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Rapid Determination of Lignin Content via Direct Dissolution and ^{1}H NMR Analysis of Plant Cell Walls

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Increasing societal demand for environmental and economic sustainability is placing a renewed focus on the agro-forest industry. This industry plays an essential role in the development of renewable energy and biofuels, especially in light of growing concerns related to energy security and climate change.[1] The economical transformation of differing sources of biomass into biofuels has become a global research theme, directed at displacing nonrenewable petroluem-based resources to reduce long-term carbon dioxide emissions.[2] Although most current bioethanol and biodiesel plants represent first-generation biorefineries, utilizing readily processable bioresources such as sucrose, starches and plant oils,[3] the efficient utilization of all components of biomass to maximize sustainable, economic development is of extreme significance. Among the different biomass sources, the use of lignocellulosics for biofuel production has shown two obvious potential advantages: higher net energy gain and lower production costs.[4] However, the use of lignocellulosics to produce liquid biofuel as a viable alternative to petroleum-based transportation fuels suffers from intrinsic recalcitrance of biomass, owing to the complicated structure of the plant cell walls, which, by their nature, are resistant to breakdown.[5] Thus, a better understanding of plant cell wall structure and its composite materials (cellulose, hemicelluloses, and lignin; see Figure 1)[6] has emerged as a crucial research focus. As the "natural glue" for plant cell walls, lignin is the second-most-abundant natural polymer, after cellulose, and is produced by enzyme-mediated radical coupling of the three monolignols (see Figure 1 D).[7] Although lignin can provide a renewable source of phenolic polymers, a high lignin content has proved to be a major obstacle not only in processing of plant biomass to biofuels,[8] but in other processes such as chemical pulping and forage digestibility also.[9] Therefore, lignin has emerged as one of the leading research fields in biofuel. With recent advances in lignin engineering via genetic modification of lignin's biosynthesis,[10] rationally designed bioenergy crops with reduced lignin content to facilitate more efficient degradation of the cell walls has been made possible. However, these programs require assessing a large numbers of "new" plants. For this purpose, precise analytic techniques for efficient lignin content assessment of a large number of samples at a microscale has become a pressing research issue.

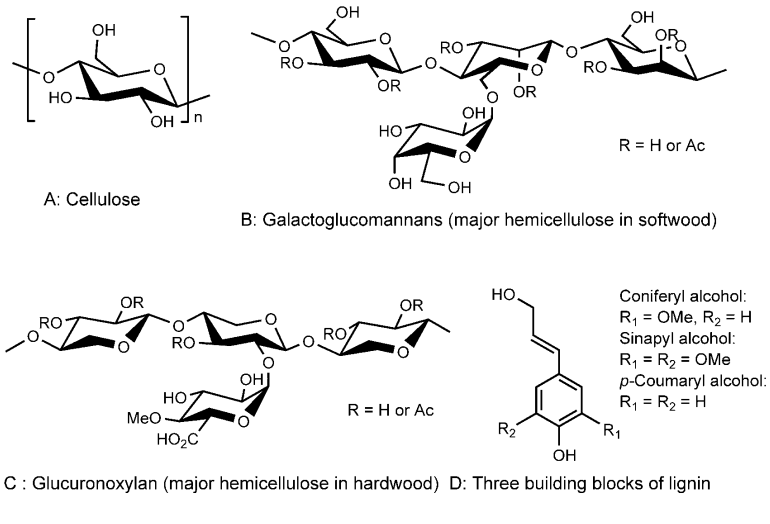

Figure 1. A–D) The structures of three major biopolymers of the plant cell walls. E) ^{1}H NMR spectra of [Hpyr]Cl-d_6 in [D_6]DMSO, and F) ball-milled poplar dissolved in 1:2 [Hpyr]Cl-d_6/[D_6]DMSO.

Many methods based on gravimetric or spectrophotometric analysis have been developed to quantitatively determine

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lignin content in plants,[11] with disadvantages including a relatively large sample size and time-consuming procedures (gravimetric method), and the difficulty in finding an appropriate calibration standard (spectrophotometric methods). Recently, a near-infrared (NIR) spectroscopy method was modified to improve the precision of lignin content determination and assess the lignin syringyl/guaiacyl (S/G) ratio,[12] and high-throughput screening of plant cell wall compositions via pyrolysis molecular beam mass spectroscopy to analyze lignin content and S/G ratio has also been reported.[13]

Owing to the limited solubility of biomass in conventional deuterated organic solvents, the use of nuclear magnetic resonance techniques for biomass analysis traditionally required tedious separation or derivatization of biomass for improved solubility, which could also induce incomplete recovery of the derivatized sample during work-up procedures and therefore lead to inaccurate results. Recently, the use of whole-cell NMR analysis by Ralph et al.[14] and Ragauskas et al.[15] has emerged as a powerful technique to supplement the above-mentioned methodologies in providing detailed structural information of lignin and hemicellulose in native plant cell walls.

The wide-spread use of ionic liquids[16] for biomass dissolution,[17] since the initial reports by Rogers and co-workers about the ability of imidazolium-type ionic liquids to dissolve various biomass resources, such as cellulose and wood sawdust, has made these compounds a promising choice for direct biomass dissolution and NMR analysis.[18] The perdeuterated pyridinium ionic liquid [HPyr]Cl-d_6 (Figure 1 E) developed by our group for direct biomass dissolution and NMR analysis has successfully revealed detailed structures of plant cell walls via both ^{13}C and heteronuclear single quantum coherence (HSQC) NMR.[15] As a supplement to the structural details acquired by whole-cell 2D NMR methods, this Communication highlights a novel protocol that enables the use of quantitative ^1H NMR analysis of ball-milled plant cell walls dissolved in the [HPyr]Cl-d_6/[D_6]DMSO bisolvent system to measure lignin content via a linear extrapolation method by the measurement of signals between 6.0–8.0 ppm (see Figure 1 F), which can be attributed to the aromatic/olefinic protons of lignin (see Figure 1 D). Furthermore, a modified procedure also allows to measure the lignin content of non-ball-milled (Wiley-milled) samples with parallel accuracy.

To facilitate the search of a suitable procedure to measure the lignin content via direct biomass dissolution and ^1H NMR analysis, a ball-milled switchgrass sample was first investigated for data analysis with 1:2 [Hpyr]Cl-d_6/[D_6]DMSO system as the solvent. [D_6]DMSO (99.9 atom%) was used as the co-solvent of [Hpyr]Cl-d_6 to reduce the solvent viscosity. Six dependent experiments were carried out to optimize the conditions for complete biomass dissolution, as shown in Table 1. Our experimental results showed that the ratio of integration of signals of lignin aromatic (signals between 6.0 ppm and 8.0 ppm) and integration of nondeuterated DMSO (2.5 ppm) increased with prolonged stirring at 25 °C, indicating incomplete biomass dissolution (Table 1, entries 1–3). In contrast, biomass dissolution carried out at 60 °C gave comparable ratios, which suggests complete biomass dissolution (Table 1, entries 4–6). Therefore biomass dissolution carried out at 60 °C for 12 h was chosen as the standard condition for further analysis of lignin content.

Table 1. Optimization of ball-milled switchgrass complete dissolution.[a]

Entry	Temperature [°C]	Time [h]	Ratio of integration[b]
1	25	6	1.35
2	25	12	1.68
3	25	24	1.83
4	60	6	2.18
5	60	12	2.22
6	60	24	2.17

[a] Dried extract-free ball-milled switchgrass cell walls (40.0 mg), 600.0 mg 1:2 [Hpyr]Cl-d_6/[D_6]DMSO ([D_6]DMSO, 99.9 atom% D) and spinbar (5 mm × 2 mm) in 5 mm NMR tube were stirred at specific temperature for the specific time for the optimization of biomass dissolution. [b] Direct ^1H NMR analysis of the resulting biomass solution afford the ratio of integration of signals (6.0 ppm–8.0 ppm, exclusively attributed to lignin aromatic signals) and integration of DMSO (2.5 ppm, non-deuterated DMSO as the internal standard).

After optimizing the conditions for completely dissolving biomass, the linear extrapolation method for the measurement of lignin content was investigated by the addition of a specific amount of isolated switchgrass lignin to the biomass solution, to examine integration ratio changes in the quantitative ^1H NMR spectra with nondeuterated DMSO as the internal standard. Thus, four samples of 40.0 mg dried extractive-free ball-milled switchgrass samples and 2.0 mg, 4.0 mg, 6.0 mg, or 8.0 mg of isolated switchgrass lignin were added into a 5 mm NMR tube containing 600.0 mg 1:2 [Hpyr]Cl-d_6/[D_6]DMSO. After 12 h of stirring at 60 °C for complete biomass dissolution, the mixture was subjected to quantitative ^1H NMR analysis with nondeuterated DMSO signal as the internal standard (see Supporting Information Figure 1). With the signals between 6.0 ppm and 8.0 ppm attributed to lignin aromatic/olefinic region, we found a good linear relationship between the ratio of integrations and the added lignin amount (Figure 2). Further data analysis[19] revealed that the lignin amount in 40 mg dry extractive-free switchgrass sample can be measured as 7.0 mg, and thus the lignin content in the switchgrass sample can be calculated to be 17.4%. To determine the accuracy of the current method, the Klason lignin content of switchgrass was also analyzed by a modified literature procedure,[20] which afforded

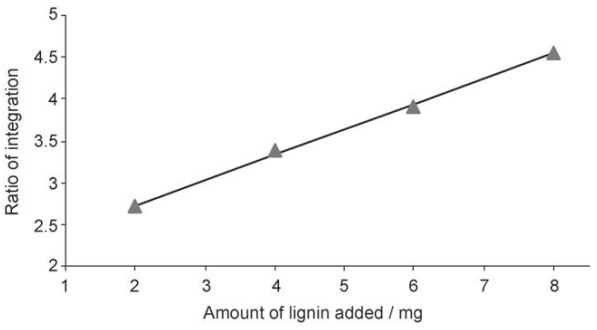

Figure 2. Linear relationship between ratios of integration of signals (integration$_{6.0–8.0ppm}$/integration$_{DMSO}$) and added lignin amount in ball-milled switchgrass samples.

the Klason lignin content of the switchgrass sample as 17.1%. Therefore, our current method proves to give a comparable result with the traditional Klason lignin content.[21]

After applying the method to analyze the switchgrass sample, a ball-milled hardwood sample (poplar) was next subjected to direct dissolution and ^{1}H NMR analysis for the measurement of lignin content. Similarly, a good linear relationship between the ratio of integrations and added lignin amount for ball-milled poplar sample, as shown in Figure 3, was used to

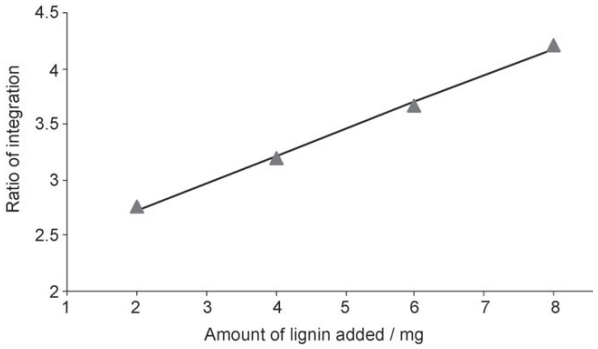

Figure 3. Linear relationship between ratios of integration of signals (integration$_{6.0–8.0ppm}$/integration$_{DMSO}$) and added lignin amount in ball-milled poplar samples.

calculate the lignin amount in 40 mg poplar sample as 9.3 mg, which affords the poplar lignin content as 23.3%, which is also comparable with independent Klason lignin content (24.1%). Therefore our current lignin content analysis via direct dissolution and ^{1}H NMR proved to be simple and accurate.[21]

Furthermore, our initial studies[15] also demonstrated that Wiley-milled samples with an average particle size of 20 mesh exhibited parallel solubility with ball-milled samples in 1:2 [Hpyr]Cl/[D$_6$]DMSO system though a prolonged dissolution. However, the complete dissolution of a Wiley-milled sample in 5 mm NMR tube proved difficult owing to less-efficient stirring. Therefore a modified method involving the use of one signal of nondeuterated pyridinium cation (signals at 9.1 ppm) as the internal standard was investigated to analyze the lignin content of these samples, in order to achieve a more convenient and effective procedure for lignin content analysis without the complications associated with the ball-milling processes. Three samples of 30.0 mg dry extractive-free Wiley-milled poplar sample (average particle size 20 mesh) and 1.0 mg, 2.0 mg, or 4.0 mg isolated poplar lignin were dissolved in a 10 mL vial containing 500.0 mg 1:2 [Hpyr]Cl-d$_6$/[D$_6$]DMSO via vigorous stirring for 12 h at 60 °C. After the biomass solution was transferred into a 5 mm NMR tube, the vial was rinsed with 2× 150 mg [D$_6$]DMSO, which was also transferred into the NMR tube for ^{1}H NMR analysis. Analysis of the ^{1}H NMR data also confirmed a good linear relationship between ratio of integrations and the added lignin amount (Figure 4), which afforded the corresponding lignin content of 23.3%.[21]

Having successfully analyzed the lignin content of hardwood and grass samples, our method was further extended to ana-

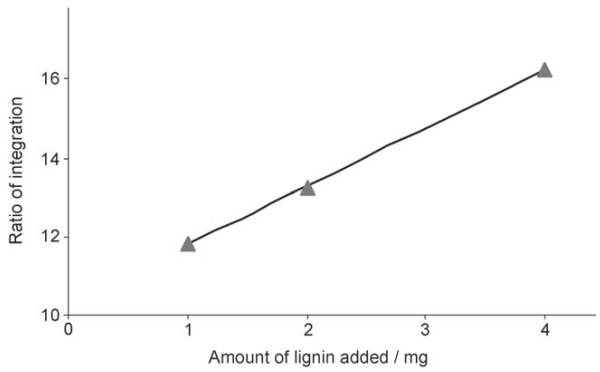

Figure 4. Linear relationship between ratios of integration of signals (integration$_{6.0–8.0ppm}$/integration$_{9.1ppm}$) and added lignin amount in Wiley-milled poplar samples.

lyze the Wiley-milled softwood sample (pine, 20 mesh) with nondeuterated pyridinium cation (signals at 9.1 ppm) as the internal standard. Our results show an excellent linear relationship between ratio of integrations and the added lignin amount (Figure 5). The corresponding pine lignin content is calculated as 28.9%, which is comparable to the independent Klason lignin content (29.4%).[21–23]

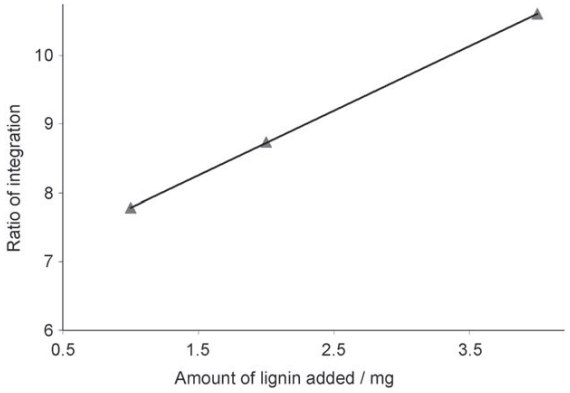

Figure 5. Linear relationship between ratios of integration of signals (integration$_{6.0–8.0ppm}$/integration$_{9.1ppm}$) and added lignin amount in Wiley-milled pine samples.

In summary, we have developed a novel methodology for efficient lignin content assessment of biomass samples (ball- or Wiley-milled) at a microscale via direct dissolution and NMR analysis of plant cell walls in 1:2 [Hpyr]Cl-d$_6$/[D$_6$]DMSO bisolvent system. More importantly, our experiment results showed comparable lignin contents as the traditional Klason lignin contents, and therefore the current direct dissolution and NMR analysis of biomass provides a new venue for rapidly assessing the lignin contents in large numbers of "new" plants during biofuel research. This protocol significantly broadens the application of whole-cell NMR analysis, as not only the lignin content and lignin *p*-hydroxyphenyl/syringyl/guaiacyl (H/S/G) ratio[25] can be acquired by 1D ^{1}H NMR and quantitative

^{13}C NMR, but the detailed structure of lignin and hemicelluloses can be ascertained by 2D HSQC NMR analysis.

Experimental Section

Materials: Poplar and switchgrass were procured from the National Renewable Energy Laboratory (NREL), and loblolly pine (*Pinus taeda*) was obtained from the University of Georgia research plot in Baldwin County, GA, all of which were Wiley-milled to pass 20 mesh screen. The samples were air-dried overnight and then Soxhlet-extracted with a benzene/ethanol (2:1, *v/v*) mixture for 24 h followed by an additional 24 h ethanol extraction. The extracted residue was then air-dried overnight and dried to constant weight under vacuum before use. Ball-milled samples were prepared by loading 0.5 g Wiley-milled material into a Retsch MM 200 Mixer Mill (equipped with a 10 mL stainless steel jar and 2 7 mm stainless steer grinding ball) and milled for 4 h (25 s^{-1}). Anhydrous [D_6]DMSO (99.9 atom% D), [D_5]pyridine (99.5 atom% D), [D_4]MeOH (99 atom% D) and all other solvents and reagents were obtained from Sigma–Aldrich.

Isolation of biomass lignin: Ball-milled lignin was isolated according to modified literature methods. In brief, the Wiley-milled samples were Soxhlet-extracted with water and benzene-ethanol (2:1, *v/v*) overnight, and then dried under vacuum overnight. The dried sample was ball-milled in a porcelain jar with ceramic balls using a rotatory ball mill running at 96 rpm for 7 days. A ceramic balls/biomass weight ratio of 30:1 was used. The ball-milled cell wall powder was then extracted twice with dioxane/water (96:4, *v/v*) under stirring for 48 h in the dark. The extracted mixture was centrifuged and the supernatant was collected. The collected dioxane/water solution was roto-evaporated at 40 °C under reduced pressure, and deionized water was added into the mixture. The mixture was freeze-dried and the crude ball-milled lignin was collected. The crude lignin was dissolved in acetic acid/water (90:10, *v/v*; 50 mg mL^{-1}) and precipitated in deionized water (200 mL), centrifuged, and freeze-dried. The solid product was further dissolved in 1,2-dichloroethane/ethanol mixture (2:1, *v/v*) and precipitated in diethyl ether (200 mL), centrifuged, washed with petroleum ether, dried overnight under vacuum at 40 °C, and stored in a desiccator over P_2O_5 prior to use.

Measurement of Klason lignin content of biomass: Measurement of Klason lignin content was carried out by a modified literature procedure.[17] In brief, the extractive-free sample was treated with 72 wt% sulfuric acid for 1 h at 30 °C and then diluted to 3 wt% sulfuric acid using deionized water and subsequently autoclaved at 121 °C for 1 h. The resulting solution was cooled to room temperature and the precipitate was filtered, dried, and weighed to get the Klason lignin content as 17.1% for switchgrass, 24.1% for poplar, and 29.4% for loblolly pine (acid-soluble lignin (ASL) calculated as 1.0%, 0.3%, and 0.1% for switchgrass, poplar, and loblolly pine, respectively).

Determination of lignin content in ball-milled biomass via direct dissolution and ^1H NMR analysis: Dry extractive-free ball-milled biomass (switchgrass or poplar, 40.0 mg), its corrosponding isolated switchgrass/poplar lignin (2.0 mg, 4.0 mg, 6.0 mg, or 8.0 mg), 600.0 mg 1:2 [Hpyr]Cl-d_6/[D_6]DMSO ([D_6]DMSO, 99.9 atom% D) and a stirring bar (5 mm × 2 mm) were carefully added to a 5 mm NMR tube, and the mixture was flushed with nitrogen for three times and then capped. The mixture was stirred at 60 °C in an oil bath for 12 h. After the stirring bar was removed, the NMR tube was cleaned and then subjected to ^1H NMR analysis by using nondeuterated DMSO as the internal standard. Signals between 6.0 ppm and 8.0 ppm, exclusively attributed to lignin, and a signal at 2.50 ppm (nondeuterated DMSO) for all four solutions were integrated. The ratios of integration of signals (6.0 ppm–8.0 ppm) and integration of DMSO (2.5 ppm) in different samples were analyzed to give the native lignin content of biomass.

Determination of lignin content in Wiley-milled biomass via direct dissolution and ^1H NMR analysis: Dry extractive-free Wiley-milled poplar (30.0 mg), isolated poplar lignin (1.0 mg, 2.0 mg, or 4.0 mg), 500.0 mg 1:2 [Hpyr]Cl-d_6/[D_6]DMSO ([Hpyr]Cl-d_6 prepared from [D_5]pyridine 99.5 atom% D) and a stirring bar (10 mm × 3 mm) were carefully added to 10 mL vial, and the mixture was flushed with nitrogen for three times and then capped. The mixture was vigorously stirred at 60 °C in an oil bath for 12 h. After the solution was transferred to a 5 mm NMR tube, the vial was further rinsed with 2 × 150 mL [D_6]DMSO, which was combined and then added into NMR tube containing the initial biomass solution. The combined biomass solution was then subjected to ^1H NMR analysis by using one signal of pyridinium cation (signal at 9.1 ppm) as the internal standard. Signals between 6.0 ppm and 8.0 ppm, exclusively attributed to lignin, and signal at 9.1 ppm were integrated for all three solutions, and the ratios were used to give the native lignin content of the biomass.

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Keywords: biofuels · biomass · ionic liquids · NMR spectroscopy · sustainable chemistry

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[19] Regression analysis was chosen as the modeling approach, because it straightforwardly follows the standard statistical theory of linear models and is easy to carry out and fit to our data analysis.

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[21] The standard error for the current NMR analysis of lignin content is 4% and the standard error for our measurement of Klason lignin content was 3%. Furthermore, our calculated lignin content by NMR method (17.4% for ball-milled switchgrass, 23.3% for ball-milled or Wiley-milled poplar, and 28.9% for Wiley-milled pine) are comparable with the combination of Klason lignin and acid-soluble lignin content (18.1% for switchgrass, 24.4% for poplar, and 29.5% for pine).

[22] Softwood (pine) shows less solubility in our current bi-solvent system, thus 25 mg Wiley-milled sample was dissolved in 700.0 mg 1:2 [Hpyr]Cl-d_6/[D_6]DMSO for complete biomass dissolution.

[23] Model compounds (*4*-methylanisole, A, as the model compound of coumaryl alcohol; *3,4*-dimethoxytoluene, B, as the model compound of coniferyl alchohol; A/B mole ratio 8:92 were used as determined by our quantitative ^{13}C NMR analysis of pine lignin) as the additive to replace isolated lignin were also investigated. The use of *4*-methylanisole and *3,4*-dimethoxytoluene, instead of coumaryl alcohol and coniferyl alcohol, is due to their olefin functional group also having signals between 6.0 and 7.0 ppm, which provides more aromatic signal per gram additive, as well as their instability. However, our results from model compounds as the additives for lignin content analysis gave lower lignin content (calculated as 22.9% from model compounds compared with 28.9% from the isolated lignin as the additive, in spite of good linear relationship between integrations and the added model compounds' amount). This lower lignin content can be attributed to substantial amount of cross-linking present in native lignin,[24] which results in a lower aromatic proton signal per gram biomass compared with the model compound in quantitative ^1H NMR.

[24] P. Sannigrahi, A. J. Ragauskas, S. J. Miller, *Energy Fuels* **2010**, *24*, 683 – 689.

[25] Our current 1:2 [Hpyr]Cl-d_6/[D_6]DMSO bi-solvent system for direct biomass dissolution and NMR analysis has shown the distinct carbon signals for lignin methoxy (C_{MeO}, 57.0 ppm), C2/6 resonance of syringyl-like lignin structures ($C_{S2/6}$, 104–109 ppm), C4 resonance of *p*-hydroxyphenyl structures (C_{H4}, 156–159 ppm), and our quantitative ^{13}C NMR spectra has be successfully used to determine H/S/G ratio in lignin as calculated as the following: $H/S/G = 2\times[\text{integration}(C_{H4})]/\text{integration}(C_{S2/6})/2\times[\text{integration}(C_{MeO}) - \text{integration}(C_{S2/6})]$. Hence, we could calibrate the lignin content accordingly in case of significant lignin H/S/G ratio changes.

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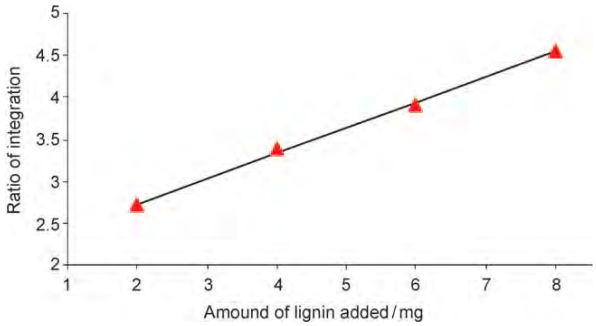
Rapid Determination of Lignin Content via Direct Dissolution and ^{1}H NMR Analysis of Plant Cell Walls



A rapid and efficient process for the measurement of lignin content in the plant cell walls is reported. The described method can be used to analyze ball- or Wiley-milled samples at a micro-scale via direct dissolution and ^{1}H NMR analysis of biomass using perdeuterated pyridinium chloride/[D_6]DMSO bisolvent system.

