OXIDATION AND QUANTIFICATION OF 
14C-LIGNIN AT DIFFERENT AGES IN WHEAT, PINE, OAK, AND KENAF

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ABSTRACT
Plant materials, containing 14C-labeled lignins, were harvested at 
intervals up to 1 year after labeling. They were oxidized with sodium 
chlorite (NaClO₂) and nitrobenzene. Age of lignin appears to limit 
the degree of solubilization of lignin by NaClO₂. Nitrobenzene 
oxidation solubilized all lignins in wheat, oak, and kenaf but not all 
lignin in some pine samples. Klasson and UV analysis of lignin content 
were compared with 14C-content to determine percent lignin soluble in 
3% sulfuric acid (Klasson analysis solvent), corrected lignin contents, 
and UV absorptivity of lignin. Ten to 20% of most lignins were soluble 
in 3% sulfuric acid. When corrected Klasson lignin contents were used, 
UV absorptivities at 280 nm were about 38 g⁻¹ 1 cm⁻¹ for wheat 
straw and kenaf lignins and 11 g⁻¹ 1 cm⁻¹ for pine lignin.

INTRODUCTION
Lignin is a major energy-rich, aromatic polymer in terrestrial 
woody-plant biomass. Economically successful biomass conversion 
processes will use nearly all of a plant, including lignin and its 
degradation products. One logical approach to using lignin is to 
separate it from other plant components. Sodium chlorite, hydrogen 
peroxide, copper oxide, and nitrobenzene are oxidants that 
preferentially oxidize lignin and degrade it to aromatic aldehydes, 
aromatic acids, aliphatic acids, and carbon dioxide.¹² Many
oxidation products have been identified and related to the original structure of the lignin. Brink et al. oxidized spruce with nitrobenzene and isolated several aliphatic acids. They suggested that formic acid and related compounds resulted from carbohydrate oxidation, and oxalic acid and related compounds resulted from lignin oxidation. Aromatic aldehydes resulting from nitrobenzene oxidation of lignin are often used to classify a plant lignin by type: guaiacyl (from gymnosperms), syringyl-guaiacyl (from dicotyledonous angiosperms) or syringyl-guaiacyl-p-hydroxyphenyl (from monocotyledonous angiosperms). Reported yields of reaction products from nitrobenzene oxidation of lignin varied from 25% of the lignin (measured by Klason analysis) to 51% of the milled wood lignin starting material. In preliminary research we discovered that all of the lignin in wheat, kenaf, oak, and pine could be solubilized by nitrobenzene oxidation. This has not been previously reported to our knowledge. To thoroughly investigate this observation, we produced $^{14}$C-containing lignins of different ages and different structures and oxidized them with nitrobenzene. $^{14}$C-lignins were also oxidized with NaClO$_2$ for comparison to the nitrobenzene oxidation results. Lignin was measured by UV, Klason, and $^{14}$C analyses before and after oxidations to determine the amount solubilized. These reaction studies were designed to show that the $^{14}$C-lignins are representative of mature plant lignin, and that nitrobenzene oxidation results are reliable.

**EXPERIMENTAL**

Wheat, kenaf, and pine were chosen to represent the three structural types of plant lignin. Oak was also tested because annual
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and perennial dicotyledonous angiosperms differ in amount and accessibility of their lignin. Wheat (Triticum aestivum L., Hard Red Spring variety) and kenaf (Hibiscus cannabinus L., Everglade 41 variety) were grown from seed and fed 50 μCi (wheat) or 100 μCi (kenaf) per plant of L-[$^{14}$C]-phenylalanine in 0.01 N HCl as received from ICI, Biomedicals, Inc., Irvine, CA.

A small area of the plant's roots were exposed by gentle washing. The exposed roots were placed in a vial containing a 0.5 ml solution of L-[$^{14}$C]-phenylalanine (50 μCi in 0.01 N HCl for wheat) and placed in the dark. With few exceptions, the phenylalanine was taken up in 3 to 5 hr, and 3 ml additional water was taken up in 17 hr or less. The roots were then replaced in the soil and the plant continued to grow. Feeding phenylalanine through a few exposed, cleaned roots allowed continued growth of the plants without injury unlike other methods of $^{14}$C-labeling. The phenylalanine was fed to wheat plants 36 days after planting and to kenaf 65 days after planting. Wheat at 36 days has leaves, and its stalks are 10 to 15 cm tall. Kenaf at 65 days has one primary stalk with a few branches and an average height of 1.3 m. Three kenaf and six wheat plants were fed phenylalanine in this way.

Four eastern white pines (Pinus strobus L.) were purchased from Hoerr Nursery, Peoria, Illinois and 4 unidentified oaks of one species were selected from the wild in Peoria County, Illinois. They were 30 to 60 cm tall. Each was fed 100 μCi of L-[$^{14}$C]-phenylalanine in 1.0 ml solution through a few exposed roots as was described above for wheat and kenaf.
Plants were harvested at specific days after initial $^{14}$C-labeling: wheat—15, 30, and 55 days (2 plants per harvest); kenaf—30, 60 and 120 days (1 plant per harvest); pine—30, 180, and 270 days (1 plant per harvest); oak—30 and 328 days (1 plant per harvest). Wheat and kenaf control plants were harvested as mature plants grown to full term without labeling. One each of the pine and oak trees was harvested immediately as a control sample with no $^{14}$C-labeling. All plants were selected for similar growth characteristics.

Harvested plants were divided into roots, stalk, and leaves, then dried at 45°C, ground in a Wiley-type mill equipped with a screen containing 2-mm-diameter holes, and extracted. The ground plant parts were extracted sequentially with benzene:ethanol (2:1), water at 4°C and treated with protease at 28°C, then washed with water and freeze-dried (Figure 1).

Nitrobenzene oxidation was performed carefully by placing freeze-dried plant material (1 g) in the bottom of a stainless steel pressure vessel, then adding nitrobenzene (6.0 ml) dropwise to thoroughly wet the sample. NaOH (120 ml, 2 N) was added last, and the vessel was flushed with $N_2$ for 30 s before sealing. The reactants were heated to $160\pm5^0$ for 2.5 hr and then cooled immediately. The reaction mixture was filtered through a polypropylene fritted funnel, and the insoluble residue was washed with water and ethanol.

For $^{14}$C-labeled samples, 100 ul of the alkali-soluble phase was counted in 10 ml of a scintillation fluid previously reported. The
washed residue was dried and then pyrolyzed to CO₂ in an oxygen combustion furnace. The CO₂ was trapped in the same scintillation fluid as mentioned above and counted (Figure 1). Separate portions of the same residue were also analyzed for lignin by Klason and UV methods.

Two procedures were used for NaClO₂ oxidation. Procedure No. 1 is similar to that commonly employed. Except for wheat, each plant sample (10 g in 400 ml H₂O) was treated at 90°C four times with 5 ml glacial acetic acid and 15 g NaClO₂ for 30 min each. Procedure No. 2, consisting of lower temperature (75°C), more dilute solution (10 g in 500 ml H₂O), less reagent (0.6 ml acetic acid and 7.5 g NaClO₂, and 3 treatments, was used to oxidize wheat lignin. Klason analysis was performed as described by Pettersen, except that a medium-porosity polypropylene filter was used to collect the insoluble lignin. Filtrates were clear but brown. After several days of standing, precipitates formed in five samples. These precipitates were collected on Whatman 541 filter paper and pyrolyzed to determine ¹⁴C content. ¹⁴C-Analysis of these additional small quantities of precipitate revealed that the polypropylene filter effectively retained 97% or more of the precipitated lignin. Soluble ¹⁴C was measured on the neutralized (by NaOH or Amberlite 45 resin), filtered, and concentrated solubles from the Klason analysis (see Figure 1).

RESULTS AND DISCUSSION

Specific activities are defined as the disintegrations per minute (dpm) of ¹⁴C in samples, as measured by a scintillation counter,
Harvested Plants

Root Stalk Leaves

1. Dry at 45°C, grind
2. Benzene:ethanol (2:1) extraction
3. Water soak, 4°C
4. Protease

Freeze-dried plant parts

UV analysis for lignin,
pyrolysis to CO₂ and ^14CO₂ counted

Klason (72% H₂SO₄)

Solubles
1. Neutralize
2. Filter
3. Concentrate

^14C counted

Insolubles

Nitrobenzene Oxidation

Solubles
1. Klason analysis

^14C counted

Insolubles
2. UV analysis for lignin

Solubles
3. Pyrolysis to CO₂ and ^14CO₂ counted

NaClO₃ Oxidation

Solubles (discard)

Figure 1. Analytical procedures.

divided by the weight of the sample (mg). Specific activities (dpm/mg)
of the extracted plant parts are listed in Table I. The plant parts
were pyrolyzed to CO₂ and the CO₂ trapped in scintillation fluid
which was then counted in a scintillation counter to determine their
^14C content. The slower uptake of nutrients by oak and the large
mass of the pine trees reduced the specific activities of oak and pine
plant parts. Thus, the roots of oak and pine were used in our study
because they had higher specific activities than other plant parts.
Even low levels of ^14C-label (9 to 100 dpm/mg) were accurately
determined by pyrolysis of 20 to 40 mg of sample and collection of the
resulting CO₂ in scintillation fluid. Four to 8 replicates were
averaged to obtain data for Table I. Lignin contents, measured by
OXIDATION AND QUANTIFICATION OF $^{14}$C-LIGNIN

TABLE I
Specific Activities, dpm per mg

<table>
<thead>
<tr>
<th>Lignin Age</th>
<th>Wheat 15</th>
<th>Wheat 30</th>
<th>Wheat 55</th>
<th>Kenaf 30</th>
<th>Kenaf 60</th>
<th>Kenaf 120</th>
<th>Oak 30</th>
<th>Oak 32R</th>
<th>Pine 30</th>
<th>Pine 180</th>
<th>Pine 210</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roots</td>
<td>2753</td>
<td>4738</td>
<td>3744</td>
<td>2970</td>
<td>1153</td>
<td>82.8</td>
<td>74.9</td>
<td>111</td>
<td>32.2</td>
<td>27.0</td>
<td></td>
</tr>
<tr>
<td>Stalk</td>
<td>232</td>
<td>294</td>
<td>322</td>
<td>420</td>
<td>213</td>
<td>204</td>
<td>24.6</td>
<td>9.1</td>
<td>13.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Branches</td>
<td>232</td>
<td>294</td>
<td>322</td>
<td>420</td>
<td>213</td>
<td>204</td>
<td>24.6</td>
<td>9.1</td>
<td>13.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaves</td>
<td>585</td>
<td>867</td>
<td>598</td>
<td>297</td>
<td>234</td>
<td>234</td>
<td>43.0</td>
<td>3.9</td>
<td>18.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Disintegrations per minute of $^{14}$C per mg of sample. |
| Days to harvest after $^{14}$C-labeling. |

Klason analysis of unlabeled control plants, were 40, 9.0, 13, and 17% for pine, kenaf, wheat, and oak, respectively. The value for oak was lower than the values reported by Petersen for oak species and reflects the low degree of lignification common for young trees.

Klason and UV analysis of a benzene:ethanol (2:1) extracted mature oak was not much higher, 20 and 22%, respectively.

Nitrobenzene oxidation of various plant parts consistently solubilized nearly all of the $^{14}$C-labeled lignin (Table II). The dark color of solubilized pine lignin did not always allow accurate scintillation counting of $^{14}$C contents in the soluble phase. However, in all cases the residual $^{14}$C contents in the solids were easily determined by pyrolysis to CO$_2$. The $^{14}$C content of undissolved residues confirmed the accuracy of solubilized $^{14}$C results in those cases where solubilized $^{14}$C results were obtained by adding up to nearly 100% of the original $^{14}$C-content. Only pine stalk lignin was incompletely solubilized (Table II). Either these data are correct and lignin was completely removed from wheat, kenaf,
TABLE II
Percent of $^{14}C$ Solubilized/Retained on Nitrobenzene Oxidation of $^{14}C$-Lignins

<table>
<thead>
<tr>
<th>Lignin Age</th>
<th>Wheat Stalk</th>
<th>Wheat Root</th>
<th>Kenaf Stalk</th>
<th>Kenaf Root</th>
<th>Oak Root</th>
<th>Oak Stalk</th>
<th>Pine Root</th>
<th>Pine Stalk</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>98.3/0.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>98.1/1.0</td>
<td>98.0/ND</td>
<td>110/1.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>99.2/0.7</td>
<td>96.3/ND</td>
<td>92.5/2.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>110/1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>180</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>270</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>328</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>101/1.3</td>
</tr>
</tbody>
</table>

1 Days to harvest after $^{14}C$-labeling.
2 Not determined.

oak, and pine root as indicated by the $^{14}C$ results in Table II or the $^{14}C$-labeled lignins tested were not representative of true plant lignins.

Unrepresentative $^{14}C$-lignin could be the result of only young lignin being labeled. If this were true, other methods of lignin labeling wherein $^{14}C$-lignin was formed and recovered in 24 to 48 hr would be even less representative than 30-day and older lignins. The plant samples discussed in this paper contained $^{14}C$-labeled lignin that was labeled and then matured 30 days or more. Wheat and kenaf were harvested at full maturity (wheat, 55 days and kenaf, 120 days after labeling). These annual crop lignins get no older, so we conclude that age of the lignin has no effect on its susceptibility to nitrobenzene oxidation. Oak and pine lignin at 1 year and at 9 months, respectively, after labeling were totally solubilized by nitrobenzene oxidation. Again age did not play a role for ages studied here.
Unrepresentative $^{14}$C-lignin might be the result of atypical biochemistry induced by feeding the plant an unusual amount of phenylalanine. Some monomeric, aromatic aldehydes and acids are bound to cell walls in wheat. The question arises as to whether a large influx of phenylalanine might be directed to this type of more labile linkage rather than more stable lignin polymers. If the biochemistry were upset, the $^{14}$C would be less representative of lignin in the plant. An alternative explanation for obtaining a non-representative $^{14}$C-lignin could be based on the fact that the biosynthesis of lignin is not completely understood. Perhaps the shikimic acid pathway leading through phenylalanine, coniferyl alcohol, and its methoxylated homologs is not the only contributing biochemical pathway to lignin. Thus, our phenylalanine would label only part of the lignin.

To test for an unrepresentative $^{14}$C-lignin, we compared the $^{14}$C-lignin to unlabeled lignins by reaction with NaClO$_2$ and 72% H$_2$SO$_4$. Biological attack and alkaline peroxide have already been shown to remove similar amounts of lignin, both $^{14}$C-labeled and unlabeled, in wheat straw and kenaf. Table III shows that NaClO$_2$ oxidation leaves about 17, 20, 17, and 10% of the original $^{14}$C-labeled lignin unsolubilized in wheat stalk, kenaf stalk, oak root, and pine root, respectively. UV analyses of the same plant materials before and after NaClO$_2$ oxidation show that 14, 16, 19, and 13% of the original lignin remained unsolubilized in the oldest plant samples. It is unlikely that the lignin left in the solid residue is still in its original form. Thus, the $^{14}$C values are
probably a better measure of residual lignin than the UV values. However, the lignin degradation products apparently retained most of their aromatic character (maximum UV absorbance at 280 μm), because both sets of data are similar. The results for only one sample (kenaf stalk-30) show much discrepancy between $^{14}$C-lignin analysis and UV analysis. The discrepancy is wider and consistent between UV and Klasson analyses of NaClO₂ oxidized samples (Table III). The residual lignin in NaClO₂-oxidized samples probably has carboxylic acid end units and fewer crosslinks, which makes them more soluble in the 3% H₂SO₄ used in the Klasson analysis.

The 30-day harvested samples were consistently lower in the amount of residual lignin after NaClO₂ oxidation than the older samples,

<table>
<thead>
<tr>
<th></th>
<th>Wheat Stalk</th>
<th>Kenaf Stalk</th>
<th>Oak Stalk</th>
<th>Pine Stalk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 35</td>
<td>30 120</td>
<td>30 32h</td>
<td>30 270</td>
</tr>
<tr>
<td>$^{14}$C-analysis</td>
<td>32.4 16.5</td>
<td>8.4 19.6</td>
<td>8.7 16.6</td>
<td>2.6 10.2</td>
</tr>
<tr>
<td>UV analysis of $^{14}$C-lignin</td>
<td>25.5 14.0²</td>
<td>19.2 16.3</td>
<td>9.8 18.7</td>
<td>4.1 12.7</td>
</tr>
</tbody>
</table>

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1. See Footnote 1, Table II.
2. NaClO₂ procedure #2. See Experimental section.
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except for wheat stalk (Table III). This is the first evidence reported that age of the lignin affects oxidative solubilization of lignin.

Another important question is how much lignin is acid-soluble and not weighed with the total lignin in the Klason analysis. We found that 10, 13, 22, and 2.2% of the $^{14}$C-lignin in 30-day harvested wheat, kenaf, oak, and pine, respectively, was soluble in the Klason analysis solvent (Table IV). Others have reported values of 12 to 15% for hardwood lignin 15,16 and a few tenths of a percent for conifers. 17 Age of the lignin did not seem to have a significant effect on the amount of lignin solubilized. One interesting calculation can be made from the percent lignin solubilized in Table IV. If percent lignin from the Klason analysis is corrected for solubles as measured by $^{14}$C-analysis, then a corrected weight percent lignin is obtained. An absorbance value ($g^{-1}l^{-1}cm^{-1}$) can be obtained from the UV absorbance, the corrected percent lignin, and the sample weight in the equation:

$$\text{Absorbance (} g^{-1}l^{-1}cm^{-1} ) = \frac{\text{Absorbance x 10}}{\text{Sample weight x corrected % lignin}}$$

Doing this calculation for Klason and UV lignin analyses of wheat (15, 30, and 55-day harvest), kenaf (30, 60, and 120-day harvest), and pine (30, 180, and 270-day harvest) gave an average absorbance of 38.8 $g^{-1}l^{-1}cm^{-1}$ for wheat straw, 38.1 $g^{-1}l^{-1}cm^{-1}$ for kenaf and 10.8 $g^{-1}l^{-1}$
TABLE IV
Percent of $^{14}C$-Lignins Soluble in 3% $H_2SO_4$

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Wheat Stalk</th>
<th>Kenaf Stalk</th>
<th>Oak Root</th>
<th>Pine Root</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>15.9</td>
<td>10.1</td>
<td>13.1</td>
<td>21.6</td>
</tr>
<tr>
<td>30</td>
<td>10.4</td>
<td>11.2</td>
<td>11.4</td>
<td>11.4</td>
</tr>
<tr>
<td>55</td>
<td>17.3</td>
<td>21.6</td>
<td>2.2</td>
<td>1.5</td>
</tr>
</tbody>
</table>

1. The Klason lignin analysis solvent is 3% $H_2SO_4$ from which insoluble lignin is collected and measured as "Klason lignin."  
2. See Footnote 1, Table II.

According to the data, $^{14}C$-lignin is solubilized in similar percentages to unlabeled lignin by $NaClO_2$, alkaline $H_2O_2$, and fungal degradation, and it is precipitated nearly completely by 3% $H_2SO_4$ as is unlabeled lignin. We have demonstrated earlier that the $^{14}C$-label is uniformly distributed among lignin structural units. Thus, we must conclude that the data in Table II are accurate and generally applicable. Nitrobenzene oxidation solubilizes nearly 100% of the lignin. Klason and UV analysis of the nitrobenzene-oxidized samples are shown in Table V. In contrast to the $NaClO_2$ oxidation results, Klason analysis is a better measure of residual lignin than is UV analysis. Although the samples are
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**TABLE V**

Percent of Original Lignin Remaining Insoluble After Nitrobenzene Oxidation

<table>
<thead>
<tr>
<th></th>
<th>Wheat Stalk</th>
<th>Kenafl Stalk</th>
<th>Oak Root</th>
<th>Pine Root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>55</td>
<td>60</td>
<td>120</td>
</tr>
<tr>
<td>UV analysis</td>
<td>5.6</td>
<td>6.5</td>
<td>11.9</td>
<td>8.2</td>
</tr>
<tr>
<td>Klassen analysis</td>
<td>0</td>
<td>0</td>
<td>2.5</td>
<td>0</td>
</tr>
</tbody>
</table>

1. See Footnote 1, Table II.

thoroughly washed, nitrobenzene or its reduction products may have been attached to residual cellulosic material. This would explain why there is a small amount of UV absorbance left but no lignin in the sample (Table V).

Nitrobenzene oxidation is a powerful tool for solubilizing all of the lignin in most plants. The mechanism of the reaction, the two-phase system, the ability of nitrobenzene to swell lignin and the electrochemical potential of the system may each play a part in the utility of the reaction. If we can find which of these parameters are most important, perhaps we can improve the system to a more practical process.

**ACKNOWLEDGMENTS**

We thank Thomas Lee and Lily Remolina for performing many of the analyses.

**REFERENCES**
