Quantitative \(^{13}\)C NMR Characterization of Lignin

I. A Methodology for High Precision

By Lawrence L. Landucci
Forest Products Laboratory, Forest Service
U.S. Department of Agriculture, Madison, WI 53705, USA

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- \(^{13}\)C NMR Spectroscopy
- Quantitation
- Lignin
- Milled Wood Lignin
- Precision
- Acetylated lignin

**Introduction**

Quantitative \(^{13}\)C NMR analysis is often a very demanding procedure. Many requirements must be met, for example, complete relaxation of all nuclei between pulses, elimination of the nuclear Overhauser effect (NOE), uniform power bandwidth over the entire spectral range, accurate and unbiased phasing of both spectrum and integral, and adequate methods of integration. These have been described in detail (Martin, Delpuech, and Martin 1980; Schoeller 1977; Sotak, Dumoulin, and Levy 1983). Analytical schemes based on \(^{13}\)C NMR have not been very popular because of the excessive instrument time required by the long relaxation delays and because several repetitions must be performed to determine the reproducibility of the results under a given set of conditions. Quantitation has frequently been applied to mixtures of relatively simple compounds, the spectra of which contain well resolved resonances (Blunt and Munro 1976). Severe peak overlap in \(^{13}\)C spectra of complex biopolymers such as lignin has been a detriment to quantitation. However, with higher field magnets in which the \(^{13}\)C nuclei resonate at a frequency of at least 50 MHz, valuable information can be obtained by integrating peak clusters representing specific carbon types. The first report of quantitative analysis of lignins by \(^{13}\)C NMR spectroscopy was by Robert and Gagnaire (1981). In this report and in subsequent reports (Robert 1982; Robert, Bardet, and Gagnaire 1983) a precision of 10% was assumed, but no replicate data were given, to support this value. Also, some integral phase errors were evident in the published spectra (Robert, Vardet, and Gagnaire 1983) which affect quantitation.

The purpose of this study was to develop an improved methodology for rapid quantitative analysis of various peak clusters in \(^{13}\)C NMR spectra of lignins and to determine the precision of such analyses by replication. The effects on precision of various acquisition and processing parameters were also determined. A single sample of a white birch (Betula papyrifera Marsh.) milled wood lignin (MWL) was used for the study to eliminate chemical effects. The MWL was acetylated to allow its dissolution in d_6-acetone, which does not contain any interfering peaks.
Experimental

Preparation of Acetylated MWL
The white birch MWL was prepared from a high-yield kraft pulp as described previously (Obst and Ralph 1983), followed by acetylation with 1/1 (v/v) acetic anhydride/pyridine for 18 hours at room temperature.

$^{13}$C NMR Experiments

The NMR spectra were obtained with a Bruker WM-250 spectrometer controlled by an Aspect 2000A minicomputer. A 28% solution of the acetylated MWL in $d_6$-acetone was used for all experiments. For large samples (700 mg) a 10 mm single frequency probe (62.89 MHz) was used, and for small samples (70 mg) a 5 mm broadband probe was used. Unless noted otherwise, with the 10 mm probe, 100–2,000 free induction decays (FID’s) of 16K data points were accumulated over a spectral width of 30,000 Hz at 310° K. Pulse widths of 7 and 14 microseconds ($\mu$sec) corresponding to a tip angle of 45° and 90° of the macroscopic magnetization, were used along with corresponding relaxation delays of 8 and 16 sec., respectively. With the 5 mm probe, 8,000 transients were accumulated and a pulse width of 6 $\mu$sec corresponding to a tip angle of 45° was used. The NOE was suppressed by a gated decoupling pulse sequence (Schoolery 1977). The FID’s were zero-filled to 32K data points and multiplied by an exponential factor corresponding to a 4 Hz line broadening prior to Fourier transformation. All lignin peaks were defined by at least five data points above half-height.

The Aspect minicomputer was programmed to run a sequence of gated decoupling experiments with different parameter levels. At the end of a sequence the program cycled back to the beginning and ran the desired number of replications. With this method, any detuning which occurred during the entire time span was averaged amongst the replications.

Results and Discussion

Areas Measured

In Figure 1 are illustrated the peak clusters which were measured in the $^{13}$C NMR spectrum of an acetylated white birch MWL. Each cluster is separated from the adjoining one by a sufficient amount of baseline (see insets) to allow precise measurement. It was found that ample baseline at both limits of the integral is essential for accurate phasing and reproducibility to within ± 10%. Also, it has been reported that these limits must be consistent throughout a series of experiments (Sotak, Dumoulin, and Levy 1983). Although higher magnet strengths could conceivably result in the definition of more integrable clusters, Figure 1 represents the maximum number for a 250 MHz instrument.

Area Ratios

Useful information pertaining to lignin structure can be obtained from ratios of the areas of peak clusters. For example, an indirect measure of the syringyl/guaiacyl ratio can be obtained from the aromatic/methoxyl ratio as follows. Assuming that hardwood lignin is composed of a mixture of three basic C$_9$ units, p-hydroxyphenyl, guaiacyl, and syringyl, as shown in Figure 2, the aromatic/methoxyl ratio (R) can be represented by:

$$ R = \frac{6h + 6g + 6s}{g + 2s} $$

where h, g, and s are mole ratios of the p-hydroxyphenyl, guaiacyl, and syringyl units, respectively. Since

$$ h + g + s = 1 $$

then

$$ R = \frac{6}{g + 2s} $$

Solving the two simultaneous equations 2 and 3 for the syringyl/guaiacyl ratio (s/g) gives:

$$ s/g = \frac{R (h - 1) + 6}{2R (1 - h) - 6} $$

which reduces to

$$ s/g = \frac{6 - R}{2R - 6} $$

if h is assumed equal to zero.

Figure 1. Areas measured in $^{13}$C NMR spectrum of acetylated white birch milled wood lignin.

Figure 2. Phenylpropane (C$_9$) units present in hardwood lignin.

Frequently, the methoxyl content per C$_9$ phenylpropane unit is reported in the literature. Since there are six aromatic carbons in a C$_9$ unit this value can be obtained by the expression:

$$ \frac{OCH_3}{C_9} = \frac{6}{R} $$
It should be noted that the relative deviation of values calculated from peak ratios is not necessarily the same as that of the ratio. For example, for a 2.8 hour run the ratio aromatic/methoxyl = 3.80 ± 1.3% (Table 1). Calculation of s/g and OCH₃/C₉ from this ratio by equations 5 and 6, respectively, gives values of 1.38 ± 8.7% and 1.58 ± 1.3%. Thus, the relative deviation remains the same for OCH₃/C₉, but is much greater for the s/g ratio.

Robert (1982) reported direct measurement of the syringyl content by integrating syringyl and guaiacyl resonances for a birch MWL. However, the high value (80%) calculated for syringyl C₉ units by this method conflicts with more typical syringyl contents not exceeding 59% based on methoxyl analyses (Sarkanen and Hergert 1971). We attempted a similar direct method but commonly found variations in excess of ± 20%. In addition to difficulties in integral phasing when inadequate baseline is present, potentially large variations can result from improper peak assignments or hidden peaks within the aromatic region. The indirect method is relatively insensitive to specific assignments.

Optimum Relaxation Delay

Generally, for valid quantitative data the relaxation delay between pulses must be at least 5T₁, where T₁ is the spin-lattice relaxation time (Martin et al. 1980). The T₁'s of typical MWL’s have been determined to range from 0.1 sec to 3 sec (Nimz 1982; Robert 1982). Therefore, for a 90° tip angle a delay of at least 15 sec should be used. In this study the effect of relaxation delay on four ratios was determined. As shown in Figure 3 the ratios which are sensitive to relaxation delay become constant after about 6 sec. which is consistent with previously determined T₁’s since a 45° tip angle was used in these experiments.

Sample Size and Accumulation Time

A small MWL sample (70 mg) and a large sample (700 mg) were run repeatedly to test the precision of four ratios (Table 1). With large samples, runs of only 2.8 hours resulted in reproducibility of all ratios within ± 5%. With 5.6 hour runs, the precision was ± 3%. With small samples, runs of fewer than 8,000 pulses gave unsatisfactory spectra which were difficult to integrate. For 8,000 pulses, 22 hours was required to give a precision of ± 10%.

Baseline Quality

During signal averaging, instrumental artifacts contribute to baseline distortions. The familiar “baseline roll” (Schoolery 1977) present in ¹³C NMR spectra of lignin prevents reproducible quantitation, so it must be corrected or prevented. Baseline correction by conventional software methods proved to be too arbitrary and resulted in unsatisfactory precision. More sophisticated software has recently been developed which minimizes operator bias (Sotak, Dumoulin, and Levy 1983). However, in the present study no baseline corrections were used. It was found that baseline imperfections could be minimized by using a spectral width about twice that needed for the lignin peaks. The effect of spectral width on the precision of the two ratios most difficult to reproduce is shown in

<table>
<thead>
<tr>
<th>Sample size (mg)</th>
<th>Number of runs</th>
<th>Pulses</th>
<th>Acetate CH₃/ Aromatic CH₃ = O</th>
<th>Aromatic Methoxyl</th>
<th>Aromatic Aliphatic</th>
<th>Acetate CH₃/ Methoxyl</th>
<th>Run time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>700</td>
<td>6</td>
<td>2000</td>
<td>1.06(0.9)</td>
<td>3.73(1.3)</td>
<td>2.22(2.7)</td>
<td>1.09(0.9)</td>
<td>5.6</td>
</tr>
<tr>
<td>700</td>
<td>6</td>
<td>1000</td>
<td>1.05(2.9)</td>
<td>3.80(1.3)</td>
<td>2.24(4.9)</td>
<td>1.09(0.9)</td>
<td>2.8</td>
</tr>
<tr>
<td>700</td>
<td>6</td>
<td>500</td>
<td>1.02(2.9)</td>
<td>3.35(2.6)</td>
<td>2.32(6.0)</td>
<td>1.08(2.7)</td>
<td>1.4</td>
</tr>
<tr>
<td>700</td>
<td>6</td>
<td>250</td>
<td>1.03(4.9)</td>
<td>3.75(5.3)</td>
<td>2.14(8.9)</td>
<td>1.06(2.8)</td>
<td>0.7</td>
</tr>
<tr>
<td>700</td>
<td>6</td>
<td>100</td>
<td>1.06(7.5)</td>
<td>3.74(6.4)</td>
<td>2.37(11.8)</td>
<td>1.08(7.4)</td>
<td>0.3</td>
</tr>
<tr>
<td>700</td>
<td>6</td>
<td>8000</td>
<td>1.05 (8.5)</td>
<td>4.07(10.1)</td>
<td>2.16(5.1)</td>
<td>1.12(2.7)</td>
<td>22</td>
</tr>
</tbody>
</table>

1The ratios for each run were determined from the average of five separate integrations. Reported values represent the averages of all runs.
2Precisions are given as ± percent deviations in parentheses.
Table 2. The striking difference in baseline quality between the two extremes in spectral width is shown in Figure 4. Another factor which affects the quality of the baseline is the initial phasing of the spectrum. It was found that the entire range of resonances must be phased as an entity. Attempts to phase sections of the spectrum individually introduced large deviations, and it was difficult to prevent biasing the data during replicate phasings.

Table 2. Effect of spectral width on precision

<table>
<thead>
<tr>
<th>Spectral width (Hz)</th>
<th>Aromatic Methoxyl (% deviation)</th>
<th>Aromatic Aliphatic (% deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15,000</td>
<td>3.62 (1.9)</td>
<td>2.05 (6.3)</td>
</tr>
<tr>
<td>20,000</td>
<td>3.79 (1.6)</td>
<td>2.25 (6.2)</td>
</tr>
<tr>
<td>30,000</td>
<td>3.75 (0.8)</td>
<td>2.21 (2.3)</td>
</tr>
</tbody>
</table>

Table 3. Effect of tip angle on precision of Aromatic/Methoxyl ratio

<table>
<thead>
<tr>
<th>Run length (hr)</th>
<th>Tip angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>45° (delay = 8 sec)</td>
<td>± 5.3% ± 9.1%</td>
</tr>
<tr>
<td>1.4</td>
<td>± 2.6% ± 6.5%</td>
</tr>
<tr>
<td>2.8</td>
<td>± 1.3% ± 1.6%</td>
</tr>
</tbody>
</table>

Integrations Methodology

Of utmost importance for precise quantitation was the maintenance of fixed integral limits for each peak cluster (Fig. 1). These limits were identical for all comparative experiments, and ample baseline at both limits for each cluster was essential for precise unbiased integral phasing. After phasing, the areas were measured over a predetermined frequency span (identical for all experiments) which did not necessarily correspond to the integral limits. The areas were obtained directly from the computer instead of by manual measurement of plotted integrals which is another potential source of variation. In Table 4 are listed the frequency limits which were used for integral phasing, along with the limits used for the area measurements for the peak clusters shown in Figure 1. Shortening the limits for area measurements served to eliminate unnecessary baseline and also certain overlapping peaks which were not representative of the carbon type in a particular cluster. For example, for the methoxyl peak a 59–50 ppm integral was necessary for reproducible phasing, but the area was measured only over the 59–56 ppm interval in order to eliminate the small peak at about 55.4 ppm which has been assigned to Cβ in pinoresinol structures (Nimz 1980). Similarly, the area measurement over the aromatic cluster was limited to exclude resonances at 95–102 ppm due to C–1 of hemicelluloses (Lapierre, Lallemand, and Monties 1982; Landucci 1984). Some causes of interference, such as vinyl carbons which appear in the aromatic region rather than with the rest of the side chain carbons, cannot be prevented by this integration technique.

Table 4. Frequency limits' for integral phasing and area measurements

<table>
<thead>
<tr>
<th></th>
<th>Acetate C = 0</th>
<th>Aromatic</th>
<th>Aliphatic</th>
<th>Methoxyl</th>
<th>Acetate CH₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integral phasing</td>
<td>175–162</td>
<td>164–95</td>
<td>95–59</td>
<td>59–50</td>
<td>22.5–17</td>
</tr>
<tr>
<td>Area measurement</td>
<td>175–167</td>
<td>164–102</td>
<td>95–59</td>
<td>59–56</td>
<td>22.5–19.4</td>
</tr>
</tbody>
</table>

Figure 4. Uncorrected baselines when a spectral width (SW) of a) 15,000, and b) 30,000 Hz were used.

Pulse Width

Although the pulse width typically used in quantitative studies corresponds to a 90° tip angle of the macroscopic magnetization, it was found that a 45° tip angle gave greater precision (Table 3). This is probably the result of a more uniform power bandwidth over the spectral range caused by the shorter pulse width.
Conclusions

It was demonstrated that various peak clusters in the $^{13}$C NMR spectra of a white birch MWL could be measured with high precision. The methodology presented in this report is partially instrument-dependent and is by no means rigid. It must be emphasized, however, that a sufficient number of replications is necessary in order to make a statistical analysis of the data, and achieve observations of the desired precision. Because of the many sources of variation in $^{13}$C NMR spectroscopy of lignin, any quantitative measurements made without sufficient replication and careful control of all phases of the experiment are highly questionable. Generally, arbitrary manipulations of the data, such as manual baseline corrections, should be avoided. Although the precision obtained in this study was high, the accuracy of the data is dependent entirely upon the validity of the peak assignments and the basic assumptions (e.g. the mole ratio of the p-hydroxyphenyl units and the absence of vinyl carbons in the aromatic region). In subsequent reports the methodology described here will be applied to problems relating to lignin characterization.

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References


