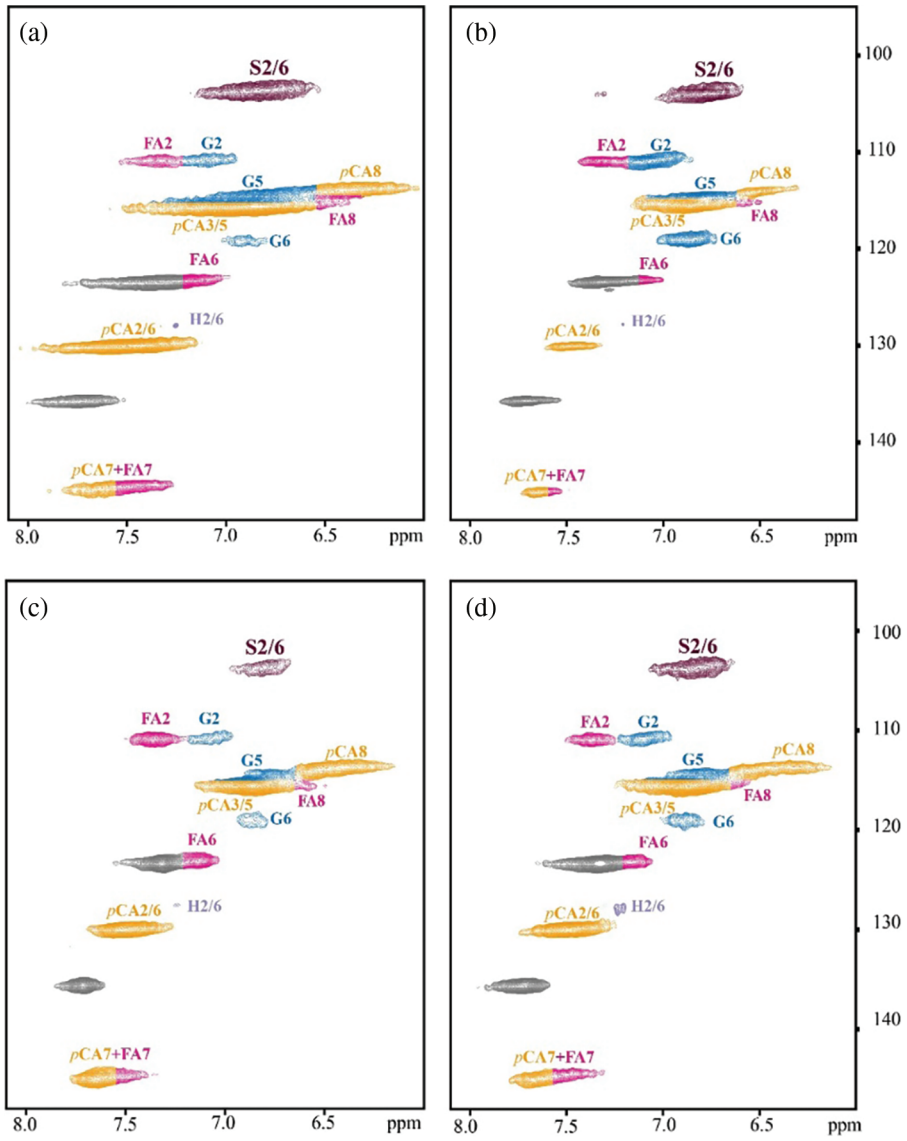

Figure 3: The aromatic region of the 2D HSQC NMR spectra of whole cell wall (a) Bagasse, (b) Wheat straw, (c) Corn stover, and (d) Corncob

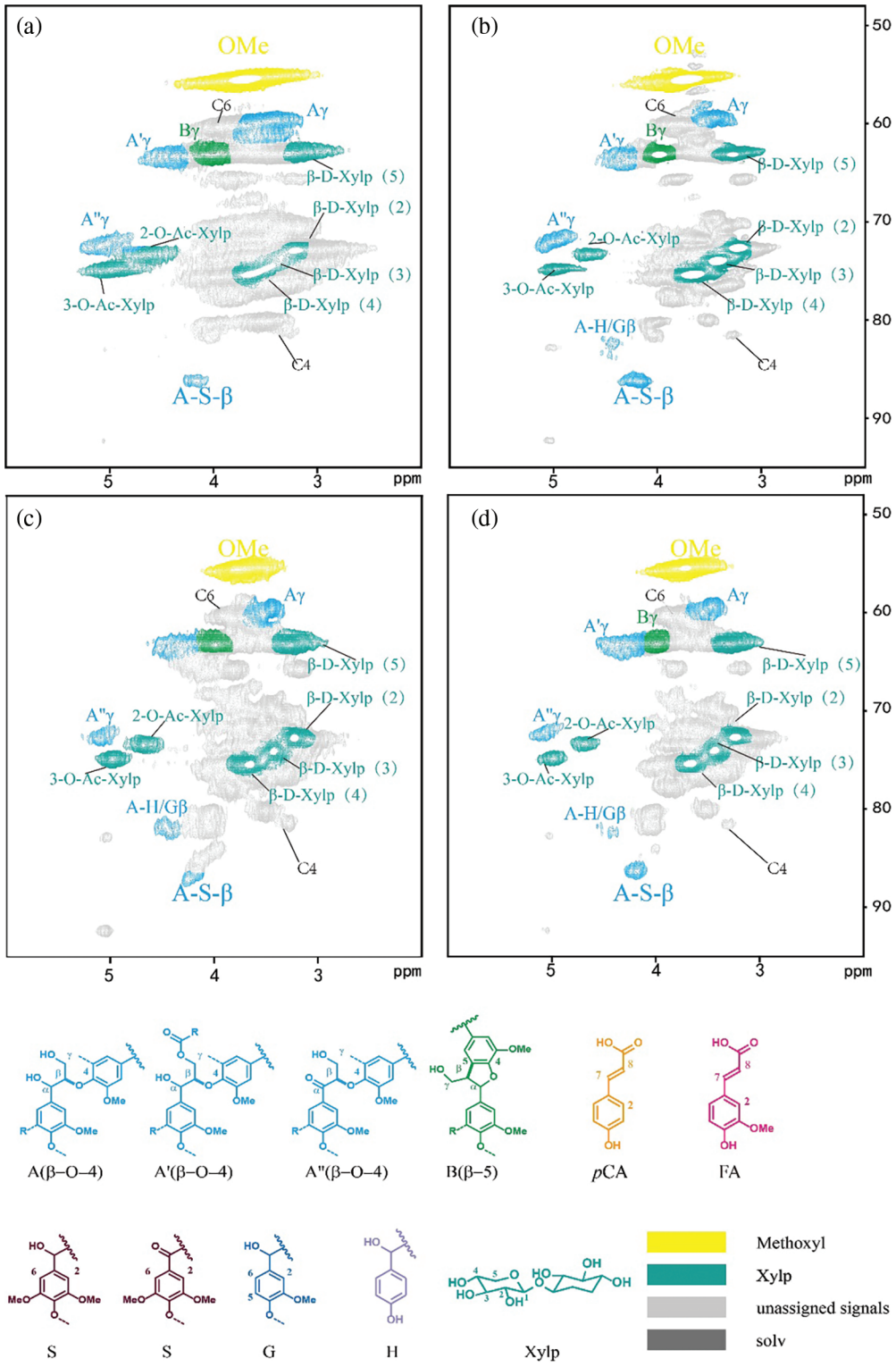

Figure 4: The aliphatic region of the 2D HSQC NMR spectra of whole cell wall (a) Bagasse, (b) Wheat straw, (c) Corn stover, and (d) Corncob

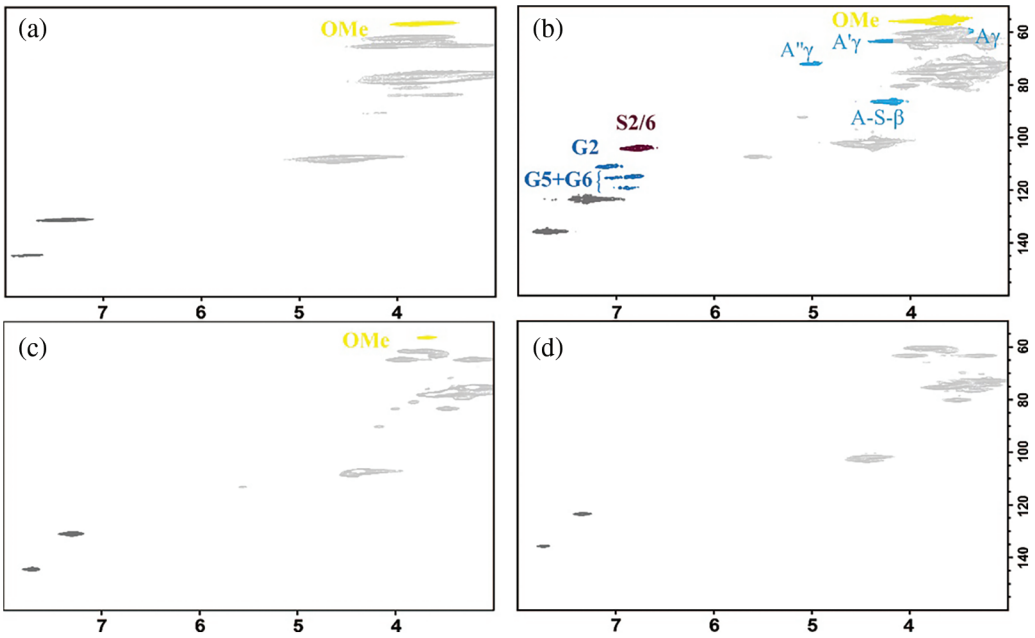

Figure 5: 2D HSQC NMR spectra of alkaline hydrolysis residue in aromatic region (a) Bagasse (b) Wheat straw (c) Corn stover (d) Corncob

3.3 2D HSQC NMR Characterization of Alkali Lignins

Alkaline hydrolysis is a common method to separate lignin and hemicellulose from lignocellulosic biomass. A large amount of lignin could be dissolved in the alkaline solution during the isolation process of FA and *p*CA from the gramineae raw materials. Depolymerization occurred to the lignin at varying temperatures during alkaline treatment. As shown in Fig. 6, the four different alkali lignins are composed of three units (G, S, and H units). The correlations of FA disappeared in the four different alkali lignins, whereas a small amount of *p*CA remained. This result indicates that FA could be completely released at 100°C with 1 M NaOH for 4 h. Although it is reported that the linkages between FA/*p*CA and lignin in gramineous raw materials are mainly ester and ether bonds, the reactivities of these two structures, to some extent, behave differently under alkali treatment as shown by the NMR spectra [21,22]. It suggests that there might be a certain amount of stubborn connection between *p*CA and lignin that remained intact during alkali treatment.

Table 3 shows the molecular weights of four different alkali lignins. The molecular weights of alkali lignins are within 1100–1300 g/mmol, which is lower than that of common alkali lignin or kraft lignin [23,24]. The polydispersity is 2.2–2.6, indicating a relatively narrow molecular weight distribution. Combined with NMR analysis in the aliphatic side-chain region, trace amount of β-O-4 characteristic signals indicate that lignin was mainly degraded and dissolved without severe condensation reaction under this condition.

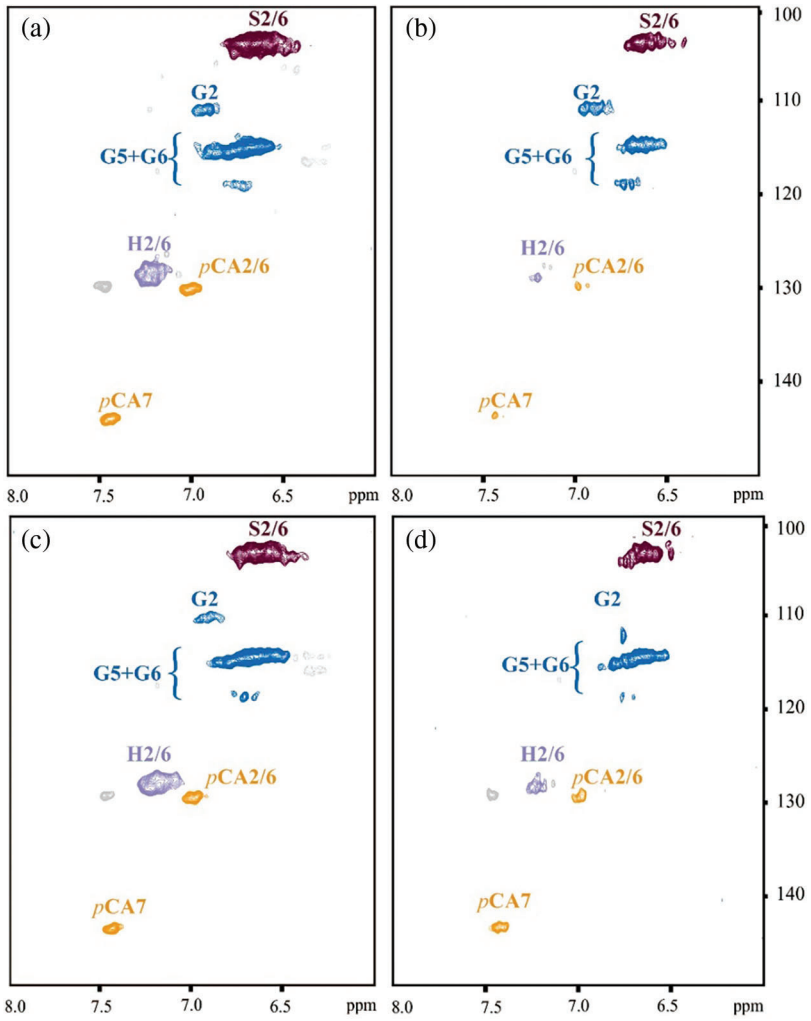

Figure 6: 2D HSQC NMR spectra of alkali lignins (a) Bagasse (b) Wheat straw (c) Corn stover (d) Corncob

Table 3: The molecular weights of alkali lignins (g/mol)

	Mw	Mn	PD
Bagasse	1164	510	2.2
Wheat straw	1300	491	2.6
Corn stover	1117	502	2.2
Corncob	1148	524	2.2

4 Conclusion

This paper explored the contents of FA and *p*CA in four different gramineous plant samples and characterized the lignin structure before and after alkali treatment. The bagasse, wheat, corn straw, and corncob released 39.8, 13.7, 28.0, 35.1 mg/g of *p*CA, and 11.5, 11.0, 11.0, 14.5 mg/g of FA, respectively, in 1 M NaOH at 100°C for 4 h. According to the characterization of the extracted lignin from the four gramineous plant materials by 2D HSQC NMR, the applied alkali treatment was able to completely

remove FA, but not the *p*CA, from the lignin. Overall, this study discloses the structure of residual lignin during the quantitative extraction of FA and *p*CA from different gramineous materials, providing a simultaneous way for the efficient isolation of *p*CA, FA, and alkaline lignin.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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