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A ^{13}C CP/MAS-Based Nondegradative Method for Lignin Content Analysis

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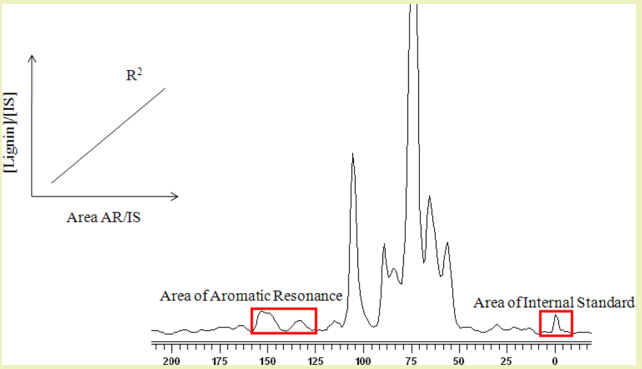
ABSTRACT: Most current lignin content analysis methods require dissociation/degradation and/or preconditioning steps. A simple yet reliable method that can be used to analyze content of various lignocellulosic biomasses without destroying their native structure is needed. In this research effort, an improved nondegradative method for determining the content of lignin in native biomass was established. By extending the scope of the solid state ^{13}C CP/MAS NMR technique, via homogeneous mixing of an internal standard (TMSP) with varying lignin contents, a standard curve was generated for calculating the lignin content of various biomasses without dissociation or degradation. The results showed good accuracy in the calculation of lignin content. Compared to chemical degradative methods, such as the acetyl bromide method, the relative difference was less than 5.00%. This method is applicable for estimating lignin content in certain samples (e.g., acid insoluble lignin) that are difficult to analyze accurately with chemical methods. By offering an alternative methodology for lignin content estimation using solid state ^{13}C CP/MAS NMR with an internal standard, this study also demonstrated the quantitative potential of solid state ^{13}C CP/MAS NMR when coupled with the internal standard (TMSP). The proposed procedure fills a current technological gap in lignocellulosic biomass analysis.



KEYWORDS: *Lignin content analysis, Nondegradative, Solid state NMR, ^{13}C CP/MAS, TMSP*

INTRODUCTION

Together with cellulose, lignin belongs to the most abundantly occurring renewable resources on earth.[1] Lignin exists in widely available biomass such as trees, plants, and agricultural crops. It serves to facilitate water transport, protect plants against chemical and biological attacks, and provide structural integrity.[2]

There are diverse structures of lignin in nature. The heterogeneity of lignin is caused by variations in the polymer composition, functional groups, and cross-linking.[1] Differences exist in the molecular composition and linkage type between the three basic phenylpropanoid monomers, *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, derived from coumaryl, coniferyl, and sinapyl alcohol precursors, respectively.[3,4] Lignins contain several chemical functional groups, such as phenolic hydroxyl or alcoholic hydroxyl, methoxyl, carbonyl, and carboxyl, in various amounts and proportions, depending on origin and the applied isolation process.[1,5] The coupling linking, such as aryglycerol-β-ether dimer (β-O-4), pino/resinol (β-5), phenylcoumaran ($\beta-\beta'$), spirodienon, diphenylethane (β-1), and dibenzodioxin, constructs an irregular three-dimensional reticulate structure of lignin.[6,7]

Because of multitudinous different lignin structures, it is not easy to analyze lignin content accurately. Currently, the most widely applied processes for lignin content analysis are the degradative methods. Generally, there are two commonly used chemical methods for estimating lignin content, the Klason method and acetyl bromide analysis (AcBr) method.[8−10] In the Klason method, biomass samples are partial degraded in 72% H_2SO_4 solution, leaving an insoluble residue.[11] However, based on Lai and Sarkanen's results, the Klason method greatly depends on the type of sample[12] and is mainly suitable for herbaceous plants, as the coprecipitation of proteinaceous substances and nonlignin inorganic components may affect the results. Acetyl bromide analysis is a widely accepted method for estimating the lignin content in various plant biomasses. It relies on solubilization of lignin fragments. However, if there is nonlignin UV-absorbing substances solubilized in the samples, significant overestimation could happen. Another concern is that it has not been established whether the extinction coefficient in AcBr analysis can be used for all lignin sources.[12] Some researchers also used monomeric compositions of lignins for the purpose of quantification, such as *pyrolysis* GC/MS,[13] nitrobenzene oxidation,[14] and thioacidolysis.[15] However, the *p*-

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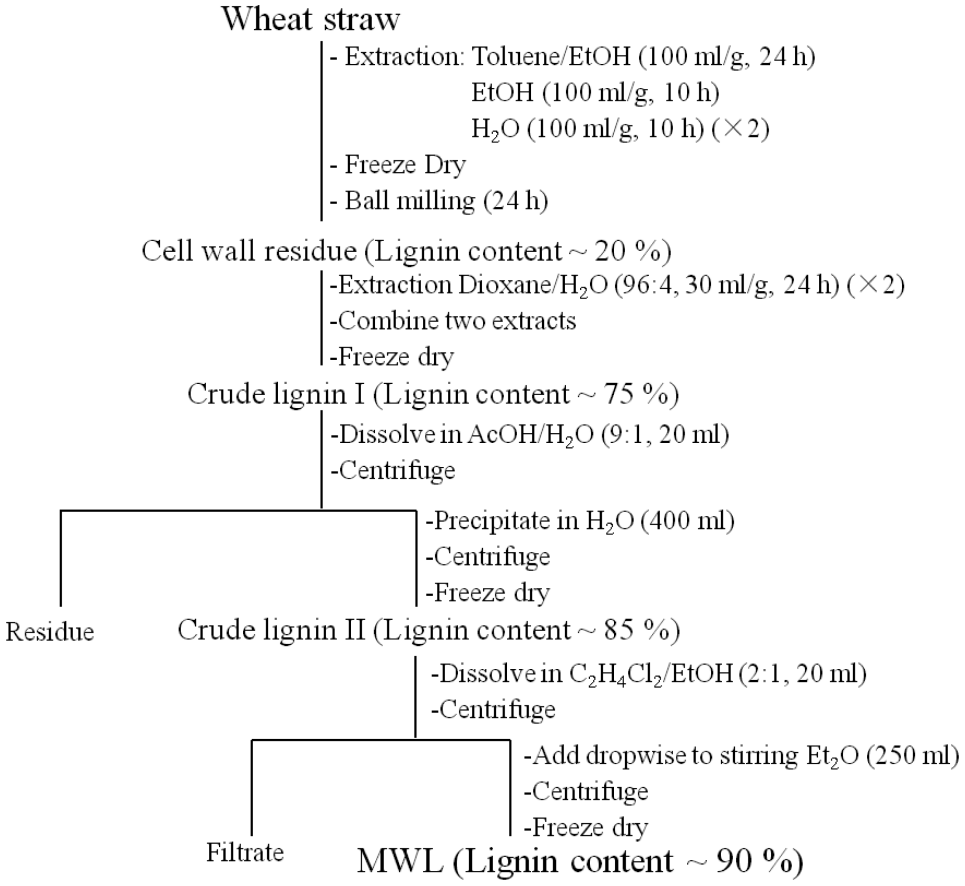

Figure 1. Modified Björkman method to extract pure lignin (MWL).

hydroxyphenyl components (H unit) of lignin cannot be accurately detected with any of these methods; accurate quantification of the S/G values is also inadequate. Recently, Patrick Louchouarn et al.[16] used gas chromatography/tandem mass spectrometry to analyze the lignin-derived phenols in standard reference materials and ocean-dissolved organic matter. Although this is not available for solid lignin, tandem mass spectrometry provided both the high sensitivity and selectivity required for identification and quantification of trace levels of dissolved lignin. Lignin monomers were also quantified by gas chromatography (GC)/flame ionization detection (FID) after release by CuO oxidation, and ^{13}C content was determined by GC coupled via a combustion interface to isotope ratio mass spectrometry (GC/C-IRMS). Haithem Bahri et al.[17] calculated the proportion of C4-derived organic carbon (OC) in lignin monomer pools by applying the isotopic mass balance equation to each lignin monomer. A further quantitative description of the turnover kinetics of different lignin monomers needs to be performed using such molecular data to develop a quantitative dynamic model.

In addition, different infrared and near-infrared techniques have been utilized for the determination of lignin content,[18] such as diffuse reflectance Fourier transform infrared spectrometry,[19] near-infrared reflectance spectroscopy,[20] and transmittance near-infrared spectroscopy.[21] Gordon G. Allison et al.[22] quantified lignin in perennial forage and energy grasses by Fourier transform infrared spectroscopy (FTIR) and partial least-squares (PLS) regression. Their study demonstrated that FTIR coupled with PLS was too sensitive for use in lignin prediction and estimation models. Although near-/infrared methods are fast, the models were not stable or accurate enough and were only appropriate for specific samples, as those used to establish the model. Similarly, other attempts were also made, such as nitrogen immobilization and the semi-micro method.[23,24]

Solid state ^{13}C CP/MAS NMR methods have been proven to be useful techniques in the investigation of lignified plant materials.[25−28] Love et al.[29] determined the aromatic lignin content in oak wood at relatively low field strength to obviate the aromatic carbons discriminated against in lignin with spinning side bands using both CP and Bloch decay or single pulse excitation (SPE). They further applied ^{13}C CP/MAS solid state NMR spectroscopy to plant materials of very low lignin content. Their work was the first to illustrate the quantitative potential of solid state ^{13}C NMR. However, the results were time consuming, and the values were considerably overestimated. The aromatic material presented was not lignin but appeared to be predominantly an anthocyanin of the cyanidin type, with the quantity corresponding to a lignin content of 0.9%.[30] Sievers et al.[31] also proved that it was possible to utilize solid state NMR to quantitatively analyze the residues from acid hydrolysis of loblolly pine wood. Recently, Capanema et al.[32] suggested an approach for the quantification of different lignin structures in milled wood lignin (MWL) by using a combination of NMR techniques. About 80% of the side chain moieties, such as different β-O-4, dibenzodioxocin, phenylcoumaran, pinoresinol, and others, were identified on the molecular structural level. Also, the presence of appreciable amounts of R-O-alkyl and C-O-alkyl ethers was suggested. A comparison of calculated results with known databases on the

spruce MWL structure showed that this approach was rather informative and comparable to information obtained from the combination of various wet chemistry lignin analysis methods.

However, the degradative step is required for all present solid state ^{13}C NMR methods. Moreover, the methods cannot be broadly applied to different biomasses and accuracy is barely satisfactory.[29−32] The precision is not as good as that obtained with the chemical methods either. Therefore, a modified methodology to directly quantify lignin and its derived compounds without dissociation or degradation of biomass presents a technical gap.

The present study was performed to address the need for accurate lignin analysis without destroying lignin's structure but also having wide applicability. In order to employ the ^{13}C NMR for quantitative analysis, adding the proper internal standard was necessary. After screening and preliminary tests, sodium-3-trimethylsilylpropionate (TMSP) was selected as the suitable internal standard (IS), which also had advantages in homogeneous mixing with the biomass samples during NMR acquisition. A simple standard curve was established using solid state ^{13}C CP/MAS NMR together with an internal standard, as described next. After establishing the standard curve and response factor under solid state ^{13}C NMR analysis with varying lignin contents, the ratio of the normalized area of lignin versus that of the internal standard was plotted linearly, and the response factor for concentration was obtained using the corresponding linear equation. Internal validation showed that the presently developed method was reliable and stable. For the tested samples, the results showed high accuracy in the determination of their lignin contents when compared with existing chemical methods. This study illustrates the quantitative potential of solid state ^{13}C CP/MAS NMR when coupled with internal standard (TMSP). This method can be used to accurately and reliably determine lignin content from various lignocellulosic biomasses without destroying their native structure.

MATERIALS AND METHODS

Preparation of Milled Wood Lignin (MWL). The modified Björkman method[33−37] was employed to obtain pure lignin (MWL) (Figure 1). The modified Bjiirkman method proved suitable for removing most of the carbohydrate fraction in the sample (less than 4% left), although some minor modifications (demethylations) seemed to take place in the resulting purified lignin. Also, this purification procedure made it possible to obtain a lignin preparation very close to the natural polymer called milled wood lignin (MWL).[38] The lignin source used in this study was wheat straw. After the wheat straw was hammer milled, the sawdust (10 mesh, 2.00 mm) was extracted with ethanol/toluene (1:2 (v/v) for 24 h), then extracted by ethanol for 10 h, and at last by distilled water for 10 h twice. After freeze-dried the sample, the extracted sawdust was then subjected to dry milling (24 h) in a zirconium planetary ball mill under a nitrogen atmosphere and with a temperature control. Then the control wheat cell wall residue (CWR) was ready (60 mesh, 0.25 mm) to be processed further. The lignin content of the CWR at this stage was about 20%.

The CWR was further extracted using dioxane/distilled water (96:4, 30 mL/g) for 24 h twice; then the two extracts were combined and concentrated. After being freeze-dried, crude lignin I was obtained, with a lignin content around 75%. Next, crude lignin I was dissolved into 20 mL AcOH/distilled water (9:1, v/v). After being centrifuged, the supernatant was added drop by drop into 400 mL distilled water to precipitate overnight in a 4 °C refrigerator. After being centrifuged and freeze-dried, around 1.2 g of crude lignin II with about 85% lignin content was extracted. In the last step, crude lignin II was dissolved in 20 mL $C_2H_4Cl_2$/ethanol (2:1, v/v); then the supernatant was added after centrifuging drop by drop into 250 mL diethyl ether to precipitate overnight in the 4 °C refrigerator. After being centrifuged and freeze-dried, finally about 0.7 g of milled wood lignin (MWL) was obtained, with a lignin content of above 90%.

Sample Preparation. The extracted 0.7 g of MWL was used along with Avicel, xylan, and pectin to prepare nine samples, as shown in Table 1. Six samples (1−6) were used as model samples for establishing the method. Three samples (I, II, and III) were used as internal validation samples to test the reliability and stability of the method samples.

Table 1. Content of Samples for Method Establishment (1−6) and Internal Validation (I−III)

no.	MWL (g)	Avicel (g)	xylan (g)	pectin (g)	total (g)
1	0.2251	0.0000	0.0000	0.0000	0.2251
2	0.1265	0.0757	0.0243	0.0255	0.2520
3	0.0755	0.1260	0.0261	0.0255	0.2531
4	0.0501	0.1492	0.0247	0.0265	0.2505
5	0.0250	0.1752	0.0255	0.0245	0.2502
6	0.0000	0.1995	0.0264	0.0255	0.2514
I	0.0776	0.1219	0.0266	0.0259	0.2520
II	0.0583	0.1417	0.0246	0.0251	0.2497
III	0.0298	0.1701	0.0251	0.0245	0.2495

Six additional samples were used as test samples, also called external validation samples (Table 2), which were used to verify the accuracy of prediction and verification of the standard curve. On the other hand, the native wheat straw and poplar samples were just hammer milled without any chemical or biological pretreatment.

Table 2. Content of Test Samples for Method Prediction and Verification

sample	origin	weight (g)
native wheat straw	wheat straw	0.2498
cell wall residue	wheat straw	0.2501
acid insoluble lignin	wheat straw	0.2491
native poplar	poplar	0.2503
white rot fungi treated	wheat straw	0.2498
Streptomyces viridosporus T7A treated	wheat straw	0.2500

Acetyl Bromide (AcBr) Analysis: Lignin Content in Samples. To quantitatively determine the lignin content, modified AcBr analysis was performed for all samples.[9,38−40] Each sample (30 mg) was individually treated with a reaction mixture consisting of 25% AcBr (v/v) in glacial acetic acid containing 4% perchloric acid and then placed in an oven at 70 °C for 30 min after sealed. Then the solutions were transferred to volumetric flasks containing 2 M NaOH (10 mL) and glacial acetic acid (12 mL). Each sample was rinsed with a minimum amount of acetic acid, making the solution up to 50 mL with glacial acetic acid, while corresponding solubilized materials were individually measured for UV absorptivity (I = 280 nm).[9,38,39] At last, an extinction coefficient of 20.09 L/g/cm[9,40] was used to estimate lignin content. The lignin content of the extracted MWL, native wheat straw, cell wall residue sample, acid insoluble lignin sample, and native poplar was analyzed, respectively.

Method in Study for Adding TMSP to Samples. For quantitative analysis using solid state ^{13}C NMR, an internal standard was added to each sample before running NMR analysis. The optimal internal standard included the following characterizations:

(1) Presents strong signal(s) in the solid state ^{13}C CP/MAS NMR spectra.
(2) Easy to analyze peak(s) of internal standard among sample spectra; peak(s) does not overlap with targeted sample peaks.
(3) Homogeneous distribution in sample.

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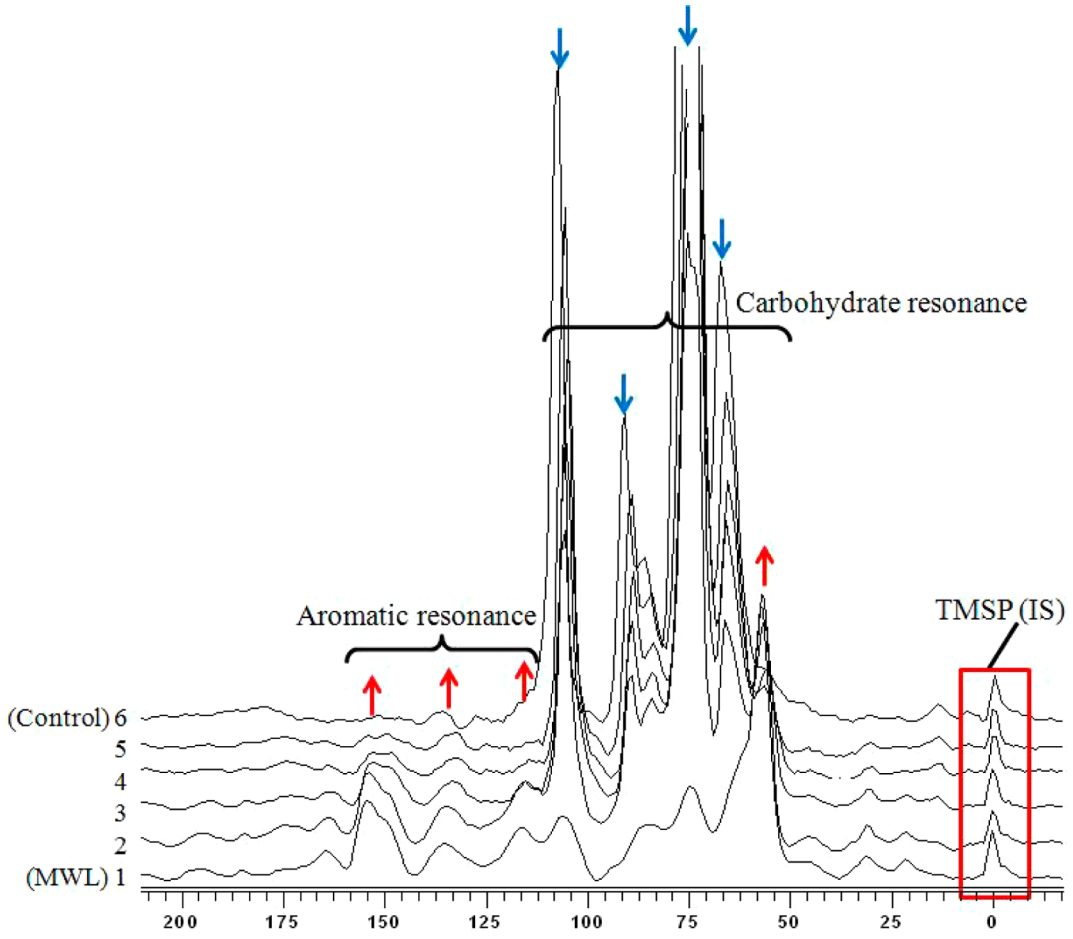

Figure 2. Solid state ^{13}C CP/MAS NMR spectra of standard curve-establishing samples.

TMSP-2,2,3,3-D4 (D, 98%) (sodium-3-trimethylsilylpropionate) was chosen in this study as the internal standard because of its structural features. The major peak of TMSP in the solid state ^{13}C CP/MAS NMR spectra is 0 ppm. The signal is adequately strong, without interference from other sample signals. In particular, TMSP is very easy to dissolve in distilled water. With TMSP as the internal standard, a simple technique was needed to address the problem of homogeneous distribution of the internal standard in each sample. The method demonstrating how TMSP was added to the samples is as follows. TMSP (0.1000 g) was weighed and added to 1.00 mL of distilled water to make the 0.100 g/mL TMSP solution. A 100.0 μL aliquot of this solution was added to each 250 mg sample, and then 0.5 to 1.0 mL of distilled water was added to make sure the water level and solid sample were at the same level. This was done in order to properly disperse the TMSP in each sample. In some cases, the distilled water did not infuse in the solid sample quickly enough; for such samples, the mixture was centrifuged for 5 to 10 s at 3000 rpm. Finally, the solid state NMR analysis was performed after the samples were freeze-dried.

Solid State ^{13}C CP/MAS NMR Analysis. Approximately 125 mg of each sample was individually packed into a 5 mm pencil-type rotor (Revolution NMR, LLC, AMP4089-001 5 mm, 160 ul, Rotor Assembly, Teflon Caps, Zirconia, CS); then the ^{13}C CP/MAS spectra of the samples were recorded under the same acquisition parameters, respectively. The solid state ^{13}C CP/MAS analyses (400 MHz) were carried out at the NMR Center, Washington State University, using a Bruker Avance 400 spectrometer; (Bruker AXS, Inc., Madison, WI, U.S.A.), equipped with a double resonance probe (Chemagnetics, Varian, Inc., Palo Alto, CA, U.S.A.). For acquisition of ^{13}C CP/MAS NMR spectra, a contact time of 0.5 ms, a proton field of approximately 40 kHz during CP and data acquisition, a relaxation delay of 4 s, and a spinning speed of 5 kHz were used. The spectrum shown in the results were derived from 20,480 scans, with the chemical shifts given as δ ppm.

RESULTS AND DISCUSSION

Acetyl Bromide (AcBr) Analysis Results. The lignin contents of all samples listed in Tables 1 and 2 were estimated using the AcBr method described earlier.[4,27] Upon applying the standard extinction coefficient to the AcBr analysis, the lignin content was found to be 91.30 ± 0.01% for the extracted pure lignin (MWL), 19.18 ± 0.03% for the native wheat straw, 18.58 ± 0.02% for the cell wall residual sample of wheat straw, 21.08 ± 0.03% for native poplar, 13.18 ± 0.01% for white rot fungi treated wheat straw, and 14.98 ± 0.01% for the *Streptomyces viridosporus T7A* (strep)-treated wheat straw, respectively. It should be noticed here that the AcBr method is not suitable for analyzing the acid insoluble lignin sample, as the result is always overestimated due to the interferential UV absorption around 280 nm.[38]

Quantitative Solid State ^{13}C NMR Standard Curve. *Solid State ^{13}C CP/MAS NMR Results.* In this experiment, MestreC software was used to analyze the NMR spectra and data. Figure 2 shows the solid state ^{13}C CP/MAS NMR spectra of the six standard curve-establishing samples after the correction with noise to signal ratios. Figure 2 also shows the increasing MWL amount of each sample from top to bottom. The top one is that of the control sample without lignin, only Avicel, xylan, and pectin were contained; the bottom one is the pure MWL sample with lignin content of 91.30%.

The spectra showed characteristic peaks that correspond to components of cellulose, hemicellulose, and lignin. The three regions outlined showed the aromatic, carbohydrate resonances, and the internal standard. In the spectra, the peaks (red arrows) show an increase, while the lignin content increases and the blue arrows (pointed peaks) decrease as the lignin content increases. From the NMR spectra, it can be seen that δ_c = 0 ppm is the internal standard (TMSP), and its peaks in the six spectra look similar, which means the collected spectra were reliable. Distinct differences could be observed around δ_c = 110−160 ppm, which is the resonance of most aromatic lignin. The results suggested the increased lignin contents in the samples (1−6) in Table 1. The results also indicated the cleavage of the lignin polymeric framework. In this range, the resonance signal around δ_c = 110 is due to unsubstituted aromatic carbons *ortho-* or *para-* to substituted carbon. The signals at δ_c = 130−140 represent C- or H-substituted aromatic carbon. The O-substituted aromatic carbon of guaiacyl units displayed around δ_c = 150−160.[41,42] Also, δ_c = 50−110 ppm is the main cellulose and hemicellulose region, which shows the carbohydrate resonances. It is clear that the peaks changed regularly, due to the lignin content variation in different samples. Moreover, Table 3[41−45] shows the chemical shift values and assignments of the lignin samples spectra in detail.

Table 3. Chemical Shift Values and Assignments of Lignin Samples' Spectra

chemical shift δ_c (ppm)	forms of carbon resonance
0	TMSP (internal standard)
15−35	aliphatic triglycerides and fatty acid derivatives
17	methyl carbon
30−32	alkyl carbon in long chain polymethylene type structures
56	methoxyl group of aromatic ring of G unit
57−60	methoxyl region
62−64	C-2, C-3, and C-5 of cellulose
65	C-6 sugar
90	C-4 sugar
50−110	cellulose and hemicellulose region
72 and 105	ring carbons of carbohydrates
110−160	lignin aromatic region
109	anomeric sugar C-1
110	unsubstituted aromatic carbons *ortho-* or *para-* to substituted carbon
130−140	C- or H-substituted aromatic carbon
150−160	O-substituted aromatic carbon of guaiacyl/syringyl units
160−175	carboxylic, amide, and ester groups
180	aromatic carbons connected to methoxy groups in syringyl units

Standard Curve Data and Establishment. Figure 3 shows the strategy for using the spectra to establish the quantitative NMR standard curve. In the aromatic resonance area, the two peaks are around δ = 150 and 130 ppm and mostly from the lignin aromatic region. Thus, the integrals of these specific areas were obtained by MestreC, and the area ratios of the aromatic resonance versus the internal standard were calculated. Then the area ratio was plotted on the *X* axis and the ratio of the concentration of lignin versus internal standard on the *Y* axis. In this manner, the standard curve was established.

Table 4 shows the data of the initial standard curve. Here, the area of the internal standard was normalized to 1.00. After the calculation of the actual lignin content in samples, the standard

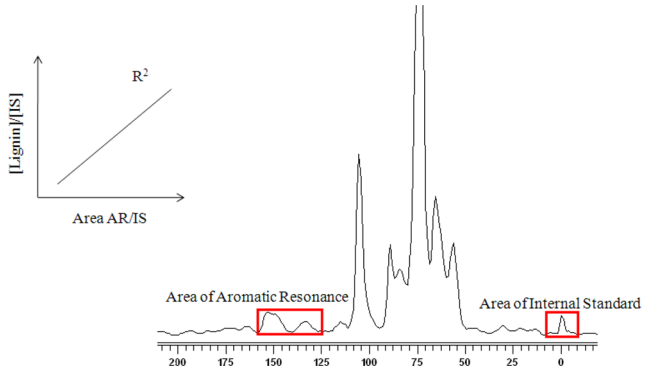

Figure 3. Strategy for lignin quantitative analysis and model establishment.

Table 4. Response Factors of Selected Aromatic Regions vs Internal Standard[a,b]

area AR	area IS[c]	area (AR/IS)	[lignin] (mg)	[IS] (mg/mL)	[lignin]/[IS] (mL)
1.13	1.00	1.13	0.00	10.00	0.00
1.11	1.00	1.11	0.00	10.00	0.00
3.23	1.00	3.23	22.83	10.00	2.28
3.32	1.00	3.32	22.83	10.00	2.28
3.93	1.00	3.93	45.74	10.00	4.57
4.06	1.00	4.06	45.74	10.00	4.57
6.37	1.00	6.37	68.93	10.00	6.89
6.48	1.00	6.48	68.93	10.00	6.89
10.70	1.00	10.7	115.49	10.00	11.55
10.76	1.00	10.7	115.49	10.00	11.55
18.08	1.00	18.08	205.52	10.00	20.55
18.41	1.00	18.41	205.52	10.00	20.55

[a]Extrapolation of ratio of area vs ratio of concentration. [b]Slope and/or gradient was calculated using the linear equation, $y = mx$. [c]Normalized IS area to 1.00.

curve with the ratio of the area and the ratio of the concentration was generated, as shown in Figure 4. The linear equation is $y = 1.1004x$, and $R^2 = 0.9869$.

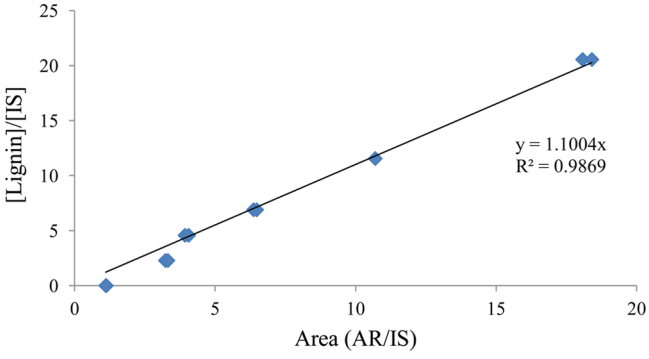

Figure 4. Initial established standard curve.

A problem that needs addressed is shown in Figure 2. The top spectrum, representing the control sample, shows the existence of extremely low intensity nonlignin-derived small peaks (down to noise level), specifically around $\delta_c \sim$ 140 ppm. Thus, it was necessary to correct the area of aromatic resonance by subtracting the area of AR of the control sample from the other five samples (Tables 5 and 6). Otherwise, when lignin content is zero, the area of aromatic resonance with the

Table 5. Corrected Area of Aromatic Resonance

area AR with 0 mg lignin	area AR	corrected area AR	[lignin] (mg)
1.13	3.23	2.10	22.83
1.11	3.32	2.21	22.83
	3.93	2.80	45.74
	4.06	2.95	45.74
	6.37	5.24	68.93
	6.48	5.37	68.93
	10.70	9.57	115.49
	10.70	9.59	115.49
	18.08	16.95	205.52
	18.41	17.30	205.52

Table 6. Corrected Response Factors of Selected Aromatic Regions vs Internal Standard

area AR	area IS	area AR/area IS	[lignin] (mg)	[IS] (mg/mL)	[lignin]/[IS] (mL)
2.10	1.00	2.10	22.83	10.00	2.28
2.21	1.00	2.21	22.83	10.00	2.28
2.80	1.00	2.80	45.74	10.00	4.57
2.95	1.00	2.95	45.74	10.00	4.57
5.24	1.00	5.24	68.93	10.00	6.89
5.37	1.00	5.37	68.93	10.00	6.89
9.57	1.00	9.57	115.49	10.00	11.55
9.59	1.00	9.59	115.49	10.00	11.55
16.95	1.00	16.95	205.52	10.00	20.55
17.30	1.00	17.30	205.52	10.00	20.55

equation of the standard curve is $y = 1.1703x + 0.4984$. Such a plot does not intercept the zero in the xy-axis.

Figure 5 shows the corrected standard curve. The value of R^2 is 0.9944 versus 0.9869 after correction.

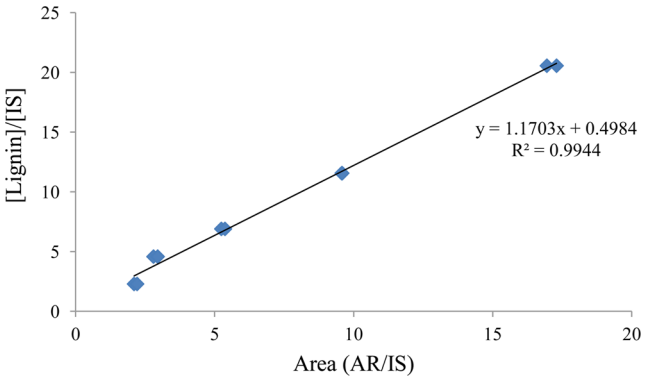

Figure 5. Corrected standard curve.

Standard Curve Verification and Prediction. In order to verify the reliability and stability of the established standard curve,[46] three internal validation samples (I, II, and III, Table 1) were used for the test. Figure 6 depicts the three NMR spectra of the internal validation samples. From top to bottom, the samples are I, II, and III, respectively, with decreasing lignin contents.

At first, the calibration method, without applying the internal standard, was employed to test this approach. In this case, the areas of the aromatic region were just integrated without normalizing the TMSP area to 1.00. Similar to Sievers et al.'s results,[31] from Figure 7, the good agreement between the estimated concentrations and the actual composition of the samples indicates that the quantification of the composition of these samples was accurate ($R^2 = 0.9994$). Therefore, it can be concluded that ^{13}C CP/MAS NMR provides quantitative information in this case.

However, the accuracy of this calibration method was not satisfactory. For the three internal validation samples, the lignin contents were within the error margin, from 1.72 to 4.42 wt %. Therefore, it is necessary to apply the internal standard to verify the reliability and stability of the established standard curve. Table 7 shows the response factors of the three samples. From Table 8, it is evident that the relative error is no more than 1.00% between the predicted results from the standard curve and the AcBr analysis results, verifying the reliability and stability of the standard curve. The results demonstrate that solid state ^{13}C CP/MAS NMR coupled with internal standard has the potential for determining lignin content precisely.

It was necessary to verify the accuracy of the standard curve. Six different test samples were used as external validation samples for the prediction and verification. Table 9 shows the actual lignin content of the test samples after calculation. As mentioned before, it is very difficult to determine the correct lignin content of the acid insoluble lignin by employing the AcBr method; thus, it is left blank here. Besides, the NMR spectra of these samples shown in Figure 8 were all achieved under similar acquisition parameters.

Subsequently, the integral values of aromatic region (δ 160–130 ppm) and internal standard region (δ 0 ppm) were recorded. After correction of AR/IS, the predicated lignin content was obtained and is shown in Table 10. Comparison of lignin content values obtained by AcBr analysis versus ^{13}C CP/MAS NMR shows that the largest absolute error in the test samples was 1.61 mg and the relative error less than 5%, indicating that the prediction from the corrected integration factor was acceptable. Meanwhile, the predicted result of different kinds of biomass (e.g., native poplar) revealed comparable values with less than 1.3% relative errors. It means that not only can the standard curve accurately quantify lignin analysis by solid state ^{13}C NMR, it also can be used to estimate the lignin content of different kinds of native and pretreated biomass (biological methods). In addition, it can provide information on lignin content for samples that are otherwise difficult to determine using chemical methods (e.g., acid insoluble lignin).

Discussion. The results of this study showed that solid state ^{13}C CP/MAS NMR coupled with an internal standard showed great potential for quantifying lignin content The method developed has clear advantages and effectiveness for lignin analysis. The solid state ^{13}C CP/MAS NMR method overcomes the problem of overestimation caused by the AcBr method. The new method does not require the degradation step in sample preparation as the samples only need to be hammer milled. The particle size of the sample does not have to be very small. The NMR analysis can be performed after adding the internal standard. The internal validation results suggested that the curve is reliable and stable; the relative error in prediction results between the model and the AcBr method was less than 5%. In summary, this method is relatively easy, simple, and accurate.

The major concern regarding this study was that the spectra used to establish the standard curve were not repeated. Although the internal and external validation showed good results, it could be much more convincing if the six method-establishing spectra were replicated with the solid state NMR

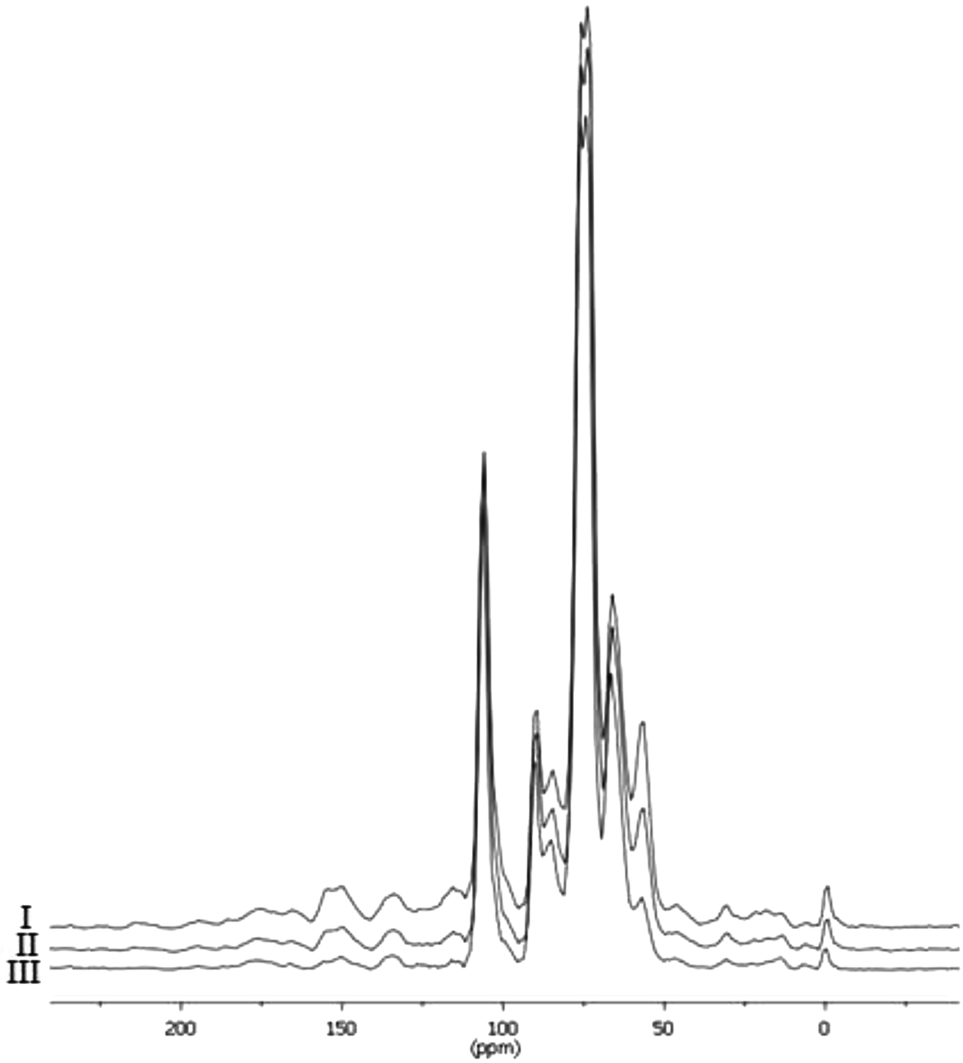

Figure 6. Solid state ^{13}C CP/MAS NMR spectra of internal validation samples.

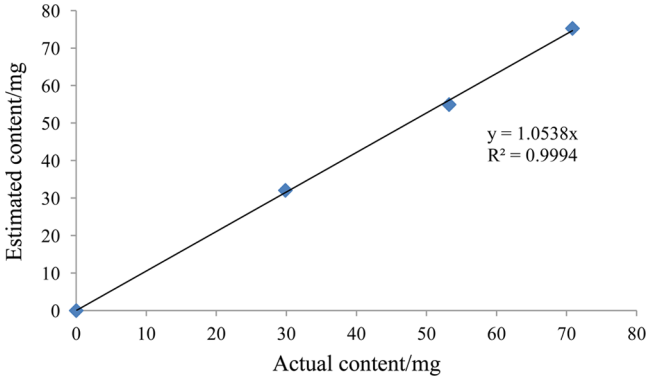

Figure 7. Calibration curve for quantitative analysis of internal validation samples.

Table 7. Response Factors of Internal Validation Samples

no.	area AR	area IS	area (AR/IS)	[lignin] (mg)	[IS] (mg/mL)
I	6.11	1.00	6.11	77.6	9.19
	6.09	1.00	6.09		
II	4.56	1.00	4.56	58.3	9.19
	4.58	1.00	4.58		
III	2.33	1.00	2.33	32.7	9.19
	2.32	1.00	2.32		

Table 8. Prediction Results of Internal Validation

no.	AcBr lignin content (mg)	predicted [lignin]/[IS]	predicted lignin content (mg)	absolute error (mg)	relative error (%)
I	70.85	7.64	70.22	−0.63	−0.89
II	53.23	5.85	53.76	0.53	1.00
III	29.86	3.22	29.60	−0.25	−0.85

under the same acquisition parameters (repeated three times at least) to obtain more data for building the standard curve. Another concern is the error range and repeatability of the AcBr method used to estimate the lignin content of the samples. Possibly, different wet chemical methods could be employed to test and compare the predicted results. Also, providing evidence for discrepancy in acquiring appropriate NMR signal intensities for internal standard samples, without the homogeneous mixing process, is recommended to prove the importance of this simple method based on the addition of the internal standard.

Currently, the limitation of the method developed in this study is that it took 24 h to run an NMR analysis; thus, further work is needed to improve its performance. First, the samples

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Table 9. Actual lignin Content of Test Samples with AcBr Analysis

test samples	weight (mg)	AcBr analysis (%)	actual content (mg)
native wheat straw	249.80	19.18	47.91
CWR−wheat straw	250.10	18.58	46.47
native poplar	250.30	21.08	52.76
acid insoluble lignin	249.10	N/A	N/A
white rot fungi treated	249.80	13.18	32.92
strep treated	250.00	14.98	37.45

Table 10. Prediction Results for Model Compared to AcBr Analysis

test samples	AcBr lignin content (mg)	predicted lignin content (mg)	absolute error (mg)	relative error (%)
native wheat straw	47.91	49.30	1.39	2.90
CWR−wheat straw	46.47	47.47	1.00	2.14
native poplar	52.76	53.44	0.68	1.29
acid insoluble lignin	N/A	184.36	N/A	N/A
white rot fungi treated	32.92	34.53	1.61	4.90
strep-treated wheat straw	37.45	38.92	1.47	3.93

in this study were run for 20,480 scans; in the future, fewer scans and shorter processing times, such as 1, 3, or 8 h, should be tested to reduce the length of time for total analysis. Second, more test samples, especially different kinds of biomass samples, are needed to verify the method. Then, with large test sample sets, method modification may be needed. Last, if software and other conditions were made available, simulation and deconvolution could be done. Spectral deconvolution would facilitate separating out and reducing the undesired resonance background of detectors, leading to more accurate experimental results. This extensive prior information would allow one to deconvolve overlapped resonances and obtain explicit concentration estimates of basic units in the corresponding biomass; these improvements would significantly reduce the manual labor associated with the ^{13}C CP/MAS NMR peak assignment and concentration/content estimation in live biomass analysis. Therefore, new correction

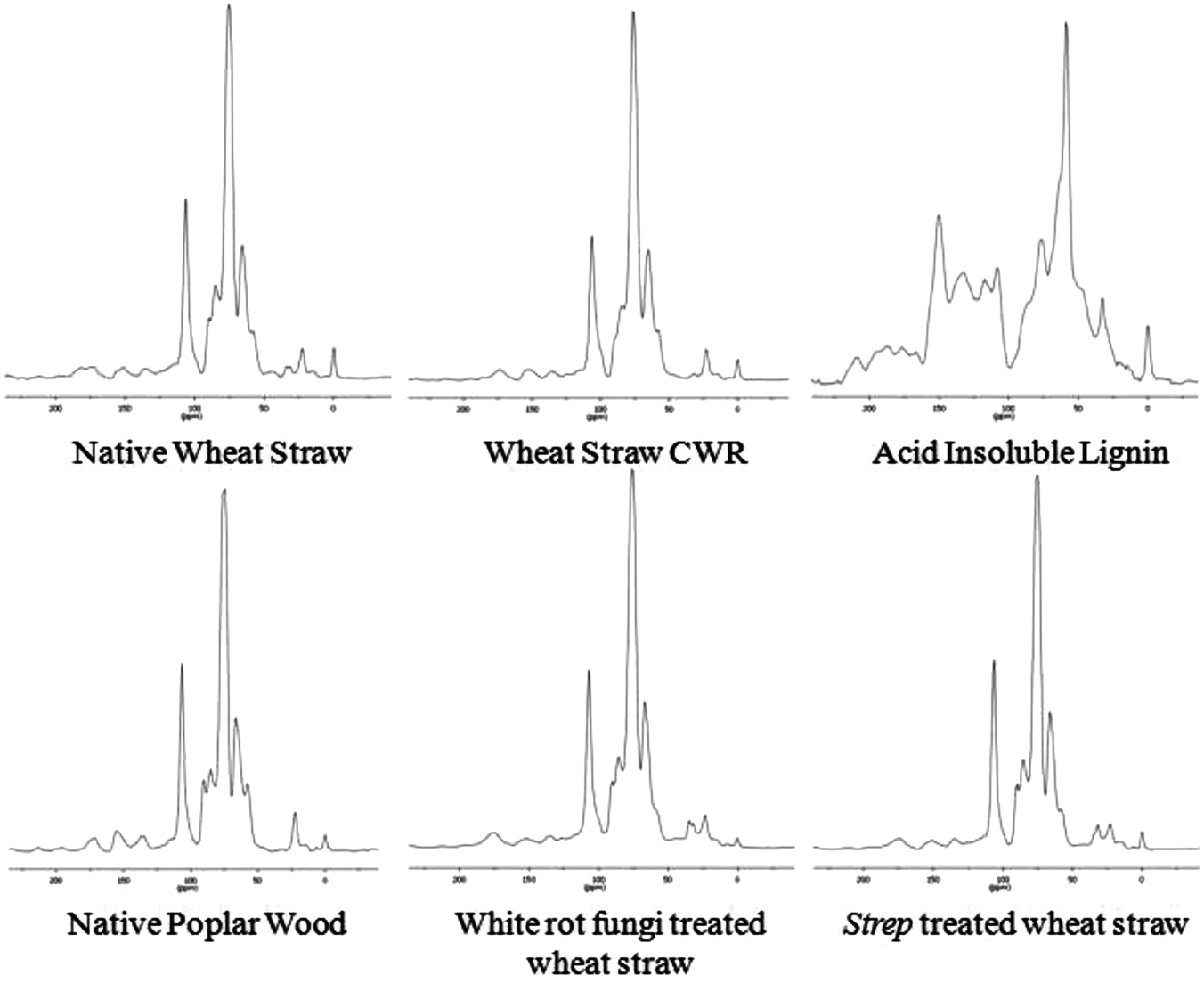

Figure 8. Solid state ^{13}C CP/MAS NMR spectra of test samples.

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factors would be incorporated in the calculation of accurate estimation of lignin content for a wide range of samples in their native form. Furthermore, in this work, the monomeric compositions or structure of lignins were not studied, although solid state 13C NMR is capable of being used for this type of work.

CONCLUSION

Solid state ^{13}C CP/MAS NMR coupled with an internal standard was used for analyzing lignin content. The internal validation results suggested that the standard curve was reliable and stable; also, the relative error of the prediction results between the model and the acetyl bromide method was no more than 5%. The method has the potential to be used for predicting lignin content in different kinds of biomass.

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Notes

The authors declare no competing financial interest.

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