LIGNIN QUANTITATION BY FT-RAMAN SPECTROSCOPY

Umesh P. Agarwal*

USDA FS, Forest Products Laboratory, Madison WI, 53726, USA.
uagarwal@fs.fed.us

ABSTRACT

Because of the structural complexity of biomass, quantitation of lignin in a variety of wood and plant cell walls is difficult. Consequently, to measure lignin in different plant species, a number of different methods exist. The methods can give different values for the same material samples and such differences result from the nature of the biomass. In the past, although there have been many applications of Raman spectroscopy in the studies of lignin, so far, for most materials, quantitation of lignin has proven to be a challenge. In the present work, a novel approach that successfully quantified lignin is described. The strategy, in part, was based upon the minimization of the contributions to the lignin band intensity at 1600 cm$^{-1}$ by those structures that contribute to it excessively, namely chromophores and aromatic-ring conjugated units in lignin. Using a variety of samples with lignin composition in the range 4.9 to 48.4% good linear correlations against Klason total lignins were developed (coefficients of determination $R^2$ 0.97 and 0.95, respectively).

Keywords: lignin, quantitation, raman spectroscopy, FT-Raman

INTRODUCTION

Over the years, a number of methods have been proposed to quantify lignin in lignocellulosic materials. However, considering that for such analysis either lignin has to be isolated completely or needs to be analyzed in its native state, most methods have proven to be less than totally satisfactory. Dence who reviewed this topic in 1992[5] had classified the available methods under the categories of direct and indirect methods. Under the former category were the methods that determined lignin amount gravimetrically after the substrates were acid hydrolyzed to remove carbohydrate components (e.g. acid-insoluble or Klason lignin determination method[1]). However, with the Klason procedure, it was found that the yield and composition of lignin depended upon the concentration of sulfuric acid and time and temperature of the treatment. Moreover, with the Klason method, depending upon the substrate, some lignin was found to be present in the filtrate from the hydrolysis stage. Such lignin was referred to as the acid soluble lignin. Moreover, if this latter lignin has a significant mass then it needs to be added, as an adjustment, to the Klason lignin amount. The methods of acid hydrolysis were further modified in the case of annual plants and forage; after the ethanol: toluene extraction the sample has to be successively treated by proteinase, dilute acid and 72% sulfuric acid. This has to be carried out for a sample where the protein content is high. Then there are the indirect methods where lignin need not be isolated, instead either lignin’s structural characteristic/functionality is measured and related to its concentration or lignin content is calculated by subtracting (from 100%) the carbohydrate content.

Falling in the indirect category is the interaction of lignin with radiation (absorption, fluorescence, scattering etc.) that has been used as a measure of lignin concentration in the material. Although most methods have limitations, microscopy (UV and interference) and spectroscopy (IR $^{13}$C NMR, Near-IR, and Raman) methods have all been used. Given the brief nature of this report, detailed discussions on the individual methods cannot be held and interested readers are referred to the literature (for a review see[2]). The other two lignin determination methods that also fall under the category of indirect methods are the acetyl bromide method and the methods used for lignin determination in unbleached chemical pulps, namely methods based on oxidant consumption. However, in this report, the author will focus on the role of Raman spectroscopy in lignin determination.

Early on, in 1987[3,4], lignin content in native and acid chlorite treated (delignified to various degrees) wood samples was determined using visible Raman spectroscopy. It was found that by excluding the data for untreated wood a good correlation between Klason lignin (or total lignin) and the relative Raman peak intensity at 1595 cm$^{-1}$ (relative with respect to band at 1098 cm$^{-1}$) could be developed. As was suggested in the publications, in the untreated wood, specific structures present in native lignin were likely to be behind the excessive contributions to the lignin band intensity at 1595 cm$^{-1}$. This was later shown to be the case, where data based on bleached mechanical pulps clearly established that when contributions of chromophores were minimized the 1601 cm$^{-1}$ band intensity declined very significantly[5]. This was also supported by the findings of an earlier study where Raman scattering at 1595 cm$^{-1}$ in untreated and bleached mechanical pulps was found to be excitation wavelength dependent[6] basically implying that the chromophoric structures in lignin were responsible for significant band intensity enhancement.

Subsequently, residual lignin in unbleached and partly bleached chemical pulps was successfully quantified using two different Raman approaches. The first approach involved use of the FT-Raman[7-9] technique whereas the work published in 2001 made use of UV-resonance Raman spectroscopy[10]. Although the residual lignin was
successfully quantified for this specific class of lignocellulosic samples (wood chemical pulps), Raman still lacked the capability of quantifying lignin across all matrices irrespective of the former’s structural features or content. The present study is intended to accomplish such an objective.

Considering that in the Raman spectrum of lignin the 1600 cm\(^{-1}\) band intensity depends upon other factors besides concentration (as mentioned above), it is important to make sure that these intensity enhancing effects/structures are either absent or their role is minimized. In the present strategy this is accomplished by combining three different chemical treatments all of which are lignin modifying but not removing. These treatments consisted of alkaline hydrogen peroxide bleaching, diimide hydrogenation, and sodium borohydride reduction. The bleaching with alkaline peroxide was preferred over other bleaching treatments because it removes/modify coniferaldehyde and quinone structures more effectively. It was expected that the peroxide-diimide combination treatment will produce the lowest 1600 cm\(^{-1}\) peak intensity, which would be the desired outcome.

Samples of 9 lignin containing materials were chosen such that they represented a wide range of lignin concentration (Klason lignin content from 4.9 to 48.4%). These consisted of 2 samples of southern pine (obtained from different sources) and 1 sample each of black spruce, aspen, white oak, partly delignified black spruce, ball milled enzyme lignin (BMEL) from black spruce, unbleached southern pine kraft pulp, and corn stalk. Corn stalk was the only herbaceous material sample in the set of 9 samples.

**EXPERIMENTAL**

**Alkaline Peroxide Bleaching**

After acetone: water (9:1) extraction 1.2 gram of each sample was washed in deionized water for oxidative bleaching using alkaline hydrogen peroxide. Bleaching conditions for the samples on an o.d. weight basis were 5% H\(_2\)O\(_2\), 5.3% total alkali, 0.1% Mg and 4% Na\(_2\)SiO\(_3\). Initial pH just after mixing the chemicals with the sample was 11.7 and bleaching was performed at 11% consistency. The samples and bleaching solution were put in a zip-lock bag and mixed by kneading. The bag was kept in a 70°C water bath for 2.5 h. Samples were remixed periodically by kneading to ensure uniform distribution of bleaching chemicals. After 2.5 h, the samples were filtered and washed first with deionized water and then with dilute sodium bisulphite solution.

**Diimide Treatment**

Diimide treatment was done on alkaline hydrogen peroxide treated samples. In 100 mL of acetone, 1 gram of dry sample was added while stirring and the mixture was aerated for 20 minutes. 160 microliters of hydrazine hydrate solution was added along with 1.2 mg of copper sulfate pentahydrate. The mixture was stirred with aeration for 2 hours, then filtered and washed with absolute ethanol.

**Sodium Borohydride Treatment**

Samples of corn stalk, unbleached kraft pulp, and partly delignified black spruce wood were treated with 0.5 M NaBH\(_4\) for 2 hours at room temperature. It was found that for these samples the borohydride treatment further reduced the intensity of the Raman band at 1600 cm\(^{-1}\).

**Chemical Analysis**

Using standard chemical methods, all of the samples were subjected to compositional analysis for Klason lignin, acid soluble lignin, and carbohydrates. This information was necessary to calibrate the Raman method.

**Raman Spectroscopy**

A Bruker RFS-100 near-IR FT–Raman spectrometer (Bruker Instruments, Inc., Billerica, MA) was used to obtain the spectra. Samples were excited using a 1064-nm Nd:YAG diode laser. The samples were pressed into pellets using a hydraulic press and 1024 scans were accumulated using 750 mW of laser power. For most samples the laser was focused on the pellet surface and a front coated mirror was positioned behind the pellet. For samples that were fluorescent when excited at 1064 nm, laser was used in the defocused mode and the number of scans accumulated was increased to get a good signal-to-noise ratio.

Peak intensities of 1600 and 1096 cm\(^{-1}\) bands were calculated using the OPUS’s (RFS-100 software) sloping background method. For this purpose, spectra were cut between 250 and 1850 cm\(^{-1}\) and baseline corrected using the rubber band correction. In each spectrum, the measured peak height for the 1600 cm\(^{-1}\) band was converted to relative intensity by dividing the former by the carbohydrate band intensity at 1096 cm\(^{-1}\) (\(I_{1600}/I_{1096}\)). This was done considering that Raman spectroscopy is a semi-quantitative technique and an internal standard is needed (for normalizing the spectra) in order for the intensities to be compared (Table 1). Moreover, because the total carbohydrate content varied between the materials (Table 1), before the (\(I_{1600}/I_{1096}\)) data can be used for lignin comparison purposes, they need to be corrected with respect to the total carbohydrate difference. For example, the total carbohydrate content of unbleached kraft pulp and partly delignified black spruce wood are 94 and 74%, respectively (Table 1). To reflect this difference, the (\(I_{1600}/I_{1096}\)) data for the unbleached kraft was multiplied by 1.27 (the ratio 94/74). This “corrected data” (\(I_{1600}/I_{1096}\)\(_{corr.}\)) was then used in the quantification of lignin analysis.

Similarly, when this total carbohydrate correction was applied to the remaining lignocellulosic materials, factors other than 1.27 had to be used due to the materials’ different total carbohydrate contents (compared to unbleached kraft; Table 1).
### Table 1. Chemical composition analysis

<table>
<thead>
<tr>
<th>Sample</th>
<th>Klason Lignin</th>
<th>Acid Soluble L.</th>
<th>Total Carbohydrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unbl. Kraft</td>
<td>4.9</td>
<td>0.8</td>
<td>94.0</td>
</tr>
<tr>
<td>Partly Delig. B. Spruce</td>
<td>8.1</td>
<td>4.3</td>
<td>74.0</td>
</tr>
<tr>
<td>Corn Stalk</td>
<td>13.8</td>
<td>5.8</td>
<td>63.0</td>
</tr>
<tr>
<td>Aspen</td>
<td>18.3</td>
<td>3.3</td>
<td>65.6</td>
</tr>
<tr>
<td>W. Oak</td>
<td>25.2</td>
<td>4.2</td>
<td>58.4</td>
</tr>
<tr>
<td>B. Spruce</td>
<td>27.2</td>
<td>0.7</td>
<td>64.1</td>
</tr>
<tr>
<td>S. Pine, 1</td>
<td>28.9</td>
<td>0.8</td>
<td>62.0</td>
</tr>
<tr>
<td>S. Pine, 2</td>
<td>28.9</td>
<td>0.8</td>
<td>62.0</td>
</tr>
<tr>
<td>BMEL, B. Spruce</td>
<td>48.4</td>
<td>1.0</td>
<td>42.7</td>
</tr>
</tbody>
</table>

*Same values are used as for S. Pine 1 sample

### RESULTS AND DISCUSSION

Whereas the bleaching of wood mechanical pulps with alkaline hydrogen peroxide is a very well researched topic\(^{[11]}\), the diimide treatment to remove conjugated structures in lignin has not been studied in detail\(^{[12]}\). To determine how long a treatment with diimide will be sufficient, 20 mesh extracted black spruce wood was treated for different durations (2, 4, 8, and 22 h). The conclusion was that after 2 h very little additional reduction occurred in the peak heights of the lignin Raman bands at 1600 and 1654 cm\(^{-1}\). Therefore, it was decided to perform the diimide reduction for 2 h.

Raman spectra of the 9 samples are shown in Fig. 1. The spectra are from those samples that have undergone a sequence of treatments which generated most decline in the intensity of the 1600 cm\(^{-1}\) band. It was found that the number of treatments needed and the sequence of the treatments was material dependent (sequences are identified in Table 2). In this respect, it is to be noted that the sample of corn stalk was the most difficult to treat (Table 2). The corn stalk treatment sequence consisted of 4 stages - alk. H\(_2\)O\(_2\), followed by diimide, then by NaBH\(_4\), followed by another diimide.

The need for different treatment sequences for accomplishing a minimized 1600 cm\(^{-1}\) intensity (without removing lignin) was probably due to the differences in the nature of the cell walls of various materials. Based on the spectra shown in Fig. 1 relative band intensities \((I_{1600}/I_{1096})\) and \((I_{1600}/I_{1096})_{\text{corr}}\) were calculated (Table 2). The latter are the intensities corrected for differences in total carbohydrate content.

### Correlations

The \((I_{1600}/I_{1096})_{\text{corr}}\) lignin intensities (Table 2) were plotted against the Klason and total lignin data (Table 1) and the two were found to be linearly correlated (Fig. 2). The coefficients of determination were 0.97 and 0.95 for of the Klason and total lignin correlations, respectively. It’s important to note that the correlations were valid over a variety of materials and a large range of lignin concentration.

### Table 2. Relative Raman band intensities \((I_{1600}/I_{1096})\)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment Sequence</th>
<th>((I_{1600}/I_{1096}))</th>
<th>((I_{1600}/I_{1096})_{\text{corr}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unbl. Kraft</td>
<td>Alk.-H(_2)O(_2) then NaBH(_4)</td>
<td>0.12</td>
<td>0.15</td>
</tr>
<tr>
<td>Partly Delig. B. Spruce</td>
<td>Alk.-H(_2)O(_2) then NaBH(_4)</td>
<td>0.39</td>
<td>0.39</td>
</tr>
<tr>
<td>Corn Stalk</td>
<td>Alk.- H(_2)O(_2) then Diimide then NaBH(_4) then Diimide</td>
<td>0.76</td>
<td>0.66</td>
</tr>
<tr>
<td>Aspen</td>
<td>Alk.- H(_2)O(_2) then Diimide</td>
<td>0.75</td>
<td>0.66</td>
</tr>
<tr>
<td>W. Oak</td>
<td>Alk.- H(_2)O(_2) then Diimide</td>
<td>1.19</td>
<td>0.94</td>
</tr>
<tr>
<td>B. Spruce</td>
<td>Alk.- H(_2)O(_2) then Diimide</td>
<td>1.54</td>
<td>1.34</td>
</tr>
<tr>
<td>S. Pine, 1</td>
<td>Alk.- H(_2)O(_2) then Diimide</td>
<td>1.46</td>
<td>1.22</td>
</tr>
<tr>
<td>S. Pine, 2</td>
<td>Alk.- H(_2)O(_2) then Diimide</td>
<td>1.61</td>
<td>1.35</td>
</tr>
<tr>
<td>BMEL, B. Spruce</td>
<td>Alk.- H(_2)O(_2) then Diimide</td>
<td>4.0</td>
<td>2.32</td>
</tr>
</tbody>
</table>

### CONCLUSIONS

The modification of a variety of lignocellulosic materials by various combinations of chemical treatments facilitated the quantitation of lignin by FT-Raman spectroscopy. Such treatments successfully reduced the original intensity of the 1600 cm\(^{-1}\) band of lignin and therefore, minimized the excessive contributions of chromophores and conjugated groups. Good correlations between the intensity of lignin’s 1600 cm\(^{-1}\) band and Klason and total lignins were obtained. Raman spectroscopy, a non-invasive method, has the potential to be developed as a lignin quantitation technique for diverse bio-materials and cell walls, irrespective of their lignin composition and content.

### ACKNOWLEDGMENTS

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Fig. 2. Correlations of lignin’s 1600 cm⁻¹ band intensity with % Klason and total lignin for various lignocellulosic materials. % Klason lignin are listed in parentheses. Black spruce MWEL (48.4), 2 samples of southern pine (28.9), black spruce (27.3), white oak (25.2), aspen (18.3), corn stalk (13.8), partially delignified black spruce (8.1), and unbleached kraft pulp (4.9)

REFERENCES


