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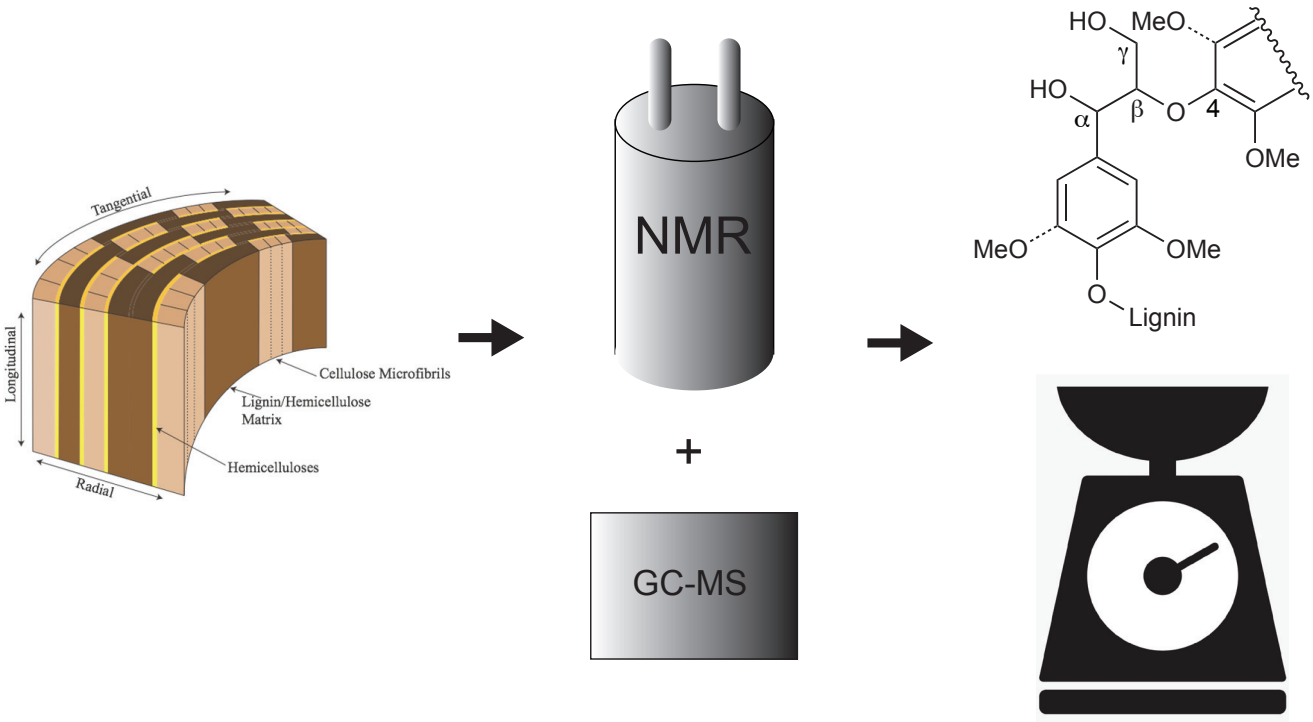
Multifaceted Approach for Determining the Absolute Values for Lignin Subunits in Lignocellulosic Materials

Daniel J. Yelle



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Abstract

Quantifying the cell wall polymers in wood and plants has been a tremendous challenge because no single method can achieve absolute quantification of any cell wall polymer. Two-dimensional NMR spectroscopic techniques have advanced this area with the introduction of wood dissolution and cryogenically cooled probes, allowing for semi-quantification. However, a more robust technique had to be developed to allow for a more absolute quantification of cell wall polymers. This study explored a multifaceted approach to accurately quantify lignin methoxyls using gas chromatography and mass spectroscopy so as to normalize the methoxyl peak in the two-dimensional NMR spectra. This made it possible for any well-dispersed peak in the spectra to be accurately quantified. Several lignin model compounds were analyzed to test the accuracy of the methoxyl analysis, and several lignocellulosic materials were tested using this approach. The results showed that the methoxyl analyses of the model compounds were useful in confirming the accuracy of the method, which allows for faster and more robust methoxyl analysis of the lignocellulosic materials. The importance of this study relates to a fundamental understanding of what specific cell wall polymers are being modified during chemical, biological, or other modification of lignocellulose.

Keywords: Lignin, methoxyls, gas chromatography, mass spectroscopy, NMR spectroscopy, absolute value, quantification

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Multifaceted Approach for Determining the Absolute Values for Lignin Subunits in Lignocellulosic Materials

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Introduction

Visualizing changes in the chemistry of wood and plant cell walls after some kind of modification (such as chemical, hydrothermal, biological) using one-dimensional (1D) NMR spectroscopy is not greatly informative. In many cases, the 1D NMR peaks in ^{13}C or ^{1}H spectra of cell wall polymer structures are not well dispersed and overlap heavily (Ralph et al. 1999). Significant improvements over the 1D NMR experiments were made with the utilization of two-dimensional (2D) heteronuclear single-quantum coherence (HSQC) applied to ball-milled wood or plants (Lu and Ralph 2003; Yelle et al. 2008a; Kim et al. 2008; Kim and Ralph 2010). These advanced techniques, in conjunction with cryogenically cooled triple-resonance probes, have allowed for significant advances in our understanding of whole cell wall chemistry in wood and plants and their modifications (Yelle et al. 2008b; Yelle et al. 2011a; Yelle and Ralph 2016). Yet, with these existing techniques there remains the challenge of absolute quantification of cell wall structures. With the introduction of adiabatic pulse sequences (Kupče and Freeman 2007), the HSQC experiment has become more quantitative. Thus, semi-quantitative information can now be obtained from these adiabatic HSQC experiments, as long as the cell wall material is prepared properly and some rules are followed in the NMR acquisition and processing (Zhang and Gellerstedt 2007; Yelle et al. 2011a; Yelle et al. 2013).

The semi-quantitative data that is achieved with these 2D NMR techniques are not without their own limitations. For example, polymeric structures in the cell wall can be quantified only relative to some large peak (or peaks) in the spectra. The aromatic guaiacyl unit (C2) and syringyl unit (C2 and C6) peaks are known to be commonly used as internal references in 2D NMR spectra because they are indicative of native lignin (Lu and Ralph 2003; Yelle et al. 2008a). Thus, selecting the guaiacyl C2 or syringyl C2/C6 aromatic peaks as internal references to help semi-quantify other peaks in the 2D NMR spectra sounded promising. However, researchers have revealed that when lignin condensation occurs at the C5-position of a guaiacyl unit during a plant cell wall modification, the newly condensed bond at C5 created a broadened group of peaks surrounding the C2 peak that could not be completely attributable to the native guaiacyl C2 peak (Yelle et al. 2008b). Furthermore, peaks arising from condensation at the C5-position in wood or plants with both guaiacyl and syringyl units may potentially be counted as part of syringyl aromatic peaks at C2 and C6, thus overestimating the amount of syringyl units and interjecting unwanted error in any subsequent quantification (Zhang et al. 2016). The lignin methoxyl group is a common peak that was chosen as an internal reference because of its good chemical stability (Lundquist and Lundgren 1972). However, in certain cell wall modifications, the lignin methoxyls are not always stable and may cleave or become demethoxylated during certain biological treatments. One such treatment is brown-rot decay of wood, where the ethers in lignin can become cleaved because of the presence of nonselective hydroxy-radicals formed during Fenton chemistry (Yelle et al. 2008b; Goodell et al. 1997). Nevertheless, even when the lignin methoxyl group is cleaved to a substantial degree during a biological treatment, methoxyl analysis using a chromatographic method has been shown to be accurately determined (Li et al. 2017). Collectively, the lignin methoxyl is currently the most promising functional group in wood and plants to be the representative internal reference for quantifying cell wall structures. Quantifying the chemical structures in wood requires an approach that involves 2D NMR experiments (such as adiabatic HSQC) and methoxyl analysis (Yelle et al. 2011b). With methoxyl analysis, the chemical structures in the NMR spectra can be quantified to give absolute values because the methoxyl peak in the NMR spectra can be normalized to this value, but this of course requires an accurate and robust methoxyl analysis method.

The methoxyl analysis of lignin not only is a fundamental characterization method of lignin but can provide indicative information on modification of the cell wall polymer chemistry. The original method was introduced by Zeisel, in which methoxyl or ethoxyl groups in compounds were treated and cleaved with refluxing hydriodic acid (HI), giving off ethyl or methyl iodide (Zeisel 1885). The most widely used modification of the Zeisel method was developed by Vieböck and Schwappach (1930) because of its incorporation of titration, rather than precipitation, which increased precision and accuracy. However, this method

is time consuming, involving an intricate apparatus, with distillation of the volatile alkyl iodide that must be trapped and then titrated.

The use of gas chromatography in methoxyl content analysis of lignin model compounds, lignin, and lignocellulosics has been developed by several researchers to help simplify the method. Shtreis and Bobkova (1973) studied methoxyl analysis of lignin model compounds, lignin, and wood using gas-liquid chromatography. Hodges et al. (1979) used hydroxypropyl methyl cellulose to study the precision of a Zeisel gas chromatographic method for methoxyl analysis and found the method gave low variability. Girardin and Metche (1983) studied the methoxyl content of poplar milled-wood lignin using gas chromatography and found alkyl iodide recovery to be >97%. Baker (1996) performed methoxyl analysis of industrially prepared lignins using gas chromatography and found that values were within 2% of those obtained by titrimetric methods. All these gas chromatographic methods have demonstrated the advantages of saving time and increasing sample throughput over those by Zeisel (1885) and Vieböck and Schwappach (1930).

Research by Goto et al. (2005, 2006) demonstrated that traditional hydriodic acid (HI) methoxyl analyses of lignocellulosic materials may be flawed in that the polysaccharides in the material may also release methyl iodide in a similar fashion to that of lignin, thus obscuring the true number of lignin methoxyls. However, they found that selected monosaccharides produced methyl iodide to the level of 5–10 µmol/g, whereas selected wood species produced 1,400–1,800 µmol/g. Therefore, the contribution of saccharides to methoxyl content was found to be less than 0.7% of the total methoxyl content. For example, if wood has 4% methoxyl content, then the highest contribution from saccharides would be 0.03%.

This paper focuses on the iodometric method and quantifying the released methyl iodide from lignin model compounds, wood, and plants. The goal is to determine the absolute values for polymeric cell wall structures in lignocellulosic materials using a gas chromatographic–mass spectroscopic (GC–MS), combined with a solution-state NMR spectroscopic, approach. Obtaining absolute values of chemical structures in wood and plants is vital, so that when modifications are performed, the specific structures involved in a cell wall modification can be quantified to a high level of confidence. Specific steps in the approach to achieve this goal are as follows:

1. Use accessible lignin model compounds to test the viability of the gas chromatographic methods described in the literature on how to determine methoxyl groups and modify methods as necessary (Hodges et al. 1979; Girardin and Metche 1983; Baker 1996; Goto et al. 2005, 2006).
2. With viable and accurate methoxyl contents of the lignin model compounds using gas chromatography and mass spectroscopy, measure methoxyl contents of several lignocellulosic materials.
3. Run two-dimensional ^{1}H–^{13}C correlation NMR experiments on selected lignocellulosic materials and integrate the major lignin subunit peaks and the methoxyl region.
4. Use the determined methoxyl content values to normalize the methoxyl peak in the spectra and calculate the absolute values for major lignin subunit polymers in wood and plant materials.

Experimental

Materials

All wood and plant samples were provided by the Forest Products Laboratory. The wood species, common North American commercial species, included *Populus tremuloides* (trembling aspen), *Liriodendron tulipifera* (yellow poplar), *Acer saccharum* (sugar maple), *Pinus taeda* (loblolly pine), and *Picea glauca* (white spruce). All wood materials were from kiln-dried lumber. The plant species, herbaceous grasses commonly used as biomass for biofuels, were *Triticum aestivum* (wheat straw) and *Heteropogon contortus* (tanglehead). The wood and plant materials were dried in a vacuum oven at 40 °C until their masses were constant. Ball-milling of the dry wood and plant materials followed a similar method as described in Yelle et al. (2008a). Briefly, the dry sound wood or plant stem material was weighed out to approximately 500 mg and then cut into approximately 5- by 5- by 5-mm (125-mm^3) cubes and ball-milled using a Retsch PM-400 planetary ball-mill (Newtown, Pennsylvania, USA) with a 50-mL ZrO_2 jar with three 20-mm ZrO_2 balls at 200 rpm for 10 h (20 min milling, 10 min pause). The pre-milled samples were then further ball-milled using approximately 200–300 mg of material into a 50-mL ZrO_2 jar with ten 10-mm ZrO_2 balls and milled at 300 rpm for a total of 10 h (20 min milling, 10 min pause) to give 400 min actual milling time. Model compounds and other chemicals used were obtained from Aldrich Chemical Company (Milwaukee, Wisconsin, USA), unless otherwise noted.

Methoxyl Analysis

The procedure for determining methoxyl content of the lignin model compounds and lignocellulosic samples was similar to that described by Hodges et al. (1979), Girardin and Metche (1983), Baker (1996), and Goto et al. (2005, 2006), but with modifications. Briefly, to a 40-mL head-space vial, approximately 5–50 mg of each model compound or ball-milled lignocellulosic sample was added, followed by 5 mL of 57% hydriodic acid via a volumetric pipette. A few carborundum crystals were added to each

vial (to suppress bumping), and the vials were purged with nitrogen. The vials were sealed with Teflon/silicone septa (with crimped caps), and reaction vials were heated in a welled hot-plate to 140 °C for 60 min. Immediately after this reaction period, the reaction vials were cooled in an ice-water bath, and 10.0 mL of carbon tetrachloride (organic solvent) was added through the septa via a 10.0-mL syringe (no. 20 size needle) to extract the organic phase. As an internal standard, 100 µL of an ethyl iodide solution (89.7 mg/mL in carbon tetrachloride) was added through the septa via a 100-µL glass syringe so that each sample vial contained 10.0 mL + 0.1 mL carbon tetrachloride with 0.888 µg ethyl iodide. The vials were shaken vigorously for 1 min using a mistral mixer and stored in a freezer (approximately –20 °C) to allow complete phase separation. The organic phase (uppermost layer containing methyl iodide) was then subjected to GC–MS analysis.

Gas Chromatography–Mass Spectroscopy

GC–MS quantitative analyses were performed using a Varian (Walnut Creek, California, USA) model 3800 gas chromatograph interfaced to a Varian model 4000 ion trap mass detector, operated in full scan EI mode at 70 eV in the range m/z = 135–175. The transfer line was maintained at 250 °C. A Restek (Bellfonte, Pennsylvania, USA) Rxi-1MS column (30 m × 0.25 mm × 0.25 µm) was temperature programmed as follows: 35 °C to 45 °C at 2 °C/min, to 250 °C at 50 °C/min. A CombiPal autosampler (CTC Analytics, Zwingen, Switzerland) equipped with a 10-µL liquid syringe was used for 1-µL injections. The injector was operated in 50:1 split mode at 200 °C. Quantification was performed on peak areas of quant ion m/z = 142 for CH_3I using Varian MS Workstation software v6.9. Processing was performed using triplicate injections of external standards with response factor RSD 0.03% and correlation coefficient 0.999%. Each lignin model compound sample analysis was replicated twice with three replicate injections per sample to give six total injections. Each lignocellulosic sample analysis was replicated three times with three replicate injections per sample to give nine total injections.

Solution-State NMR Spectroscopy

The polymeric structures in the lignocellulosic materials were further analyzed using solution-state NMR spectroscopy. Approximately 30 mg of each ball-milled sample was dissolved directly into a 5-mm diameter NMR tube (7 in. (178 mm) length) using 500 µl of DMSO-d_6 and sonicated at 35 °C for 1 h. In some cases, the ball-milled samples required 1-methylimidazole-d_6 (NMI-d_6) to facilitate dissolution; a solution of 400 µl of DMSO-d_6 with 100 µl of NMI-d_6 was added directly to the sample in a 5-mm NMR tube. The dissolved samples were homogeneous and clear solutions. NMR spectra were acquired on a Bruker-Biospin (Rheinstetten, Germany) AVANCE III HDTM 500 MHz spectrometer fitted with a nitrogen-cooled 5-mm ProdigyTM TCI gradient cryoprobe with inverse geometry. One-bond ^{1}H–^{13}C correlation (HSQC) spectra were acquired using adiabatic Bruker pulse program hsqcetgpsisp2.2 and processed as previously described (Yelle et al. 2008a) using Bruker TopSpin 3.6.2 software.

Quantification of Methoxyls

Quantifying the methoxyls (OMe) in each sample was performed using the data from gas chromatography. Methoxyl content was calculated by the following equations:

(1) OMe (mg) = [Ave µg/µL CH_3I found with GC] × [10.1 mL total volume] × [1 mmol CH_3I/141.95 mg CH_3I] × [1 mmol OMe/1 mmol CH3I] × [31.03 mg OMe/1 mmol OMe]

(2) % OMe = [OMe (mg)] / [weight of wood sample or compound (mg)] × 100

(3) µmol OMe/g wood = [OMe (mg)] × [mmol OMe/ 31.03 mg] × [1000 µmol/1 mmol] × [integral of subunit peak relative to OMe peak] / [weight of wood sample (g)]

Results and Discussion

GC-MS Analysis

Lignin Model Compounds

The purpose of measuring the methoxyl content of lignin model compounds was to test the accuracy of the GC–MS technique with low-molecular-weight structures that are similar to structures found in lignin. Table 1 shows the model compounds selected for these initial experiments along with the methoxyl analysis results. These model compounds represent a variety of functionality at the Cα, Cβ, and Cγ positions as well as the potential hydroxyls or methoxyls at the C3, C4, and C5 positions. From the GC–MS results of these model compounds, the theoretical values for methoxyl concentration were compared to the measured values. The comparison showed that the technique was accurately measuring the methoxyl content for all the model compounds tested here with relative standard deviation (RSD) values of less than or equal to 3.6.

Lignocellulosics

With the accuracy of lignin model compounds established, the focus shifted to the analysis of lignocellulosic materials, such as isolated lignins, holocellulose, and ball-milled wood and plants. Table 2 summarizes the methoxyl analysis results for several lignocellulosic materials. The results of the analysis gave low variability overall, with the highest variability seen in aspen. Aspen is known to have high amounts of tension wood, a reaction wood tissue that is high in cellulose content and low in lignin. This may lead to higher variability because it is difficult to control where reaction wood tissues reside in lumber, and once milled, all anatomical features are destroyed.

Table 1—Lignin model compounds analyzed for methoxyl content using GC–MS[a]

Model compound	Formula weight (g/mol)	Weight (mg)	CH_3I (theory) (µg/µL)[b]	CH_3I (average, measured) (µg/µL)	OMe (theory) (%)	OMe (average, measured) (%)	RSD (%)
Vanillin methyl ether	166.18	9.62	1.627	1.536 (0.0281)	37.35	35.25 (0.64)	1.8
Syringaldehyde methyl ether	196.20	10.44	2.244	2.374 (0.0833)	47.45	49.71 (1.70)	3.5
4-hydroxy-3-methoxy cinnamaldehyde	192.21	10.19	0.745	0.792 (0.0121)	16.14	16.99 (0.26)	1.5
Ferulic acid	208.21	5.31	0.358	0.361 (0.0130)	14.90	15.01 (0.54)	3.6
Syringaldehyde	182.17	10.78	1.663	1.641 (0.0110)	34.07	33.61 (0.23)	0.7
Vanillin	152.15	11.16	1.031	1.073 (0.0318)	20.39	21.23 (0.63)	3.0
Syringic acid methyl ether	212.20	10.06	1.999	2.018 (0.0307)	43.87	44.29 (0.67)	1.5
Veratric acid	182.17	10.87	1.677	1.646 (0.0508)	34.07	33.43 (0.10)	3.1
Benzyl alcohol methyl ether	198.22	6.24	1.327	1.225 (0.0490)	46.96	43.34 (1.73)	1.4
Vanillyl alcohol	154.16	11.78	1.074	1.064 (0.0376)	20.13	19.94 (0.70)	3.5

[a]Each model compound sample was replicated twice, with three separate injections per sample, to give six total injections. Standard deviations are given in parentheses.
[b]Theory = (mg weighed/10.1 mL) × (1/formula weight of compound) × (no. OMe) × (formula weight of methyl iodide).

Table 2—Ball-milled lignocellulosics analyzed for methoxyl content using GC–MS[a]

Sample	Weight (mg)	CH_3I (average, measured) (µg/µL)	OMe (average, measured) (%)	RSD (%)
Trembling aspen (*Populus tremuloides*)	49.65	1.072 (0.1606)	4.8 (0.33)	6.9
Yellow poplar (*Liriodendron tulipifera*)	30.09	0.723 (0.0248)	3.5 (0.10)	3.0
Sugar maple (*Acer saccharum*)	29.40	0.698 (0.0153)	5.2 (0.11)	2.2
Loblolly pine (*Pinus taeda*)	30.30	0.507 (0.0062)	3.7 (0.05)	1.5
White spruce (*Picea glauca*)	49.06	1.049 (0.0518)	4.7 (0.27)	5.8
Wheat straw (*Triticum aestivum*)	10.83	0.151 (0.0161)	3.1 (0.17)	5.6
Tanglehead (*Heteropogon contortus*)	30.60	0.347 (0.0032)	2.5 (0.09)	3.7

[a]Each lignocellulosic sample was replicated three times, with three separate injections per sample, to give nine total injections. Standard deviations are given in parentheses.

The syringyl/guaiacyl (S/G) ratio of hardwoods can substantially influence the amount of lignin methoxyls. Hardwoods have both syringyl units (two methoxyls) and guaiacyl units (one methoxyl), but the lignin content of hardwoods (18% to 25% of dry wood) are typically lower than softwoods (25% to 35% of dry wood). Therefore, methoxyl contents of softwoods can be equivalent to those found in hardwoods, unless the syringyl content is high (that is, S/G ratios are >2).

The monocotyledon grasses wheat straw and tanglehead showed the lowest methoxyl contents. Grasses also have the least amount of lignin (averaging 15% to 22% of dry grass). Grasses, like hardwoods, have syringyl units. However, they also have *p*-coumaryl units, or H-units, that have no methoxyls. They also have hydroxycinnamates, such as ferulate (one methoxyl) and *p*-coumarates (no methoxyls), and tricin (two methoxyls), a flavonoid that was found to be covalently incorporated into the lignin polymer in monocots (Lan et al. 2015).

Solution-State NMR Spectroscopy

Two-dimensional (2D) NMR analysis was performed on nonderivatized samples to visualize the structural chemistry of the lignin. For this study, trembling aspen, loblolly pine, and wheat straw were selected for spectral analyses. Figures 1 to 3 show the ^{1}H–^{13}C HSQC spectra for these lignocellulosic samples. For simplicity, only the aliphatic regions are displayed in the figures because the aliphatic region is where that portion of the lignin subunits are visualized. In the aliphatic region, the major lignin subunit linkages (that is, β-aryl ethers, phenylcoumarans, and resinols) and the lignin methoxyls were analyzed by integrating the contour peaks representing the Hα-Cα positions of these subunits. Figure 4 displays the chemical structures of these three lignin subunits that are referenced in the spectra in Figures 1 to 3. The methoxyl contour peak, typically one of the largest peaks in wood and plant cell wall NMR spectra, was normalized using the GC–MS data of methoxyl content for each lignocellulosic sample. With the methoxyl peak normalized, the lignin subunit contour peaks in the 2D NMR spectra were integrated to calculate the amounts of the major lignin subunits. Table 3 summarizes the NMR integral data and absolute quantification results for the three major lignin subunits found in trembling aspen wood, loblolly pine wood, and wheat straw.

From the results in Table 3, the quantities of cell wall polymer structures in wood and plants can be easily compared to each other. The aspen was found to have more than double the amount of β-aryl ethers and approximately 20 times as many resinols of both the pine and the straw. On the other hand, the pine was found to have approximately 18 times and 8 times as many phenylcoumarans as aspen and straw, respectively. This analysis gives a clearer understanding of how these major lignin subunits are distributed in different wood and plant species.

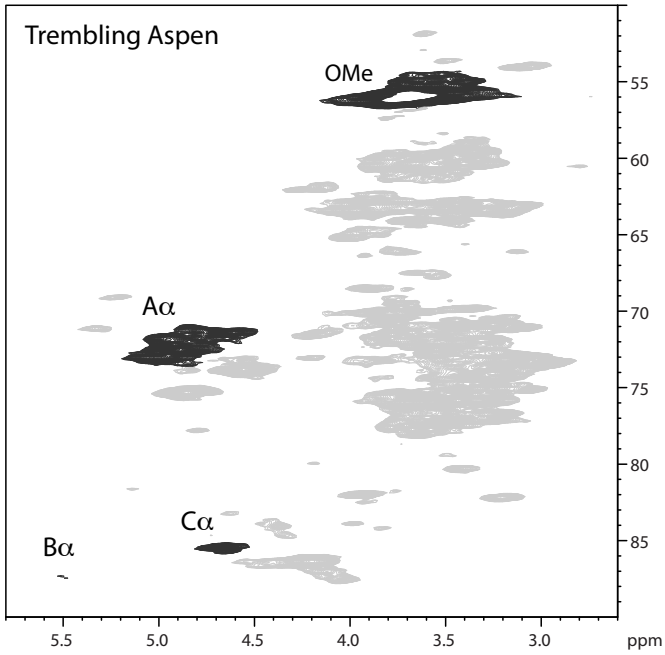

Figure 1—Partial 2D NMR spectrum of nonderivatized trembling aspen wood cell walls. The horizontal axis represents the ^{1}H dimension, and the vertical axis represents the ^{13}C dimension. The letters A, B, and C (along with their respective darkened contour peak) represent the lignin subunit structures as shown in Figure 4. The methoxyl (OMe) Aα, Bα, and Cα peaks were integrated, and these values were placed into Table 3.

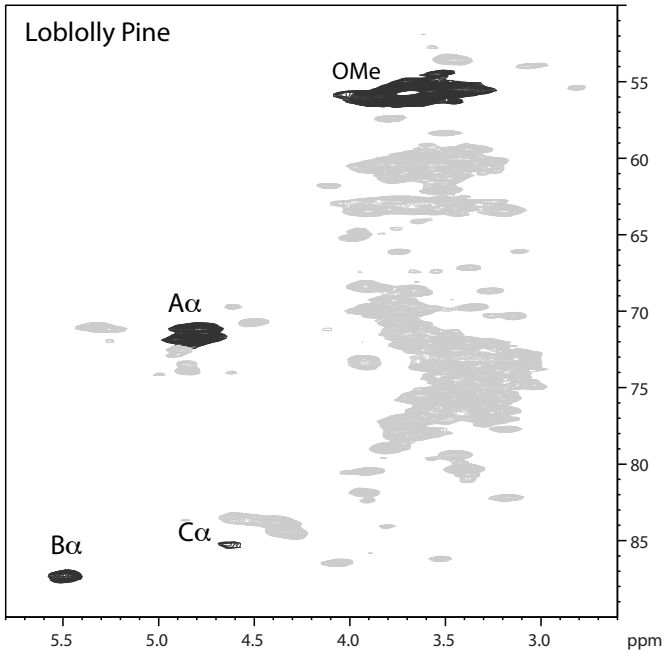

Figure 2—Partial 2D NMR spectrum of nonderivatized loblolly pine wood cell walls. The horizontal axis represents the ^{1}H dimension, and the vertical axis represents the ^{13}C dimension. The letters A, B, and C (along with their respective darkened contour peak) represent the lignin subunit structures as shown in Figure 4. The methoxyl (OMe) Aα, Bα, and Cα peaks were integrated, and these values were placed into Table 3.

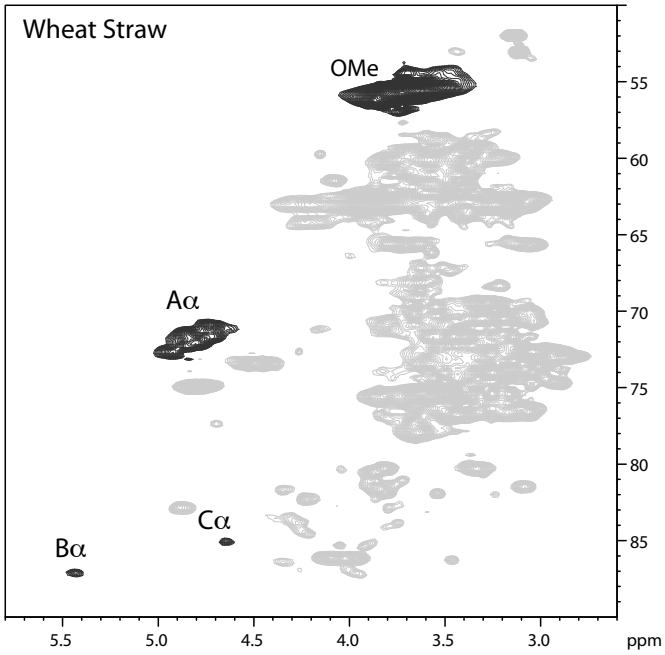

Figure 3—Partial 2D NMR spectrum of nonderivatized wheat straw plant cell walls. The horizontal axis represents the ^{1}H dimension and the vertical axis represents the ^{13}C dimension. The letters A, B, and C (along with their respective darkened contour peak) represent the lignin subunit structures as shown in Figure 4. The methoxyl (OMe) Aα, Bα, and Cα peaks were integrated, and these values were placed into Table 3.

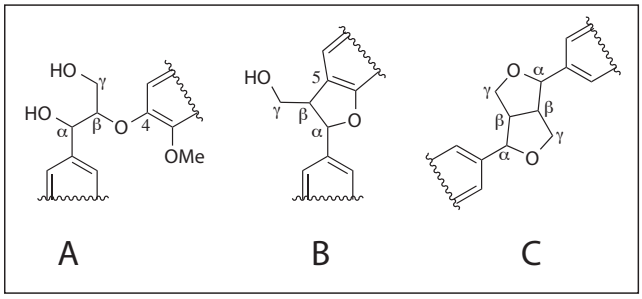

Figure 4—The three major subunits found in lignin. These chemical structures are represented in Figures 1 to 3 and in Table 3. Structure A corresponds to b-aryl ethers, structure B corresponds to phenylcoumarans, and structure C corresponds to resinols.

Further research is needed to expand this analysis to explore more wood and plant species. Moreover, other tissues (such as earlywood, latewood, reaction wood), cell types (such as vessel, tracheid, parenchyma), and structures in the cell wall will need to be explored and quantified so that a more comprehensive understanding of cell wall chemistry can be established.

Conclusions

Quantifying the detailed chemistry of wood and plant cell walls has been one of the most challenging tasks faced by wood and plant chemists. The technique described here allows researchers to use the accuracy and precision of a well-established wet chemical method, the titrimetric methoxyl analysis, but use modern GC–MS instrumentation to increase sample capacity, speed, and robustness of the analysis. Several wood and plant cell wall species were able to be analyzed for methoxyl content with good accuracy and precision. When the GC–MS methoxyl analysis was combined with 2D NMR spectroscopy, the analysis of cell wall structures became much more quantitative. GC–MS–NMR analysis of trembling aspen, loblolly pine, and wheat straw cell walls allows for a more quantitative approach to understanding the structural chemistry of the cell wall. With this technique, any major or minor changes that might occur to the cell wall during modifications (such as biological decay, chemical modification, biomass pre-treatments) can be determined.

Literature Cited

Baker, S.M. 1996. Rapid methoxyl analysis of lignins using gas chromatography. Holzforschung. 50(6): 573–574.

Girardin, M; Metche, M. 1983. Fast micro-determination of alkoxyl groups by gas chromatography: application to lignin. Journal of Chromatography. 264: 155–158.

Goodell, B.; Jellison, J.; Liu, J.; Daniel, G.; Paszczynski, A.; Fekete, F.; Krishnamurthy, S.; Jun, L.; Xu, G. 1997. Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal

Table 3—Absolute quantification of lignin subunits from selected wood and plant cell wall lignocellulosics[a]

	Trembling aspen		Loblolly pine		Wheat straw	
Lignin structure	Integral Hα/Cα peak	Absolute value (µmol/g wood)	Integral Hα/Cα peak	Absolute value (µmol/g wood)	Integral Hα/Cα peak	Absolute value (µmol/g wood)
β-aryl ether (A)	0.5157	797	0.2874	343	0.2919	291
Phenylcoumaran (B)	0.0018	2.8	0.0447	53	0.0063	6.3
Resinol (C)	0.0522	81	0.0036	4.3	0.0045	4.5

[a]Absolute values were calculated by the following equation:
Lignin subunits (µmol/g wood) = [integral of Hα/Cα peak relative to OMe peak] × [ave. OMe content (µmol OMe/g wood)]

biodegradation of wood. Journal of Biotechnology. 53(2–3): 133–162.

Goto, H.; Koda, K.; Tong, G.; Matsumoto, Y.; Meshitsuka, G. 2005. Formation of methyl iodide from methoxyl-free compounds by hydriodic acid treatment. Journal of Wood Science. 51: 312–314.

Goto, H.; Koda, K.; Tong, G.; Matsumoto, Y.; Meshitsuka, G. 2006. Interference of carbohydrates in the determination of the methoxyl content of lignin in woody samples. Journal of Wood Chemistry and Technology. 26: 81–93.

Hodges, K.L.; Kester, W.E.; Weiderrich, D.L.; Grover, J.A. 1979. Determination of alkoxyl substitution in cellulose ethers by Zeisel-gas chromatography. Analytical Chemistry. 51: 2172–2176.

Kim, H.; Ralph, J. 2010. Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d6/pyridine-d5. Organic and Biomolecular Chemistry. 8(3): 576–591

Kim, H.; Ralph, J.; Akiyama, T. 2008. Solution-stage 2D NMR of ball-milled plant cell wall gels in DMSO-d6. Bioenergy Research. 1: 56–66.

Kupče, E.; Freeman, R. 2007. Compensated adiabatic inversion pulses: broadband INEPT and HSQC. Journal of Magnetic Resonance. 187: 258–265.

Lan, W.; Lu, F.; Regner, M.; Zhu, Y.; Rencoret, J.; Ralph, S.A.; Zakai, U.I.; Morreel, K.; Boerjan, W.; Ralph, J. 2015. Tricin, a flavonoid monomer in monocot lignification. Plant Physiology. 167(4): 1284–1295.

Li, H.; Yelle, D.J.; Li, C.; Yang, M.; Ke, J.; Zhang, R.; Liu, Y.; Zhu, N.; Liang, S.; Mo, X.; Ralph, J. 2017. Lignocellulose pretreatment in a fungus-cultivating termite. Proceedings of the National Academy of Sciences. 114(18): 4709–4714.

Lu, F.; Ralph, J. 2003. Non-degradative dissolution and acetylation of ball-milled plant cell walls: high-resolution solution-state NMR. Plant Journal. 35: 535–544.

Lundquist, K.; Lundgren, R. 1972. Acid degradation of lignin. Part VII. The cleavage of ether bonds. Acta Chemica Scandinavica. 26(5): 2005–2023.

Ralph, J.; Marita, J.M.; Ralph, S.A.; Hatfield, R.; Lu, F.; Ede, R.M.; Peng, J.P.; Quideau, S.; Helm, R.F.; Grabber, J.; Kim, H.; Jimenez-Monteon, G.; Zhang, Y.S.; Jung, H.J.; Landucci, L.L.; MacKay, J.; Sederoff, R.R.; Chapple, C.; Boudet, A.M. 1999. Solution-state NMR of lignins. In: Argyropoulos, D. (ed.). Advances in lignocellulosics characterization. Atlanta, GA: TAPPI Press: 55–108.

Shtreis, G.B.; Bobkova, V.V. 1973. Determination of methoxyl groups in wood and lignin and its model compounds by gas-liquid chromatography. Khimiya Drevesiny 14: 72–75.

Vieböck, F.; Schwappach, A. 1930. Eine neue Methode zur massanalytischen Bestimmung der Methoxyl- und Athoxylgruppe I. Makro-Analyse. Berichte der Deutschen Chemischen Gesellschaft. 63: 2818–2823.

Yelle, D.J.; Ralph, J. 2016. Characterizing phenol-formaldehyde adhesive cure chemistry within the wood cell wall. International Journal of Adhesion and Adhesives. 70: 26–36.

Yelle, D.J.; Ralph, J.; Frihart, C.R. 2008a. Characterization of nonderivatized plant cell walls using high-resolution solution-state NMR spectroscopy. Magnetic Resonance in Chemistry. 46: 508–517.

Yelle, D.J.; Ralph, J.; Lu, F.C.; Hammel, K.E. 2008b. Evidence for cleavage of lignin by a brown rot basidiomycete. Environmental Microbiology. 10: 1844–1849.

Yelle, D.J.; Ralph, J.; Frihart, C. 2011a. Delineating pMDI model reactions with loblolly pine via solution-state NMR spectroscopy. Part 2. Non-catalyzed reactions with the wood cell wall. Holzforschung. 65(2): 145–154.

Yelle, D.J.; Wei, D.; Ralph, J.; Hammel, K.E. 2011b. Multidimensional NMR analysis reveals truncated lignin structures in wood decayed by the brown rot basidiomycete Postia placenta. Environmental Microbiology. 13(4): 1091–1100.

Yelle, D.J.; Kaparaju, P.; Hunt, C.G.; Hirth, K.; Kim, H.; Ralph, J.; Felby, C. 2013. Two-dimensional NMR evidence for cleavage of lignin and xylan substituents in wheat straw through hydrothermal pretreatment and enzymatic hydrolysis. BioEnergy Research. 6: 211–221.

Zeisel, S. 1885. Über ein Verfahren zum quantitativen Nachweis von Methoxyl. Monatshefte für Chemie. 6: 989–996.

Zhang, L.M.; Gellerstedt, G. 2007. Quantitative 2D HSQC NMR determination of polymer structures by selecting suitable internal standard references. Magnetic Resonance in Chemistry. 45: 37–45.

Zhang, W.; Sathitsuksanoh, N.; Simmons, B.A.; Frazier, C.E.; Barone, J.R.; Renneckar, S. 2016. Revealing the thermal sensitivity of lignin during glycerol thermal processing through structural analysis. RSC Advances. 6(36): 30234–30246.