

RESEARCH ARTICLE

Finely Pulverized Low-Moisture (FPL) Gel-State NMR for Near-Completely Solubilized Whole Plant Cell Walls

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ABSTRACT

High-resolution NMR analysis of heterogeneous, insoluble lignocellulosic materials is a significant challenge because poor magnetic field homogeneity and restricted molecular mobility often hinder the acquisition of reproducible solution-state NMR spectra. In studies of lignocellulosic biomass and plant cell walls, gel-state NMR methods have enabled important advances. However, practical limitations in sample preparation, shimming stability, and spectral broadening have constrained method robustness and routine use. Here, we present a finely pulverized low-moisture (FPL) gel-state NMR method that enables the reliable acquisition of high-quality two-dimensional (2D) HSQC spectra from intact whole plant cell walls without chemical pretreatment. Systematic evaluation of drying protocols, ball-milling efficiency, and solvent composition shows that moisture control and particle fineness are the primary factors influencing gel homogeneity and magnetic field shimming performance. Use of a DMSO-*d*₆/DMF-*d*₇ (4:1, v/v) solvent system produces gels with reproducible chemical shifts, narrow linewidths, and well-resolved heteronuclear correlations. This stability allows standard automated shimming and acquisition protocols to be applied reliably. The method is validated across diverse types of biomass, establishing a robust NMR sample-preparation framework for whole plant cell wall analysis and extending the practical reach of solution-state NMR methodologies.

1 | Introduction

A detailed understanding of the composition and structure of lignocellulosic biomass is essential for elucidating the molecular architecture of plant cell walls (CWs) and the chemical features governing polymer interactions. Plant CWs consist of intricately interwoven polymers, cellulose, hemicelluloses, and lignin, forming a chemically complex and physically recalcitrant matrix. Traditional analytical approaches, including degradative chemical methods such as thioacidolysis, nitrobenzene oxidation, and derivatization followed by reductive cleavage (DFRC) method, as well as spectroscopic techniques such as MS, UV, IR, and solid-state NMR, provide only partial structural information and often require extensive sample processing that can alter the native CW components

[1, 2]. Although fractionated lignin preparations from ball-milled CWs, such as milled wood lignin (MWL) and cellulosytic enzyme lignin (CEL), have historically enabled detailed solution-state NMR characterization [3–5], these preparations are time-consuming and yield only a partial representation of the whole CW composition.

To address these limitations, a gel-state NMR method was developed, in which ball-milled biomass is swollen directly in DMSO-*d*₆ to form a gel suitable for solution-state NMR analysis, especially 2D ¹H–¹³C HSQC. This minimally disruptive approach provided a highly informative structural fingerprint of whole CWs without derivatization or fractionation, preserving native lignin and polysaccharide features [6]. Subsequent refinements introduced a DMSO-*d*₆/pyridine-*d*₅

(4:1, v/v) solvent system that improved sample mobility, simplified preparation, and enhanced spectral resolution [7, 8]. The method was developed for the comprehensive analysis of whole plant CWs, representing a major advance in lignin chemistry and biomass research due to its analytical efficiency. Since its introduction, it has been broadly used to uncover unprecedented structural insights into lignin, including aromatic units and interunit linkages, as well as polysaccharides, all directly from unfractionated CWs [9–14]. Alternative solvent systems, such as DMSO-NMI (*N*-methylimidazole), have been explored for the dissolution and structural characterization of lignocellulosic biomass, including chemical modification strategies and full-dissolution approaches for whole CWs [15–19]. However, many of these methods require additional derivatization steps, involve toxic reagents, or introduce spectral interferences. For example, even though the use of DMSO- d_6 /NMI- d_6 (4:1 v/v) allowed improved ^1H - ^{13}C contour dispersion of the polysaccharide anomeric region, some residual ^1H signals from NMI- d_6 solvent obscured the lignin aromatic region [16]. Ionic liquids and organic electrolyte solvents capable of dissolving cellulose and lignin have also been investigated; although these systems can achieve substantial biomass dissolution, their high viscosity, strong solvent–solute interactions, and potential for chemical modification of biomass structures often complicate direct structural analysis by solution-state NMR [17, 20, 21].

In contrast, gel-state NMR operates on a fundamentally different paradigm based on controlled swelling rather than complete dissolution. This method allows for the structural characterization of minimally modified plant CWs while preserving the native structural features of lignin and associated polysaccharides. Critically, gel-state NMR is governed by the exceptional swelling capacity of DMSO, not true polymer solubilization [6, 22, 23]. Conflation of swelling with dissolution obscures important chemical and methodological differences.

Although successfully utilized, the original gel-state NMR methods [6, 7] still faced limitations in data quality and reproducibility. These issues often arose from the solvent system, variability among biomass species, incomplete ball milling, and persistent difficulties in achieving reliable shimming during NMR acquisition. For example, the DMSO- d_6 /pyridine- d_5 (4:1, v/v) system produces noticeable chemical-shift offsets relative to the standard DMSO- d_6 solvent [7], which complicates direct comparison with model compound data acquired in DMSO- d_6 . Inadequate ball milling also reduced gel homogeneity and resulted in broadened spectral signals. Shimming remained a critical factor, influenced by biomass type, particle size distribution, solvent viscosity, and probe configuration. Across solvent systems, partially solubilized gels can lead to poor spectral homogeneity that reduces spectral resolution. These limitations underscore the need for a more robust and broadly applicable gel-state NMR approach. Addressing the limitations requires a sample-preparation strategy capable of delivering consistent gel homogeneity and reliable magnetic field shimming across diverse lignocellulosic materials.

2 | Experimental

2.1 | General and Plant Materials

Aspen (*Populus tremuloides*, an angiosperm) and loblolly pine (*Pinus taeda*, a gymnosperm) wood samples were obtained from archived materials maintained at the USDA Forest Products Laboratory (FPL). Corn stover (*Zea mays* L., Poaceae) was harvested in 2023 at the farm of the US Dairy Forage Research Center (DFRC), Prairie du Sac, Wisconsin, USA. Whole CW gel-state NMR samples were prepared following the established DMSO-based protocols with modifications as described below [6, 7].

All biomass samples were air-dried and then ground using a Wiley mill and sieved through a 40-mesh screen. Samples were then sonicated (30 min $\times$ 3) in distilled water and 80% ethanol sequentially to remove extractives, followed by centrifugation (8000 rpm, 30 min) after each solvent cycle. The extractives-free biomass was initially air-dried at room temperature, and oven-dried and freeze-dried samples were additionally prepared prior to ball-milling.

For oven-drying, biomass samples were dried at 50°C. The dried materials were immediately transferred to a desiccator over anhydrous calcium sulfate (Drierite) and stored until ball-milling to minimize moisture uptake from ambient humidity. The milling vessels are designed to remain tightly sealed throughout the milling process to limit exposure to atmospheric moisture. After milling, the finely ground samples were stored in sealed vials prior to NMR sample preparation. Plant CW materials readily absorb moisture and cannot be completely free of tightly bound water. Therefore, the drying procedure was intended to minimize moisture content prior to ball-milling rather than achieve complete dehydration.

The dried CWs were finely milled using a Fritsch planetary micro mill PULVERISETTE 7 premium line (Germany) at 600 rpm with zirconium dioxide (ZrO_2) vessels (20 mL) containing ZrO_2 grinding balls (10 mm $\times$ 10). Moisture content of the CW samples was measured using a Veritas i-Thermo G64AM graphical moisture analyzer (BEL Engineering s.r.l., Italy).

1. Aspen

Extractive-free Wiley-milled CW (300 mg) was ball-milled for 5 h and 37 min (grinding time: 15 min $\times$ 15, interval break: 8 min $\times$ 14).

2. Pine

Extractive-free Wiley-milled CW (300 mg) was ball-milled for 7 h and 20 min (grinding time: 20 min $\times$ 15, interval break: 10 min $\times$ 14).

3. Corn stover

Extractive-free Wiley-milled CW (300 mg) was ball-milled for 3 h and 40 min (grinding time: 10 min $\times$ 15, interval break: 5 min $\times$ 14).

2.2 | Lignin Isolation

The enzyme lignins (ELs) were prepared from extractive-free CWs as detailed in previous publications [12, 14]. Concisely, the extractive-free ball-milled CWs were treated with cellulases (Cellulysin, EC 3.2.1.4, activity > 10,000 units/g, EMD Millipore Corp.) from *Trichoderma viride*. About 300 mg of CWs were suspended in NaOAc buffer (pH 5), and 9 mg of Cellulysin was added. The reaction mixture was shaken on a rotary incubator shaker at 35°C for 48 h. The residue was collected by centrifugation (8000 rpm, 30 min), and the enzyme digestion process was repeated three times. The collected residue was sonicated and washed with deionized (DI) water (20 mL) three times and lyophilized. EL yields were measured and used as an indirect indicator of cell-wall accessibility and degree of polymer disruption achieved under different drying and milling conditions (Table S1).

2.3 | 2D HSQC NMR

NMR samples were prepared by dissolving of ELs or gelation of ball-milled whole CWs in 0.6-mL NMR solutions. About 40 mg of samples was directly prepared into 5-mm NMR tubes. Spectra were acquired using a Bruker (Billerica, MA, USA) 500-MHz AVANCE III NMR spectrometer equipped with a Prodigy N₂-cooled 5-mm gradient TCI cryoprobe (¹H/¹³C/¹⁵N, inverse configuration). All NMR experiments were performed using Bruker standard pulse programs; an adiabatic ¹H–¹³C two-dimensional heteronuclear single-quantum coherence (2D HSQC) experiment (hsqcetgpsisp2.2; phase-sensitive gradient-edited 2D HSQC using adiabatic pulse sequences for inversion and refocusing) at 25°C [24]. The central dimethyl sulfoxide (DMSO) solvent peak (δ_C 39.5, δ_H 2.49 ppm) was used as the internal reference.

The earlier version of gel-NMR experiments was performed in DMSO-*d*₆:pyridine-*d*₅ (4:1, v/v) or DMSO-*d*₆ for the air-dried whole CW samples as previously described [6, 7, 12]. Isotopically enriched pyridine-*d*₅ (“100”; min. 99.94 atom% D) was needed to avoid interference between the residual solvent peaks and aromatic correlations. The 2D HSQC experiments were acquired from 11.0 to –0.5 ppm in F2 (¹H) with 1148 data points (acquisition time 100 ms), 215 to –5 ppm in F1 (¹³C) with 512 increments (F1 acquisition time 9.25 ms) of 111 scans (NS) with a 500-ms interscan delay (D1). The *d*₂₄ delay was set to 0.89 ms (1/8J, J = 145 Hz). The total acquisition time was approximately 10 h. The shimming process was skipped.

The new finely pulverized low-moisture (FPL) gel-state NMR experiments using DMSO-*d*₆/DMF-*d*₇ (dimethyl sulfoxide-*d*₆/N,N-dimethylformamide-*d*₇, 4:1, v/v) were applied to oven- and freeze-dried whole CW and EL samples. Standard Bruker 2D HSQC experiments were acquired in the range of 11.0 to –0.5 ppm (11.5 ppm spectral width) in F2 (¹H) with 2298 data points (acquisition time, 200 ms) and 215 to –5 ppm (220 ppm spectral width) in F1 (¹³C) with 512 increments (F1 acquisition time, 9.25 ms) of 56 scans (NS) with a 1-s interscan delay (D1). The *d*₂₄ delay was 0.89 ms (1/8J, J = 145 Hz). The total acquisition time for each sample was about 10 h. EL data could also be acquired in 5 h 30 min (NS = 32). Data processing employed

typical matched Gaussian apodization (GB = 0.001, LB = –0.1) in F2 and a squared cosine-bell function (SSB = 2) with one level of linear prediction (LPfc, 40 coefficients) in F1. The gradient shimming process was performed via TopShim as normal.

Volume integration of HSQC peaks was performed using Bruker TopSpin 4.5.0 software (Mac OS version). No correction factors were applied; therefore, the reported data represent raw integral volumes. For semiquantification of aromatic H/G/S signals, the **H**_{2/6} and **S**_{2/6} correlations were integrated, and the **G**₂ integrals were doubled to normalize on the same aromatic atom basis (**S**_{2/6} + **S'**_{2/6} + **H**_{2/6} + 2**G**₂ = 100%). For the aliphatic interunit linkage analysis, well-resolved α-C/H peaks were measured, and relative percentages were calculated on a 2**A**_α + 2**B**_α + **C**_α = 100% basis (**A**, β-O-4 ether; **B**, β-5 phenylcoumaran; **C**, β-β resinol) (Table 1). The 2D HSQC NMR enables identification of specific carbon-hydrogen connectivities that cannot be identified using either one-dimensional ¹³C or ¹H spectra. It should be noted that HSQC spectra are inherently semiquantitative because peak intensities are influenced by several factors including ¹H *T*₁ (spin–lattice) relaxation effects, ¹H and ¹³C *T*₂ (spin–spin) relaxation effects, variations in heteronuclear coupling constants (¹J_{CH}), and homonuclear proton coupling effects. Because these factors can vary among different functional groups and chemical environments, the integrated HSQC peak volumes reported in this study represent relative abundance estimates rather than absolute quantitative measurements [2, 8, 25]. To ensure reliable comparison among samples, identical acquisition parameters and processing procedures were applied throughout the study. Moreover, the use of adiabatic pulse sequences in the HSQC spectra, as performed in this study, has the advantage of *J*-independence and offset insensitivity over an essentially unlimited active bandwidth, improving the reliability of relative quantification [24, 26]. Although such determinations by 2D HSQC are not fully quantitative, the results can provide reliable information about relative abundances of various functionalities based on per 100 aromatic or aliphatic units. Highly mobile pendent groups tend to be overrepresented. As such, the aromatic data are presented on an **S** + **S'** + **G** + **H** = 100% basis as *p*CA, *p*HBA, and FA units are always considered to be terminal groups and are also considered as overestimated by NMR. Therefore, they were excluded from the total lignin sum and are reported separately as percentages relative to that total.

2.4 | NMR Assignments

All lignin and polysaccharide peak assignments were based on values reported in previous literatures and verified plant CW model compounds [6, 7, 11, 12, 27]. Although no new peak assignments are anticipated in this work, efforts to expand and refine the assignments are currently underway.

2.5 | Gel-Permeation Chromatography (GPC)

For the GPC analysis, aspen ELs were previously acetylated and dissolved in tetrahydrofuran (THF). GPC was performed on a Shimadzu Prominence-i LC-2030 3D GPC system (Shimadzu, Kyoto, Japan) equipped with a photodiode array (PDA) detector, using the following conditions: column, PLgel 5 μm MIXED-D,

TABLE 1 | Semiquantitative analysis of lignin subunits and interunit linkages in aspen, pine, and corn biomass samples, based on 2D HSQC spectra of whole cell walls (CWs) and isolated enzymatic lignins (ELs).

Biomass species	Sample type	NMR solvent	Drying method	Ball-milling time	Lignin aromatic subunits (%)							Linkages (%)		
					S	G	H	pHBA	pCA	FA	Tricin	A	B	C
Aspen	EL	D	Air	10 + 5	64.70	35.21	0.09	5.12	—	—	—	85.19	5.85	8.96
	EL	D	Air	15 + 8	65.63	34.23	0.14	4.80	—	—	—	85.56	5.63	8.81
	CW	DD	Oven 16h	15 + 8	67.65	32.29	0.06	3.92	—	—	—	87.22	4.37	8.41
	EL	D	Oven 16h	15 + 8	65.59	34.26	0.15	4.91	—	—	—	83.85	6.15	10.00
	EL	D	Oven 48h	20 + 10	64.64	35.24	0.12	4.93	—	—	—	83.68	6.23	10.09
Pine	CW	DD	Freeze	15 + 8	68.29	31.82	0	3.96	—	—	—	87.69	4.53	7.78
	CW	DD	Oven 48h	20 + 10	—	99.34	0.66	—	—	—	—	72.19	21.83	5.98
	EL	D	Oven 48h	20 + 10	—	99.48	0.52	—	—	—	—	68.79	24.08	7.12
Corn	CW	DD	Oven 16h	10 + 5	62.51	33.41	4.08	—	64.34	27.35	9.30	90.60	9.26	0.14
	EL	D	Oven 16h	15 + 8	61.61	36.30	2.09	—	52.09	5.32	7.16	90.91	7.58	1.51

Note: Ball-milling time expressed as grinding + break (e.g., 10 + 5 = 10 min grinding followed by 5 min break); total grinding time was adjusted according to the sample amount. For semiquantification of aromatics, $H/G/S$ signals were estimated as the total $(S_{2/6} + S'_{2/6} + H_{2/6} + 2G_2 = 100\%)$. pCA , $pHBA$, FA , and $tricin$ units were estimated based on the total $H/G/S$ aromatics. The aliphatic interunit linkages were calculated on a $2A_\alpha + 2B_\alpha + C_\alpha = 100\%$ basis (A , β -O-4 ether; B , β -5 phenylcoumaran; C , β - β resinol). Abbreviations: A , β -O-4; Air, air-dried; B , β -5; C , β - β . D, DMSO- d_6 ; DD, DMSO- d_6 /DMF- d_7 (4:1, v/v); FA, ferulate; G, guaiacyl; H, hydroxyphenyl (p -coumaryl); Oven 16h, oven-dried for 16h; Oven 48h, oven-dried for 48h; pCA , p -coumarate; $pHBA$, p -hydroxybenzoate; S, syringyl.

7.5×300 mm (Agilent Technologies, UK); THF as eluent; flow rate, 0.5 mL min⁻¹; temperature, 40°C; sample detection, PDA response at 280 nm. The data acquisition and computation used LabSolution GPC software version 5.82 (Shimadzu). The molecular weight calibration was via polystyrene standards (Mw range from 5.8×10² up to 3.24×10⁶ Da, Agilent Technologies).

2.6 | Scanning Electron Microscopy (SEM)

A sample of ball-milled CW material (Table S1) was mounted on a carbon tape that was attached to an aluminum stub, and sputter-coated with 5 nm of gold. The samples were imaged using a Zeiss Evo 15 (LaB6) SEM (Carl Zeiss LLC, UK) with an accelerating voltage of 2–3 kV. The SEM observation showed that the oven-dried preparation yielded smaller and finer CW particles after ball milling.

3 | Results and Discussion

3.1 | Development of a DMSO-*d*₆/DMF-*d*₇ Cosolvent System for Improved Gel-State NMR

To address the limitations of the previous gel-state NMR methods, we developed the FPL gel-state NMR method, which integrates an improved DMSO-*d*₆/DMF-*d*₇ (4:1, v/v) cosolvent system, moisture-controlled drying, and optimized ball milling process.

After revisiting the preliminary solvent screening studies for earlier gel-state NMR methods [6–8], we reexamined DMF-*d*₇ as a cosolvent to DMSO-*d*₆ and found that it effectively reduces viscosity, stabilizes chemical shifts, and preserves native structural features such as natural acetyl and ester groups without introducing spectral interference of the CW. This combination of DMSO-*d*₆/DMF-*d*₇ markedly improves spectral reproducibility and resolves long-standing shimming issues associated with earlier solvent systems, resulting in better spectral quality across diverse biomass types. DMF-*d*₇ is an effective cosolvent that lowers the viscosity of gel samples and provides more stable NMR chemical shifts. We also evaluated dimethylacetamide-*d*₉ (DMA-*d*₉) and obtained similar results, but DMF-*d*₇ was preferred due to its wider availability and lower cost. Importantly, chemical shifts obtained using DMSO-*d*₆/DMF-*d*₇ (4:1, v/v) closely match those of model compounds dissolved in DMSO-*d*₆, enhancing confidence in peak assignments. In contrast, the earlier DMSO-*d*₆/pyridine-*d*₅ (4:1, v/v) system causes 0.1–0.2 ppm downfield proton shifts compared with both DMSO-*d*₆-only and DMSO-*d*₆/DMF-*d*₇ systems and is highly sensitive to small variations in solvent ratio. The DMSO-*d*₆/DMF-*d*₇ (4:1, v/v) system, by comparison, remains stable and provides consistent, reliable spectra.

3.2 | Effect of Residual Moisture on Ball Milling and Gel Homogeneity

Another key enhancement of the FPL gel-state NMR methods is maintaining low residual moisture, which is critical for improving ball-milling efficiency to obtain finer, more uniform

CW powders. We evaluated three drying approaches, air drying, oven drying at 50°C, and freeze drying, to assess their effect on moisture removal and gel formation. Oven drying consistently produced biomass with lower residual moisture (~2%–4%) than air drying (~5%–8%), whereas freeze-dried samples showed the lowest moisture levels (~1%–2%). SEM imaging confirmed that oven-dried samples yielded smaller, more uniformly fractured CW particles than air-dried material (Figure S1), consistent with their improved swelling behavior and greater gel homogeneity. Both oven-dried and freeze-dried samples formed uniform, translucent gels upon swelling in DMSO-*d*₆/DMF-*d*₇ (Figure 1), and these gels exhibited improved shimming performance and enhanced spectral resolution (Figure S2). For aspen, oven-drying for 16 h and 48 h produced essentially identical NMR spectral quality, indicating that overnight drying (~16 h) is sufficient. All oven-dried samples also showed free induction decay (FID) relaxation profiles comparable to those of isolated lignins, which allowed the use of standard Bruker NMR acquisition parameters without the specialized conditions, such as shorter acquisition times, required in earlier gel-state NMR methods. This compatibility enhances experimental throughput and routine NMR data acquisition.

Air-dried samples often resulted in incomplete swelling and poor gel formation, especially for grasses and softwoods. These observations suggest that reducing moisture content enhances both the efficiency of ball milling and solvent swelling, ultimately improving gelation and spectral quality. In contrast, oven-dried or freeze-dried samples undergo more efficient ball milling, producing finer powders that swell easily and produce highly homogeneous, near-completely solubilized gels. Therefore, reducing moisture improves particle uniformity and solvent accessibility, both of which are essential for achieving reproducible gel formation.

Since ball-milling efficiency was significantly affected by residual moisture, the milling protocol was further optimized.



FIGURE 1 | Ball-milled aspen cell wall (CW) samples in NMR tubes from different preparation conditions (ball milling condition: 15 min grinding×11 and 8 min break; total grinding time: 4 h 5 min for 200 mg CW): (a) Freeze-dried CW in DMSO-*d*₆/DMF-*d*₇ (4:1, v/v), (b) oven-dried (48 h) CW in DMSO-*d*₆/DMF-*d*₇ (4:1, v/v), (c) oven-dried (16 h) CW in DMSO-*d*₆/DMF-*d*₇ (4:1, v/v), (d) air-dried CW in DMSO-*d*₆/DMF-*d*₇ (4:1, v/v), and (e) air-dried CW in DMSO-*d*₆.

For aspen, a revised milling cycle consisting of 15 min grinding followed by an 8 min break outperformed the previous shorter duration of 10 min grinding with 5 min breaks. Moreover, when combined with oven-drying at 50°C for over 15 h, this protocol reliably generated powders that formed highly homogeneous gels.

3.3 | Improved Magnetic-Field Homogeneity and Shimming Performance

Shimming is a crucial step for obtaining high-quality NMR data [28, 29] and has been a major limiting factor for spectral quality in earlier gel-state NMR methods under suboptimal moisture control or milling conditions. Earlier methods mostly failed to shim under automated routines such as IconNMR (Bruker), leading operators to skip the shimming step. In contrast, the optimized method presented here, combining controlled drying, improved milling, and the DMSO- d_6 /DMF- d_7 (4:1, v/v) solvent system, significantly improved magnetic-field homogeneity and spectral reproducibility by successfully ensuring the shimming process in the Bruker TopSpin operating system.

3.4 | Robustness and Generality of the FPL Gel-State NMR Method Across Biomass Types

To assess the robustness and generality of the FPL gel-state NMR method, representative lignocellulosic biomass samples, aspen (hardwood), pine (softwood), and corn (grass), were examined (Figures 2 and 3). Oven-dried CWs consistently produced sharper proton (Figure S2) and 2D HSQC spectra than air-dried samples, regardless of the solvent systems used. Aspen was selected as a reference species for evaluating the FPL gel-state NMR method with DMSO- d_6 /DMF- d_7 (4:1, v/v) due to its chemically diverse CW compositions and well-resolved NMR spectral features (Figure 2). The resulting spectra revealed well-resolved signals corresponding to both lignin and polysaccharide components. The lignin region displayed characteristic syringyl (S) and guaiacyl (G) units, along with distinct *p*-hydroxybenzoate (*p*HBA) γ -ester conjugates predominantly associated with S-units [27]. The enhanced spectral resolution achieved with the DMSO- d_6 /DMF- d_7 (4:1, v/v) system also allowed simultaneous detection of complex polysaccharide structures along with lignin features, demonstrating comprehensive whole CW profiling capacity. Pine CW spectra showed a simpler lignin pattern dominated by G-units with minor H-units, which are only visible at

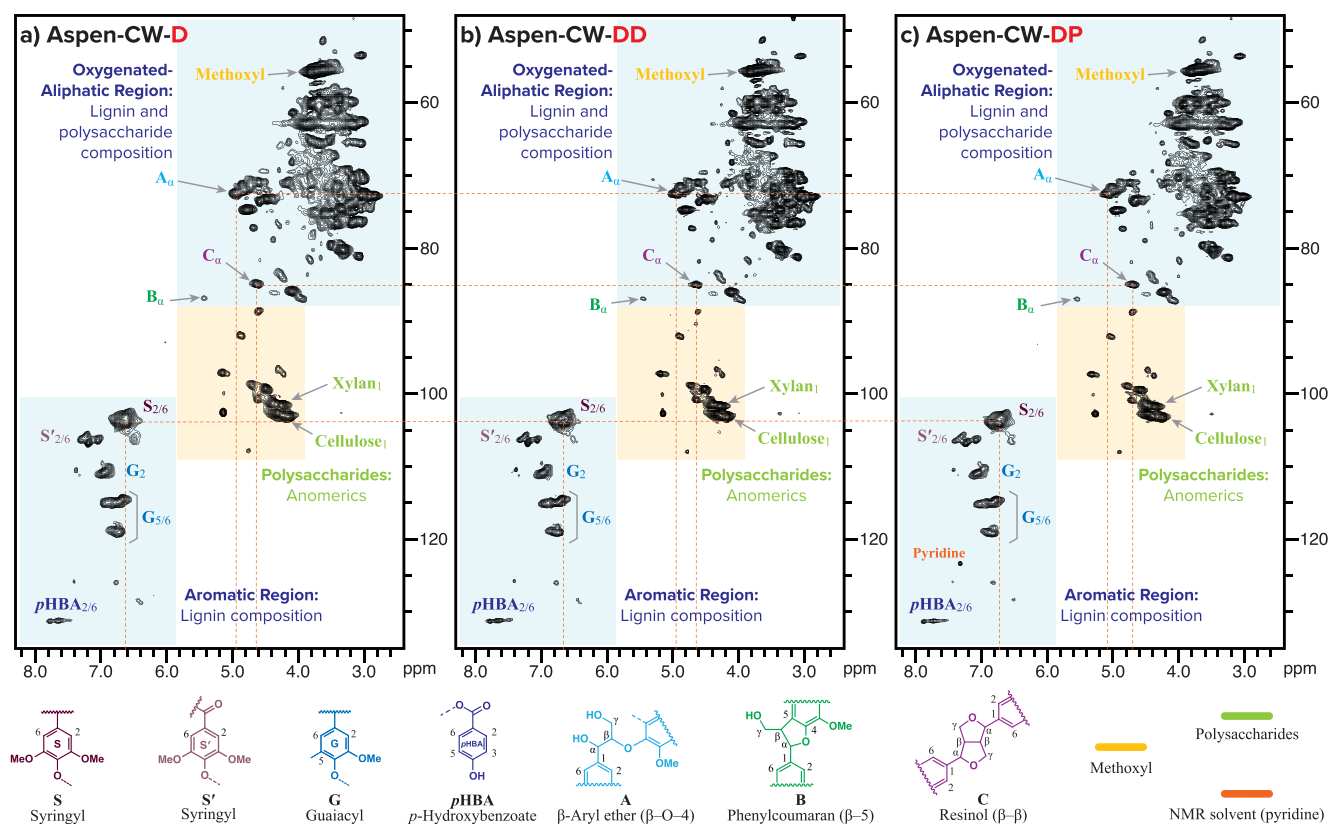


FIGURE 2 | 2D HSQC spectra of aspen gel-state NMR samples prepared from finely pulverized low-moisture cell wall (CW) after oven drying, analyzed in different NMR solvent systems: (a) DMSO- d_6 (D) using modified gel-state NMR parameters (F2 acquisition time = 100 ms, F1 interscan delay = 500 ms, NS = 111, total EXPT = 10 h) without shimming, (b) DMSO- d_6 /DMF- d_7 (DD, 4:1, v/v) using standard Bruker NMR parameters (F2 acquisition time = 200 ms, F1 interscan delay = 1 s, NS = 56, total EXPT = 10 h) with gradient shimming, and (c) DMSO- d_6 /pyridine- d_5 (DP, 4:1, v/v) using modified gel-state NMR parameters (F2 acquisition time = 100 ms, F1 interscan delay = 500 ms, NS = 111, total EXPT = 10 h) without shimming. The central DMSO solvent peak (δ_C 39.5, δ_H 2.49 ppm) was used as internal reference. Correlations obtained from (a) DMSO- d_6 and (b) DMSO- d_6 /DMF- d_7 (4:1, v/v) systems are well matched, but those from DMSO- d_6 /pyridine- d_5 (4:1) appear slightly downfield (0.1–0.2 ppm), indicating solvent-dependent chemical-shift variations that may lead to misassignments.

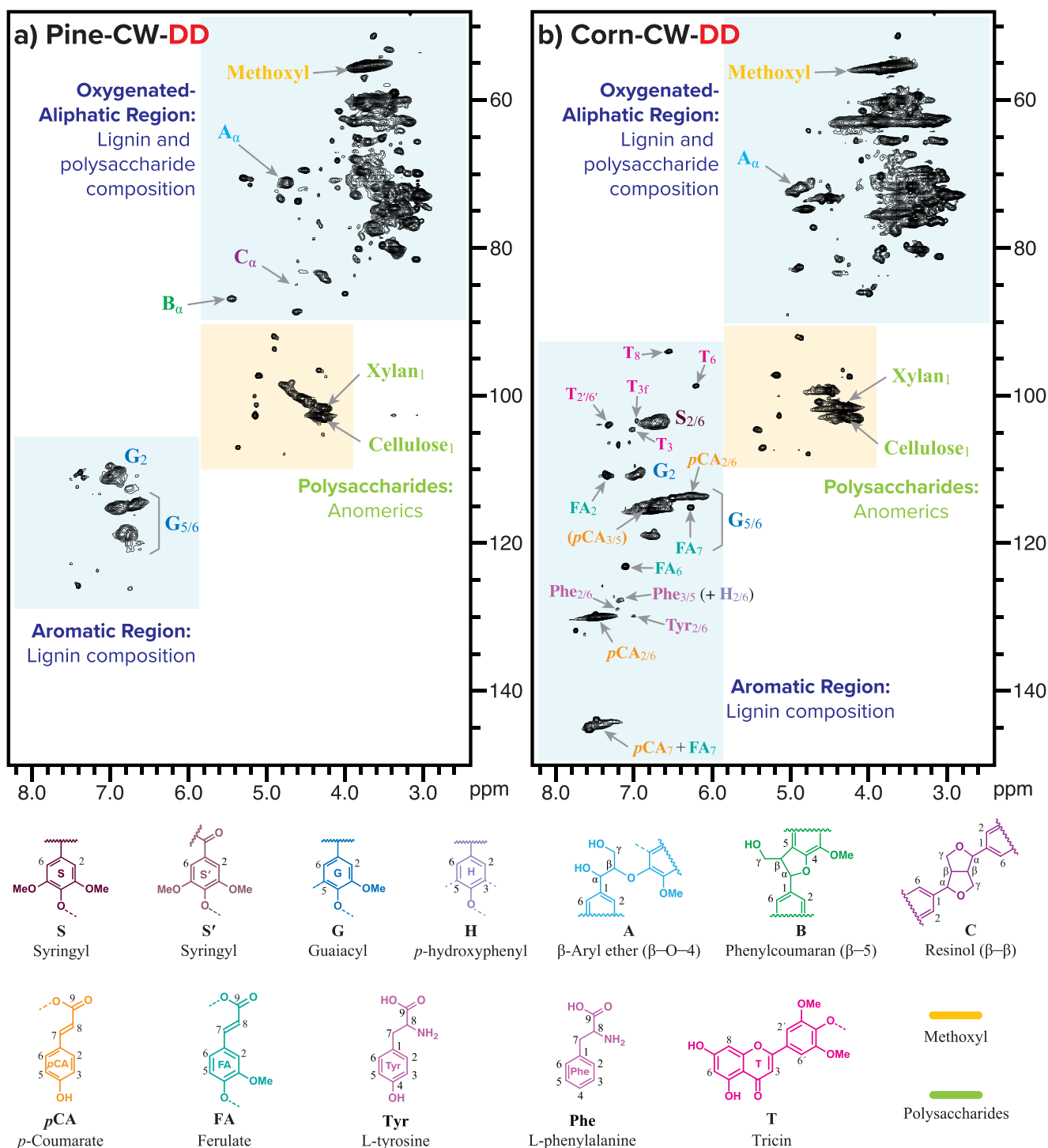


FIGURE 3 | 2D HSQC NMR spectra of (a) pine cell wall (CW) and (b) corn cell wall (CW) gels in DMSO- d_6 /DMF- d_7 (4:1, v/v) acquired using standard Bruker NMR parameters after successful shimming.

lower contour levels at δ_C/δ_H 127.8/7.18 ppm, and well-resolved β -O-4, β - β , and β -5 unit linkages (Figure 3a). Corn stover exhibited characteristic grass (Poaceae) lignin features, including γ -acylating *p*-coumarate (*p*CA) and ferulate (FA) esters involved in lignin–polysaccharide cross-linking, along with major S-, G-, and minor H-units (Figure 3b) [30, 31]. Additionally, triclin, a flavonoid that serves as one of the potential initiation sites for lignification, was also evident [13, 32]. Collectively, these results demonstrate that the DMSO- d_6 /DMF- d_7 -based FPL

gel-state NMR method enables simultaneous detection of lignin subunits, interunit linkages, and polysaccharide structures across hardwood, softwood, and grass species, highlighting broad applicability and high structural resolution.

To confirm the preservation of native lignin structures during the preparation of whole CW samples, ELs were isolated and analyzed using the original DMSO- d_6 -only system for direct comparison with CW samples analyzed by the FPL gel-state

NMR method. Their molecular-weight distributions were further evaluated by GPC (Figure S3). The mechanical ball-milling facilitated the enzymatic hydrolysis of CWs, yielding ELs that were fully soluble in DMSO and produced high-quality 2D HSQC spectra (Figure 4). Comparative analyses revealed that CW spectra (Figure 4a–c) obtained with the DMSO- d_6 /DMF- d_7 (4:1, v/v) closely matched EL spectra (Figure 4d–f), exhibiting virtually identical chemical shifts. Minor differences, such as a slightly lower β -O-4 abundance (3.4%) in EL samples compared with CW samples, likely resulted from the removal of residual polysaccharides in the CW matrix that overlapped in the α -position region of lignin. Several peaks corresponding to acetylated xylan and mannan also appeared in the same region of the CW spectra. The S/G ratios in aspen CW and EL samples were 2.09 and 1.91, respectively, indicating close agreement (Table 1). GPC analysis of aspen ELs indicated moderate depolymerization, with Mw reduced by 25.6% and Mn reduced by 28.5%, whereas the polydispersity remained essentially unchanged at $\sim$ 2.7, suggesting relatively uniform chain cleavage

without substantially altering the overall molecular-weight distribution (Figure S3).

Softwood (pine) and grass (corn) ELs exhibited similar trends in the NMR analysis. In pine, the β -O-4 content in EL was 3.4% lower than in CW, which is similar to the observation in aspen (Table 1). Corn EL spectra revealed typical grass lignin features, high β -O-4 content, low β - β levels, and aromatic units including S, G, H, *p*CA, and tricin. Importantly, the DMSO- d_6 /DMF- d_7 (4:1, v/v) system clearly resolved tricin and *p*CA correlations in corn lignin, reinforcing the method's suitability for analyzing grass-type lignins (Figure 3). These results confirm that the FPL gel-state NMR method provides reliable, high-resolution whole CW spectra directly comparable to isolated lignins, preserving native structural features across hardwood, softwood, and grass species.

The DMSO- d_6 /DMF- d_7 (4:1, v/v) cosolvent system not only enhances lignin resolution but also preserves and reveals

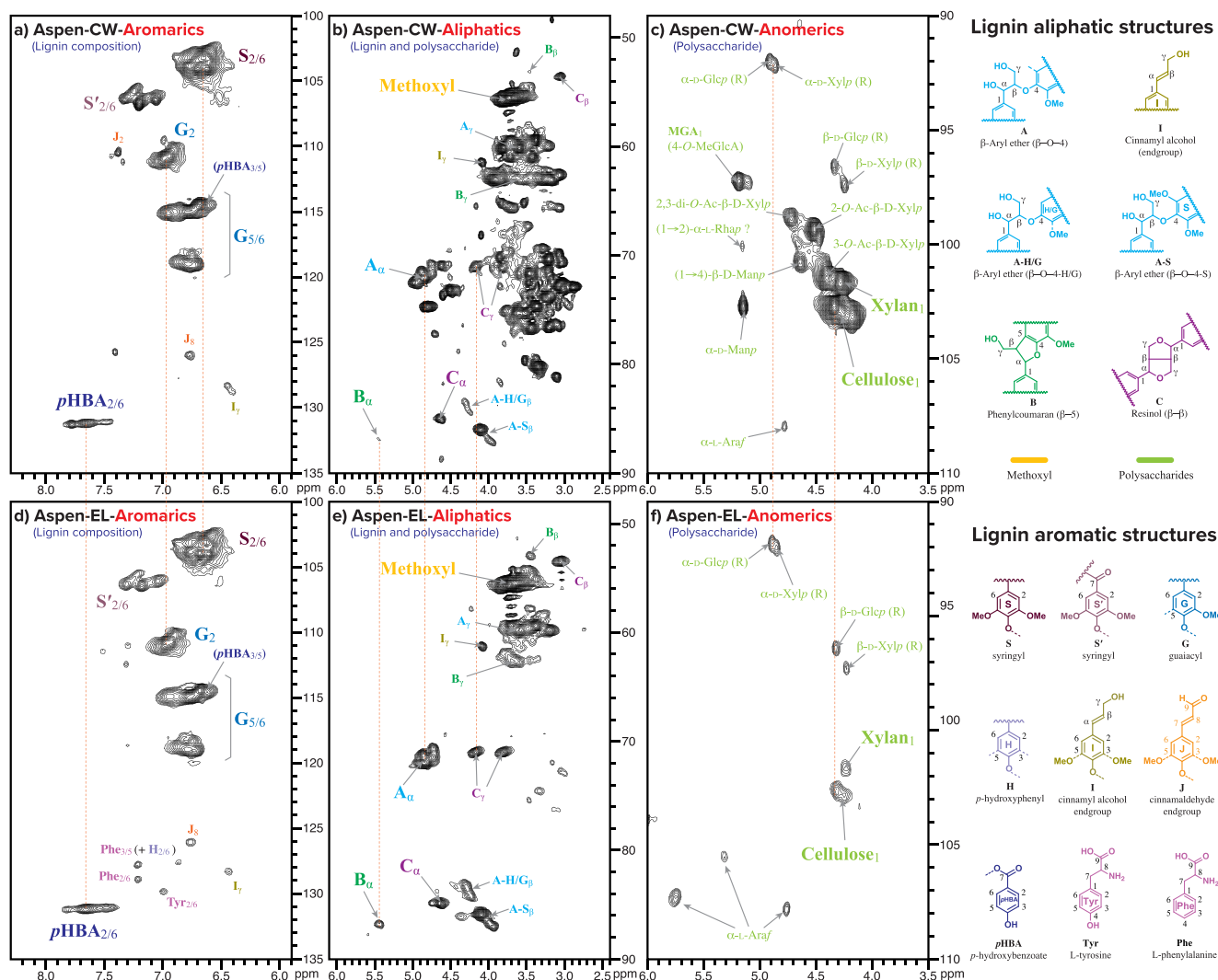


FIGURE 4 | Comparison of the 2D HSQC spectra of aspen cell wall (CW) in DMSO- d_6 /DMF- d_7 (4:1, v/v) and enzymatic lignin (EL) in DMSO- d_6 : (a) CW aromatic region, (b) CW aliphatic region, (c) CW polysaccharides anomeric region, (d) lignin aromatic region, (e) lignin aliphatic region, and (f) lignin polysaccharide anomeric region, which typically includes polysaccharide residues that remain after enzyme digestion. A direct comparison of the proton chemical-shift deviation between the CW in DMSO- d_6 /DMF- d_7 (4:1, v/v) and the EL in DMSO- d_6 shows that all corresponding correlations are in well-mannered agreement.

polysaccharide features in whole CW samples. The aspen CW samples exhibited equal or higher signal intensities than those obtained with DMSO- d_6 -only or the DMSO- d_6 /pyridine- d_5 systems (Figure 2), reflecting the improved gelation and shimming in the new FPL gel-state method. Across all species, characteristic anomeric correlations and reducing ends of cellulose, xylan, and 4-O-methyl- α -D-glucuronic acid (4-O-MeGlcA) were clearly resolved in the δ_C/δ_H 90–110/3.5–6.0 ppm region (Figures 3 and 4c), consistent with earlier gel-state NMR data [6, 7]. Species-specific differences were also evident. Aspen showed typical acetylated 4-O-methylglucuronoxylan [33], along with minor glucomannan and pectin, whereas the main cellulose correlation appeared just below the main xylan signal (Figure 4c). Pine CW displayed galactoglucomannan, arabinoglucuronoxylan, and pectin correlations characteristic of softwoods (Figure 3a) [6, 7, 11, 33], whereas corn contains polysaccharide components similar to aspen but with a simpler profile (Figure 3b). Nonanomeric polysaccharide signals (C2–C6/H2–H6) were observed in the aliphatic region (δ_C/δ_H 50–90/2.5–6.0 ppm) and were well resolved alongside lignin sidechain signals.

Overall, the FPL gel-state NMR method significantly improves spectral quality and shimming reproducibility compared with earlier approaches, allowing simultaneous and balanced detection of both lignin and polysaccharide components within intact CWs. Although crystalline cellulose domains remain only partially swollen and thus underrepresented, the new method still allows comprehensive characterization of major polysaccharides alongside lignin structures.

4 | Conclusion

The FPL gel-state NMR method using DMSO- d_6 /DMF- d_7 (4:1, v/v) cosolvent system was developed to enhance 2D NMR analysis of whole CWs and biomass, enabling effective and nondestructive structural characterization across diverse plant species. This approach allows simultaneous observation of lignin subunits, interunit linkages, and polysaccharide structures directly from intact biomass. Controlled drying and fine pulverization proved critical for efficient cell-wall swelling and gel formation. Using oven-dried samples and finely pulverized powders prepared from planetary ball milling, the FPL gel-state NMR workflow consistently produced high-quality 2D HSQC spectra with enhanced signal intensities and markedly improved resolution across aromatic, sidechain, and carbohydrate regions after successful shimming. No significant chemical-shift deviations were observed between DMSO- d_6 -only and DMSO- d_6 /DMF- d_7 gels, confirming their compatibility. Controlled drying reduces residual moisture, increases particle brittleness, and improves milling efficiency, producing fine powders that swell uniformly and achieve near-complete solubilization. Overall, the optimized FPL gel-state NMR method delivers sharper, more reproducible spectra, effectively extending solution-state NMR to direct whole CW analysis. This development offers a reliable, high-resolution platform for detailed biomass characterization and establishes a basis for future research connecting physical processing parameters with gelation behavior and spectral performance.

Author Contributions

Hoon Kim: conceptualization, investigation, funding acquisition, writing – original draft, methodology, validation, visualization, writing – review and editing, formal analysis, supervision, resources, data curation, software. **Yimin Mao:** validation, methodology, writing – review and editing, formal analysis. **Daniel J. Yelle:** methodology, writing – review and editing. **Jorge Rencoret:** methodology, writing – review and editing. **José C. del Río:** writing – review and editing. **Peter Kitin:** methodology.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Peer Review

For transparency, the peer review documents associated with this article are available at <https://doi.org/10.1002/mrc.70106>.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Effect of drying method and ball-milling conditions (Table S1) on the morphology of aspen cell wall particles. The SEM images show cell wall particles (arrows) on a carbon tape substrate. (a) Air-dried with 3 h and 40 min total milling time. (b) Oven-dried (16 h) with 5 h and 37 min total milling time. (c) Oven-dried (16 h) with 7 h and 20 min total milling time. **Figure S2:** ^1H NMR spectra (500 MHz) of aspen cell wall (CW) in DMSO- d_6 /DMF- d_7 (4:1, v/v) and enzymatic lignin (EL) in DMSO- d_6 illustrating the effects of drying and shimming process on spectral resolution (DMSO = δ_{H} 2.49 ppm). (a) Air-dried CW without shimming: a typical proton spec-trum showing extremely broad peaks due to poor magnetic field homo-geneity. (b) Oven-dried CW with shimming: well-resolved and sharp peaks reveal distinct signals from lignin and polysaccharide compo-nents. (c) Enzymatic lignin (EL) with shimming: Isolated lignin fea-tures with sharp peaks are clearly observed after enzymatic removal of polysaccharides. **Figure S3:** GPC spectra of aspen enzyme lignins (ELs) from differently dried cell wall (CW) samples. (a) EL from air-dried CW sample (yield = 14.9%; Mw = 13,118, Mn = 5080, Mw/Mn = 2.6). (b) EL from 16 h oven-dried CW sample (yield = 15.0%; Mw = 9760, Mn = 3631, Mw/Mn = 2.7). Ball milling conditions: 15 min grinding ($\times 15$) with 8 min break; total grinding time = 5 h 37 min for 300 mg of cell wall ma-terial. **Table S1:** Effect of drying method and ball-milling conditions on cellulolytic enzyme lignin (EL) yields from cell-wall samples. About 300 mg of extractives-free Wiley-milled biomass was finely pulverized using a Fritsch planetary micro mill PULVERISETTE 7 premium line under the indicated grinding and interval cycles. Air- and oven-drying treatments were applied as moisture-control steps prior to milling. Total ball-milling times include all grinding and interval break periods. Results show that prolonged milling significantly reduced EL yield in aspen, whereas pine and corn maintained relatively consistent yields across drying conditions.