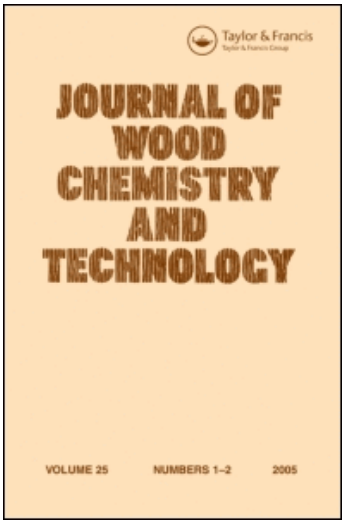

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OXIDATION AND QUANTIFICATION OF ^{14}C-LIGNIN AT DIFFERENT AGES IN WHEAT, PINE, OAK, AND KENAF

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ABSTRACT

Plant materials, containing ^{14}C-labeled lignins, were harvested at intervals up to 1 year after labeling. They were oxidized with sodium chlorite ($NaClO_2$) and nitrobenzene. Age of lignin appears to limit the degree of solubilization of lignin by $NaClO_2$. Nitrobenzene oxidation solubilized all lignins in wheat, oak, and kenaf but not all lignin in some pine samples. Klason and UV analysis of lignin content were compared with ^{14}C-content to determine percent lignin soluble in 3% sulfuric acid (Klason analysis solvent), corrected lignin contents, and UV absorptivity of lignin. Ten to 20% of most lignins were soluble in 3% sulfuric acid. When corrected Klason 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.[1,2] Many

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oxidation products have been identified and related to the original structure of the lignin.[3] Brink et al. oxidized spruce with nitrobenzene and isolated several aliphatic acids.[4] They suggested that formic acid and related compounds resulted from carbohydrate oxidation, and oxalic acid and related compounds resulted from lignin oxidation.[4] 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)[1] to 51% of the milled wood lignin starting material.[5] 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

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-[U-^{14}C]-phenylalanine in 0.01 N HCl as received from ICN, 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-[U-^{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.[6] 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 uCi of L-[U-^{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[6] 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±5° 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.[7] The

washed residue was dried and then pyrolyzed to CO_2 in an oxygen-combustion furnace. The CO_2 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[8] and UV[9] methods.

Two procedures were used for $NaClO_2$ oxidation. Procedure No. 1 is similar to that commonly employed. Except for wheat, each plant sample (10 g in 400 ml H_2O) was treated at 90°C four times with 5 ml glacial acetic acid and 15 g $NaClO_2$ for 30 min each. Procedure No. 2, consisting of lower temperature (75°C), more dilute solution (10 g in 500 ml H_2O), less reagent (0.6 ml acetic acid and 7.5 g $NaClO_2$, and 3 treatments, was used to oxidize wheat lignin.[10] Klason analysis was performed as described by Pettersen,[8] 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 ^{14}C content. ^{14}C-Analysis of these additional small quantities of precipitate revealed that the polypropylene filter effectively retained 97% or more of the precipitated lignin. Soluble ^{14}C 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 ^{14}C in samples, as measured by a scintillation counter,

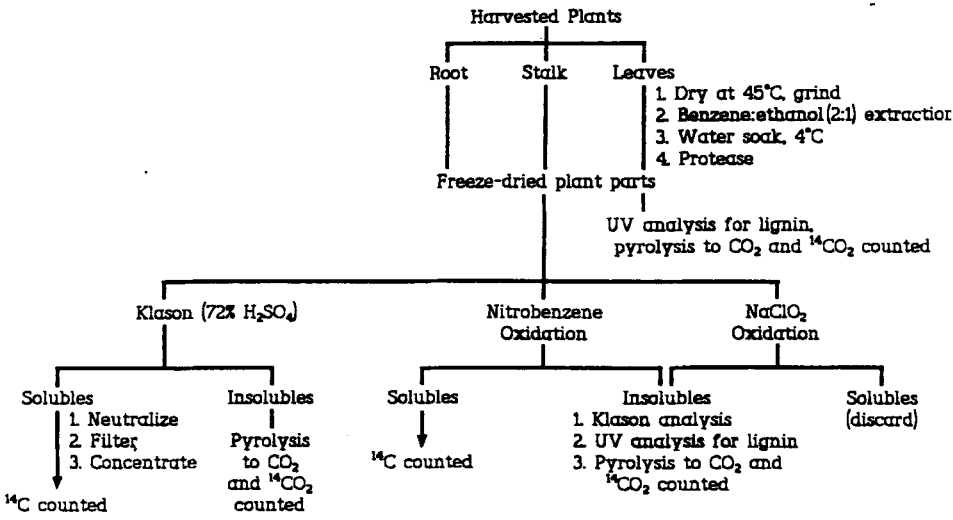

Figure 1. Analytical procedures.

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 CO_2 and the CO_2 trapped in scintillation fluid which was then counted in a scintillation counter to determine their ^{14}C 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 ^{14}C-label (9 to 100 dpm/mg) were accurately determined by pyrolysis of 20 to 40 mg of sample and collection of the resulting CO_2 in scintillation fluid. Four to 8 replicates were averaged to obtain data for Table I. Lignin contents, measured by

TABLE I
Specific Activities, dpm per mg[1]

Lignin Age[2]	Wheat			Kenaf			Oak		Pine		
	15	30	55	30	60	120	30	328	30	180	270
Roots	2729	2753	4738	3744	2970	1153	82.8	74.9	111	32.2	27.0
Stalk	332	294	322	420	213	204	24.6	9.1	13.9	——	——
Branches	——	——	——	——	——	——	30.0	——	15.8	——	——
Leaves	585	847	598	297	234	97	43.0	3.9	18.4	——	——

[1]Disintegrations per minute of ^{14}C per mg of sample.

[2]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[11] 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

	Wheat	Kenaf		Oak	Pine	
	Stalk	Root	Stalk	Root	Root	Stalk
Lignin Age[1]						
15	98.3/0.9					
30	98.1/1.0	96.0/ND[2]	110/1.3	102/1.8	ND/2.5	ND/24
55	99.2/0.7					
60		96.3/ND	92.5/2.0			
120			110/1.1			
180					ND/1.0	
270					ND/3.0	
328				101/1.3		

[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.[12] 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_2SO_4. Biological attack[13,14] and alkaline peroxide[2] 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

TABLE III
Percent of Original Lignin Remaining Insoluble After $NaClO_2$ Oxidation

Age[1]	Wheat Stalk		Kenaf Stalk		Oak Root		Pine Root	
	30	55	30	120	30	328	30	270
^{14}C-analysis	32.4	16.5	8.4	19.6	8.7	16.6	2.6	10.2
UV analysis of ^{14}C-lignin	25.5	14.0[2]	19.2	16.3	9.8	18.7	4.1	12.7

	Benzene/Ethanol (2:1)-Extracted Mature Plants			
	Wheat Stalk	Kenaf	Oak Stalk	Pine Stalk
UV analysis	28.7	14.8	15.5	19.1
Klason lignin analysis	4.3	3.1	0.33	0.67

[1]See Footnote 1, Table II.

[2]$NaClO_2$ procedure #2. See Experimental section.

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 um), 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 Klason analyses of $NaClO_2$ oxidized samples (Table III). The residual lignin in $NaClO_2$-oxidized samples probably has carboxylic acid end units and fewer crosslinks, which makes them more soluble in the 3% H_2SO_4 used in the Klason analysis.

The 30-day harvested samples were consistently lower in the amount of residual lignin after $NaClO_2$ oxidation than the older samples,

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} 1 cm^{-1}) can be obtained from the UV absorbance, the corrected percent lignin, and the sample weight in the equation:

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

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} 1 cm^{-1} for wheat straw, 38.1 g^{-1} 1 cm^{-1} for kenaf and 10.8 g^{-1}

TABLE IV
Percent of ^{14}C-Lignins Soluble in 3% H_2SO_4

Age[2]	Wheat Stalk			Kenaf Stalk			Oak Root		Pine Root		
	15	30	55	30	60	120	30	328	30	180	270
	15.9	10.1	10.4	13.1	11.2	17.3	21.6	11.4	2.2	1.5	11.6

[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.

1 cm^{-1} for pine lignins. The value for kenaf is 9% higher than the value reported in the literature.[9] Our calculated values were obtained on a limited number of samples (approx. 6 each) and the variation between different plants is great enough to warrant a more extensive study before the above values can be accepted as generally applicable.

According to the data, ^{14}C-lignin is solubilized in similar percentages to unlabeled lignin by $NaClO_2$, alkaline H_2O_2,[2] and fungal degradation,[13] 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.[6] 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

TABLE V
Percent of Original Lignin Remaining Insoluble After Nitrobenzene Oxidation

Age[1]	Wheat Stalk		Kenaf Stalk		Oak Root		Pine Root		
	30	55	60	120	Control	328	30	180	270
UV analysis		5.6	6.5	11.9	8.2	18.7	7.5	8.5	16.3
Klason analysis	0	0	2.5	0	0	0	1.4	0.31	0.47

[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.

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