

# Estimation of Syringyl Units in Wood Lignins by FT-Raman Spectroscopy

Umesh P. Agarwal,<sup>\*,†</sup> Sally A. Ralph,<sup>†</sup> Dharshana Padmakshan,<sup>‡</sup> Sarah Liu,<sup>‡</sup> and Cliff E. Foster<sup>§</sup>

<sup>†</sup>USDA, Forest Service, Forest Products Laboratory, Madison, Wisconsin 53726, United States

<sup>‡</sup>DOE Great Lakes Bioenergy Research Center, Wisconsin Energy Institute, University of Wisconsin–Madison, Madison, Wisconsin 53726, United States

<sup>§</sup>DOE Great Lakes Bioenergy Research Center, Michigan State University, East Lansing, Michigan 48824, United States

## Supporting Information

**ABSTRACT:** Syringyl (S) lignin content and the syringyl-to-guaiacyl (S/G) lignin ratio are important characteristics of wood and lignocellulosic biomass. Although numerous methods are available for estimating S lignin units and the S/G ratio, in this work, a new method based on Raman spectroscopy that uses the 370 cm<sup>-1</sup> Raman band-area intensity (370-area) was developed. The reliability of the Raman approach for determining S content was first tested by the quantitative analysis of three syringyl lignin models by sampling them, separately, in dioxane and in Avicel. Good linear correlations between the 370 cm<sup>-1</sup> intensity and model concentrations were obtained. Next, the percent syringyl (%S) lignin units in various woods were measured by correlating the 370 cm<sup>-1</sup> Raman intensity data with values of S units in lignin determined by three regularly used methods, namely, thioacidolysis, DFRC, and 2D-HSQC NMR. The former two methods take into account only the monomers cleaved from  $\beta$ -O-4-linked lignin units, whereas the NMR method reports S content on the whole cell wall lignin. When the 370-area intensities and %S values from the regularly used methods were correlated, good linear correlations were obtained ( $R^2 = 0.767$ , 0.731, and 0.804, respectively, for the three methods). The correlation with the highest  $R^2$ , i.e., with the 2D NMR method, is proposed for estimating S units in wood lignins by Raman spectroscopy as, in principle, both represent the whole cell wall lignin and not just the portion of lignin that gets cleaved to release monomers. The Raman analysis method is quick, uses minimal harmful chemicals, is carried out nondestructively, and is insensitive to the wet or dry state of the sample. The only limitations are that the sample of wood contains at least 30% S and not be significantly fluorescent, although the latter can be mitigated in some cases.

**KEYWORDS:** cell wall, DFRC, NMR, syringyl-to-guaiacyl lignin ratio, percent syringyl, thioacidolysis

## INTRODUCTION

In woody plants and other lignocellulosics, lignin is one of the three main structural components of cell walls. An understanding of its structure and composition is important in a wide variety of fields. Lignin, an aromatic biopolymer, is biosynthesized in plants from the polymerization of the three types of *p*-hydroxycinnamyl alcohols that produce *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units of the polymer.<sup>1</sup> Lignin not only plays a significant role in the growth and development of plants but is also important in the industrial utilization of lignocellulosic biomass. Lignin detrimentally affects the ease of industrial processing.<sup>2</sup> Successful manipulation of its composition and structure can have beneficial outcomes for several biomass-based processes, including technologies focused on improving sugar release for biofuel production,<sup>3</sup> delignification in pulping and bleaching,<sup>4,5</sup> digestibility of forages in ruminants,<sup>6</sup> and valorization of lignin into high-value chemicals and materials.<sup>2,7,8</sup>

The lignin monomer composition, and therefore its S content, is an important parameter for lignocellulosic biomass characterization and utilization. For example, in chemical pulping, the S content has been shown to have a significant impact on lignin removal and the bleachability of pulp.<sup>9,10</sup> Similarly, Yoo et al. reported the positive impact of higher S

content on a biomass-to-ethanol conversion process.<sup>11</sup> Considering that lignin composition can influence the utilization of biomass, various techniques have been used to estimate the H, G, and S monomer composition of lignins. Such techniques consist of both wet-chemical methods (e.g., analytical thioacidolysis<sup>12–14</sup> and nitrobenzene oxidation<sup>15,16</sup>) and spectroscopic methods (e.g., 2D-HSQC (heteronuclear single-quantum coherence) NMR,<sup>17,18</sup> FT-IR,<sup>19–21</sup> and NIR<sup>22,23</sup>). The chemical approaches are limited in that the monomer compositional information provided is strictly only on the monomers released by cleaving  $\beta$ -O-4 bonds in lignin units, for example.<sup>14</sup> They can be time-consuming, labor-intensive, and require use of hazardous chemicals. 2D-HSQC NMR analyzes the whole cell wall lignin but is not absolutely quantitative.<sup>17,18</sup>

In the field of Raman spectroscopy, the FT-Raman technique, which involves sample excitation by a 1064 nm laser, has some advantages.<sup>24</sup> In particular, the technique is highly appropriate for *in situ* analysis of lignocellulosic

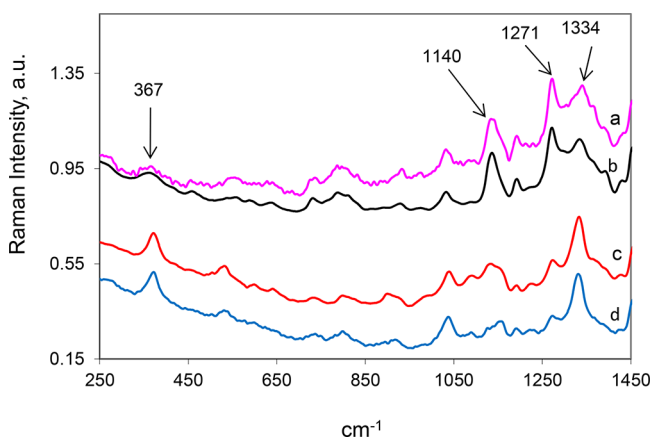
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materials because spectra with minimal fluorescence and high signal-to-noise ratio are typically obtained.<sup>25,26</sup> The technique is nondestructive, meaning that the sample can be recovered for further analysis. We have previously reported FT-Raman spectra of woods,<sup>25,27</sup> milled-wood lignins (MWLs),<sup>28</sup> dehydrogenation polymer (DHP) lignins,<sup>29</sup> and lignin models.<sup>30</sup> In hardwood MWLs, DHP lignins, and S lignin unit models, a band at approximately  $370\text{ cm}^{-1}$  was found to be associated with the S unit and was significantly more intense than in softwood-MWLs and models of G- and H-lignins.<sup>28–30</sup> From Figure 1, in which Raman spectra of MWLs, in the



**Figure 1.** Raman spectra of milled-wood lignins in the region  $250\text{--}1450\text{ cm}^{-1}$ : (a) loblolly pine, (b) black spruce, (c) sweetgum, and (d) aspen. Reproduced with permission from ref 28. Copyright Taylor & Francis 2011.

region  $250\text{--}1450\text{ cm}^{-1}$ , are compared,<sup>28</sup> it can be clearly noted that the spectral contributions at  $370\text{ cm}^{-1}$  from black spruce and loblolly pine (both softwood) MWLs were much reduced compared to those of aspen and sweetgum (both hardwood) MWLs. In the Raman spectra of black spruce and loblolly pine MWLs (Figure 1), weak broader features were detected, respectively, at  $361$  and  $367\text{ cm}^{-1}$ .<sup>28</sup> These are likely to have arisen from the guaiacyl (G)-lignin. In the Raman spectra of woods,<sup>25,27</sup> prominent contributions from cellulose are present at  $380$  and  $350\text{ cm}^{-1}$  (Figure S1 shows a Raman spectrum of Avicel, a pure cellulose). Previously, for hardwoods, in the context of cellulose crystallinity estimation by the so-called 380-Raman method, the role of the  $370\text{ cm}^{-1}$  band was investigated.<sup>27</sup> It was reported that this band impacted the apparent crystallinity of syringyl-lignin-containing lignocellulosics. In the present situation, the roles are reversed and one has to remove the interference of cellulose and minor G-lignin peaks from the wood spectra so that only the syringyl contribution remains. This was accomplished by subtracting the Raman spectrum of black spruce (which has no syringyl content) from the spectra of the selected hardwoods.

Previously, Raman spectroscopy in conjunction with thioacidolysis has been used to develop PLS (partial least-squares) models for predicting lignin S/G ratios.<sup>31</sup> However, this multivariate approach lacks simplicity and specificity and involves first developing a predictive model based on a large number of samples. In another FT-Raman approach,<sup>32</sup> to determine lignin S/G, first, S and G bands in the spectra of lignin models were identified. To separate S and G contributions in the spectra of lignocellulosic substrates, spectral deconvolution between  $1220$  and  $1530\text{ cm}^{-1}$  was

carried out. By integrating the intensities of the deconvoluted Raman bands that represented the S and G units, the Raman S/G ratio was calculated. Next, a regression model was constructed between the FT-Raman and pyrolysis-GC/MS S/G ratio results and was used to estimate final S/G ratios.<sup>32</sup> However, sample spectral regions assigned to S, G, and H lignin units had significant interference not only due to sample fluorescence but also from overlap with cellulose and hemicellulose contributions. The deconvolution approach used is a subjective method, and the obtained values may not be reliable. Our approach is simply to remove the interfering contributions by spectral subtraction.

The objective of the present study was to use FT-Raman spectroscopy to estimate the S content of hardwoods based upon the intensity of  $370\text{ cm}^{-1}$  band of lignin. The S data determined from Raman spectroscopy were then correlated with the values of %S obtained by the methods of thioacidolysis, DFRC (derivatization followed by reductive cleavage), and 2D-HSQC NMR. The correlations show the usefulness of the Raman methodology for estimating the %S of hardwood lignins.

## MATERIALS AND METHODS

**Materials.** To develop the %S lignin units quantitative method, woods listed in Table 1 were selected. Except for black spruce, all of

**Table 1.** Woods Used and Their Lignin Contents

| wood                                     | type     | % lignin <sup>a</sup> |
|------------------------------------------|----------|-----------------------|
| black spruce; <i>Picea mariana</i>       | softwood | 30.0                  |
| aspen; <i>Populus tremuloides</i>        | hardwood | 15.1                  |
| cottonwood; <i>Populus deltoides</i>     | hardwood | 25.7                  |
| hickory; <i>Carya ovata</i>              | hardwood | 23.2                  |
| madrone; <i>Arbutus menziesii</i>        | hardwood | 17.7                  |
| sweetgum; <i>Liquidambar styraciflua</i> | hardwood | 19.8                  |
| white birch; <i>Betula papyrifera</i>    | hardwood | 15.4                  |
| willow; <i>Salix babylonica</i>          | hardwood | 24.4                  |

<sup>a</sup>Based on the acetyl bromide lignin method (see ref 34).

the woods were hardwoods. These woods were either available in our laboratory or were obtained from other researchers at the Forest Products Laboratory. The air-dried wood chips were Wiley milled and sieved to pass a 1 mm screen. The milled woods were then extracted with an acetone/water (9:1) mixture as previously described.<sup>25</sup> Post extraction, the wood samples were air-dried under ambient conditions.

**Lignin Models and Other Chemicals.** The two simple S models, sinapic acid and syringic acid, were purchased from Sigma-Aldrich (MO). Syringylglycol- $\beta$ -guaiacyl ether (dimer) was prepared following a literature procedure.<sup>33</sup> Avicel (microcrystalline cellulose derived from wood pulps) was from FMC Corporation (Newark, Delaware). All other solvents and chemicals, unless stated otherwise, were from Sigma-Aldrich. The commercial analytical reagent grade chemicals and solvents were used without further purification.

**Lignin Determination by the Acetyl Bromide Method.** Percent lignin, listed in Table 1, was determined using the acetyl bromide method which involves measuring UV-vis absorption at  $280\text{ nm}$  after derivatization and dissolution of whole cell wall samples.<sup>34,35</sup> The dry extract-free whole cell walls ( $2\text{--}4\text{ mg}$ , weighted accurately) were suspended in acetyl bromide ( $0.5\text{ mL}$ ,  $25\%\text{ v/v}$  acetyl bromide in acetic acid). The suspension was heated at  $50\text{ }^{\circ}\text{C}$  for  $2\text{ h}$ . The samples were then cooled in an ice bath and transferred to a  $10\text{ mL}$  volumetric flask using acetic acid. The acetyl bromide was quenched with  $2\text{ M}$  NaOH ( $2\text{ mL}$ ) using ice water to keep the reaction cool. Then,  $0.5\text{ M}$  hydroxylamine ( $0.35\text{ mL}$ , prepared freshly from the hydrochloride) was added to the flask. The sample was then diluted to the  $10\text{ mL}$

mark with acetic acid. The resulting solution was filtered through a 0.2  $\mu\text{m}$  PTFE filter, and the absorbance at 280 nm was measured. Using an extinction coefficient of 20 for all the samples, the concentration of dissolved lignin was determined.<sup>34</sup> The percent standard error (%SE) associated with the acetyl bromide method has been reported to be 11.4%.<sup>35</sup>

**Carbohydrate Content of Woods.** The wood samples listed in Table 1 were analyzed chemically to quantitate the amount of carbohydrates.<sup>36</sup> The standard deviation of this method was <1%.

**Raman Spectroscopy.** A MultiRam FT-Raman spectrometer (Bruker) was used to obtain Raman spectra of the lignin models and woods. The Raman instrument was equipped with a 1064 nm, 1000 mW Nd:YAG laser. In most cases, spectra with good signal-to-noise ratios were obtained using 660 mW laser power and 1024 scans. Bruker OPUS 7.2 software was used to process the spectral data which involved normalization of spectra, selection of a spectral region, background correction, and band integration. Background correction was performed using a 64 points OPUS “rubberband option”. For plotting purposes, the spectra were converted to ASCII format and exported to Excel.

**Quantitation of Lignin Models.** For S unit quantitation in the models, calibration curves were generated by FT-Raman spectroscopy. Five different concentrations (w/w; 20, 10, 5, 2.5, and 1.25%) of the three models were prepared as solutions in dioxane and as mixtures in Avicel (by adding a specific volume of model-dioxane-solution to solid Avicel). A 20% (w/v) solution of each compound was prepared with dioxane for stock solutions. Dilutions were prepared from the stock solution with fresh dioxane. Mixed samples of the models and Avicel were prepared by adding dioxane solution of a model (containing 20 mg of a model) to 100 mg of Avicel. For each model, a stock mixture was prepared by mixing a methanol solution of the model compound with Avicel which was then tumbled under vacuum for mixing and drying. Dilutions were made by mixing known amounts of the stock mixture with pure Avicel.

The dioxane solutions were sampled in capped NMR tubes and the “model-in-Avicel” sample mixtures were made into pellets for Raman analysis. Using a hydraulic press, a sample pellet was made from ~100 mg of a material. In the Raman spectra of the models, the 488  $\text{cm}^{-1}$  band from dioxane and the 1096  $\text{cm}^{-1}$  band from cellulose were used as internal standards for the solutions and the Avicel pellets. The use of the internal standards addressed the problem of Raman band intensity variations caused by the instrument and sampling issues. The spectra of the models in dioxane and in Avicel were normalized, respectively, to the 488 and 1096  $\text{cm}^{-1}$  bands. Moreover, due to nonhomogeneous distribution of the models in Avicel, a solid matrix, several pellets made from each sample mixture were prepared and analyzed. In the calibration plots of the lignin models, from the normalized spectra, the average intensities of the 370  $\text{cm}^{-1}$  band were plotted against the model concentrations. The 370  $\text{cm}^{-1}$  band intensities were determined by drawing a horizontal baseline under the 370  $\text{cm}^{-1}$  peak from the 350  $\text{cm}^{-1}$  position.

**Estimating S Groups in Hardwood Lignins.** For Raman spectroscopy analysis of black spruce (BS, softwood) and the hardwoods (Table 1), a set of 3 pellets from ~100 mg of each wood was made from the extracted ground wood. In all cases woods were analyzed in triplicate and spectra with good signal-to-noise ratios were obtained using a 660 mW laser power and 1024 scans. To estimate the S levels in hardwood lignins, the 370  $\text{cm}^{-1}$  band area intensity (370-area) was calculated from the processed wood spectra. All spectral processing was carried out using Bruker’s OPUS 7.2 software, and the spectra, in the 250 to 1850  $\text{cm}^{-1}$  region, were subjected to baseline correction and normalization at 1096  $\text{cm}^{-1}$  (primarily a cellulose band although hemicelluloses contribute to this band).<sup>25</sup> Next, a similarly processed and 1096  $\text{cm}^{-1}$ -normalized spectrum of BS was subtracted from each of the hardwood pellet spectra (hardwood – BS). Then, the area intensity at 370  $\text{cm}^{-1}$  (370-area) was measured from the subtracted spectrum by drawing a sloped straight line between 385 and 330  $\text{cm}^{-1}$ . This approach is based on the fact that BS lignin contains no S groups<sup>37</sup> (also see later the %S from NMR and DFRC), and therefore, in the 385 to 330  $\text{cm}^{-1}$

region of the BS Raman spectrum, primarily the cellulose contribution is detected. However, in the case of hardwoods, both cellulose and S lignin units’ contributions coexist in this spectral region. Nevertheless, by the process of spectral subtraction, the former contribution is removed from the spectra, thereby leaving only the S lignin units’ contribution. This assumes that, in BS and the hardwoods, both the content of cellulose/carbohydrates and their supramolecular states are similar. The latter is a reasonable assumption, but the former is not because cellulose/carbohydrate compositional differences have been reported in woods.<sup>27</sup> Lastly, the 370-area Raman intensities were corrected for differences in lignin (see Table 1) and total carbohydrate (see later) contents between the hardwood samples.

**Thioacidolysis.** The lignin monomer composition was determined using the thioacidolysis method described by Harman-Ware et al.<sup>13</sup> To 2 mg of dried extractive free wood meal, 1.0 mL of a reaction mixture containing 2.5% (v/v) boron trifluoride diethyl etherate and 10% (v/v) ethanethiol in 1,4-dioxane was added. The reaction vial headspace was purged with dry nitrogen, and the vial was tightly capped and heated in a heating block at 100  $^{\circ}\text{C}$  for 4 h. After it was cooled on ice, 400  $\mu\text{L}$  of the reaction mixture was transferred from the reaction vial and placed into a glass culture tube to which 250  $\mu\text{L}$  of 1 M sodium bicarbonate was added for neutralization. The pH was adjusted back to acidity with 100  $\mu\text{L}$  of 2 M HCl in order to maximize thioacidolysis monomer extraction efficiency. The thioacidolysis products were extracted between 1 mL of water and 800  $\mu\text{L}$  of ethyl acetate. After the layers were settled, 100  $\mu\text{L}$  of the organic layer was transferred to a GC/MS vial with micro inserts and further derivatized with the addition of 50  $\mu\text{L}$  of N,O-bis(trimethylsilyl)-acetamide and 10  $\mu\text{L}$  of pyridine. The GC/MS vials were allowed to sit at room temperature for 2 h in order to complete the derivatization before being injected into the GC/MS. The GC/MS analysis was carried out on an Agilent 6890/5975B system with a 15 m SLB-5MS capillary column.

**DFRC.** Another method to degrade lignins to low molecular weight compounds and to obtain structural information is called derivatization followed by reductive cleavage, or DFRC.<sup>38</sup> The procedure described by Lu and Ralph<sup>38</sup> was used on Wiley-milled woods after the samples had been solvent-extracted.

The DFRC analysis was performed as follows. Dry extractive-free whole cell walls (10–15 mg) were treated with a solution of acetyl bromide in acetic acid (1:4, v/v, 2 mL) at 50  $^{\circ}\text{C}$  for 2.0 h. The acetylated and benzyl-( $\alpha$ -carbon)-brominated lignin solution was evaporated to dryness using a SpeedVac concentrator (35 min, 50  $^{\circ}\text{C}$ ). The dry film was treated with absolute ethanol (1 mL), and then the ethanol was removed on a SpeedVac concentrator (15 min, 50  $^{\circ}\text{C}$ ). The dry sample was then immediately dissolved in a mixture of 1,4-dioxane/acetic acid/water (5:4:1, by volume, 5 mL), and zinc nanopowder (200 mg) was added to the vial. The reaction was stirred for 22 h at room temperature and then quenched with saturated ammonium chloride. The quenched reaction crude was spiked with internal standards of diethyl 5,5'-diferulate diacetate (DEDF) and bis-1,1-(*p*-hydroxyphenyl)ethane (BPO, which is analyzed as BPA). The organics were extracted with dichloromethane (DCM) (4  $\times$  15 mL), and the combined organic fractions were dried over anhydrous sodium sulfate. The DCM was removed under vacuum, and the free hydroxyl groups were acetylated using a mixture of acetic anhydride and pyridine (1:1, 2 mL). The excess acetic anhydride and pyridine were removed on a rotary evaporator, after which the crude product was loaded onto a Supelco Supelclean LC-SI SPE tube (Sigma-Aldrich part no. 505048) with the aid of ethyl acetate (0.5  $\times$  3 mL). The purified product was eluted using a mixture of hexanes/ethyl acetate (1:1, 5 mL) and concentrated to dryness. The dry film was dissolved in DCM (1 mL), and aliquots were injected into a GC-MRM-MS for quantitative analysis (using calibration curves derived from synthetic standards).

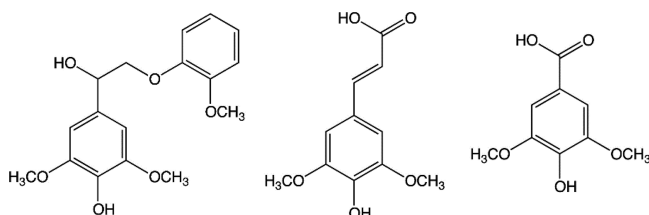
The molar percent of H (*p*-hydroxycinnamyl alcohol), G (coniferyl alcohol), and S (sinapyl alcohol) lignin units, all as their peracetates, were determined from the *Z*- and *E*-isomers of the corresponding *p*-hydroxycinnamyl alcohols released by DFRC.

**2D-HSQC NMR.** In 2008, a new NMR method capable of analyzing gelatinous cell walls swelled in deuterated-DMSO was published.<sup>39</sup> The unique aspect of this technique is that the woody cell wall can be analyzed without significant structural modification beyond what is caused by ball-milling and ultrasonication steps. It is quite well established that ball-milling induces bond cleavage in lignins, which reduces the average molecular weight and increases their content of free phenolic groups, but the H–G–S nature of the lignin units are retained.<sup>40,41</sup> The NMR method was used to analyze lignin in the samples of the ball-milled woods, and the experimental details were similar to those provided in the reference cited above. The method was subsequently improved, producing more mobile solutions and superior spectra, by using 4:1 DMSO/pyridine.<sup>17,18</sup> This is the method used here.

The Wiley-milled extractive-free wood powders (50 mg) were suspended in a solution of DMSO-*d*<sub>6</sub>/pyridine-*d*<sub>5</sub> (4:1, 500  $\mu$ L). The HSQC spectra were acquired on a Bruker Biospin (Billerica, MA) Avance 700 MHz NMR spectrometer equipped with a 5 mm quadrupole-resonance <sup>1</sup>H/<sup>31</sup>P/<sup>13</sup>C/<sup>15</sup>N QCI gradient cryoprobe with inverse geometry (proton coils closest to the sample). The central DMSO solvent peak was used as an internal reference ( $\delta_C$  39.5,  $\delta_H$  2.49 ppm). The percentage of syringyl and guaiacyl lignin was quantified by measuring the contour volume integrals of the C–H pairs in G2, S2/6, and S'2/6. In the partial short-range <sup>1</sup>H–<sup>13</sup>C (HSQC) correlation spectra (aromatic regions only), G2 refers to the C–H correlation at position 2 in the guaiacyl ring. Similarly, in syringyl units, S2/6 refers to the C–H correlations at positions 2 and 6 in the syringyl ring. S'2/6 are from oxidized syringyl units.<sup>18</sup> The % H was too low for meaningful quantitation by the NMR method and was neglected in the calculation of %S. Thus, calculated %S is represented below (see later).

## RESULTS AND DISCUSSION

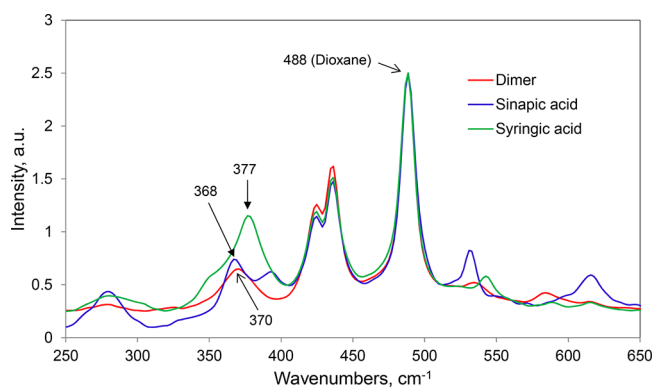
**Estimation of S Groups in Lignin Models.** First, to check the suitability for quantitation of the S unit by using the Raman band at 370 cm<sup>−1</sup>, three different S lignin models were selected (Figure 2). For quantitation purposes, the models



**Figure 2.** Lignin models from left to right: syringylglycol- $\beta$ -guaiacyl ether (dimer), sinapic acid, and syringic acid.

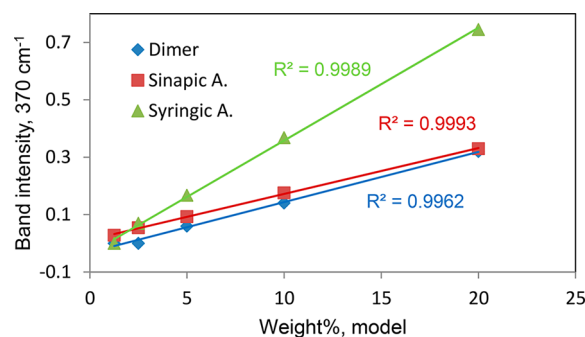
were analyzed in dioxane and Avicel in the concentration range 1.25–20% (w/w). The highest model concentration of 20% (w/w) was chosen, keeping in mind that in hardwoods, on a

mass basis, the %S is not likely to exceed 20% (w/w) of the wood; Table 1 shows total lignin content of hardwoods and Table 2 lists the molar G and S levels. Figure 3 shows the



**Figure 3.** Raman spectra of syringylglycol- $\beta$ -guaiacyl ether (dimer), sinapic acid, and syringic acid in dioxane at 20% (w/w) concentration. The band at 370 cm<sup>−1</sup> due to the S unit is annotated along with the 488 cm<sup>−1</sup> dioxane peak.

Raman spectra of the models at 20% (w/w) concentration in dioxane. In all three cases, excellent linear correlations between the 370 cm<sup>−1</sup> band intensity and the model concentrations were obtained (Figure 4,  $R^2 = 0.996$ ,  $0.999$ , and  $0.999$ ).

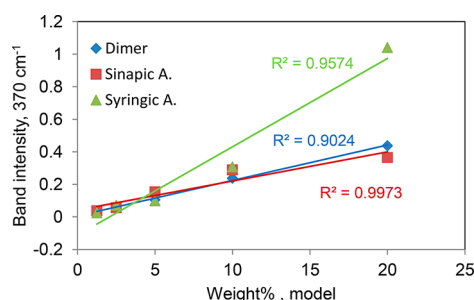


**Figure 4.** Quantitation of S lignin units models in dioxane using Raman spectroscopy. The models were syringylglycol- $\beta$ -guaiacyl ether (dimer), sinapic acid, and syringic acid.

However, the correlations for Avicel samples were slightly lower (Figure 5,  $R^2 = 0.902$ – $0.997$ ). This implied that between the dioxane and Avicel samples, the former gave better correlations and reflected the fact that, in dioxane, the models were homogeneously distributed at the molecular level. This was not the case with Avicel sampling, and therefore,

**Table 2.** Percent Molar Ratio of G and S Units by Standard Methods

| wood         | DFRC         |              | 2D HSQC NMR  |              | thioacidolysis |              |
|--------------|--------------|--------------|--------------|--------------|----------------|--------------|
|              | %G, $\pm$ SD | %S, $\pm$ SD | %G, $\pm$ SD | %S, $\pm$ SD | %G, $\pm$ SD   | %S, $\pm$ SD |
| cottonwood   | 42 $\pm$ 1   | 57 $\pm$ 1   | 45 $\pm$ 1   | 55 $\pm$ 1   | 41 $\pm$ 1     | 58 $\pm$ 1   |
| sweetgum     | 27 $\pm$ 1   | 73 $\pm$ 1   | 25 $\pm$ 1   | 75 $\pm$ 1   | 27 $\pm$ 3     | 73 $\pm$ 2   |
| willow       | 33 $\pm$ 1   | 66 $\pm$ 1   | 37 $\pm$ 2   | 63 $\pm$ 2   | 36 $\pm$ 1     | 64 $\pm$ 2   |
| aspen        | 33 $\pm$ 0   | 67 $\pm$ 0   | 29 $\pm$ 2   | 71 $\pm$ 2   | 29 $\pm$ 2     | 70 $\pm$ 4   |
| madrone      | 14 $\pm$ 0   | 86 $\pm$ 0   | 8 $\pm$ 3    | 92 $\pm$ 3   | 12 $\pm$ 0     | 88 $\pm$ 3   |
| white birch  | 21 $\pm$ 0   | 78 $\pm$ 2   | 21 $\pm$ 0   | 79 $\pm$ 0   | 22 $\pm$ 1     | 77 $\pm$ 2   |
| hickory      | 25 $\pm$ 2   | 75 $\pm$ 1   | 29 $\pm$ 2   | 71 $\pm$ 2   | 27 $\pm$ 1     | 72 $\pm$ 3   |
| black spruce | 96 $\pm$ 1   | 0 $\pm$ 0    | 100 $\pm$ 0  | 0 $\pm$ 0    | 96 $\pm$ 1     | 2 $\pm$ 0    |



**Figure 5.** Quantitation of S lignin units models in Avicel using Raman spectroscopy. The models were syringylglycol- $\beta$ -guaiacyl ether (dimer), sinapic acid, and syringic acid.

considering the heterogeneity in model distribution, at least 10 spectra were obtained from different pellets. The average intensity in Figure 5 was calculated from the 10 spectra (instead of the usual 3) obtained from different pellets. The standard deviations (SD) are reported in the Supporting Information (Table S1).

Within the models, when the SD data associated with the intensity measurements were taken into account (Table S1), it became clear that, in Avicel, although both syringic acid and sinapic acid were nonhomogeneously distributed, the latter's distribution was more heterogeneous. Nevertheless, this is not recognized by Figure 5 where a better correlation was obtained for sinapic acid compared to syringic acid ( $R^2 = 0.99$  vs  $0.96$ ). Additionally, the SDs for the dimer were lower compared to either of the monomer acids (Table S1), but for the dimer, a lower correlation was obtained (Figure 5). The reasons for these discrepancies are not clear. The higher heterogeneity is likely to have arisen from the models' specific structural characteristics and the nature of the cellulose matrix. In any case, the conclusion from the models' studies was that Raman method based on the  $370\text{ cm}^{-1}$  can be used to quantify the S models.

**Comparative Measurements of %S by DFRC, 2D-HSQC NMR, and Thioacidolysis.** The results of DFRC analysis of the 8 wood samples are reported in Table S2 and Table 2. Because %H was negligible (Table S2) only %G and %S are listed in Table 2. It can be noted that madrone showed the highest %S (86%) and none of the hardwoods had a molar %S of less than 50%. The %S in BS, a softwood, on the other hand, was zero, as expected.<sup>37</sup> From the %S data in Table 2, it can be noted that the hardwoods with the highest and lowest %S were madrone and cottonwood. The data also indicated that there was hardly any H lignin units in the hardwoods, whereas BS, a softwood, had 4% H units. The S/G ratios of the wood lignins obtained by DFRC are reported in Table S2.

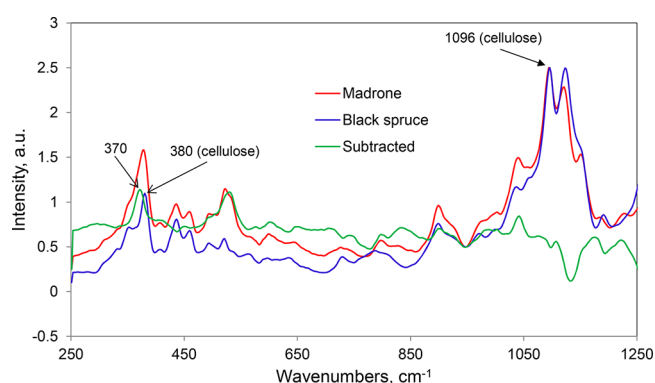
The results of the whole cell wall characterization method 2D HSQC NMR are reported in Table S3 and Table 2. Because %H was negligible (Table S3), only %G and %S are listed in Table 2. Results of the hardwoods were similar to what was obtained in the DFRC analysis (Table 2), although %S and %G values were slightly different. As was the case in DFRC analysis, the hardwoods that showed the highest and lowest %S values were, respectively, madrone and cottonwood. Comparison of the DFRC and NMR data for madrone indicated that, whereas in NMR %S was slightly higher (92% vs 86%, Table 2), %G values were somewhat higher in DFRC (14% vs 8%, Table 2). The difference in %S may have to do with the fact that the whole cell wall is analyzed by the NMR

method and not just the isolated/liberated lignin fraction, as is the case in DFRC.

The results of thioacidolysis analysis of the liberated H, G, and S lignin units monomers from the woods are reported in Table S4 and Table 2. Because %H was negligible (Table S4), only %G and %S are listed in Table 2. Except for BS, H monomers were present at very low levels as was the case in the DFRC analysis (Table S2 vs Table S4). The %S showed a trend that was similar to those earlier noted from Table 2 for the cases of DFRC and NMR. As can be seen from Table 2, all three methods give remarkably similar values, even though the DFRC and thioacidolysis data derive only from the monomers released by cleaving  $\beta$ -ethers, whereas the NMR data are from the whole lignin in the wood.

### Raman Spectroscopic Estimation of S Groups.

Estimation of %S by Raman spectroscopy was carried out using the spectra obtained by subtracting the BS spectrum from each of the hardwood spectra. As an example, in Figure 6,



**Figure 6.** Raman spectra in  $250\text{--}1250\text{ cm}^{-1}$  region: madrone, black spruce, and (madrone – black spruce). The band at  $370\text{ cm}^{-1}$  due to the S unit is annotated along with the cellulose peaks at  $380$  and  $1096\text{ cm}^{-1}$ .

the normal spectra of madrone and BS along with the subtracted spectrum “madrone – BS” are shown. In Figure 6, the  $380$  and  $1096\text{ cm}^{-1}$  peaks of cellulose are annotated along with the S lignin units band at  $370\text{ cm}^{-1}$ . The  $370$ -area intensities, reported in Table 3, were measured from the (hardwood – BS) subtracted set of Raman spectra. Because the lignin and total carbohydrate contents of the hardwoods were different (Table 1 and Table 3), the Raman intensities thus obtained were further corrected. For this, the following two equations were used, i.e., eq 1 for lignin correction and eq 2 for total carbohydrate correction.

corrected intensity I

$$= (\text{measured } 370\text{-area intensity}) \times 100 / (\text{lignin content}) \quad (1)$$

corrected intensity II

$$= (\text{corrected intensity I}) \times (\text{total carbohydrate ratio}) \quad (2)$$

where total carbohydrate ratio is the ratio of total carbohydrate amounts between hardwood and black spruce (Table 3) and varies only minimally between the wood samples. Therefore, it is not likely to play a significant role in the %S determination. The corrected intensity II reflects the intensity of the S units per 100 lignin units and is further normalized with respect to

Table 3. Carbohydrate Composition and 370 cm<sup>-1</sup> Band Intensities of Various Wood Samples

| wood         | % glucan | % total carbohydrate <sup>a</sup> | total carbohydrate correction factor <sup>b</sup> | 370-area, a.u. | corrected 370-area <sup>c</sup> , a.u. |
|--------------|----------|-----------------------------------|---------------------------------------------------|----------------|----------------------------------------|
| cottonwood   | 46.3     | 63.8                              | 1.02                                              | 3.34 ± 0.07    | 13.19                                  |
| sweetgum     | 41.5     | 60.6                              | 0.96                                              | 5.87 ± 0.49    | 28.59                                  |
| willow       | 40.6     | 54.9                              | 0.87                                              | 5.04 ± 0.51    | 18.07                                  |
| aspen        | 46.9     | 65.6                              | 1.04                                              | 3.67 ± 0.35    | 25.42                                  |
| madrone      | 39.8     | 59.6                              | 0.95                                              | 5.47 ± 0.20    | 29.32                                  |
| white birch  | 40.4     | 63.0                              | 1.0                                               | 4.54 ± 0.19    | 29.54                                  |
| hickory      | 44.5     | 62.8                              | 1.0                                               | 5.41 ± 0.62    | 23.32                                  |
| black spruce | 41.8     | 62.8                              | NA <sup>d</sup>                                   | NA             | NA                                     |

<sup>a</sup>Total carbohydrate is sum of glucan, xylan, mannan, arabinan, and galactan. Because black spruce has glucomannan, part of its glucan is coming from the glucomannan. <sup>b</sup>Obtained by dividing the total carbohydrate contents of hardwoods by that of black spruce. <sup>c</sup>Corrected for differences in lignin and total carbohydrate amounts between hardwoods. This is same as area intensity II in eq 2. <sup>d</sup>Not applicable.

the total carbohydrate content of the woods. These corrected intensities (eq 2, called “corrected 370-area intensity” in Figures 7–9) were plotted against the %S determined by the

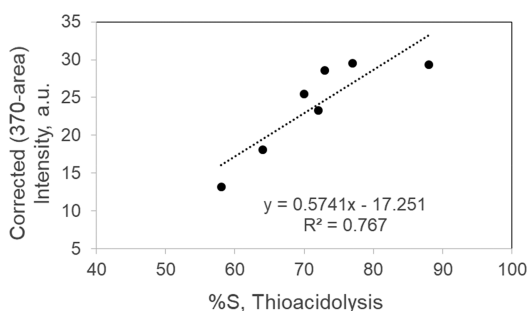


Figure 7. Correlation of the corrected 370 cm<sup>-1</sup> Raman band intensities (Table 3) and %S determined using the thioacidolysis method (S/(G + S + H)).

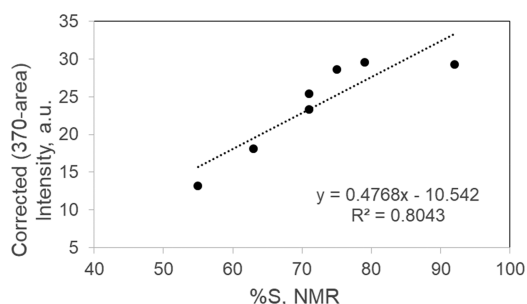


Figure 8. Correlation of the corrected 370 cm<sup>-1</sup> Raman band intensities (Table 3) and %S determined by 2D-HSQC NMR (S/(S + G)).

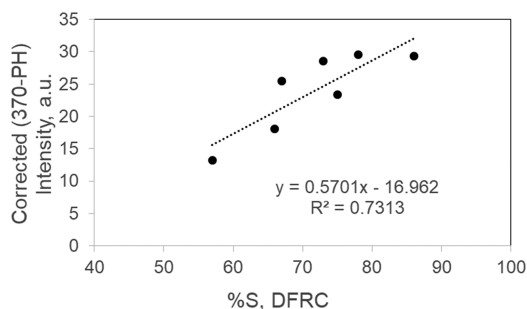


Figure 9. Correlation of the corrected 370 cm<sup>-1</sup> Raman band intensities (Table 3) and %S determined by DFRC (S/(G + S + H)).

normally used methods. The three correlations corresponding to thioacidolysis vs Raman, NMR vs Raman, and DFRC vs Raman are shown in Figures 7–9.

The correlations coefficients for %S obtained from Figures 7–9 and between the regularly used methods are summarized in Table 4. In the former case ( $R^2$  between 0.804 and 0.731), it

Table 4. %S Correlation Coefficients between Raman Spectroscopy and Traditional Methods

| method              | Raman | 2D-HSQC NMR | thioacidolysis | DFRC  |
|---------------------|-------|-------------|----------------|-------|
| Raman, spectroscopy | —     | 0.804       | 0.767          | 0.731 |
| 2D HSQC-NMR         | —     | —           | 0.994          | 0.926 |
| thioacidolysis      | —     | —           | —              | 0.949 |

is apparent that the Raman spectroscopy method showed good correlation with the other commonly used methods. The highest correlation ( $R^2 = 0.804$ ) was between the 2D-HSQC NMR and the Raman methods (Table 4). That is perhaps expected because both methods analyzed the syringyl content of the whole cell walls. Therefore, it is recommended that this correlation, eq 3 below, be used to estimate %S by Raman spectroscopy. In this equation,  $I_{370\text{-area}}$  is the corrected intensity II (eq 2) of the 370 cm<sup>-1</sup> band.

$$\%S_{\text{Raman}} = (I_{370\text{-area}} + 10.542)/0.4768 \quad (3)$$

#### S/G Ratio Estimation by Raman Spectroscopy.

Although G groups could not be directly determined by Raman spectroscopy, considering the H lignin units data from the other methods (Tables S2 and S4), it is safe to assume that the H lignin units are negligible in the hardwood lignins analyzed here. In that case, %G units can be simply calculated by subtracting %S from 100 (eq 4). Thus, estimated values of %G and S/G ratio (designated as  $(S/G)_{\text{Raman}}$  are listed in Table 5.

$$\%G = (100 - \%S) \quad (4)$$

The %S and S/G data from different methods are compared in Table 5. It can be noted that, overall, these values were similar to what was obtained from the standard methods.

In summary, to estimate %S in hardwood lignins, a method based on the intensity of the 370 cm<sup>-1</sup> band in the Raman spectra of the woods was developed. Although the 370-area Raman intensities correlated well with the %S data obtained using 2D-HSQC NMR, thioacidolysis, and DFRC methods, the intensities correlated best with the NMR data. Additionally, with the assumption that no H lignin units are present in

Table 5. %S, %G, and S/G by Raman Spectroscopy

| wood        | %S <sup>a</sup> | %G <sup>b</sup> | S/G (Raman) <sup>c</sup> | S/G (NMR) | S/G (DFRC) | S/G (thioacidolysis) |
|-------------|-----------------|-----------------|--------------------------|-----------|------------|----------------------|
| cottonwood  | 49.3            | 50.7            | 1.0                      | 1.23      | 1.36       | 1.40                 |
| sweetgum    | 84.2            | 15.8            | 5.3                      | 3.06      | 2.70       | 2.74                 |
| willow      | 65.5            | 34.5            | 1.9                      | 1.70      | 2.0        | 1.79                 |
| aspen       | 73.1            | 26.9            | 2.7                      | 2.40      | 2.03       | 2.38                 |
| madrone     | 86.9            | 13.1            | 6.6                      | 11.5      | 6.14       | 7.13                 |
| white birch | 83.9            | 16.1            | 5.2                      | 3.87      | 3.71       | 3.45                 |
| hickory     | 71.0            | 29.0            | 2.4                      | 2.45      | 3.0        | 2.68                 |

<sup>a</sup>From eq 3. <sup>b</sup>From eq 4. <sup>c</sup>Assuming no H lignin units present.

these hardwood lignins, G values and S/G ratios can be calculated based on the Raman data. The main advantages of the Raman method are its user-friendliness, simplicity, and avoidance of use of various chemicals, some of which are harmful. Nevertheless, an inability to detect low syringyl contents (<30%) and a limitation on analyzing woods (and other lignocellulosics) that produce high fluorescence upon laser excitation are two main disadvantages of the Raman method. In any case, like 2D-HSQC NMR, the Raman method provides an estimation of %S based on the whole cell wall. Therefore, the Raman method developed in this work appears to be useful for estimating %S (and S/G ratios) in hardwoods.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b06707.

Raman spectrum of Avicel (Figure S1); Raman band intensities and SDs of the models (Table S1); DFRC: percent molar ratio of H, G, and S aromatic units (Table S2); 2D HSQC NMR: percent molar ratio of G and S units (Table S3); and thioacidolysis: percent molar ratio of H, G, and S aromatic units (Table S4) (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

\*E-mail: uagarwal@fs.fed.us. Tel.: 608-231-9441. Fax: 608-231-9538.

### ORCID

Umesh P. Agarwal: 0000-0002-5509-1961

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## ■ ABBREVIATIONS USED

ASCII, American standard code for information interchange; BS, black spruce; 2D, two-dimensional; CA, coniferyl alcohol; DFRC, derivatization followed by reductive cleavage; DHP, dehydrogenation polymer; FT, Fourier transform; G, guaiacyl; GC/MS, gas chromatography–mass spectrometry; H, *p*-hydroxyphenyl; HA, *p*-hydroxycinnamyl alcohol; HSQC, heteronuclear single quantum coherence; MWL, milled-wood

lignin; Nd:YAG, neodymium-doped yttrium aluminum garnet; NMR, nuclear magnetic resonance; PLS, partial least-squares; PTFE, polytetrafluoroethylene; S, syringyl; SA, sinapyl alcohol; SD, standard deviation; S/G, syringyl/guaiacyl (ratio); %S, percent syringyl

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