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Solution-state 2D NMR of Ball-milled Plant Cell Wall Gels in DMSO-d_6

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Abstract Although finely divided ball-milled whole cell walls do not completely dissolve in dimethylsulfoxide (DMSO), they readily swell producing a gel. Solution-state two-dimensional (2D) nuclear magnetic resonance (NMR) of this gel, produced directly in the NMR tube, provides an interpretable structural fingerprint of the polysaccharide and lignin components of the wall without actual solubilization, and without structural modification beyond that inflicted by the ball milling and ultrasonication steps. Since the cellulose is highly crystalline and difficult to swell, the component may be under-represented in the spectra. The method however provides a more rapid method for comparative structural evaluation of plant cell walls than is currently available. With the new potential for chemometric analysis using the 2D NMR fingerprint, this method may find application as a secondary screen for selecting biomass lines and for optimizing biomass processing and conversion efficiencies.

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Abbreviations

Ac_2O	acetic anhydride
DMSO	dimethylsulfoxide
DMSO-d_6	perdeutero-dimethylsulfoxide
2D	two-dimensional
FA	ferulate
G	guaiacyl
H	*p*-hydroxyphenyl
HSQC	heteronuclear single quantum coherence
HMQC	heteronuclear multiple quantum coherence
NMR	nuclear magnetic resonance (spectroscopy)
NMI	*N*-methylimidazole
NS	number of scans
PCA	*p*-coumarate
PB	*p*-hydroxybenzoate
S	syringyl
TLC	thin layer chromatography

Introduction

Efficient utilization of plant cell walls is becoming an important topic in the escalating biomass to biofuels industries. In attempting to select the best biomass substrates, or to optimize biomass conversion processes, rapid methods are needed to assay the composition of plant cell wall materials. Additionally, methods are required to provide more detailed (chemical) structural analysis. NMR methods, including the one described here, fall into this latter class. NMR methods are far from rapid and not as sensitive

as other spectroscopic methods, but NMR provides unparalleled structural information on the complex polymers of the cell wall. Emerging chemometrics methods utilizing 2D NMR "fingerprints" of the cell wall [12] hold promise for relating cell wall compositional and structural characteristics to the performance of the walls in a variety of processes.

Plant cell walls are natural composites of three major components: cellulose, hemicelluloses, and lignins with complex structures. Cellulose, a linear polymer chain of β-(1→4)-linked D-glucosyl units, is the major structural component of the secondary cell walls in higher plants. The DP (degree of polymerization) of plant cellulose is about 7,000 to 15,000 [9]. Hydrogen bonding between regular cellulose chains results in highly crystalline fibers. Xylans and glucommannans are classified as hemicelluloses. They have lower DPs than cellulose and are branched [9]. Lignins in the plant cell wall are synthesized by radical coupling reactions of phenolic monomers, primarily in endwise cross-coupling reactions with the growing polymer chain. The monomers are primarily the three monolignols (the hydroxycinnamyl alcohols *p*-coumaryl, coniferyl, and sinapyl alcohols), but also various other available monomers [4, 37, 41]. As lignin is synthesized after the polysaccharide matrix has been laid down, isolation of pure lignin is problematic. Industrial fractionation of plant cell walls to cellulose requires the costly removal of lignin, e.g. via chemical pulping to produce pulp and paper or via ethanolysis [27] to produce cellulose for saccharification to glucose for fermentation to ethanol. In other processes that ferment polysaccharide-derived sugars to biofuels, lignin remains a key limiting factor due to its association with the polysaccharides in the wall.

NMR methods are important for characterizing the complex plant cell wall, at least in a bulk sense—the method is too insensitive to use at the cellular level, so the data derives from homogenizing all the cell types in a given sample. In the past, except by certain degradative analyses, characterization of the individual polymers (or polymer fractions) required separation of the components. For example, lignins from finely divided (ball-milled) materials were usually isolated, not entirely free of polysaccharide contaminants, by their dissolution into solubility-parameter-matched solvent mixtures such as 96:4 dioxane:water [3]. The yields of such lignins (relative to the total lignin in the plant sample) range from ~10% to some 65%, depending on the nature of the plant material; for example, typical softwoods yield ~15% so-called "milled wood lignin" (MWL) whereas softwoods such as poplar or dicots such as kenaf may yield much higher fractions.

2D-NMR techniques in lignin and cell wall research have improved over the past decade, as reviewed [33, 38]. More recently, by utilizing the resolution and dispersion of high-resolution and high-field 2D and 3D NMR, considerable structural information is available from dissolution of the cell wall without the need to isolate fractions [22, 32]. This method also has the advantage of fully representing all components without the selective fractionation that might occur during component isolation. However, complete dissolution of the entire cell wall component without any structural modification is not an easy task. Whole-cell-wall-dissolution systems based on hydrogen-bond-disrupting solvents such as DMSO/tetrabutylammonium fluoride and DMSO/N-methylimidazole (NMI) perform well to completely dissolve finely divided (ball-milled; considerable depolymerization results from ball-milling) plant cell wall material [22]. Certain ionic liquids also dissolve the wall [10, 28, 46] although 2D spectra have not been published to date to determine how non-destructive the dissolution is. Following acetylation using the DMSO/NMI system, acetylated wall material is soluble in $CDCl_3$ and therefore convenient for high-resolution 2D [22], or even 3D [32], NMR studies. Chemical modification of the wall, even simple derivatization, leads to the loss of some information. For example, natural acetylation in the wall is masked when the sample is per-acetylated. Also, as has been discovered previously, there is some complementarity between NMR data for acetylated wood components (in $CDCl_3$, DMSO-d_6, or acetone-d_6) and data for unacetylated components in DMSO—the dispersion of various signals can be greater in one system than the other, allowing more substantive structural interpretation. For example, the β-C/H correlations of β-ether units resolve significantly better in underivatized lignins in DMSO [5].

The idea of utilizing gel-state samples with traditional solution-state NMR techniques is a novel concept, although various other studies may have, perhaps inadvertently, exploited similar methods. For example, milled wood cellulolytic enzyme lignin preparations (produced following polysaccharidase treatments to partially remove polysaccharides; the entire lignin fraction is retained, but the preparation may still be comprised of 50:50 polysaccharides:lignin) have been subjected to NMR following prolonged "dissolution" in DMSO, often with heating [17]. Even without actual dissolution, swelling of polymers improves molecular mobility allowing the application of high-resolution solution-state NMR methods. The literature describes few gel-state NMR studies, most of which obtained simple proton or carbon spectra, predominantly via solid-state NMR spectroscopy which provides limited information [11, 25, 26]. Samples can be prepared for gel-state NMR without the usual sample preparation requirements, such as dissolving samples in complex solvents, filtering, and drying, with apparently no significant loss of cell wall structural information compared to other dissolution systems using various solvents [1, 15, 19]. The gel-

state 2D NMR method for ball-milled plant cell wall material, reported here, has the potential to be used as an independent or complimentary research tool.

DMSO-d_6 was chosen for gel-state NMR experiments here because it is not only one of the popular NMR solvents [2, 5, 13], but also an effective swelling reagent for cellulose and other wall components. Investigations into the swelling of cellulose, wood and fibers in a series of organic solvents revealed that DMSO, butylamine, formamide, and ethylene glycol are good swelling reagents for cellulose and sulfite pulps [24]. Our recent studies on whole cell wall dissolution and chemical modification also used DMSO as the primary solvent [22].

The objective of this work was to determine whether a simplified system could be derived to produce cell wall samples for detailed structural analysis by NMR. As will be shown, considerable simplification is indeed achievable in a process that also has significant advantages, including the ability to acquire 2D spectra on essentially the entire cell wall component in as little as 30 min.

Materials and Methods

General

Solvents used were AR grade and supplied by Fisher Scientific (Atlanta, GA, USA). Reagents were from Aldrich (Milwaukee, WI, USA). The ultrasonic bath was a Branson (Danbury, CT, USA) 3510EMT, tank capacity 5.7 L, with mechanical timer) was used for homogenization of the gel-state NMR sample.

Plant Materials

Dry plant materials were pre-ground for 2 min in Retsch (Newtown, PA, USA) MM301 cryogenic mixer mill at 30 Hz, using corrosion-resistant steel screw-top grinding jars (50 ml) containing a stainless steel ball bearing (1× 30 mm). The pre-ground cell walls were extracted with distilled water (ultrasonication, 1 h, three times) and 80% ethanol (ultrasonication, 1 h, three times). Isolated cell walls were ball-milled using a Retsch PM100 vibratory ball mill vibrating at 600 rpm, using zirconium dioxide (ZrO_2) vessels (50 ml) containing ZrO_2 ball bearings (10×10 mm). The grinding times are dependent on the plant cell wall type and the amount (see below); samples here were ground on a fairly large scale, but grinding times for ~200 mg of cell wall material is typically only ~1 h.

Loblolly Pine Pre-ground (Wiley mill, 2 mm screen) cell wall material (1 g) was solvent-extracted and ball-milled as described above for 10 h 20 min (in 20 min grinding/10 min interval cycles to avoid excessive heating). Lignins were also isolated from the pine sample using method described previously [35]. Briefly, lignins were prepared by ball-milling solvent-extracted cell walls, digesting away most of the polysaccharides with crude cellulases, and extracting with dioxane:H_2O (96:4). The lignins (~60 mg, non-acetylated) were dissolved in DMSO-d_6 for NMR.

Aspen Pre-ground cell wall material (800 mg) was ball-milled as described above for 4 h 10 min (in 10 min on/5 min interval cycles).

Kenaf Bast Fiber Pre-ground cell wall from freshly harvested 1 year old Tainung2 kenaf (200 mg) was ball-milled as described above for 45 min (in 5 min on/5 min off intervals).

Corn Stalks The source material was that used in a prior study [34]. Pre-ground cell wall (507 mg) was ball-milled as described above for 1 h 45 min (in 5 min on/5 min off intervals).

NMR Sample Preparation

Gel-state NMR cell wall samples prepared in a simple way. Ball-milled cell walls (50–70 mg of each) were transferred into 5 mm NMR tubes. The sample was distributed as well as possible off the bottom and up the sides of the tube. DMSO-d_6 (1–2 ml) was carefully added down the side of the NMR tube. The NMR tubes were then placed in a sonicator and sonicated for 1–5 h (depending on the sample), until the gel became clear-looking, but still turbid, and apparently homogeneous. Note that as the NMR active volume requires less than 0.5 mL of solution in a 5 mm NMR tube on the spectrometers used, less material and a smaller volume of DMSO can be used; the cell wall concentration used here for NMR is less than 50 mg/ml implying that less than 25 mg of cell wall is actually in the NMR-active volume. Despite this low concentration, spectra can be acquired with excellent sensitivity in as little as 30 min using a cryoprobe-equipped spectrometer.

Acetylated cell wall samples were prepared by the cell wall dissolution and acetylation method described previously [22].

NMR Experiments

NMR spectra were acquired on either a 750 MHz (DMX-750) or a 500 MHz (DRX-500) Bruker Biospin (Rheinstetten, Germany) instruments, each equipped with inverse (proton coils closest to the sample) gradient 5-mm TXI $^1H/^{13}C/^{15}N$ cyroprobes for high sensitivity. The central

DMSO solvent peak was used as internal reference (δ_C 39.5, δ_H 2.49 ppm). An adiabatic HSQC experiment (hsqcetgpsisp) [16] at 750 MHz typically had the following parameters: 16-Transient spectral increments were acquired from 11 to −1 ppm in F2 (^{1}H) using 1078 data points for an acquisition time of 60 ms, an interscan delay of 750 ms (for a total scan recycle time of 810 ms), 196 to −23 ppm in F1 (^{13}C) using 480 increments (F1 acquisition time: 5.78 ms), with a total acquisition time of 1 h 48 min. The standard Bruker implementation (invietgssi) of the gradient-selected sensitivity-improved inverse (^{1}H-detected) HSQC experiment [45] was used at 500 MHz and had the following parameters: 32-Transient spectral increments were acquired from 9 to 1 ppm in F2 (^{1}H) using 1,200 data points, 160 to 10 ppm in F1 (^{13}C) using 512 increments (F1 acquisition time: 13.6 ms) of 32 NS, with a total acquisition time of 5 h 35 min. Processing used typical matched Gaussian apodization in F2 and a squared cosine-bell in F1.

We used a Bruker Avance 360 MHz instrument equipped with an inverse (proton coils closest to the sample) gradient 5-mm ^{1}H/broadband gradient probe for structural elucidation and assignment authentication for the model compounds, and for the corn stalk Ac-CW spectrum in Fig. 1f. The standard Bruker implementations of the traditional suite of 1D and 2D (gradient-selected, ^{1}H-detected, e.g. COSY, HMQC, HMBC) NMR experiments were used. Normal HMQC (inv4gptp) experiments at 360 MHz were used for model compounds and had the following parameters. 32-Transient spectral increments were acquired from 10 to 0 ppm in F2 (^{1}H) using 1400 data points, 200 to 0 ppm in F1 (^{13}C) using 128 (or 256) increments (F1 acquisition time: 35.3 ms), with a total acquisition time of 1 h and 23 min.

Lignin Model Compounds

Model compounds were prepared and their NMR spectra were acquired in DMSO to enable assignments made in this paper. Coniferyl alcohol and sinapyl alcohol were prepared using borohydride exchange resin [14]. *p*-Coumaryl alcohol was prepared as described previously [29]. Coniferyl alcohol dimers were synthesized from *in vitro* radical coupling with MnO_2 in dioxane:H_2O (1:1, v/v) [42]. Sinapyl alcohol dimers were prepared with $FeCl_3 \cdot 6H_2O$ in dioxane:H_2O (5:2, v/v) [43]. *p*-Coumaryl alcohol dimers were synthesized via horseradish peroxidase and hydrogen peroxide in acetone:water (1:10, v/v) and also with $FeCl_3 \cdot 6H_2O$ in acetone:H_2O (5:1, v/v). Each reaction was stirred for 1 to 4 h, except for peroxidase reactions that take about 15 h, and monitored by TLC. Reaction solutions were poured into EtOAc, and washed with saturated NH_4Cl. EtOAc solutions were dried over anhydrous $MgSO_4$, and concentrated. Model dimers were separate on preparative TLC plates with $CHCl_3$:MeOH (10:1, v/v). The fully authenticated NMR data for model compounds will be deposited in the "NMR Database of lignin and cell wall model compounds" available via the internet [39]. Also the complete models study in DMSO-d_6 will be published elsewhere.

Polysaccharide Samples

Cellulose Cellulose (Aldrich, ~20 μm; 200 mg) was ground with a Retsch PM100 ball mill for 8 h 40 min as described above. Oat spelts arabinoxylan was obtained from Sigma.

Locust Bean Gum Locust Bean Gum (galactomannan polysaccharide, polymers of β-D-mannopyranose + α-D-galactopyranose), from seeds of *Ceratonia Siliqua* L., (Sigma, 1 g) was ground with a Retsch PM100 ball mill for 20 h as described above.

Results and Discussion

Sample Preparation

Ball milling of plant material and swelling with DMSO-d_6, directly in the NMR tube, are described in the Experimental Section. The prepared samples usually have some mobility, but the high viscosity (Fig. 1g) is initially disturbing when contemplating traditional solution-state NMR experiments. NMR shimming becomes particularly insensitive; an advantage is that the sample scarcely needs shimming. Little material was actually soluble; extended extraction of the ball-milled material into DMSO solubilized only 9–19% of the material. NMR of the soluble fractions produced particularly high-resolution spectra (not shown) demonstrating that this fraction was enriched in lignin.

2D NMR Experiments

Despite the insolubility and viscosity of the samples, HMQC experiments acquired on a 360 MHz NMR instrument in 14 to 15 h provided well dispersed spectra of the cell walls comparable to those from acetylated whole cell walls using recently developed cell wall dissolution methods. Excellent 2D HSQC spectra were obtained in just hours using higher-field spectrometers fitted with cryogenically cooled probes that enhance the sensitivity four- to fivefold. The rapid relaxation due to the high viscosity allows short acquisition times and rapid scan repetition rates. We did not optimize (or minimize) repetition times here due to concerns regarding the power duty cycle on cryogenic probes, but settled on acquisition times as short

as 60 ms, and interscan relaxation delays of 750 ms giving total scan repetition times of 810 ms. As we have now confirmed with our NMR facility, repetition times can be reduced to ~200 ms (by lowering the interscan delay to 140 ms) which would allow the same quality spectra to be acquired in just 27 min instead of the 1 h 48 used here on the 750 MHz instrument. Although resolution declines as the relaxation rate increases (and the repetition time can decrease) producing broader correlations, the adequate resolution and dispersion from these gel-state samples highlights another advantage over true solution-state NMR on actual cell wall solutions—the time required to acquire spectra can be drastically reduced. Thus, with cryoprobe instrumentation, spectra adequate for most purposes, including for chemometrics, can clearly be acquired in under an hour, even in 30 min, implying that acquiring spectra is not the barrier to obtaining data from 20 to perhaps 50 samples per day.

Preliminary experiments determined whether spectra resulted from the gel or simply from soluble components in the gel. Following extensive extraction of the cell wall with DMSO, the insoluble residue (81–91% of the cell wall) provided weaker spectra of essentially the same quality as from the whole cell wall sample.

Our interest has traditionally been in the lignin component. Despite the relatively large amounts of polysaccharides (cellulose and hemicelluloses) in the sample, many of the lignin resonances are well resolved in 2D NMR. Figures 1 and 2 show HSQC or HMQC (1-bond ^{13}C–^{1}H correlation) spectra from various samples. The spectra in Fig. 1 show the polysaccharide and lignin side chain area (i.e. aliphatic region) of the whole cell wall samples along with an isolated lignin and an acetylated cell wall for comparison. Aromatic regions, shown in Fig. 2, logically represent the entire lignin component as compared to the fraction isolated for the pine lignin spectrum (Fig. 2a).

Polysaccharide Correlations

Many of the correlations in the δ_C/δ_H 50–120/2.5–5.5 ppm region, Fig. 1, belong to the polysaccharide components. Regrettably, there is a paucity of NMR data relating to underivatized polysaccharides in DMSO so more substantial assignment of the many dispersed contours needs to be relegated to future studies. NMR data from cellulose, oat spelts arabinoxylan, and locust bean gum containing polymers of β-D-mannopyranose and α-D-galactose [6] were obtained by the same procedure as for plant cell walls preparations that are described in the Experimental Section to make preliminary assignments only of the anomeric (C1) correlations. In the case of pine cell walls, for example, the anomeric C/H correlation for β-D-glucosyl (Glu) residues, which is mostly due to cellulose, appears at δ_C/δ_H 101.7/4.24 ppm; the anomeric from β-D-mannosyl (Man) residues appears at δ_C/δ_H 98.9/4.72 ppm. The α-D-galactosyl units in locust bean gum appear at δ_C/δ_H 100.6/4.49 ppm—the galactose (Gal) assignments in Fig. 1 remain tentative at this point. Despite the limited assignments made here for polysaccharides, it is clear from Fig. 1 that the polysaccharide anomeric correlations are well dispersed and should eventually succumb to more complete assignment. More importantly, the anomeric correlation profiles are significantly different between the various plant types suggesting that the data will be valuable for characterization of polysaccharide polymers as well as for lignins.

One feature of the gel-state NMR may require caution. If the cellulose component remains crystalline in the gel, it is unlikely that solution-state NMR signals from this crystalline component will be visible. Cellulose may therefore be under-represented in these spectra compared to the more mobile components, the hemicelluloses, non-crystalline cellulose, and lignin.

Lignin Sidechain Regions

The aliphatic side chain region, Fig. 1, characterizes the types and distribution of interunit bonding patterns of the lignin fraction. This area also contains abundant polysaccharides, but the dispersion is sufficient that many lignin peaks are well resolved. Correlations for lignin methoxyl groups, along with the diagnostic β-ether units **A**, phenylcoumaran units **B**, and resinol units **C**, can be readily assigned. The relative concentration of the components will vary depending on the species.

The isolated pine lignin, a predominantly guaiacyl lignin, was readily dissolved in DMSO-d_6. A clear picture of the bonding patterns in this softwood lignin are seen in Fig. 1b. β-Ether (β-O-4) units **A** are the major interunit structure, followed by phenylcoumaran (β-5) units **B**. Minor amounts of pinoresinol (β-β) units **C**, and dibenzodioxocin (5–5/β-O-4) units **D** are also well resolved from the traces of polysaccharides. Cinnamyl alcohol endgroups **X1** are also discernable from their γ-C/H correlation.

Gel-state 2D NMR of pine cell walls, Fig. 1a, provides a remarkably well resolved spectrum that can be compared to that from the isolated pine lignin (Fig. 1b) as the standard. Basically the same structural information is available from the lignin except that the dibenzodioxocin D component can not be seen until lower contour levels (not shown) are examined. Polysaccharide contours are logically dominant because this is a whole plant cell wall sample. There is also an acetate peak at δ_C/δ_H 20.9/2.01 ppm which likely belongs to polysaccharides rather than to lignin, because only a trace of the acetate contour can be found in the extracted pine lignin (Fig. 1b). Pine hemicelluloses are known to be acetylated, particularly on xylan and mannan components [9, 47].

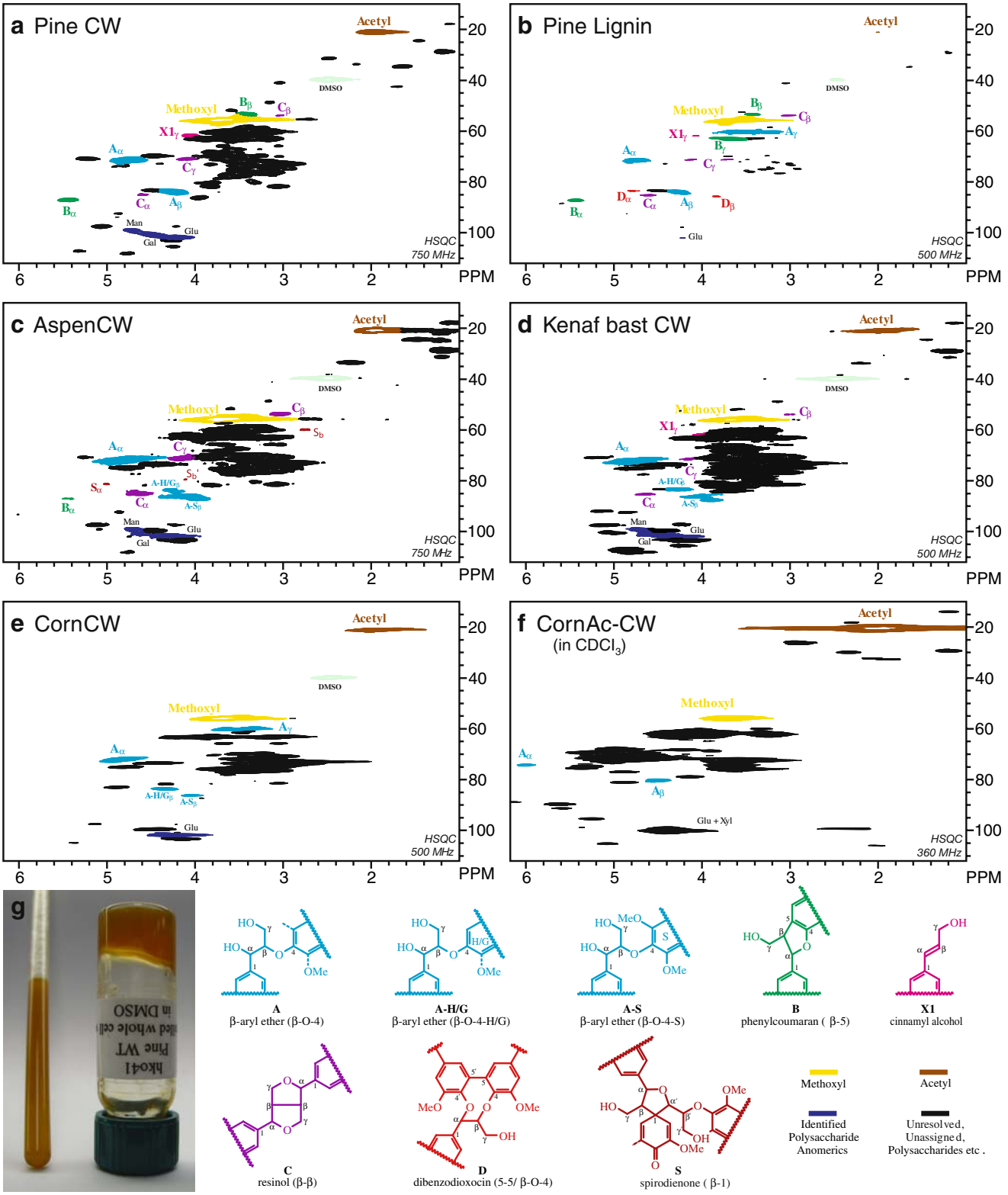

Fig. 1 Aliphatic regions of Gel-state NMR spectra. 2D ^{13}C-^{1}H correlation (HSQC or HMQC) spectra, gel-state in DMSO-d_6 (unless otherwise noted) from cell walls and lignins from various samples. **a** Pine, **b** Pine isolated lignin, **c** Aspen, **d** Kenaf bast fiber, **e** Corn stems, **f** Corn acetylated cell walls, from DMSO/NMI dissolution, in $CDCl_3$. **g** This picture shows (*left*) gel cell wall sample (~70 mg in 1 ml DMSO-d_6) in a 5-mm NMR tube and (*right*) gel cell wall sample in DMSO in a vial (upside down to demonstrate its viscosity). Lignin structures are *colored and labeled* using established conventions [33, 38]

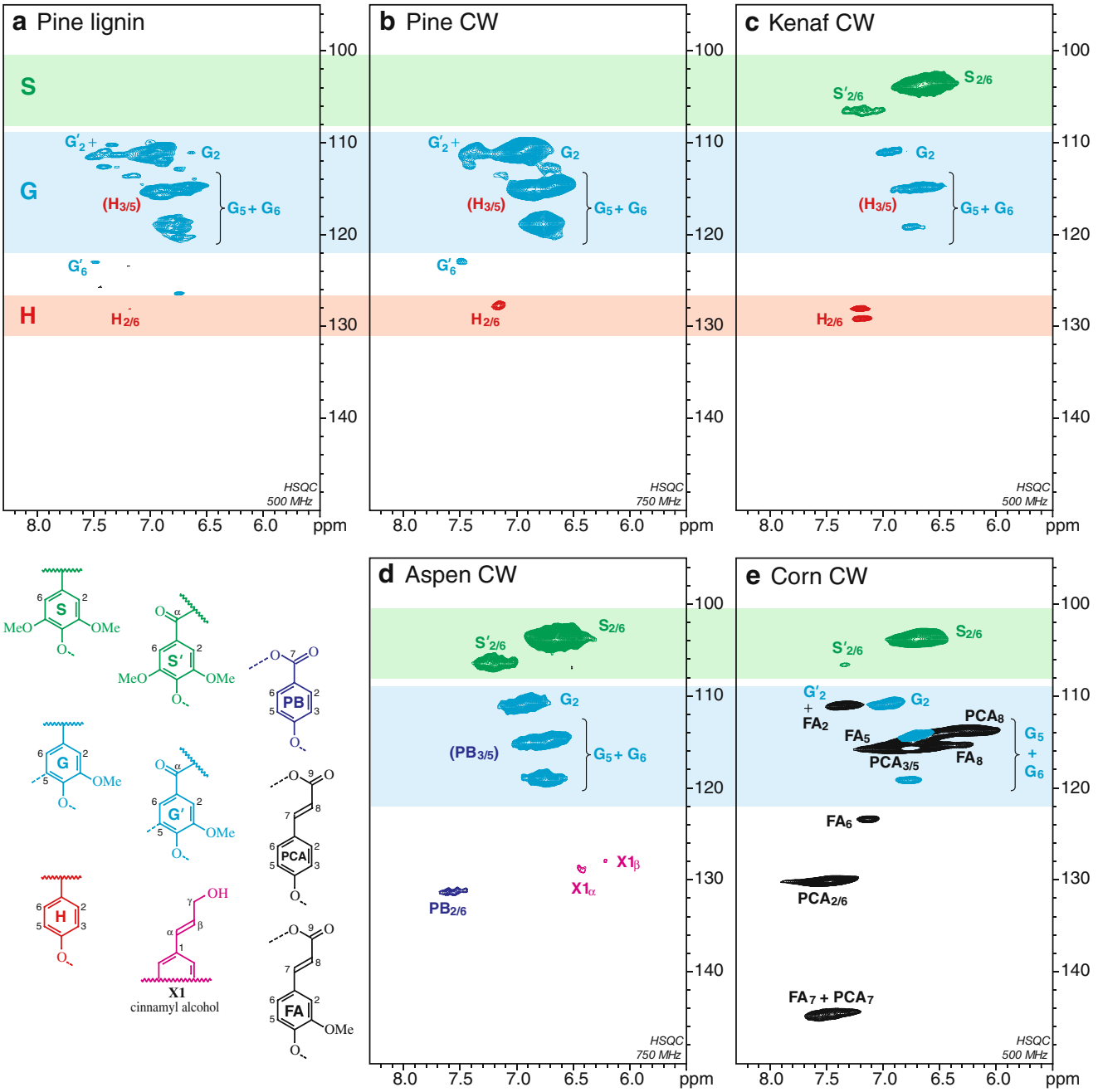

Fig. 2 Aromatic regions of gel-state NMR spectra. 2D ^{13}C-^{1}H correlation (HSQC or HMQC) spectra, gel-state in DMSO-d_6 (unless otherwise noted) from cell walls and lignins from various samples. **a** Pine isolated lignin, **b** Pine, **c** Kenaf bast fiber, **d** Aspen, **e** Corn stems. Correlations from the aromatic rings are well dispersed and can be categorized by the type of aromatic units (syringyl S, guaiacyl G, and *p*-hydroxyphenyl H, as well as *p*-hydroxybenzoates PB in aspen, and *p*-coumarates PCA and ferulates FA in corn. Preliminary assignments are primarily based on model compound data in the NMR database of Lignin and Cell Wall Model Compounds [39]

Angiosperm lignins, including that from aspen shown in Fig. 1c, are typically guaiacyl–syringyl lignins synthesized from both coniferyl and sinapyl alcohols. The HSQC spectrum of the aspen cell wall resolves most of the correlations for the various lignin linkage types as already seen in pine. However, there are clear differences in interunit proportions between the different species. Since both the 3- and 5-positions of syringyl units are substituted with methoxyl groups, there are relatively limited probabilities of generating phenylcoumaran (β-5) units **B** which can be only made when a monolignol couples to a guaiacyl (G) or *p*-hydroxyphenyl (H) unit. As a result, there are relatively small amounts of phenylcoumaran units **B** compared to the major β-ether units **A** in aspen cell wall lignins. Similarly, dibenzodioxocin **D** units result from coupling of a monolignol with 5–5-coupled units that can

not be formed via syringyl units; dibenzodioxocins are therefore more minor. On the other hand, resinol (β-β) **C** correlations are easily detected because sinapyl alcohol favors this dimerization product. β-Ether (β-O-4) units **A** exhibit two well dispersed β-position correlations depending on the sidechain substitution. If the β-position is connected to a guaiacyl (or *p*-hydroxyphenyl) unit (β-O-4-H/G) **A-H/G**, the $^{13}C–^{1}H$ correlation is centered at δ_C/δ_H 83.8/4.26 ppm. If the β-position is connected to a syringyl unit (β-O-4-S) **A-S**, the $^{13}C–^{1}H$ correlation is at δ_C/δ_H 86.1/4.09 ppm. The **A-S** correlation is more intense than that from **A-H/G** in this aspen cell wall sample, implying that more of β-ether interunits in aspen are assembled via monolignols cross-coupling with syringyl units than with guaiacyl units. Several spirodienone (β-1) **S** peaks can be found; it is not possible to see all of the $^{13}C–^{1}H$ correlations but characteristic peaks are clear—**S**-α at δ_C/δ_H 81.1/5.00 ppm, **S**-β′ at δ_C/δ_H 79.4/4.11 ppm, and **S**-β at δ_C/δ_H 59.8/2.75 ppm. Such units were only rather recently discovered in lignins; their $^{13}C–^{1}H$ correlation assignments are based on previous work [48, 49].

Kenaf bast fiber lignin is a guaiacyl-syringyl lignin that is particularly syringyl-rich (see below). The lignin is seen as being rich in β-ether (β-O-4) units **A**, with no visible phenylcoumaran **B** or dibenzodioxocin **D** units, and little resinol **C**. Syringyl β-ether units in kenaf bast fiber lignins are heavily γ-acetylated according to previous studies [7, 8, 21, 30, 31, 40]; further model compound studies are required to assign these correlations.

The spectrum from corn stalk cell walls shown in Fig. 1e provides an example of a grass and another example of a guaiacyl-syringyl lignin. The lignin content of this sample is considerably lower than in the other materials. The lignin is only seen as being rich in β-ether (β-O-4) units **A**, with no phenylcoumaran **B**, dibenzodioxocin **D**, or resinol **C** units visible in this spectrum. An NMR spectrum from corn acetylated cell walls is given in Fig. 1f for comparison.

Aromatic Regions, and Integration of Aromatic Contours (to Provide Measures of H:G:S)

Differences in the *p*-hydroxyphenyl:guaiacyl:syringyl (H:G:S) distribution in the lignins are readily visualized from the aromatic regions of the 2D $^{13}C–^{1}H$ correlation spectra. Figure 2 shows the significant variations in the nature of the lignin polymers and other aromatic constituents in the wall. Integration of contours in HSQC/HMQC plots are conveniently carried out using Bruker's Topspin 2.0 software. Here we are able to provide simple syringyl:guaiacyl (S/G) and even *p*-hydroxyphenyl:guaiacyl:syringyl (H/G/S) integral ratios for the cell wall spectra. However, accurate quantification of the aromatic unit ratios requires improved NMR methodologies (to minimize the effects of coupling-constant differences, etc.) and should follow complete assignment of the aromatic rings based on model compound studies and comparison of aromatic regions between the cell wall samples. For now, integrals from the well-dispersed 2- or 2,6-positons of each kind of aromatic types can be used (with guaiacyl integrals being logically doubled since it involves only a single (2-) correlation rather than the two in H and S units).

Pine lignin is basically a guaiacyl (G) lignin (derived from coniferyl alcohol) with traces of *p*-hydroxyphenyl (H) units (derived from *p*-coumaryl alcohol), as seen in the spectra from the isolated lignin (Fig. 2a) or the cell wall (Fig. 2b). The $^{13}C–^{1}H$ correlations from the G aromatic rings are well resolved in DMSO-d_6. The correlation for the 2-position is at δ_C/δ_H ~111.3/6.95 ppm except for the 2-position of oxidized α-ketone structures G′2 (δ_C/δ_H ~110.9/7.38 ppm). Typically, the 5-position is at δ_C/δ_H ~115.0/6.77 ppm, and most of the 6-position correlations are at δ_C/δ_H 118.8/6.76 ppm except for the 6-postion of the oxidized α-ketone structure G′6 (δ_C/δ_H 122.9/7.48 ppm). A minor 2,6-aromatic correlation from *p*-hydroxyphenyl (H) units is visible at δ_C/δ_H 127.8/7.16 ppm, but the 3,5-position correlations overlap those from guaiacyl 5-positions. The measured H/G integral value is 0.008 (0.8%). The α and β-correlations from cinnamyl alcohol endgroups **X1** are also detectable at lower contour levels (not shown) at δ_C/δ_H 128.5/6.42 and 128.5/6.21 ppm.

Pine cell walls, via the gel-state 2D NMR spectrum in DMSO-d_6, also provide remarkably well resolved peaks aromatic correlations, comparable to those in the isolated pine lignin (Fig. 2b). The α- and β-position correlations from cinnamyl alcohol end-groups **X1** can also be detected at the lower contour levels (not shown) in this region, as they can in the extracted lignin spectrum.

In the lignin from kenaf bast cell walls, syringyl correlations predominate over the relatively weak guaiacyl correlations (Fig. 2c). Kenaf's high S/G value (the integral ratio is 11.0 here) has been noted previously [30]. Spectra from the freshly harvested and milled Tainung2 kenaf also appeared to have considerable amounts of H units, Fig. 2c. Kenaf's H/G integral was 0.52, giving a total H:G:S ratio of 4:8:88. H units have been noted previously in Kenaf, although at a lower level [7], and were scarcely detected in earlier studies [20]. We might have assumed that the peaks here corresponded to some other unidentified component except that the twin correlations bear a striking similarity to those obtained from HCT-deficient pine tracheids [44], which have elevated H-levels. Cinnamyl alcohol endgroups **X1** can scarcely be detected even at the lowest contour levels.

Aspen lignin's balanced syringyl-guaiacyl composition is clearly evidenced in the aromatic region in this gel-state 2D NMR spectrum (Fig. 2d). Above the well resolved G aromatic ring correlations, there is a substantial correlation

for the 2,6-positions of syringyl (S) units at δ_C/δ_H 103.9/6.67 ppm. Also, the 2,6-position of oxidized (α-ketone) structures S′ can be found at δ_C/δ_H 106.5/7.22 ppm. A measured S/G integral of 2.4, is higher than the 1.8 determined from the acetylated cell wall sample [22] and the 2.2 by thioacidolysis [18]. Hopefully an eventual examination of the integrability of adiabatic-pulsed NMR variants [16], determination of relative response factors, and other measures will improve the reliability of the integrals. The α- and β-position correlations from cinnamyl alcohol end-groups **X1** can also be discerned. Of particular note is that the **X1** endgroups in aspen are derived solely from coniferyl alcohol, not sinapyl alcohol, just as in pine lignin; the correlations for a coniferyl alcohol end-group are distinctly different from those from sinapyl alcohol in DMSO-d_6 (not shown in this paper, but will be published elsewhere). Another readily evidenced feature of aspen lignin is the *p*-hydroxybenzoates (PB) that acylate lignin side chains [23].

Corn stalks have more interesting correlations in the aromatic region compared to the other samples because of the hydroxycinnamates, PCA and FA (Fig. 2e). As expected, syringyl correlations dominate those from guaiacyl units, with an S/G integral of 3.1. There are also oxidized α-ketone structure of syringyl and, possibly (overlapped with other correlations) guaiacyl units. However, the most interesting features are the clearly revealed intense *p*-coumarate (PCA) and ferulate (FA) peaks. PCA 2,6-correlations are at δ_C/δ_H 130.2/7.47 ppm, and the 3,5-correlations at δ_C/δ_H 115.7/6.76 ppm are overlapped with those from guaiacyl units. The PCA 7 correlation (δ_C/δ_H 144.6/7.44 ppm) and 8-correlation (δ_C/δ_H 113.8/6.26 ppm) are also noted. There is also strong evidence for ferulate in this NMR spectrum. The peak at δ_C/δ_H 123.4/7.13 ppm belongs to 6-position of FA, and the FA 8-correlation at 115.3/6.29 ppm is also partially resolved. FA 2-correlation at δ_C/δ_H 111.1/7.35 ppm overlaps with the G2 α-ketone, G′2. The 5-correlation also overlaps with G5-correlations. The ratio between cinnamates and normal lignin units was 47:53. This value is also consistent with an integration with an acetylated whole maize cell wall sample in $CDCl_3$ (as will be published elsewhere). However, response factors are presumably required here as other evidence suggests that the corn lignin is only about 18% *p*-coumarate by weight [34] and ferulate levels are much lower. At present, without methods to determine actual lignin compositions (and hydroxycinnamate levels) in these gel-state samples, we simply note that the comparative values delineate the well-known distinctive structural differences between each species. As such, 2D NMR fingerprinting, combined with chemometrics methods, should allow the *p*-hydroxycinnamate and *p*-hydroxybenzoate levels to be discriminated, along with the variations in traditional lignin units and polysaccharides. What is striking about this spectrum (Fig. 2e) is that ferulates have normally been very difficult to detect by NMR in grasses due to their being unresolved from *p*-coumarate signals in acetylated samples. The ready determination of ferulates here may render gel-state NMR (of underivatized materials) particularly valuable for grasses.

Conclusion

Although the ball-milled cell wall samples were essentially not dissolved in the NMR solvent (DMSO-d_6), detailed ^{13}C–^{1}H correlation spectra are obtained via solution-state 2D HSQC and HMQC NMR experiments. The quality of the spectra is almost as good as for the previously developed cell wall dissolution method (with acetylation), even though these gel-state NMR spectra likely represent only the amorphous components of the cell wall. At present, we have not determined if the samples represent true gels (as opposed to suspensions). As documented, however, there is little material truly in solution, and solid, unswollen material that has little mobility is essentially invisible to solution-state NMR methods; that detailed spectra are seen here suggests that most of the material is in a swollen but undissolved state sufficient to denote it as a gel at this point.

One of the significant advantages of this method is the simple and rapid sample preparation. The solvent used, DMSO, also allows further solubilization methods to be used sequentially. For example, simply adding NMI-d_7 [47] produces the full wall dissolution along the lines described in the original method [22]. Adding acetic anhydride (into the sample in DMSO/NMI solution), followed by normal workup [22], produces the acetylated cell wall materials that dissolve in $CDCl_3$ for further NMR work. Spectra from underivatized walls have better dispersion of some correlations that allow more substantive assignments. In the case of ferulates, these important cross-linking agents in grass cell walls [36] are readily distinguished for the first time in these spectra.

A major limitation of this method and prior methods is in the process of ball-milling. Fine milling breaks bonds. The weakest bonds (glycosidic linkages in polysaccharides, and β-aryl ethers in lignins) are the ones that are preferentially cleaved. However, even though the major polymer structures remain intact, the ball-milling process reduces the DP (logically creating new endgroups) and causes some oxidation. The ultrasonication to produce the gel could also induce minor structural changes. Another limitation is that the prepared gel-state NMR samples are suitable only for short-range experiments, i.e. HMQC or HSQC. Long-range experiments (e.g. HMBC) are impossible because magnetization is lost by the rapid relaxation in such samples during the significant pulse delays before the actual signal acquisition

begins (or before the necessary echo can evolve). Another minor impediment that should be overcome is the difficulty of handling the gel material. Using gel NMR sample tubes and accessories may offset these difficulties.

Rapidly expanding research areas, such as biofuel and biomass research, and transgenic plant production, need to structurally assay plant cell wall composition and structure in an efficient way. NMR will likely never provide massive scale screening capabilities of other methods, but the unparalleled structural detail from the complex cell wall polymers makes NMR a valuable tool. This gel-state NMR technique might provide an ideal secondary screening method—it allows more rapid processing and analysis of samples than could be previously accomplished. Regardless, the method itself has considerable merit as an independent analytical method. Additionally, there is emerging potential for chemometric analysis of biological samples using the 2D NMR fingerprint. The ability to examine the raw plant cell wall materials without structural modification (beyond that caused by ball-milling) is another noteworthy advantage. Finally, this approach may open up new avenues for the use of 2D NMR to study other polymers in a similar manner.

In conclusion, the rapid and simple NMR sample preparation, without extra processes associated with dissolution and derivatization of the plant cell wall, provides significant improvements in the efficiency with which detailed structural data on the cell wall can be obtained. We suspect that this method will find a role not only in traditional plant cell wall analyses but also in secondary screening methods important for current biomass related research.

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