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COMMUNICATION

^{13}C cell wall enrichment and ionic liquid NMR analysis: progress towards a high-throughput detailed chemical analysis of the whole plant cell wall

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The ability to accurately and rapidly measure plant cell wall composition, relative monolignol content and lignin–hemicellulose inter-unit linkage distributions has become essential to efforts centered on reducing the recalcitrance of biomass by genetic engineering. Growing ^{13}C enriched transgenic plants is a viable route to achieve the high-throughput, detailed chemical analysis of whole plant cell wall before and after pretreatment and microbial or enzymatic utilization by ^{13}C nuclear magnetic resonance (NMR) in a perdeuterated ionic liquid solvent system not requiring component isolation. 1D ^{13}C whole cell wall ionic liquid NMR of natural abundant and ^{13}C enriched corn stover stem samples suggest that a high level of uniform labeling (>97%) can significantly reduce the total NMR experiment times up to ~220 times. Similarly, significant reduction in total NMR experiment time (~39 times) of the ^{13}C enriched corn stover stem samples for 2D ^{13}C–^{1}H heteronuclear single quantum coherence NMR was found.

Introduction

In response to recent and widespread concerns regarding the need to develop more sustainable technologies to meet global energy demands, reducing the world's dependency on conventional natural resources while minimizing environmental impact has led to considerable research devoted to the production of lignocellulosic biofuels.[1] Biomass, a readily available and low-cost material feedstock, is one of the few single-source materials which could possibly replace petroleum in the global energy and material market.[2] Currently, the high processing cost associated with the deconstruction of cellulose to sugar for further downstream fermentation to biofuels has significantly retarded the large-scale commercialization of second generation cellulosic bioethanol.

The structure of the plant cell wall has been cited as a multifaceted micro-structural system composed of a lignin and hemicellulose matrix encapsulating and supporting cellulose fibrils packed into bundles.[3] The complex chemistry and morphology of the plant cell wall as specifically designed *via* millions of years of evolution as an effective protective and structural element make the cell wall difficult and expensive to deconstruct and process.[1,2,4] This inherent ability of the plant cell wall to resist deconstruction is referred to as recalcitrance and has been cited as the major barrier to large-scale commercial biological production of bioethanol, and biofuels in general.[1,2]

There have been a variety of proposed routes to overcome biomass recalcitrance; however we believe that one of the most effective plans involve genetic manipulation of an energy crop which already displays advantageous attributes such as high yields, broad cultivation range, low agronomic input requirements and tolerance to a variety of environmental stressors.[1,4,5] The goal of the genetic manipulation in this field is to lower a feedstock's recalcitrance by targeting specific genotypes and phenotypes linked to overall sugar yields from biological deconstruction.[4] Any solution utilizing genetic manipulation requires not only a deep understanding of the relationships between plant cell wall chemistry and morphology with recalcitrance but also the effect of the genetic manipulation on cell wall properties and plant development.[5]

A recent study by Fu *et al.* showed that down-regulation of the caffeic acid *O*-methyl transferase gene in switchgrass produces a viable plant which displayed increased sugar yields from enzymatic hydrolysis and fermentability to ethanol by both simultaneous saccharification and fermentation with yeast and *Clostridium thermocellum* with no added enzymes.[6] This is not the only example of genetic modification to improve fermentable sugar yields for biofuel production. In a precursor to the above study, Chen and Dixon investigated down-regulating lignin biosynthesis in alfalfa.[7] These types of studies seemingly prove the efficacy of utilizing genetic manipulation to overcome biomass recalcitrance, clearly demonstrating that plant system biologists have the genetic tools to effectively alter the plant cell wall structure. The current knowledge gap in this area of research does not pertain so much to these genetic tools but rather centers on which genes to alter and how those alterations will affect not only recalcitrance but cell wall properties and plant development.[4,5] The methodology to identify recalcitrance related genes such as association studies and quantitative trait locus analysis requires the statistical analysis of phenotypic data from large sample sizes.[1,5,8,9] Moreover, the inherent uncertainty associated with the genetic manipulation of complex biological systems such as plants and the numerous potential modifications and manifestation of those modifications require screening of a large number of mutant plants. As a result, the availability of high throughput analytical techniques

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to characterize the composition, chemical structure and morphology of a biofuel feedstock and how it changes throughout the stages of plant development and downstream biomass processing are critical to the above route utilizing genetic manipulation to overcome biomass recalcitrance.

The use of modern robotics and analytical techniques in biotechnology has rapidly grown the field of laboratory automation or high-throughput (HTP) screening.[8,9] Recent examples of HTP screening of biomass include systems to optimize pretreatment conditions and enzymatic formulations and determine plant cell wall composition. Selig *et al.* reported the use of a novel 96-well multi-plate reactor for the comparative analysis of biomass recalcitrance by micro-scale hydrothermal pretreatment and enzymatic hydrolysis.[8] Similar research has also shown the viability of a high-throughput platform for genetically engineering energy crops, reducing pretreatment severity, and minimizing enzyme loading.[9–11] Mutant screening using any of the above HTP methods can provide critical phenotypic data such as relative level of recalcitrance and xylan, glucan and acid insoluble lignin content. Other analytical techniques, for instance Fourier-transform infrared spectroscopy,[12,13] near infrared spectroscopy,[14] mass spectrometry[15] and monoclonal antibodies microarrays which have binding specificities for cell-wall components,[16,17] have also been employed in the HTP analysis of biomass to provide information on the presence of general chemical functionality. However, these techniques often lack the ability to generate detailed chemical information on a broad distribution of not only chemical functionality but specific monomeric and inter-unit moieties. One of the few methods which have been shown to accomplish this without extensive isolation is nuclear magnetic resonance (NMR) spectroscopy. There are examples of ^{1}H NMR in HTP analysis of sugar distribution in hydrolysate resulting from hydrothermal pretreatment or enzymatic hydrolysis.[18] ^{1}H NMR, much like the other above techniques, acquires its data relatively rapidly,[18] because of this HTP NMR analysis by other nuclei, *e.g.*, ^{13}C, which has a high chemical shift dispersion and can differentiate more chemical structures. However, ^{13}C NMR suffers generally from low natural abundance and long relaxation times, accordingly never being utilized in HTP screening for biofuel research.

Solution state NMR spectroscopy of whole plant cell samples was first reported by Ralph *et al.*,[19,20] and more recently we have reported similar methodology which relies on a novel bi-solvent system consisting of perdeuterated pyridinium molten salt and DMSO-d_6.[21–23] This solvent system facilitates the swelling or partial dissolution of both ball-milled and Wiley-milled biomass for direct ^{1}H, ^{13}C and 2D ^{1}H–^{13}C correlation NMR analysis, not requiring cell wall component isolation. In this study, we utilize this novel bi-solvent system designed for whole plant cell wall solution NMR on ^{13}C enriched and natural abundant corn stover stems to demonstrate the usefulness of ^{13}C labeling in biofuel research specifically for HTP screening by the detailed chemical analysis of the plant cell wall but also including *in situ* destruction studies and metabolite analysis. The methodology and data analysis proposed here are similar to work by Kikuchi *et al.* utilizing stable isotope labeling of plants to perform multidimensional NMR-based metabolomics; however, this manuscript clearly demonstrates a route for the application of the ionic liquid NMR methodology to the HTP analysis of plant cell wall chemistry.[24–31] We choose corn stover stems to demonstrate the efficacy of this approach primarily because its ^{13}C enriched version is readily commercially available.

Experimental

Feedstock

Ground (0.147–0.837 mm), >97 atom%, ^{13}C enriched corn stover (*Zea mays*) stems were purchased from Iso*Life* (Wageningen, The Netherlands). Extractives were subsequently removed from ground samples by placing the biomass into an extraction thimble in a Soxhlet extraction apparatus. The extraction flask was filled with 1 : 2 ethanol-benzene mixture (150 mL) and then refluxed at a boiling rate which cycled the biomass for at least 24 extractions over a 4 h period. The preparation and analysis of samples for carbohydrate constituents and acid-insoluble residue (Klason lignin) analysis used a two-stage acid hydrolysis protocol based on TAPPI methods T-222 om-88 with a slight modification[32] following the exact experimental conditions in cited literature.[33]

NMR analysis

1D ^{13}C and 2D ^{13}C–^{1}H heteronuclear single quantum coherence (HSQC) experiments were carried out at 50 °C in a Bruker Avance-500 spectrometer equipped with an *xyz*-gradient triple resonance indirect detection probe. The spectral widths were 11.0 and 180.0 ppm for the ^{1}H and ^{13}C dimensions, respectively. The 1D ^{13}C analysis was performed using a standard inverse gated Bruker pulse sequence with a 5 μs (90°) carbon pulse, 10 s recycle delay and 32 or 8 K scans. The HSQC analysis was performed using a standard gradient enhanced Bruker pulse sequence with a 5 μs (90°) proton and carbon pulse, a 0.11 s acquisition time, a 1.5 s recycle delay, a $^{1}J_{C-H}$ of 145 Hz, acquisition of 256 complex data points and 4 or 256 scans. NMR samples were prepared as follows: 10–60 mg milled dry sample was added to 0.60 g perdeuterated pyridium chloride–DMSO-d_6 (1 : 4, w/w) solution and stirred at 60 °C for 1 h after dry N_2 purge. The resulting solution was transferred directly into a 5 mm NMR tube and backfilled with a dry N_2 purge.

The solid-state NMR samples were prepared from isolated cellulose added into 4 mm cylindrical ceramic MAS rotors. Solid-state NMR measurements were performed on a Bruker Avance-400 spectrometer for ^{13}C in a Bruker double-resonance MAS probe head at spinning speeds of 10 kHz. CP/MAS experiments utilized a 5 μs (90°) proton pulse, 1.5 ms contact pulse, 4 s recycle delay, and 16 or 4 K scans.

Results and discussion

1D ^{13}C whole cell wall ionic liquid NMR could provide quantitative information about cellulose, lignin, and hemicellulose contents, methoxy content, acetyl content, the ratio of major monolignols such as syringyl (S) and guaiacyl (G), to *p*-hydroxyphenyl-type units and verify the presence of various carbonyl, carboxyl, ether, aliphatic and aryl functionalities. This NMR method on natural abundant material utilizes ~60 mg of material in 600 mg of the bi-solvent system, requiring no component isolation or extraction.[21,23,34] Our group found in a study examining the application of select ionic liquids as aprotic green solvents for lignin that up to 20 wt% solutions could be generated in 1-hexyl-3-methylimidazolium trifluoromethane sulfonate, 1,3-dimethylimidazolium methyl sulfate and 1-butyl-3-methylimidazolium methyl sulfate.[22] 1D ^{13}C NMR analysis of lignin and model compounds in these ionic liquids showed that ^{13}C chemical shifts were only slightly shifted up-field with respect to lignin in

dimethyl sulfoxide (DMSO) as the solvent.[22] However, obscuring signals from the ionic liquids–DMSO, and long acquisition times yielding low signal to noise (*S*/*N*) made chemical analysis by this 1D method difficult. There are also citations pertaining to the dissolution of polysaccharides in ionic liquid solvent systems to analyze their structure by 1D ^{13}C NMR.[35]

The use of ^{13}C enriched substrates in ^{13}C NMR analysis, as shown in Fig. 1, shows a clear advantage in sensitivity over natural abundant substrates, thus resulting in significantly reduced acquisition times on a sample of ~10 mg of material in 600 mg of the bi-solvent system. Unlike synthetic polymer systems, in which isotopic labeling is rarely done because of the extreme cost and effort associated, plants are relatively easy and inexpensive to enrich with ^{13}C nuclei *via* growth in an environment doped with ^{13}C enriched CO_2 and cell wall incorporation by photosynthesis. The measured ^{13}C spectral *S*/*N* ratio for natural abundant corn stover (Fig. 1) is about one-fourth that of the commercially available ^{13}C enriched corn stover, yet requiring 216 times longer data acquisition period. Even at long acquisition time the spectrum of the natural abundant corn stover lacks the detail to analyze any significant cell wall chemistry and is dominated by solvent peaks attributed to perdeuterated pyridium chloride and DMSO. The solvent peaks in spectrum of the ^{13}C enriched corn stover on the other hand account for a much smaller proportion of the background. Even more importantly the reduced data acquisition period allows for a long recycle delay between scans ensuring the quantitative nature of the spectra which in natural abundant systems may have required an excitation pulse below 90° and/or chemical relaxation agents. Likewise to demonstrate the broad usefulness of ^{13}C enrichment ^{13}C solid-state cross-polarization magic angle spinning (CP/MAS) spectrum is as shown in the bottom of Fig. 1, taking ~10 times longer to acquire a spectrum of similar *S*/*N* on natural abundant corn stover.

The spectra of ^{13}C enriched corn stover stem samples are presented in Fig. 1 and peak assignments based on literature values summarized in Table 1. Spectral regions of particular interest for HTP analysis include: methoxy content (~δ58 to 55 ppm), acetyl content (~δ21 ppm), aliphatic carbonyl can be seen at δ173–168, peaks at δ168–166 are assigned to conjugated carbonyl structures such as *p*-coumarates and felurates along with the ratio of the integrations of C_1 carbon related carbohydrates (~δ103 to 90 ppm) to the aromatic region (δ162–103) can be indicative of the lignin to carbohydrate contents.[36–39] Assignments which confirm the presence and relative ratio of a monolignol unit such as *p*-hydroxycinnamic acid (*P*CA), guaiacyl (G) and syringyl (S) units can be identified by the peaks in the regions δ162–160 (PCA_4), 112–110 (G_2) and 108–103 ($S_{2/6}$) ppm respectively.[36–38] This analysis indicated a 2.1/1.0/0.9 PCA/G/S ratio in the ^{13}C enriched corn stover stem samples.[36–38] Similarly, methoxy, acetyl, and lignin contents along with S/G ratio can also be approximated by the ^{13}C solid-state CP/MAS spectrum also seen in Fig. 1 by following published analysis procedures.[40,41]

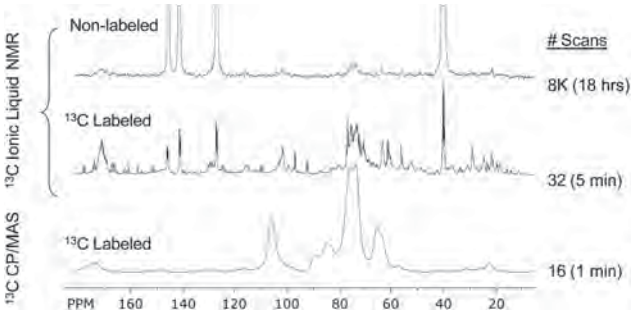

Fig. 1 Stacked plot of ^{13}C spectra of ^{13}C enriched and control natural abundant corn stover stem samples in a deuterated ionic liquid–DMSO mixture. The bottom spectrum displays a ^{13}C solid-state CP/MAS spectrum of ^{13}C enriched corn stover stem samples. Peaks at δ127.5, 146.4 and 141.4 ppm are from pyridinium chloride and δ39.5 ppm belongs to DMSO.

2D ^{13}C–^{1}H heteronuclear single quantum coherence (HSQC) whole cell wall ionic liquid NMR can also semi-quantitatively probe a number of lignin and hemicellulose structures and inter-unit linkages. The major advantage in using HSQC NMR is the additional nuclear dimension, which helps further resolve the highly overlapping spectral features commonly associated with the complex plant cell wall. The ^{13}C–^{1}H HSQC NMR spectral profiles of natural abundant and ^{13}C enriched corn stover are shown in Fig. 2 and 3. Dominant ^{13}C–^{1}H lignin resonances due to methoxyl and acetyl groups were observed at δ_C/δ_H 55.7/3.8 and 20.5/1.9 ppm. The region from approximately δ_C/δ_H 85.0–55.0/5.0–3.0 ppm is due to various polysaccharide and lignin aliphatic α-, β- and γ-carbons typically associated with lignin inter-unit linkages. A prominent resonance and spectral signature of high interest when analyzing lignin structure for biofuel production is related to β-aryl ether linkages (A); however significant traces of phenyl coumaran (B) and resinol (C) can also be detected in a variety of biomass samples *via* C_α-H correlations at δ_C/δ_H 72.0/4.8 (A_α), 87.5/5.3 (B_α) and 85.0/4.6 (C_α) ppm respectively.[20] For example, this methodology was utilized on switchgrass pretreated by steam, acid and lime procedures, indicating that degradation of lignin was observed in all pretreatments. The technique was however able to distinguish the type and extent of the degradation depending on the pretreatment method.[23] Other major polysaccharide related correlations appear for C_2-acetylated (2-O-Ac-β-D-Xylp) and C_3-acetylated xylan (3-O-Ac-β-D-Xylp) at δ_C/δ_H 74.0/4.5 (C_2/H_2) and 75.0/4.8 (C_3/H_3) ppm, respectively (observed while processing at higher magnification).[20] Tracking these types of spectral signature could be critical in future biofuel research, because the function, biosynthesis and reduction/removal of cell wall hemicellulose and lignin seem invariably linked.

In the polysaccharide anomeric region, from approximately δ_C/δ_H 90.0–105.0/4.4–5.5 ppm, fairly well resolved resonances are observed. The partial characterization of glucan (cellulose) and xylan (hemicellulose) can be accomplished by observation of cross peaks for internal anomeric signals of the (1-4) linked β-D-glucopyranoside (β-D-glucop^{I}) centered at δ_C/δ_H 102.4/4.4 ppm and (1-4) linked β-D-xylopyranoside (β-D-xylp^{I}) at 102.5/4.5 ppm.[20]

The final HSQC region, seen from approximately δ_C/δ_H 140.0–100.0/7.5–6.3 ppm, is attributed to aromatic lignin units, where the presence of major syringyl (S), guaiacyl (G), benzyl oxidized α-ketone and *p*-hydroxyphenyl-type unit structures (such as ferulates, *p*-coumarates and *p*-hydroxycinnamic acids) can be confirmed by the existence of overlapping contours.[19,20,23] In particular S and G units are identified by resonances for $S_{2,6}$, G_2, and G_6 at δ_C/δ_H 103.3/6.6 ($S_{2,6}$), 111.0/7.0 (G_2), and 119.5/6.9 (G_6) respectively.[20] A variety of other structures and their corresponding chemical shift information can be found in these series of literature references by Ralph *et al.*, many of which are detailed in Table 1.[19,20,42–44] These studies use a similar cell wall swelling technique with a mixture of perdeuterated DMSO and pyridine, identifying these above assignments related to

Table 1 ^{13}C NMR chemical shift assignments for biomass dissolved in a deuterated ionic liquid–DMSO mixture (ref. 36–39)[a]

Chemical shifts (ppm)	Assignments
173–168	Aliphatic –*C*OOR
168–166	Conjugated –*C*OOR
162–160	pCA_4
155–142	$S_{3,5}$ and $G_{3,4}$
142–122	$S_{4,1}$ and G_1
120–117	G_6
117–113	G_5
112–110	G_2
108–103	$S_{2,6}$
105–90	C_1 carbohydrates
89–70	C_4 carbohydrates and lignin side chains: C_β in β-O-4, C_α
83–70	$C_{2,3,4}$ xylan, $C_{2,3,5}$ glucan and lignin side chains: C_α
68–59	C_5 xylan, C_6 glucan and lignin side chains: C_γ
58–55	Methoxy (OMe)
21	Acetyl (OAc)

[a] *p*CA: *para*-coumaric acid; G: guaiacyl; S: syringyl.

various polysaccharide and lignin structures using model compounds and a variety of 2D NMR techniques on isolated cell wall components.

The spectra of natural abundant and ^{13}C enriched corn stover in Fig. 2 and 3 have a slightly different appearance, with a different modulation of peak intensities observed across the entire 2D spectrum. This is to be expected as the natural abundant and ^{13}C enriched corn stover samples are not clones of one another, though showing similar compositional results (Table 2). Ultimately, HTP screening studies can evaluate qualitatively the presence or absence of a variety

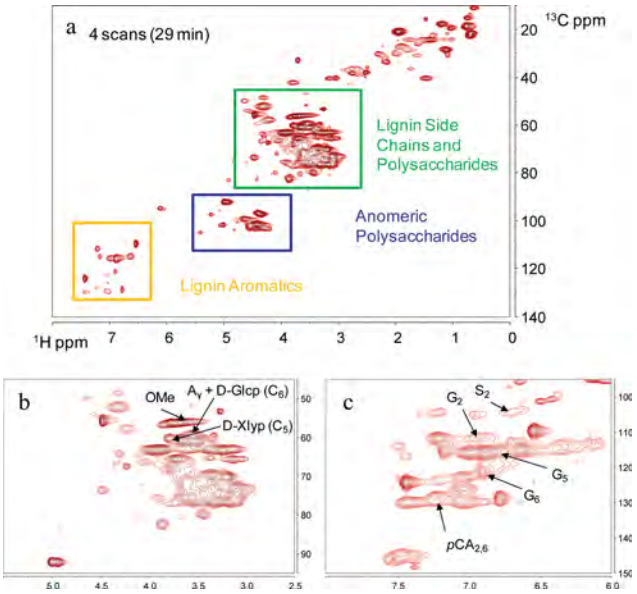

Fig. 2 Whole plant cell wall 2D ^{13}C–^{1}H heteronuclear single quantum correlation (HSQC) NMR spectra of ^{13}C enriched corn stover stem samples in a deuterated ionic liquid–DMSO mixture. (a) Full spectrum; (b) lignin side chain and polysaccharide region; (c) lignin aromatic region. A: β-aryl ether linkage; β-D-glucp: glucopyranoside units; β-D-Xylop: xylopyranoside units; S: syringyl; and G: guaiacyl; cross-peaks at δ127.5/8.14, 146.4/8.69, and 141.4/9.05 are from pyridinium chloride.

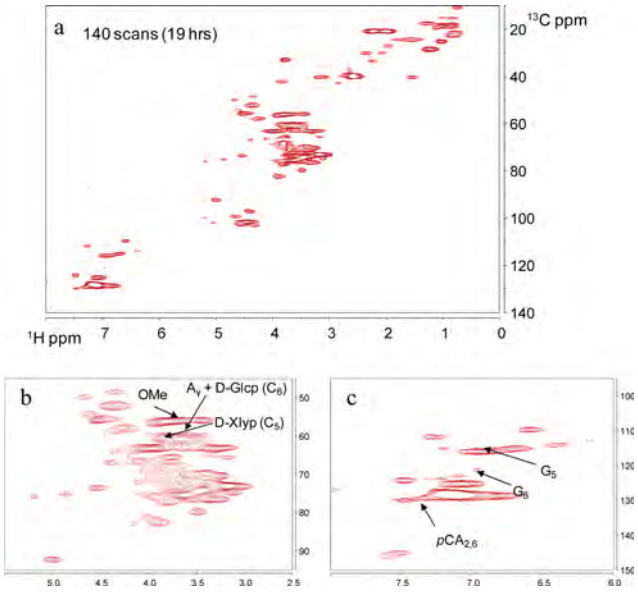

Fig. 3 Whole plant cell wall 2D ^{13}C–^{1}H heteronuclear single quantum correlation (HSQC) NMR spectra of a control natural abundant corn stover stem sample in a deuterated ionic liquid–DMSO mixture. (a) Full spectrum; (b) lignin side chain and polysaccharide region; (c) lignin aromatic region. Cross-peaks at δ127.5/8.14, 146.4/8.69, and 141.4/9.05 are from pyridinium chloride.

of resonances, quantitatively or semi-quantitatively the normalized integration of a variety of resonances[23] or through multivariate statistical or principal component analysis, a mathematical procedure converting a set of observations of possibly correlated variables into a set of linearly uncorrelated data, similar to a chemometric analysis tool proposed by Hedenstrom and Ralph (Table 3).[45,46]

A critical component of this proposed ^{13}C NMR based HTP screening is the efficient, stable and uniform isotopic labelling of biomass. A number of publications in the literature clearly demonstrate the ease with which ^{13}C enriched biomass can be generated *via* incorporation by photosynthesis in a ^{13}C enriched CO_2 environment.[24–29,31,47–53] As evident in the ^{13}C enriched corn stover stems used in this study as a proof of concept, which were >97 atom% ^{13}C enriched (verified by *via* combustion analysis and mass spectrometry of evolved CO_2 by the vendor), high levels of ^{13}C enrichment are possible. This high level of enrichment not only improves the resulting NMR *S*/*N* but also ensures uniform labelling across the variety of chemical moieties found in the plant cell wall. Though true, in any future HTP work, an important step must be verifying ^{13}C enrichment level.

The economics of next generation biorefineries and biofuel, biomaterial production is extremely dependent on the overall yields and energy/material cost associated with biomass deconstruction. Therefore, it is important to gain a detailed understanding of the physico-chemical structure of the cell wall, mechanisms of efficiently

Table 2 Compositional analysis of the ^{13}C enriched corn stover

	%Glucan	%Xylan	%Klason lignin
Control natural abundant corn stover	30	23	26
^{13}C enriched corn stover	38	25	20

Table 3 ^{13}C–^{1}H HSQC NMR chemical shift assignment for biomass dissolved in a deuterated ionic liquid–DMSO mixture[20,23]

Chemical shifts δ_C/δ_H (ppm)	Assignments
20.5/1.9	Acetyl (OAc)
75.0/4.5	C_2/H_2 in 2-OAc-β-D-xylp
75.0/4.8	C_3/H_3 in 3-OAc-β-D-xylp
97.5/5.2	4-O-MeGlcA
96.5/4.5	(1-4)-β-D-Xylop (reducing end)
102.5/4.3	(1-4)-β-D-Glcp (C_1H_1)
102.5/4.5	(1-4)-β-D-Xylp (C_1H_1)
92.0/5.0	(1-4)-α-D-Xylp (reducing end)
54.0/3.8	Methoxy (OMe)
60.1/3.7	C_γ in β-aryl ether (A_γ)
86.0/4.1	C_β in β-aryl ether (A_β)
72.0/4.8	C_α in β-aryl ether (A_α)
87.5/5.3	Phenylcoumaran (B_α)
85.0/4.6	Resinol (C_α)
103.3/6.7	$C_{2,6}/H_{2,6}$ in etherified syringyl units (S)
111.0/7.0	C_2/H_2 in guaiacyl units (G)
115.0/6.8	C_5/H_5 in guaiacyl units (G)
119.5/6.9	C_6/H_6 in guaiacyl units (G)
130.0/7.3	$C_{2,6}/H_{2,6}$ in *p*-coumarate (*p*CA)
123.5/7.2	C_6/H_6 in ferulate units (FA)

overcoming biomass recalcitrance and methods of biomass deconstruction/conversion. A major component of our recent research program has been the development of a NMR toolkit utilizing partial or uniform ^{13}C isotopic enrichment. The elegance of this system resides in the fact any analysis of conversion processes or biomass-derived material will also benefit from this labeling. In the absence of conducting a true HTP screening study, we believe that this study clearly lays out the framework by which the HTP screening of detailed chemical structures in the plant cell wall by 1D and 2D ^{13}C solution NMR can be achieved and shows qualitatively that no gross changes occurred in cell wall chemistry due to ^{13}C labeling. This proposed methodology will rely on: (1) an ionic solvent system for the swelling or partial dissolution of intact cell wall materials for direct ^{1}H, ^{13}C and 2D ^{1}H–^{13}C correlation NMR analysis, not requiring time-consuming cell wall component isolations, which may alter the cell wall chemistry and (2) ^{13}C enrichment which is achieved relatively easily and inexpensively *via* growth in an environment doped with ^{13}C enriched CO_2 and cell wall incorporation by photosynthesis.

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