Research Article

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Structural characteristics of plant cell wall elucidated by solution-state 2D NMR spectroscopy with an optimized procedure

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Abstract: A method was developed for rapid qualitative determination of lignocellulose in the tobacco cell wall by utilizing 2D heteronuclear single quantum coherence NMR spectra (2D HSQC NMR). Traditional methods for analyzing the structure of lignocellulose involve many steps of separation and extraction, which is labor-intensive. In this work, the whole cell wall was milled and dissolved in deuterium solvent. The solvent dimethylsulfoxide (DMSO- d_6) containing hexamethylphosphoramide (HMPA- d_{18}) enhanced swelling of the sample and gave high-resolution spectra. The tobacco samples are ball milled at different ball milling times, and the state of the particles is observed through an electron microscope, and then the probability of the particles being less than 5 μm is counted. Through the comparison of the abundance and integration of the peak signals in the spectra under different transmittances, it was determined that when the milling time was 6 h, the quality of the NMR spectra was the best. The optimum conditions of characterizing tobacco structure were DMSO- d_6 /HMPA- d_{18} solution and 6 h milling time. Under these conditions, complete representation of the structure of lignocellulose and simplified process could be achieved.

Keywords: lignocellulose, 2D HSQC NMR, DMSO- d_6 /HMPA- d_{18} , transmittance, tobacco

Abbreviations

2D NMR	two-dimensional nuclear magnetic resonance spectroscopy
DMSO- d_6	deuterated dimethylsulfoxide
HMPA- d_{18}	deuterated hexamethylphosphoramide
HSQC	(^1H - ^{13}C) heteronuclear single-quantum coherence
WCW	whole cell wall

1 Introduction

Plant cell walls, also known as lignocellulosic biomass, are mainly composed of cellulose, hemicellulose, and lignin [1]. Cellulose is a linear macromolecular compound formed by the linkage of 1,4-glucoside [2]. Hemicelluloses is a class of highly branched polysaccharides and is a heterogeneous glycan composed of heteropolysaccharides containing different pentoses and hexoses [3]. Lignin is a phenolic polymer formed from phenylpropane units through aryl ether bonds and carbon-carbon bonds, consisting of three lignin units called *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) [4]. Hemicellulose and lignin in plant cell walls form a network structure through covalent bonds, and cellulose is embedded in the network and makes the lignocellulose form a tight interaction [5,6]. In the field of tobacco, the structural changes of lignocellulose will cause the changes of tobacco properties during the process of tobacco production and transformation, so characterizing structural state of tobacco fast will provide guidance for the reduction of tobacco damage [7].

Most traditional methods for characterizing lignocellulose involve complicated separation of cellulose,

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lignin, and hemicellulose followed by characterizing the individual components. These methods are not only time-consuming and laborious but more importantly, they may modify the native structure of cell wall components [8]. Chemical functional groups and some molecules in plant cell walls have been revealed by wet chemistry, but these studies require cell wall component isolation to determine the source of each specific chemical functional group [9]. Degradative methods such as thioacidolysis [10], nitrobenzene oxidation [11,12], and derivatization followed by reductive cleavage [13,14] are also well-understood and are used to determine lignin monomer distribution and bonding pattern. All of them break the β -ether bond of the lignin molecular chain through chemical degradation, release different forms of products, and then analyze the components of products utilizing spectrum and chromatography. Unfortunately, these methods are typically limited to the analysis of monomeric or dimeric units that result from the degradative processes. Spectroscopic methods include ultraviolet spectroscopy [15], Fourier transform infrared spectroscopy [16,17], and nuclear magnetic resonance techniques [18,19]. UV spectra provide information about lignin through two absorption peaks at 200–208 and 268–287 nm. However, due to the interference of self-absorption and fluorescence of colored substances, the characterization of lignin structure by ultraviolet spectroscopy produces a large deviation [20]. Infrared spectroscopy, known as compound fingerprinting, is an effective way to characterize molecular structures [21]. The guaiacyl and syringyl absorption peaks are 1,270 and 1,330 cm^{-1} , respectively [22]. The relative content of guaiacyl and syringyl units can be obtained by calculating the relative area ratio of the two absorption peaks; however, the vibration coupling of the chemical bonds of different polymers in the cell wall will cause overlapping spectral peaks [23], so infrared technology has a large error in the determination of lignin monomers [24]. The solid-state nuclear magnetic resonance is hampered by poor resolution and overlapping resonances, but recently, the use of dynamic nuclear polarization technology has improved the sensitivity of solid-state nuclear magnetic resonance [25,26]. However, the solution two-dimensional NMR can not only obtain detailed information of the structure of inherent lignin without isolating the components, but it can also provide information for many polysaccharides and other compounds related to the cell wall, such as hydroxycinnamates (ferulates and *p*-coumarates) and hydroxybenzoate [27–29]. Solution two-dimensional nuclear magnetic resonance affords the visualization and

assessment of chemical constituents in the plant cell wall by sufficiently dissolving or swelling biomass, but due to the high crystallinity of cellulose and the complexity of cross-linked cell wall construction, the nondestructive dissolution of the plant cell wall in solvent has become a problem [30]. Lu et al. initially reported the derivatization method to perform solution nuclear magnetic resonance analysis on plant cell wall material [31]. In the present study, the biomass was dissolved using a binary solvent system of dimethyl sulfoxide with tetrabutyl ammonium fluoride (TBAF) or *N*-methylimidazole (NMI) after ball grinding. However, these methods promote the dissolution of cell walls by generating derivatives that alter or mask the intrinsic structure and bonding of cell walls, such as the oxidation of hemicellulose because of the addition of acetic anhydride to DMSO/TBAF [31]. In recent years, studies on two-dimensional nuclear magnetic resonance spectroscopy (2D NMR) of cell wall swelling with underivatized solvent have attracted wide attention. In these cases, significant expansion of cell wall material provides sufficient molecular fluidity for the cell wall polymer, the dipolar couplings are significantly reduced, the nuclear relaxation times are increased, and high-resolution liquid NMR spectra similar to those of entirely dissolved polymer systems can be obtained [32]. Ralph et al. utilized DMSO- d_6 to swollen plant cell walls and showed that it interrupts some of the hydrogen bonds of cellulose to a certain extent [33]. However, due to the high crystallization of cellulose, effectively swelling biomass in DMSO- d_6 is difficult and generates a highly viscous suspension, which affects the quality of the two-dimensional NMR map, that is, the abundance and intensities of the signals in the map [34,35]. Kim and Ralph reported that a solvent system consisting of DMSO- d_6 /pyridine- d_5 significantly improves the resolution of the NMR spectrum [36]. Compared with the sample dissolved in DMSO- d_6 , the advantages of DMSO- d_6 /pyridine- d_5 are decreased viscosity of solution, enhanced polymer chain mobility, and improved signal-to-noise ratio of the 2D NMR spectra. HMPA- d_{18} as a cosolvent effectively increases the solubility of lignocellulose in DMSO- d_6 . HMPA is a highly polar aprotic solvent, the P–O bond is highly polarized, and the oxygen atom has a strong negative charge. HMPA- d_{18} effectively blocks some of the hydrogen bonds of cellulose [37].

In the abovementioned nonderivative solvent systems, the crystalline region of cellulose in the cell wall is difficult to dissolve, so mechanical ball milling is often applied to reduce the crystallinity of cellulose and increase the solubility of the cell wall [38]. Under the

action of ball milling, compact structures comprised of lignin, cellulose, and hemicellulose in the cell wall are broken down, the solvent effectively penetrates the cell wall and the solubility of the cell wall material increases. However, excessive ball grinding alters the construction of cell wall components, for example by oxidizing the (1 → 4)- β -glucan and fragmenting β -O-4 structures in lignin, so it is extremely critical to control the degree of ball milling [39]. Kim and Ralph investigated the optimal ball milling time for samples of different masses [36]. The optimal particle size is based on whether the samples at different milling times are completely dissolved in alkali or acid. While this method indeed provides guidance for optimizing ball milling conditions for different quality samples, this does not fundamentally determine the relationship between particle size and optimized ball milling time. Because when the ball milling time reaches a certain level, the particle size is almost unchanged. Cheng *et al.* reported the relationship between milling time and particle size distribution by milling lignocellulosic material (*Miscanthus × giganteus*) for different durations and finally determined the best milling time of samples by combining the abundance and intensities of signals at different milling times under 2D (^1H - ^{13}C) heteronuclear single-quantum coherence (HSQC) NMR lignocellulosic material (*Miscanthus × giganteus*) [40]. However, when the ball milling conditions, equipment, intensities, and other factors are different, the ball milling time cannot be directly employed to measure the degree of ball milling to compare its impact on the structure of lignocellulose [41]. This paper determines the probability of a sample size of less than 5 μm (hereinafter referred to as “transmittance”) at different ball milling times, which can directly explain the impact of particle size changes on the construction of lignocellulose from a microscopic perspective.

To improve the quality of the 2D HSQC NMR spectrum, it is vital to select an efficient solvent and investigate the conditions of ball milling. In this paper, the ball-milling samples were dissolved in deuterium DMSO- d_6 , DMSO- d_6 /pyridine- d_5 , and DMSO- d_6 /HMPA- d_{18} solvents, then the optimal solvent was selected according to the signal abundance and intensities of the two-dimensional spectra. Moreover, the relationship between different ball milling times and particle size distribution was also explored from the microscopic perspective and use the transmittance to explain the effect of ball milling on the structure of lignocellulose. Plant samples with different transmittances were first dissolved in DMSO- d_6 /HMPA- d_{18} , then the optimal particle size of the samples was determined by comparing abundance and volume

integrations of sample signals under different transmittances with 2D HSQC NMR technology.

2 Materials and methods

2.1 Materials

Tobacco leaf and tobacco stem were collected from Zhengan, Guizhou province, China. Samples were dried at 60°C for 16 h and ground to pass through a 10–40 mm screen. The samples were extracted by toluene–ethanol (v/v, 2:1) in a Soxhlet extractor for 6 h, and the extractive-free samples were air-dried at 60°C for storage. Sodium hydroxide, ethanol, toluene, and hydrochloric acid were purchased from the China National Medicines Corporation Ltd. as analytical reagents. Sodium chlorite (80%), deuterated dimethyl sulfoxide (DMSO- d_6 , D, 99.9%), and deuterium pyridine (pyridine- d_5 , D, 99.5%) were purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd, deuterated hexamethylphosphoramide (HMPA- d_{18} , D, 99.6%) was purchased from Guangzhou Puen Scientific Instrument Co., Ltd. Cellulase (Cellic® CTec2, 100 FPU/mL) was purchased from Sigma.

2.2 Ball-milling treatment

The dewaxed plant samples (tobacco stem, 1 g; tobacco leaf, 1 g) were milled in a DECO-PBM planetary ball mill at room temperature. It was equipped with agate jars (4 × 0.1 mL) containing agate ball bearings (10 mm × 10 mm, 10 mm × 5 mm). The milling was rotated horizontally at a constant milling speed of 600 rpm for different durations (2, 4, 6, and 8 h). To avoid overheating plant powders during the ball milling process, the ball mill ground for 10 min followed by a 20 min rest.

2.3 Scanning electron microscopy characterization

Electron microscopy experiments on samples at different ball milling times were performed at Schottky field emission scanning electron microscope (SIRION200). At

each ball milling time, we counted the particle size of the sample particles at different positions (at least 9) at a magnification of 1,000 times. Particle size measurement was performed in Nano measurer 1.2 software. The number of particles in different particle size intervals was counted, and the particle size distribution curve was plotted.

2.4 Lignin isolation

The extractive-free ball-milled plant sample (10 g) was suspended in acetate buffer (200 mL, pH = 4.8) with a loading of 5.0 mL of Cellic[®] CTec2 (100 FPU/mL). The reaction mixture was incubated at 50°C in a rotary shaker (XW-80A, Shanghai Huichuang Chemical Instrument Co., Ltd) for 48 h [42], the supernatant was removed by centrifugation, and the solid residue was hydrolyzed again with fresh enzyme mixture under the same conditions. A large number of carbohydrates were removed from the biomass through two-step hydrolysis, and the solid residue was washed with water, centrifuged, dried, and then further purified with 96% dioxane with a solid-to-liquid ratio of 1:10 (g/mL) at room temperature for 48 h. The mixture was filtered and washed with the same solvent until the filtrate was clear. The filtrate was collected and concentrated on a rotary evaporator and then the concentrated filtrate was dropped into three volumes of 95% ethanol, centrifuged and freeze-dried to obtain cellulolytic enzyme lignin CEL. The experimental process is shown in Figure S1.

2.5 Cellulose and hemicellulose sample preparation

The extractive-free ball-milled plant sample (10 g) was delignified in acidic solution (pH 4.2) containing sodium chlorite (10 g) at 75°C for 2 h, adjusted with 10% acetic acid, centrifuged, and washed, and the residue was dried in an oven at 50°C for 16 h [43]. Part of dried residue was dissolved in 150 mL of 2.5 mol/L HCl solution at 90°C for 2 h, centrifuged, washed, and dried to obtain a cellulose sample. Another part of the dried residue was extracted with 17.5% and 8.75% NaOH for 2 h after filtration. The sample was then extensively washed with 1% acetic acid, and the pH of the solution was adjusted to pH 6.0 with anhydrous acetic acid. The sample solution was dropped into three volumes of 95% ethanol

with continuous stirring. The pellet rich in hemicellulose was washed with 70% ethanol, air-dried and stored for analysis.

2.6 Nuclear magnetic resonance analysis

The cell wall sample, isolated lignin, cellulose, and hemicellulose were subjected to nuclear magnetic analysis. A total of 70 mg of each sample was dissolved in DMSO- d_6 , DMSO- d_6 /pyridine- d_5 , and DMSO- d_6 /HMPA- d_{18} (0.5 mL; v/v, 4:1). The mixture was vortexed for 1 h to promote the formation of a uniform gel-like sample between biomass and NMR solvents. After sonication for 1–2 h, the sample was swelled and dissolved in a deuterated solvent. Two-dimensional (2D) ^1H - ^{13}C heteronuclear single quantum coherence (HSQC) spectra were recorded at 298 K on a Bruker Avance III 400 MHz spectrometer (Bruker, Germany) fitted with a cryogenically cooled 5 mm gradient probe with inverse geometry using Bruker's standard pulse sequence "hsqcetgpsisp2." The spectral widths were 11 and 190 ppm for the ^1H and ^{13}C dimensions, respectively. 2D HSQC NMR experiments were carried out using the following parameters: spectral width of 11 ppm in F2 (^1H) with data points 2,048 and 190 ppm in F1 (^{13}C) with 256 data points; scan number (SN) of 64; interscan delay (D1) of 1 s; acquisition time of 10 h; 64 scans (NS), and 1 s scan delay (D1). The $^1J_{\text{CH}}$ used was 145 Hz. The central DMSO solvent peak was used as an internal reference ($\delta_{\text{H}}/\delta_{\text{C}} = 2.49/39.5$ ppm). Volume integration of the

Figure 1: Dissolving state of tobacco stem cell walls in different solvents: (a) DMSO- d_6 , (b) DMSO- d_6 /pyridine- d_5 , (c) DMSO- d_6 /HMPA- d_{18} .

signals in the HSQC spectrum was performed in the MestreNova 6.1.1 software.

3 Results and discussion

3.1 The effects of different solvents on two-dimensional nuclear magnetic spectra

To compare the effect of different solvent systems on the 2D HSQC NMR spectrum of the sample, plant cell wall samples were ball-milled and dissolved in DMSO- d_6 , DMSO- d_6 /pyridine- d_5 , and DMSO- d_6 /HMPA- d_{18} . The

swelling states of tobacco stem and tobacco leaf samples in three solvent systems are presented in Figure 1 and Figure S2. As shown, the DMSO- d_6 /HMPA- d_{18} solvent system can significantly swell biological samples, and the solutions were more uniform than those formed by DMSO- d_6 and DMSO- d_6 /pyridine- d_5 . The wall of the nuclear magnetic tube where DMSO- d_6 /HMPA- d_{18} was located was clear and clean, indicating that the sample has better mobility. Thus, the DMSO- d_6 /HMPA- d_{18} solvent had the best solubility for the sample.

The 2D HSQC NMR spectra of tobacco stem and tobacco leaf samples in DMSO- d_6 , DMSO- d_6 /pyridine- d_5 and DMSO- d_6 /HMPA- d_{18} are shown in Figure 2. The spectra of plant cell wall material from the 2D HSQC NMR can be divided into three regions: lignin aromatic

Tobacco stem

Tobacco leaf

Figure 2: 2D HSQC NMR spectra of plant cell walls in different solvents: (a) DMSO- d_6 , (b) DMSO- d_6 /pyridine- d_5 , (c) DMSO- d_6 /HMPA- d_{18} .

(δ_{H} 6.0–8.0 ppm, δ_{C} 100–150 ppm), oxygenated aliphatic (lignin side-chain and polysaccharide, δ_{H} 2.5–6.0 ppm, δ_{C} 50–90 ppm), and polysaccharide anomeric (δ_{H} 3.5–6.0 ppm, δ_{C} 90–110 ppm). In the aromatic region of the 2D HSQC spectrum of tobacco stem, cross-peaks of syringyl (S) and guaiacyl (G) signals were identified. Four types of lignin linkages appeared in the oxygenated aliphatic region, including β -aryl ether (β -O-4) units, phenylcoumaran (β -5), resinol (β - β), and dibenzodioxocin (5-5/4-O- β) (not shown). Glucan [(1 $\rightarrow$ 4)- β -D-Glcp] and xylan [(1 $\rightarrow$ 4)- β -D-Xylp] in the polysaccharide anomeric region were also detected. The correlation peaks of these components were assigned on the basis of the published chemical shifts [33,36]. The detailed assignments of resonances of tobacco stem are listed in Table S1. Compared with the correlation peaks assigned from DMSO- d_6 or DMSO- d_6 /pyridine- d_5 , DMSO- d_6 /HMPA- d_{18} has the most abundant signals. Additionally, similar results were also discovered in the NMR signals of tobacco leaf. The tobacco stem sample contains a large number of S and G and trace amounts of H signals (not shown). The correlation for the S_2 position is at

104.1/6.71 ppm, except for the 2-position of oxidized acetone structures S_2 (106.3/7.19 ppm). The G_5 signals appear at 115.0/6.75 ppm while G_6 signals generally appear at 118.6/6.93 ppm. However, the signals of 6-position in phenylcoumaran (β -5) units also appear at 115.1/6.57 ppm, which overlaps with most of the G_5 signals. Therefore, it is difficult to clearly distinguish the signals of G_5 and G_6 . Interestingly, there was an unexpected discovery of herbal lignin, and the aromatic regions of tobacco stem and tobacco leaf samples did not present signals corresponding to ferulic acid and coumaric acid [44]. In the oxygenated aliphatic region of tobacco leaf, the polysaccharide region signal is very rich, but this does not affect the signal distribution of the two-dimensional spectrum of tobacco leaf. The signal assignments of the leaf are shown in Table S2. The 2D HSQC NMR spectrum in DMSO- d_6 can provide information on the main components of plant cell wall materials, such as methoxy, β -aryl ether (β -O-4) linkages, S, G, and p -hydroxybenzoate (PB) units.

The signals of plants in different regions of the two-dimensional NMR spectra are shown in Figure 3. The

Figure 3: 2D HSQC NMR spectra of lignin side chain regions polysaccharide regions (a and b) and aromatic regions (c and d) of plant cell walls in DMSO- d_6 /HMPA- d_{18} .

signals of S and G in the lignin side chain region and aromatic region of the plant sample dissolved in DMSO- d_6 /pyridine- d_5 were significantly enhanced, but the signals of the hydroxyphenyl (H) were absented. Signals of H were detected at near the noise level in 2D NMR spectra when DMSO- d_6 /HMPA- d_{18} was used (not shown), especially the signals from oxygenated aliphatic (the lignin side chain and non-anomeric carbon of polysaccharide) regions (Figure 3a and b). Additionally, polysaccharide anomeric regions become more obvious, and a strong signal at 102.7/4.28 ppm corresponding to the anomeric ^{13}C - ^1H correlation of (1 $\rightarrow$ 4)- β -D-linked glucan in DMSO- d_6 /HMPA- d_{18} was substantially stronger when compared with DMSO- d_6 or DMSO- d_6 /pyridine- d_5 (Figure 2). On the other hand, the chemical shifts of S and G in DMSO- d_6 /HMPA- d_{18} of tobacco stem and tobacco leaf were identical to those observed from DMSO- d_6 and DMSO- d_6 /pyridine- d_5 (Tables S1 and S2). However, the α -position correlation for the β -ether unit (LA α) in DMSO- d_6 shifted from 83.1/4.29 to 82.1/4.19 ppm in DMSO- d_6 /pyridine- d_5 (Table S2). These observations indicated that different nuclear magnetic solvents have differential solvent effects on LA α in plant cell walls, resulting in differing chemical environments for components from the same plant. The LA-S correlations are much more intense than those from LA-H/G in tobacco stem cell walls, implying that more β -ether units in tobacco stem are assembled via monolignols cross-coupling with syringyl units rather than with guaiacyl and hydroxyphenyl units. β -O-4 unit is the main linkage structure of lignin in tobacco stem sample, followed by the phenylcoumarin (β -5) unit, resinol (β - β) unit, and dibenzodioxocin (5-5/4-O- β) (not shown). The end groups of cinnamyl alcohol can be detected by γ -C/H correlation. Compared with tobacco stem, the signal of the polysaccharide region in tobacco leaf was stronger (Figure 3b), relating closely to the large amount of polysaccharide in tobacco leaf. In comparison, signals of the lignin components in tobacco leaf are weaker than those in tobacco stem, indicating that the lignin components in tobacco leaf was lower than those in tobacco stem. More importantly, the solution-state 2D NMR spectrum in DMSO- d_6 /HMPA- d_{18} clearly shows the basic subunits of lignin and the cross-linking of lignin-polysaccharide in tobacco stem and tobacco leaf containing low content of lignin, and the signals are complete. Therefore, DMSO- d_6 /HMPA- d_{18} is suitable for solanaceae cell wall material nuclear magnetic resonance analysis. The effective dissolution of DMSO- d_6 /HMPA- d_{18} on the cell wall increased the quality of the two-dimensional map, and the two-dimensional NMR

map in DMSO- d_6 /HMPA- d_{18} provided rich and intense signals. In summary, DMSO- d_6 /HMPA- d_{18} was selected as the solvent to dissolve plant cell wall materials in this study.

3.2 Size optimization

The degree of ball milling determines the particle size of the sample and the process of dissolution and swelling, which further affects the quality of the two-dimensional map. After ball grinding for 2, 4, 6, and 8 h, the samples of tobacco stem and tobacco leaf were characterized by scanning electron microscopy (SEM). The SEM images of tobacco stem and tobacco leaf are shown in Figures S3 and S4. Both pictures showed that with increased ball grinding time, the particle size of samples decreased, and the particle size distributions were more uniform. The particle size distribution of the samples were nearly constant between 6 and 8 h. Take tobacco leaf as an example, When the ball grinding time increased from 0 to 4 h, the average particle size of the ball milling tobacco leaf decreased significantly from 7.15 to 4.56 μm . Due to extension of the ball grinding time, the tobacco leaf lignocellulose polymerization degree decreased. When the milling time reached 8 h, the transmittance increased from 66.7% to 91.9%. When the grinding time of tobacco leaf increased from 4 to 6 h, the transmittance increased by 10.4%, corresponding to a growth rate of 12.8%. On the other hand, when the ball grinding time increased from 6 to 8 h, the transmittance of tobacco leaf increased by only 0.9% with a growth rate of 0.98%. This suggests that the particle size distribution and transmittance of the sample were uniform and stable after 6 h of ball milling. The particle size distribution of tobacco leaf and stems is shown in Table 1. Figure 4 shows the particle size distribution of plants at different milling times. With the increase of ball grinding time, the median particle size of the sample gradually decreased

Table 1: Particle size analysis of plant cell wall samples after 0, 2, 4, 6, and 8 h ball milling

Samples		Ball milling time (h)				
		0	2	4	6	8
Tobacco	Mean diameter (μm)	7.15	5.17	4.56	3.37	3.06
Leaf	% of particles < 5 μm	66.7	74.9	81.5	91.9	92.8
Tobacco	Mean diameter (μm)	8.10	6.49	3.17	2.29	1.80
Stem	% of particles < 5 μm	54.2	73.2	85.7	94.6	95.8

Figure 4: Particle size distribution of plant cell walls at different milling times: (a) tobacco stem and (b) tobacco leaf.

and measures of dispersion of the particle size distribution curve were reduced. The particle size distribution range of tobacco leaf before ball grinding was between 0 and 30 μm ; the measures of dispersion and median particle sizes of tobacco leaf significantly decreased, and the peak area within the range of 0–5 μm increased after a 2 h milling process. Compared with the tobacco leaf sample, the dispersion degree and median particle size of tobacco stem were significantly reduced and the peak area of 0–5 μm increased after 4 h ball grinding. This demonstrated that the effective milling time may vary depending on the materials and the initial particle sizes [45]. When the ball grinding time of plant samples was 6 and 8 h, the two distribution curves were almost identical, indicating that the particle size range of samples stabilized after ball grinding treatment for 6 h.

To explore the influence of particle size on the quality of two-dimensional NMR spectra, samples with different transmittance were dissolved into $\text{DMSO-d}_6/\text{HMPA-d}_{18}$ and then analyzed by 2D HSQC NMR. Figure 5 shows the two-dimensional nuclear magnetic spectra of tobacco leaf with different transmittance. When the ball grinding time was from 2 to 4 h, the number and intensity of cell wall components of tobacco leaf increased gradually. When the milling time was 6 h, the transmittance was 91.9%, and the signal intensity of the components of the lignin aromatic region, lignin side-chain region, and polysaccharide region in the map tended to be stable (Figure S5). In a two-dimensional NMR spectrum with a ball milling time of 2 h, the signals from the anomeric region (δ_{H} 3.5–6.0, δ_{C} 90–110 ppm) were not detected and the signal of three target

components – including LC_β , LC_γ and $\text{S}'_{2/6}$ were absent. Therefore, no comparison was made between the HCQC spectrum under 2 h of ball grinding and the HSQC spectra under 4, 6, and 8 h of ball grinding. As shown in Figure 5, compared with the spectrum of the 4 h ball-milling sample, the spectrum of the 6 h ball-milling sample showed more fingerprints of lignocellulosic structures, such as 4-*O*-methylglu-curonic acid, arabinofuranosyl residues, mannopyranosyl residues, as well as resinol and β -aryl ether. After 6 h of ball-milling, the homogeneity of the solution significantly improved the signal-to-noise ratio of the 2D HSQC NMR spectrum. When the ball grinding time was 8 h, some unassigned correlations (101.2/4.93 and 103.1/4.12 ppm) became stronger, suggesting that ball milling not only depolymerizes cellulose but also causes slight oxidation of (1 $\rightarrow$ 4)- β -glucan. Therefore, the undistributed signals in the spectrum may be the product of cellulose oxidation. Therefore, when the ball grinding time was 6 h, lignocellulose had the highest quality spectrum due to the least structural changes. The signal assignments of 2D HSQC NMR spectrum of cell wall components of tobacco leaf under different ball grinding times are shown in Table 2. Since the solution contains the same loading of lignocellulosic biomass, the volume integral of each characteristic signal should represent the concentration of the corresponding component dissolved in the solvent. The quantity of lignin can be determined by the integral volumes of their representative aromatic peaks corresponding to 2- and 6-positions of ^{13}C – ^1H correlation [46]. Based on these calculations, the volume integral results of lignin in tobacco stem and tobacco

Figure 5: 2D HSQC NMR spectra of ball-milled tobacco leaf cell walls at different milling times: (a) lignin side-chain and polysaccharide regions, (b) aromatic regions, and (c) anomeric regions.

leaf samples are shown in Figure 6. The lignin volume integral of tobacco stem and tobacco leaf samples peaked when the transmittance was 94.6% and 91.9% (corresponding to the ball grinding time of 6 h). It can be

inferred that with increased ball grinding time, increased sample surface area increases the contact area of the solid–liquid phase, which promotes the solubility of the sample in solution. The spectrum of the 6 h ball-milled

Table 2: Assignments of cross-peaks in the 2D HSQC NMR spectra of tobacco leaf cell walls at different ball milling times

Components	Time (h)			
	2	4	6	8
Methoxyl	55.6/3.78	55.4/3.78	55.4/3.72	55.8/3.76
LA _γ	60.34/3.63	60.6/3.46	60.3/3.55	60.6/3.57
LB _γ	63.6/3.88	63.4/3.89	63.0/3.85	63.6/3.86
LC _γ	71.8/4.18	71.9/4.10	71.2/4.12	71.6/4.18
LA-G _(β)	82.9/4.35	83.8/4.37	83.8/4.32	83.9/4.39
LC _α	83.7/4.69	84.5/4.69	84.9/4.66	84.7/4.68
LA _α	83.5/4.27	83.7/4.29	83.4/4.29	83.6/4.37
LA-S _(β)	ND	86.7/4.05	86.1/4.08	86.4/4.07
LB _α	87.4/5.63	87.5/5.63	87.5/5.65	87.7/5.66
S _{2/6}	103.4/6.67	103.9/6.64	103.8/6.69	103.7/6.59
S' _{2/6}	ND	ND	106.3/7.20	106.5/7.26
G ₂	111.4/6.94	110.4/6.94	111.3/6.97	111.5/6.95
G ₅	114.7/6.78	114.8/6.78	114.9/6.77	115.5/6.79
G ₆	118.2/6.79	118.5/6.93	119.0/6.95	119.4/6.93
PB	131.9/7.57	131.3/6.56	131.5/6.67	131.6/6.69

ND, not detected.

Figure 6: 2D NMR contour integral of lignin from tobacco stem and tobacco leaf cell walls with different transmittances. Lignin = Integral (S_{2/6}) + Integral (G₂ + G₆) + Integral (PB_{2/6}).

sample when compared with the 4 h ball-milled sample, the tobacco leaf signal from lignin was enhanced by two fold. The lignin content is almost constant between 6 and 8 h. Therefore, when the transmittance of tobacco leaf is 91.9% (corresponding to the ball grinding time of 6 h), the two-dimensional NMR spectrum signal of tobacco leaf is the strongest and the resolution is the highest. In contrast, when the milling time of tobacco stem was 4 h, the signal at 83.4/4.26 ppm which is from LA_α was absent. When the milling time increased from 2

to 4 h, the signal of lignin oxygenated aliphatic was stable, but the signal of lignin aromatic area was absent. When the milling time was 6–8 h, the signal intensity of the lignin aromatic area, lignin side chain area and polysaccharide area tended to be stable (Figure S6). The signal attribution of tobacco stem is summarized in Table S3. Thus, according to the electron microscope distribution and two-dimensional spectrum signals, when the transmittance of tobacco leaf was 98.22% (corresponding to the ball grinding time of 6 h), the quality of the two-dimensional NMR map of tobacco leaf was the highest. In summary, when the sample was ground for 6 h, the particle size range tended to be stable, the lignin structure changed the least, and the quality of the two-dimensional map was the highest. Therefore, the best grinding time for the sample is 6 h.

3.3 Comparison of 2D HSQC NMR spectra of plant cell wall material and isolated material

Under optimized conditions with the DMSO-d₆/HMPA-d₁₈ solvent and milling time of 6 h, the direct characterization of cell wall materials by 2D NMR technology facilitates the preparation process for cell wall samples, making this an attractive method. To explore whether the two-dimensional NMR spectra analysis of plant cell wall is sufficient to elucidate the characteristics of the

Tobacco leaf

Figure 7: 2D HSQC NMR spectra of isolated lignin from tobacco leaf in DMSO- d_6 /HMPA- d_{18} .

substances compared with separate plant substances, the lignin, cellulose, and hemicellulose of tobacco leaf were isolated and characterized by 2D HSQC NMR in DMSO- d_6 /HMPA- d_{18} . The results are shown in Figure 7. According to the comparison between Figures 3b and 7a, the chemical environment signals for LA- S_β , LC α , and LC γ derived from the lignin side chain (δ_H 2.5–6.0, δ_C 50–90 ppm) between isolated lignin for tobacco leaf and lignin in the cell wall of tobacco leaf, especially the minor LC β (δ_H 3.02, δ_C 53.7 ppm) also presented signal in Figure 3b. The signals of LA α and phenylcoumarin LB α in the cell wall of tobacco leaf were weaker than those in the isolated tobacco leaf lignin, but these peaks still showed strong signals in the cell wall of tobacco leaf. The signal intensities of S and G in the lignin aromatic region (δ_H 6.0–8.0, δ_C 100–150 ppm) were almost the same in both cell wall material and isolated material. In the nonanomeric region of tobacco leaf (δ_H 2.5–6.0, δ_C 50–90 ppm), the internal cellulose units (Cl $_2$, Cl $_3$, Cl $_5$, and Cl $_6$) and the cellulose nonreducing ends (CNR $_3$ and CNR $_5$) showed good signals from isolated cellulose. The xylan internal units (Xl $_2$, Xl $_3$, Xl $_4$, and Xl $_5$) and the xylan reducing/non-reducing ends units (XR α_4 , XR β_4 , XNR $_2$) from isolated hemicellulose could be found in 2D NMR spectra of tobacco leaf cell wall. On the other hand, comparing the anomeric regions of the tobacco leaf cell wall with those of separated cellulose and hemicellulose (Figure 8), the signals of polysaccharides from the cell wall were more abundant than those of separated

cellulose and hemicellulose. The internal cellulose [(1 $\rightarrow$ 4)- β -D-Glcp], xylan [(1 $\rightarrow$ 4)- β -D-Xylp] could be observed in the anomeric regions of polysaccharides on both spectra. In addition, arabinofuran residues (α -L-Araf), 4-*O*-methyl- α -D-glucuronic acid (4-*O*-MeGlcA), 2,3-di-*O*-methyl- α -D-glucuronic acid (2,3-di-*O*-MeGlcA), and acetylated xylosyl residues (2-*O*-Ac- β -D-Xylp) were also observed in the cell wall map. Additionally, the signal assignments of polysaccharides in cell walls and separation systems are listed in Table S4. The chemical environment of polysaccharide signal in the two systems was almost the same. Moreover, dibenzodioxocin (D) in tobacco leaf appeared close to the noise level in the cell wall spectrum (not shown) but had a strong signal in isolated lignin. This information revealed that dibenzodioxocins are mainly concentrated in the low molecular weight lignins, which are easy to extract during lignin isolation. By comparison, the structural information of the cell wall material characterized by 2D HSQC NMR under the optimized conditions was consistent with that of the isolated lignin, cellulose, and hemicellulose. Additionally, 2D HSQC NMR analysis of plants not only accurately determines the structure of lignin in the plant cell wall, but it also provides more abundant polysaccharide signals (Figure 8b). Therefore, 2D HSQC NMR under optimized conditions obtains plant cell wall material information consistent with the structural characteristics of the separated material without separation, and this approach simplifies sample preparation.

Figure 8: Anomeric regions of HSQC spectra of tobacco leaf cell walls and isolated cellulose and hemicellulose from tobacco leaf in DMSO- d_6 /HMPA- d_{18} . (a) Non-anomeric region, (b) anomeric region.

In summary, 2D HSQC NMR under the optimized procedure can be widely used in the structural characterization of plant cell wall materials.

4 Conclusions

In this paper, a solution-state 2D HSQC NMR analysis of the tobacco cell wall material under a combination of solvent optimization and particle size optimization is proposed. By comparing the 2D HSQC NMR spectra of tobacco in different solvents, the abundance and intensities of the spectra in DMSO- d_6 /HMPA- d_{18} were better than those in DMSO- d_6 and DMSO- d_6 /pyridine- d_5 . Therefore, DMSO- d_6 /HMPA- d_{18} was selected as the solvent for

dissolving lignocellulose. Through analysis of the particle size distribution curve, it was found that the milling time of 6 h resulted in stable particle size transmittance. Analysis of abundance and signal integration of 2D HSQC NMR spectra showed that when the transmittance of tobacco was at least 90% (i.e., the milling time was 6 h), the quality of 2D HSQC NMR spectra was the highest. By comparing the spectra of cell wall material and isolated cell wall material, it was determined that solution-state 2D HSQC NMR can characterize the complete structure of tobacco cell wall material. So, the optimized 2D HSQC NMR method is an innovative and green method for the structural analysis of plant cell walls. Therefore, the optimized two-dimensional HSQC NMR method is an innovative and green analysis method, which can be widely used in the structural analysis of plant cell wall materials.

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