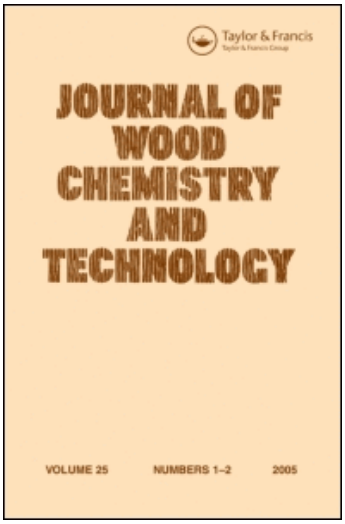


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# PREPARATION OF ENZYMATICALLY LIBERATED LIGNIN FROM NATURALLY BROWN-ROTTED WOOD

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## ABSTRACT

Brown rots from various conifer species were sieved (<60 mesh) and alcohol and water extracted to yield crude preparations of "naturally enzymatically liberated lignin" (NEL) containing 61.4-91.7% of Klason plus soluble lignin and 0.5-2.6% ash. The <200 mesh fractions were generally best represented in <60 mesh preparations and contained highest Klason plus soluble lignin percentages (86.6-92.2%). Carbohydrates varied in these fractions between 3.7 and 8.0% and contained glucose, mannose, galactose, xylose, and arabinose, decreasing generally in that order. Methoxyl contents were lower and oxygen contents higher than in milled wood lignin (MWL) suggesting some oxidative demethylation. Treatment with Cuoxam increased Klason plus soluble lignin content to 93.0-95.8%, decreased carbohydrates to 1.7-5.0%, increased methoxyl and decreased oxygen contents by removal of a part of cellulose and hemicelluloses and of more degraded lignin fractions. Infrared spectra of the preparations showed a small increase in carboxyls and possibly in phenyl conjugated double bonds and a decrease in aromatic structures, as compared with MWL. In thermogravimetric analysis the curves for MWL and Cuoxam treated NEL preparations (CuNEL) were very similar when run in nitrogen. In air, however, MWL lost weight appreciably slower. In differential scanning calorimetry in air and oxygen, MWL exhibited less intensive exotherms below 500°C than NEL and left higher amounts of char. The different behaviour of MWL in thermal analysis in oxidative atmosphere was explained by its lower molecular weight and corresponding low glass transition temperature (Tg). It was concluded that preparation of enzymatically liberated lignin from natural brown rot represents a convenient procedure where larger amounts of lignin are required; such lignins are somewhat more degraded than MWL in terms of functional groups present, but are possibly closer to protolignin in terms of molecular weight.

## INTRODUCTION

In spite of a large amount of work performed in the general field of lignin chemistry, the isolation of this polymer for chemical and other studies still requires a compromise. Ideally, the isolation of lignin in its unchanged form should meet the following criteria; (a) isolated lignin should be representative of the entire lignin contained in the lignocellulosic starting material; (b) isolated lignin should contain no carbohydrates or extraneous materials; (c) isolated lignin should not have been changed in the course of its isolation; and (d) the isolation procedure should not involve a disproportionally high cost and long time. In actuality no lignin preparation meets all four criteria. The preparation of an unchanged, carbohydrate-free lignin appears to be even theoretically impossible due to the necessity to break lignin-carbohydrate bonds and resulting changes in the lignin structure. Of the more common lignin preparations, the commercially available by-products of wood-utilizing industries, such as sodium and ammonium lignosulfonates contain carbohydrates and are changed too drastically for many uses. Klason lignin, while essentially carbohydrate-free, is appreciably changed by the sulfuric acid used in its preparation. Cuoxam and periodate lignins, while changed less, require considerable time and cost. Various milled wood lignin preparations, as well as preparations involving the use of cellulases (e.g., Onozuka), while generally considered to be changed least, require even longer time for their preparation and are acceptable only in cases requiring small amounts of material.

In our experimentation in the area of oxidative, surface activated bonding of lignocellulosic materials we required larger than usual amounts of lignin. While the laboriousness of some of the methods of lignin preparation eliminated these from consideration, the drastic changes in lignin structure, as well as presence of non-lignin materials, also made others unattractive. The examination of all available alternatives led finally to the

conclusion that isolation of lignin from natural brown rot might offer the best compromise.

That the enzymes of brown-rotting fungi selectively hydrolyze the carbohydrate portion of lignocellulosic materials has been known for a long time and many attempts have been made to utilize this for isolation of lignin in its close to native state. The procedures used can be separated in two groups: a more popular one and likely to favor lower molecular weight fractions is the one in which the lignin is isolated by extraction from the brown-rotted wood using polar organic solvents, and the other which is based on purification and refining of the insoluble residue left after exhaustive rotting of wood. In a predominant number of cases the starting material represented laboratory rotted wood rather than the natural brown rot. While artificial rotting is more laborious, it has an advantage in selection of the fungus as well as in the possibility of removing extraneous materials prior to rotting and leads in this way to a purer lignin. On the other hand, the use of natural brown rot has an advantage in its ready availability in large amounts; furthermore, the extractive problem can be eliminated by use of wood low in extraneous materials (sapwood, wood of Abies species), as well as by solvent extraction of the natural brown rots.



Nord and his co-workers[1,2] were probably the first that used brown-rotting fungi for solubilizing lignin. The yields obtained amounted to as much as 22.7% of the total lignin present. While it was claimed that the obtained lignin preparations were identical with the protolignin, other work demonstrated conclusively that brown rotting leads to a partial loss of methoxyl groups of lignin, as well as to other changes. Thus, as early as 1924, Bray and Andrews[3] showed that brown-rotting of spruce and fir wood resulted in an appreciable loss in methoxyl groups from lignin. More recently, Cowling[4] using wood of sweetgum, Liquidambar styraciflua L, found that the percent of fine particulate matter, as well as solubility in water and organic solvents, increased with the progress of rotting. As expected, the Klason

lignin content of the rotted wood increased to 69.8%. The results were recently substantiated by Danninger, et al.[5] Brown, et al.[6] investigated the chemical properties of lignin liberated by fungal attack of sapwood of sweetgum and Sitka spruce, Picea sitchensis, and found that the percentage of Klason plus acid soluble lignin was somewhat higher in the milled wood lignins (94.2-96.6%) than in the enzymatically liberated lignins (83.7-94.5%); furthermore, enzymatically liberated lignins contained appreciably more acid-soluble lignin (12.7-23.4% vs 4.0-12.5%). Methoxyl content of the enzymatically liberated lignins was also considerably lower (9.9 and 11.4% with spruce) than that of milled wood lignins (15.9%), while the infra-red spectra were rather similar. Pew[7,8] and Pew and Weyna[9] reported 70.5 to 83.8% Klason lignin contents for the rotted wood of spruce and aspen, with methoxyl contents of the Klason lignin decreasing to 11.5% as the rotting progressed in the case of spruce wood. Similar results were obtained by Grohn and Deters,[10] and by Apenitis, et al.[11] Leopold[12] found that rotting of spruce wood led to a decrease in methoxyl in lignin, formation of carboxyls, increase in oxygen content, easier sulfonation, and reduced yield of the products of the nitrobenzene oxidation. On the other hand, the content of phenolic hydroxyls, as well as composition of the products of the nitrobenzene oxidation was unchanged. Leopold concluded that the main portion of lignin in the decayed wood is only slightly changed, although some fractions of the lignin seem to be severely attacked.

Other, more recent studies substantiated overall the earlier findings.[13-17] Kirk, et al. reported an increase in phenolic hydroxyls and the presence of o-diphenolic moieties in the lignin extracted from rotted spruce wood,[14,16] and concluded that fungal degradation is largely oxidative and that loss of methoxyls from phenolic and non-phenolic units is the major degradation reaction.

In the present work, we report on the experiments aimed at developing acceptable methodology for preparing lignin from the natural brown rots which would be sufficiently close to protolignin for some uses. While we had no illusions of being able to end

up with a product identical with protolignin or even as unchanged as milled wood lignin, the ready availability of the starting material and the simple nature of the proposed refining methods made the possibilities attractive enough. The experiments performed cover the effect of the differences in particle size and of the treatment of the brown rots with Cuoxam on the lignin content, as well as on the chemical character of lignin. The comparisons were made on the basis of Klason lignin and ash contents, elemental and methoxyl analyses, composition and amount of hydrolyzed carbohydrates, infrared spectroscopy and thermal analysis.

Use of Cuoxam for isolation of lignin stems from Freudenberg.[18,19] Unfortunately, use of sound wood sawdust requires repeated prehydrolysis with diluted sulfuric acid and Cuoxam treatment, and is rather laborious.



## EXPERIMENTAL

The samples of the cubical brown-rot collected for the present investigation represented larger pieces with well preserved wood structure. Dust and smaller particles were discarded. No specific effort was made to identify the fungus, although in some cases information was available. The tree species were identified in all cases, however. The brown rots used came from two trees of California red fir, Abies magnifica A. Murr. (analyzed separately), one tree of white fir, Abies concolor (Gord. & Glend.) Lindl., one tree of Douglas-fir Pseudotsuga menziesii (Mirb.) Franco, one tree of Monterey cypress, Cupressus macrocarpa Hartw. (fungus - probably Polyporus basilaris Overh.), one tree of incense cedar, Calocedrus decurrens (Torrey) Florin (fungus - Polyporus amarus Hedgcock) with one sample collected in the root and the other in the stem area, and one tree of sugar pine, Pinus lambertiana Dougl. (fungus - Fomes officinalis (Vill. ex Fries) Faull.

The brown rots were ground in a mortar as fine as possible. The resulting powders containing between 10.5 and 35.9% moisture

were dried under vacuum over calcium chloride and sieved using 60-mesh or 40 mesh sieves (openings 0.25 and 0.425 mm in diam., respectively) to give 68-98% yield of fine, brown powders. The powders were Soxhlet extracted with ethanol for 8 hours, vacuum dried, extracted with cold water and dried again to yield 72.5-87.7% of refined materials. The total yields based on the weight of the original dry brown rots ranged from 58.8-73.2%. Klason lignin contents and ash contents were determined using the conventional procedures; the amount of acid soluble sulfuric acid lignin was determined by the UV absorption at 205 mm (Beckman Acta III spectrophotometer).[20] Carbon/hydrogen and methoxyl analyses were performed by the Microchemical Analysis Laboratory of the College of Chemistry, University of California, Berkeley. Infrared spectra were determined using Perkin-Elmer Model 457 grating spectrophotometer and KBr pelletizing methodology. Thermal analysis was conducted using DuPont 990 thermal analyzer (DSC) and 951 thermogravimetric analyzer (TG). Composition of monosaccharides in the Klason lignin hydrolyzates were determined by the aldononitrile acetate method.[21]

Cuoxam solutions were prepared by (a) dissolution in 600 ml of 30% $NH_3$ of cupric hydroxide prepared by addition of 200 ml of 10% sodium hydroxide to a solution of 59 g of $CuSO_4 \cdot 5H_2O$ in 1700 ml of water, centrifuging and washing with water until negative reaction with $BaCl_2$ solution. The resulting ammonia solution was diluted to a concentration of 1.5% $Cu^{++}$ and 20% $NH_3$, and (b) dissolution of 29.5 g of $CuSO_4 \cdot 5H_2O$ in 345 ml of 29% $NH_3$, followed by 9.5 g of NaOH and dilution to 500 ml with water. After displacement of the air by nitrogen 25 mg of Cu metal powder was added to absorb dissolved oxygen. The resulting concentration of $Cu^{++}$ was 1.5% and that of $NH_3$ 20%. In both cases deaerated water was used and the operations were performed under nitrogen. The Cuoxam treatment of the various brown rots was in all cases similar to the one described below.

Optimal Procedure for Preparation of Cuoxam-Treated Naturally Enzymatically Liberated Lignin (CuNEL)

Larger pieces of cubical brown rot are collected preferably from a freshly felled tree. The material is powderized in a mortar and dried first in air and then in a desiccator. The resulting material is sieved using a 200 mesh screen. The <200 fraction is extracted for 8 hours with ethanol in a Soxhlet and then five times with cold water and filtered under suction. The resulting material is added under nitrogen to a Cuoxam solution prepared by the method (b) using 10g (dry equivalent) for 150 ml of Cuoxam, stirred for 30 min and filtered or centrifuged under nitrogen. The undissolved part is washed under nitrogen with 10% $NH_3$ solution until colorless and then several times with distilled water followed by 1% acetic acid. Finally, the product is allowed to stand in 2% HCl solution at ambient temperature for 30 min, filtered, washed with water until neutral, and dried.

Treatment with 2% HCl dissolves cupric oxides and ash present and thus decreases the ash content. Cupric oxides cannot be removed by acetic acid.



## Preparation of Ethanol Soluble NEL

A 188g portion of <60 mesh cubical brown rot from *P. lambertiana* containing 9.5% moisture was extracted with ethanol for 8 hours in a Soxhlet extractor. Ethanol extract was concentrated to 200 ml, cooled, and treated with 500 ml of ethyl ether. The precipitate was washed with ethyl ether until the solvent was colorless. The resulting powder was treated overnight with 400 ml water, filtered and washed with water until solvent was colorless. The dried product weighed 23.8g and contained 8.6% moisture, yield, 13.0%.

## Influence of Methods of Cuoxam Preparations and of Times of Treatment

NEL <60 mesh from *A. concolor*, *P. menziesii*, and *C. macrocarpa* were treated by Cuoxam solutions prepared according to (a) and (b). On the average the yields of CuNEL were 1.7% higher and

TABLE 1. Lignin Content of <60 Mesh Naturally Enzymatically Liberated Lignins

| Source | NEL | | | | CuNEL | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Klason Lignin | Soluble Lignin | Total Lignin | Ash in Original | Yield | Klason Lignin | Soluble Lignin | Total Lignin | Ash in Original |
| *A. magnifica* I | 80.5 | 6.0 | 86.5 | 1.3 | 58.3 | 89.6 | 3.2 | 92.8 | 0.2 |
| *A. magnifica* II | 80.1 | 2.0 | 82.1 | 1.5 | 74.6 | 90.8 | 2.7 | 93.5 | 0.3 |
| *A. concolor* | 59.2 | 2.2 | 61.4 | 2.6 | 64.9 | 87.1 | 2.3 | 89.4 | 0.3 |
| *P. menziesii* | 75.2 | 3.4 | 78.8 | 0.5 | 82.0 | 79.7 | 2.2 | 81.9 | 0.2 |
| *P. lambertiana* | 88.8 | 2.9 | 91.7 | 0.6 | 78.9 | 92.8 | 2.7 | 95.5 | tr |
| *C. macrocarpa* | 71.6 | 2.6 | 74.2 | 2.2 | 68.8 | 87.1 | 3.7 | 90.8 | 0.3 |
| *C. decurrens*, roots, I | 87.0 | 1.7 | 88.7 | 0.5 | 71.7 | 87.4 | 2.4 | 89.8 | 0.2 |
| *C. decurrens*, stem II | 88.7 | 2.5 | 91.3 | 1.1 | 82.9 | 91.1 | 3.2 | 94.3 | 0.3 |
| Mean | 78.9 | 2.9 | 81.8 | 1.3 | 72.8 | 88.6 | 2.8 | 91.9 | 0.2 |

Klason plus soluble lignin contents 0.85% lower for method (b). The differences were judged to be insignificant.

NEL <60 mesh from A. magnifica I, A. concolor, and C. decurrens (roots) were treated with Cuoxam for 30 min and for 24 hours. The 30 min treatment gave on the average 22% higher yields of CuNEL, while Klason plus soluble lignin contents were 1.8% higher. The latter was judged as not significant.

## RESULTS

### Maximization of the Lignin-to-Carbohydrate Ratio

The Klason plus soluble lignin and ash contents of the naturally enzymatically liberated lignins (NEL) <60 mesh particle



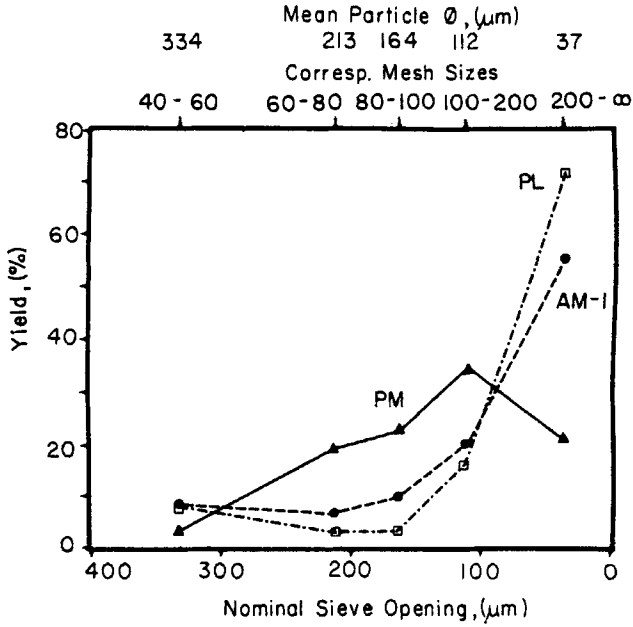


FIGURE 1. Sieve analysis of several NEL preparations. PL - P. lambertiana; AM-I - A. magnifica sample 1; PM - Ps. menziesii. Mean particle diameter was approximated by $NSO_n+NSO_m/2$ where $NSO_n$ and $NSO_m$ are nominal sieve openings of the two sieves used for isolating a fraction.

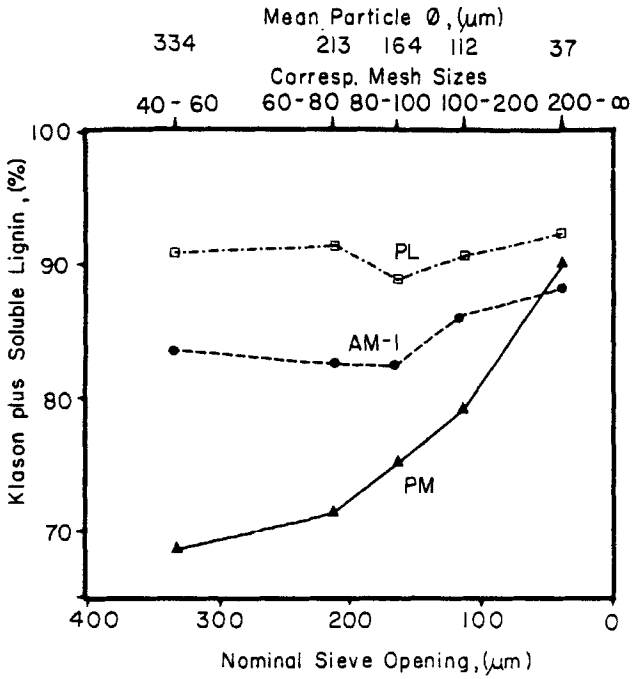


FIGURE 2. Klason plus soluble lignin contents of sieve fractions. Abbreviations as in Figure 1.

size from eight sources are listed in Table 1. The total lignin varied between 61.4 to 91.3% with an ash content of NEL of 0.5 to 2.6%. The upper values for the total lignin contents are within the range of MWL preparations, although ash content is generally rather high. Treatment with Cuoxam gave CuNEL preparations in 58.3 to 82.9% yield. The total lignin contents of CuNEL preparations increased to 81.9 to 95.5% with the preparations with the lower lignin contents tending to show highest increase (correlation coefficient R = -0.902, signif. <1.0%, df=6). The ash contents at the same time decreased to nearly trace amounts, most likely due to the final acid treatment.

Sieve analysis of the A. magnifica, P. lambertiana and P. menziesii samples of NEL (Figs. 1 and 2) indicated that the <200 mesh particles were most abundant in the first two samples,

whereas 100–200 mesh fraction was most abundant in P. menziesii sample. The finest particles were in all cases highest in total lignin content; the effect was less pronounced with A. magnifica I and P. lambertiana samples but was quite strong in the P. menziesii sample. The increase in the <200 mesh fraction following the brown rot decay of sweetgum by Poria monticola was observed earlier by Cowling[4] and attributed to the increased depolymerization of cellulose. Our results indicated that the <200 mesh fractions of NEL were particularly suitable for use as lignin preparations due to their higher lignin content and were investigated in more depth.

The results of the analysis of the <200 mesh NEL and CuNEL preparations are listed in Tables 2-4. The carbohydrate monomers fluctuated between 3.7 and 8.0% in NEL, and were composed of more than 50% glucose, followed by mannose, xylose, galactose and arabinose, commonly in that order. The total lignin content fluctuated between 86.6 and 92.2% and the ash content was relatively high, between 0.3 and 2.2%. Treatment with Cuoxam lowered the carbohydrate contents to as low as 1.7% and ash contents to negligible amounts with the total lignin reaching 95.8%. In actuality the percentages of lignin should be higher by a few percent, since the water produced from lignin during Klason lignin preparation is not accounted for in the lignin determination by the Klason method.

In the carbohydrate fraction of CuNEL glucose still represented the most important sugar, followed by mannose, galactose, xylose, and arabinose. At the same time the ratio of glucose to other sugars dropped from 65 to 38% with P. lambertiana and from 66 to 37% with A. magnifica I; this could be explained by preferential removal by the Cuoxam reagent of free cellulose stemming in part from wood and in part from fungal mycelia. No such effect was noticed, however, with P. menziesii sample where the ratio between glucose and other sugars remained practically unchanged, 58 and 65%, respectively. The presence of glucose in larger amounts and xylose in small amounts in NEL and CuNEL, is

TABLE 2. Chemical Characteristics of <200 Mesh NEL Preparations

| Source | Percent in <60 Mesh Fraction | Klason plus Soluble Lignins | Percent Ash | Elemental Composition** | | | | Formula of Phenyl-propane Unit | | | | Elemental Composition of Corresponding Klason Lignin | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | C | H | O | $OCH_3$ | C | H | O | $OCH_3$ | C | H |
| A. magnifica I | 55.5 | 88.2 | 1.6 | 60.02 | 5.02 | 34.96 | 11.06 | 9 | 7.5 | 3.6 | 0.7 | 60.71 | 4.86 |
| A. concolor*** | 53.4 | 86.6 | 2.2 | 60.08 | 5.00 | 34.92 | 11.92 | 9 | 7.4 | 3.5 | 0.8 | 61.53 | 5.05 |
| P. menziesii | 20.8 | 90.2 | 0.4 | 59.39 | 4.94 | 35.67 | 9.20 | 9 | 7.8 | 3.7 | 0.6 | 60.31 | 4.81 |
| P. lambertiana* | 77.1 | 92.2 | 0.5 | 61.26 | 5.51 | 33.23 | 10.70 | 9 | 8.4 | 3.3 | 0.7 | 63.34 | 5.26 |
| C. decurrens I | 60.9 | 89.0 | 0.3 | 61.93 | 5.18 | 32.89 | 12.12 | 9 | 7.5 | 3.1 | 0.7 | 61.71 | 4.97 |

* Data for ethanol (95%) soluble NEL from P. lambertiana: Klason plus soluble lignins - 94.4, $OCH_3$ - 10.1.

** Corrected for ash and carbohydrates. Oxygen by difference. Nitrogen was between 0.07 and 0.15% and was neglected in the calculations.

***Data for A. concolor MWL: C, 64.61; H, 6.19; O, 29.20; $OCH_3$, 15.88 (21). Klason lignin: C, 64.10; H, 5.52, O, 30.38.

TABLE 3. Chemical Characteristics of <200 Mesh CuNEL Preparations

| Source | Yield after Cuoxam (%) | Klason plus Soluble Lignin | Elemental Composition* | | | | Ammonia in Lignin | Phenylpropane Unit | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | C | H | O | $OCH_3$ | | C | H | O | $CH_3O$ |
| *A. magnifica* I | 79.1 | 94.4 | 61.60 | 4.92 | 33.48 | 12.60 | 1.35 | 9 | 7.0 | 3.2 | 0.8 |
| *P. menziesii* | 84.0 | 93.0 | 62.05 | 4.78 | 33.17 | 11.31 | 1.43 | 9 | 6.8 | 3.2 | 0.7 |
| *P. lambertiana* | 85.0 | 95.8 | 63.30 | 5.32 | 31.38 | 12.94 | 0.98 | 9 | 7.5 | 2.9 | 0.8 |

*Corrected for carbohydrates, ammonia, and ash; the latter was negligible. Oxygen by difference.

TABLE 4. Monosaccharide Composition and Content of NEL and CuNEL <200 Preparations*

| Monosaccharides in NEL and CuNEL (%) | P. lambertiana NEL | C. decurrens I NEL | A. concolor NEL | A. magnifica I NEL | P. menziesii NEL | P. lambertiana CuNEL | A. magnifica I CuNEL | P. menziesii CuNEL |
|---|---|---|---|---|---|---|---|---|
| Arabinose | 0.07 | 0.16 | 0.27 | 0.20 | 0.23 | 0.06 | 0.14 | 0.10 |
| Xylose | 0.43 | 0.67 | 1.23 | 0.48 | 0.66 | 0.14 | 0.40 | 0.28 |
| Mannose | 0.48 | 0.35 | 0.68 | 1.30 | 1.04 | 0.40 | 0.50 | 0.67 |
| Glucose | 2.40 | 6.52 | 4.48 | 5.26 | 3.75 | 0.58 | 0.81 | 2.90 |
| Galactose | 0.33 | 0.32 | 0.26 | 0.78 | 0.76 | 0.34 | 0.35 | 0.53 |
| Total | 3.71 | 8.02 | 6.92 | 8.02 | 6.44 | 1.52 | 2.20 | 4.48 |
| Glucose in carbohydrates (%) | 64.7 | 81.3 | 64.7 | 65.6 | 58.2 | 38.2 | 36.8 | 64.7 |

*Calculated for anhydrounits

in sharp contrast with the results of analysis of milled wood lignin and cellulolytic enzyme liberated lignin, by Fišerová and Šutý[22] and by Lee et al.[23] where xylose accounted for most carbohydrates; on the other hand, the results agree with the analysis of the cellulolytic enzyme liberated lignin (CEL) by Pew where the order of the percentages of individual sugars was the same as ours.[7] The results are in partial agreement with those of Brown, et al.[6] on sugars present in the lignin obtained by artificial brown-rotting of sweetgum and Sitka spruce by Lenzites trabea Pers. ex Fr., who reported glucose as the most important monosaccharide, followed by xylose, mannose, galactose and arabinose.

## Changes in Lignin Structure

The NEL and CuNEL preparations of lignin represented brown powders appreciably darker than MWL and practically insoluble in neutral organic solvents. The materials remained insoluble even upon acetylation or palmitylation; they dissolved, however, in ethylene glycol containing sodium bisulfite, at 160°C. This laboratory variation of acidic pulping for solubilization of lignin was judged to be too drastic, however, for the analytical procedures involving GPC, NMR and UV ($\Delta\varepsilon$).

The carbon/hydrogen/oxygen contents and the elemental compositions of the $C_9$ units of the five <200 NEL preparations are given in Table 3. The carbon and hydrogen values are somewhat below and oxygen values above the literature values for MWL from softwoods (61.6-64.0; 5.6-6.3; 29.8-32.5, respectively[24]). Crawford related this to the oxidative loss of methoxyl and introduction of oxygen, mainly as carboxylic groups.[13] This is probably only partially true, however, in view of the work of Chang, et al. who reported C, H, O values for the cellulolytic enzyme liberated lignin (CEL) in better agreement with ours, the preparation of CEL involving no oxidation.[25] It is possible that this reflects the larger amount of higher molecular weight lignin fractions in CEL and NEL. In all cases nitrogen occurred in trace amounts only,

fluctuating between 0.07 and 0.15%. This is in agreement with an earlier work.[6]

The methoxyl values of the NEL preparations were appreciably lower than those of MWL or CEL preparations, with the losses amounting to between 3 and 6.5%. This was not unexpected as the loss of methoxyl in brown rotting has been well documented, particularly in more recent work.[6-11,14-16] Cuoxam treatment appreciably increased the carbon and hydrogen percentages. Carbon values fell within the range of MWL from softwoods, while hydrogen values were still somewhat below. The effect is most likely due to removal of carbohydrates and to some extent of more degraded lignin fractions by ammonia. At the same time about 1.0-1.5% of ammonia became incorporated into lignin and could not be removed by the acidic extraction procedure used.

Treatment with Cuoxam also substantially increased the methoxyl values in all cases reaching 12.9% in the case of P. lambertiana CuNEL preparation. This is still well below the 14.3-16.2% commonly found with softwood MWL.[24] At the same time this methoxyl value is not very far from 13.7% reported by Chang et al.[25] for the higher molecular weight lignin fraction of CEL obtained by dioxane-water 50/50 v/v extraction. While there is little doubt that brown rotting somewhat reduces the methoxyl values of lignin, it is possible that the low $OCH_3$ values reported in Tables 3 and 4 (and by Chang et al.), as well as previously mentioned differences in C, H and O values are due, in part, to the inclusion in our preparations of the higher molecular weight lignin fractions. The lower $OCH_3$ could be the result of a more intensive exposure of these fractions to the attack of the oxidative enzymes of wood during lignification process; the result of this would be also a more extensive oxidative methoxyl loss, analogous to the one taking place during the attack of lignin by the oxidative enzymes produced by brown rotters. The methoxyl loss following the attack of dioxane lignin from Fagus crenata Bl. by oxidative enzymes peroxidase and laccase has been reported

by Oki, et al.[26]; both of these enzymes have been regarded as involved in biosynthesis of lignin by oxidative polymerization.[27]

Infrared spectra of MWL from A. concolor, NEL and CuNEL preparations from P. menziesii, P. lambertiana, A. magnifica I (<200 mesh) and L. decurrens I (<60 mesh) were determined using KBr pellet procedure. The spectra were qualitatively very similar to the spectra of softwood MWL preparations, CEL preparations, and lignins isolated from artificially rotted softwoods.[6,14] The expected maxima or shoulders at 1715 (unconjugated COOH or keto), 1670 (conjugated carbonyls), 1595 (aromatic), 1510 (aromatic), 1460 (methylene), and 1420 $cm^{-1}$ (vinyl or aromatic),[28] were discernible in all cases, but were generally sharper in MWL in conformity with its less condensed nature. In addition, a shoulder appeared in all NEL and CuNEL preparations at about 1630 $cm^{-1}$, which was absent in MWL and was assigned to phenyl conjugated double bonds.

In their studies of ozonization of lignosulfonic acids Tupureine, et al.[29] indicated that infrared bands at 1420, 1140, 1090, and 1040 $cm^{-1}$ do not change in absorbance following the loss of methoxyl and increase in oxygen content of lignin and are suited as internal standards for determining the relative absorbance of various lignin bands. We calculated the relative absorbance based on 1420 $cm^{-1}$ absorbance set as 1.00 for 1715, 1670, 1630, 1595 and 1510 $cm^{-1}$ bands (Table 5) for various NEL, CuNEL, and MWL preparations. The relative absorbance of all bands with the exception of 1510 $cm^{-1}$ band was generally higher in NEL and CuNEL preparations, and the obtained differences were statistically significant in all cases except for the band 1670 $cm^{-1}$. The increased absorption of 1715 $cm^{1}$ and 1670 $cm^{-1}$ suggests the increased content of conjugated and nonconjugated carboxyl and/or keto groups in NEL and CuNEL, although the differences are small, appreciably below those reported by Oki, et al.[26] The increased intensity at 1630 $cm^{-1}$ indicates an increase in conjugated double bonds in NEL and CuNEL, while the reduced relative absorbance at 1510 $cm^{-1}$ is due to their slightly lower aromaticity; this is in

TABLE 5. Relative Absorbance of Infrared Bands*

| Lignin Type | 1715 $cm^{-1}$ | 1670 $cm^{-1}$ | 1630 $cm^{-1}$ | 1595 $cm^{-1}$ | 1510 $cm^{-1}$ |
|---|---|---|---|---|---|
| MWL, *Abies concolor* | 0.47 | 0.62 | 0.50 | 0.92 | 1.33 |
| *P. lambertiana* <200, NEL | 0.59 | 0.54 | 0.79 | 0.93 | 1.22 |
| *P. lambertiana* <200 CuNEL | 0.66 | 0.64 | 0.70 | 0.99 | 1.32 |
| *P. menziesii* <200, NEL | 0.56 | 0.68 | 0.97 | 1.03 | 1.11 |
| *P. menziesii* <200, CuNEL | 0.56 | 0.74 | 0.92 | 1.11 | 1.13 |
| *A. magnifica* I <200, NEL | 0.52 | 0.66 | 0.83 | 1.10 | 1.28 |
| *A. magnifica* I <200 CuNEL | 0.59 | 0.66 | 0.77 | 1.01 | 1.25 |
| *C. decurrens* I <60, NEL | 0.49 | 0.57 | 0.68 | 0.95 | 1.22 |
| *C. decurrens* I <60, CuNEL | 0.33 | 0.65 | 0.82 | 1.07 | 1.25 |

*Absorbance calculated in relation to the absorbance of the 1420 $cm^{-1}$ band, as 1.00.

TABLE 7. Results of Differential Scanning Calorimetry

| Lignin Preparation | T (Peak A) (°C) | Ordinate $(\frac{cm}{mg})^*$ | T (Peak B) (°C) | Ordinate $(\frac{cm}{mg})^*$ | Starting Weight (mg) | Weight at 500° (mg) | Percent Weight Loss | Relative ΔH** (per 1 mg used) | Relative ΔH** (per 1 mg consumed) | Relative ΔH** (per 1 mg of C+H consumed) |
|---|---|---|---|---|---|---|---|---|---|---|
| Oxygen Atmosphere | | | | | | | | | | |
| MWL (A. concolor) | 295 | 2.08 | 365 | 2.78 | 1.37 | 0.49 | 64.2 | 0.565 | 0.879 | 0.864 |
| Klason lignin (A. concolor) | 310 | 5.81 | 363 | 6.44 | 1.42 | negl. | 100.0 | 1.000 | 1.000 | 1.000 |
| P. lambertiana*** | 315 | 5.27 | 360 | 5.37 | 1.22 | negl. | 100.0 | 0.925 | 0.925 | 0.951 |
| P. menziesii*** | 315 | 5.81 | 360 | 6.17 | 1.40 | 0.07 | 95.0 | 0.918 | 0.966 | 1.033 |
| A. magnifica I*** | 305 | 5.47 | 362 | 6.12 | 1.37 | negl. | 100.00 | 0.960 | 0.960 | 1.022 |
| Air Atmosphere | | | | | | | | | | |
| MWL (A. concolor) | 300 | 1.00 | 342 | 1.42 | 1.57 | 0.72 | 54.1 | 0.453 | 0.641 | 0.630 |
| Klason lignin (A. concolor) | 325 | 4.58 | 384 | 6.24 | 1.32 | 0.31 | 76.5 | 1.000 | 1.000 | 1.000 |
| P. lambertiana*** | 320 | 4.01 | 380 | 5.16 | 1.34 | 0.37 | 72.4 | 0.902 | 0.953 | 0.980 |
| P. menziesii*** | 325 | 4.13 | 385 | 5.31 | 1.23 | 0.24 | 80.5 | 0.938 | 0.892 | 0.954 |
| A. magnifica I*** | 320 | 4.10 | 384 | 5.36 | 1.30 | 0.33 | 74.6 | 0.917 | 0.941 | 1.002 |

* Per mg starting weight.
** Relative to Klason lignin = 1.0.
***CuNEL, < 200.

TABLE 7. Results of Differential Scanning Calorimetry

| Lignin Preparation | T (Peak A) (°C) | Ordinate $(\frac{cm}{mg})$* | T (Peak B) (°C) | Ordinate $(\frac{cm}{mg})$* | Starting Weight (mg) | Weight at 500° (mg) | Percent Weight Loss | Relative ΔH** (per 1 mg used) | Relative ΔH** (per 1 mg consumed) | Relative ΔH** (per 1 mg of C+H consumed) |
|---|---|---|---|---|---|---|---|---|---|---|
| Oxygen Atmosphere | | | | | | | | | | |
| MWL (A. concolor) | 295 | 2.08 | 365 | 2.78 | 1.37 | 0.49 | 64.2 | 0.565 | 0.879 | 0.864 |
| Klason lignin (A. concolor) | 310 | 5.81 | 363 | 6.44 | 1.42 | negl. | 100.0 | 1.000 | 1.000 | 1.000 |
| P. lambertiana*** | 315 | 5.27 | 360 | 5.37 | 1.22 | negl. | 100.0 | 0.925 | 0.925 | 0.951 |
| P. menziesii*** | 315 | 5.81 | 360 | 6.17 | 1.40 | 0.07 | 95.0 | 0.918 | 0.966 | 1.033 |
| A. magnifica I*** | 305 | 5.47 | 362 | 6.12 | 1.37 | negl. | 100.00 | 0.960 | 0.960 | 1.022 |
| Air Atmosphere | | | | | | | | | | |
| MWL (A. concolor) | 300 | 1.00 | 342 | 1.42 | 1.57 | 0.72 | 54.1 | 0.453 | 0.641 | 0.630 |
| Klason lignin (A. concolor) | 325 | 4.58 | 384 | 6.24 | 1.32 | 0.31 | 76.5 | 1.000 | 1.000 | 1.000 |
| P. lambertiana*** | 320 | 4.01 | 380 | 5.16 | 1.34 | 0.37 | 72.4 | 0.902 | 0.953 | 0.980 |
| P. menziesii*** | 325 | 4.13 | 385 | 5.31 | 1.23 | 0.24 | 80.5 | 0.938 | 0.892 | 0.954 |
| A. magnifica I*** | 320 | 4.10 | 384 | 5.36 | 1.30 | 0.33 | 74.6 | 0.917 | 0.941 | 1.002 |

* Per mg starting weight.
** Relative to Klason lignin = 1.0.
***CuNEL, < 200.

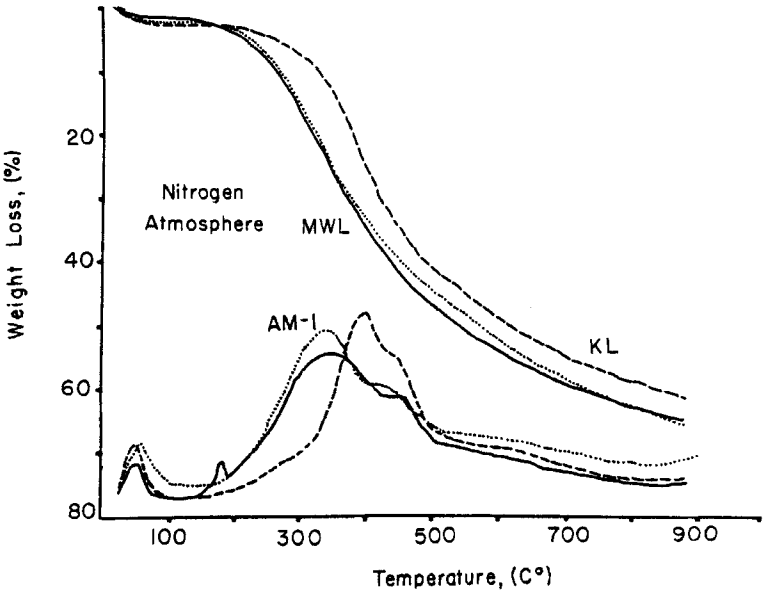


FIGURE 3. TG/DTG analysis of lignin preparations. Nitrogen atmosphere. MWL - milled wood lignin of A. concolor (10.4 mg); KL - Klason lignin of A. concolor (10.6 mg); AM-1 - A. magnifica <200 mesh CuNEL (10.4 mg). Gas flow rate: 50 ml/min. Rate of heating, 10°C/min.



accordance with the work of Kirk[14] although the differences we obtained were appreciably smaller again. The increase in aromatic absorption at 1595 $cm^{-1}$ in NEL and CuNEL is difficult to interpret in view of that just said; it is possible that it results from the appreciable increase in the 1630 $cm^{-1}$ shoulder. Contrary to the relatively clear results with MWL the IR differences between NEL and CuNEL preparations were inconclusive and statistically nonsignificant in all cases.

Finally, we compared the various lignin preparations by thermal analysis using thermogravimetry (TG), differential thermogravimetry (DTG) (Table 6) and differential scanning calorimetry (DSC) (Table 7). The results of the TG/DTG analysis in nitrogen atmosphere suggested that there is little difference between

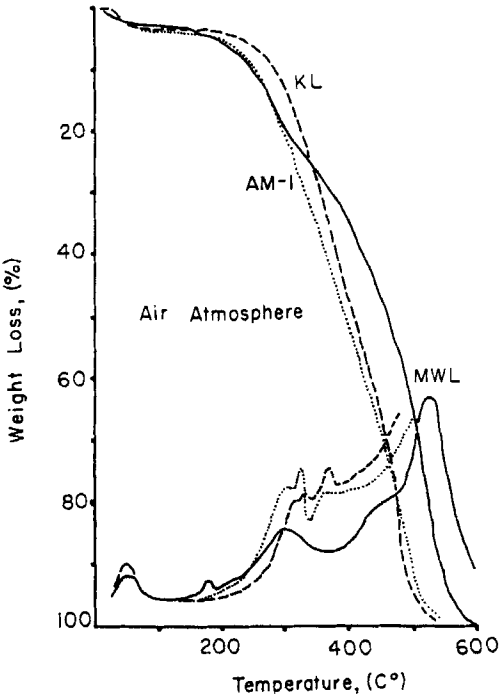


FIGURE 4. TG/DTG analysis of lignin preparations. Air atmosphere. MWL - 10.36 mg; KL - 10.76 mg; AM-1 - 10.52 mg. Abbreviations and conditions as in Figure 3.



individual preparations of CuNEL, as well as between CuNEL and NEL from A. magnifica I. There was also a good agreement between MWL and CuNEL and NEL preparations. Klason lignin, on the other hand, lost weight appreciably slower (Fig. 3). Not much difference between CuNEL preparations was found in TG/DTG analysis in air atmosphere (Fig. 4). Klason lignin lost weight somewhat slower as before particularly at lower temperatures. MWL on the other hand, while losing weight at a rate comparable to that of CuNEL up to about 300°C, transformed between 300 and 350°C into a form stabler than either Klason or CuNEL.

The DSC analyses were run between 30° and 500°C in nitrogen, air and oxygen atmospheres using open aluminum pans. The nitrogen

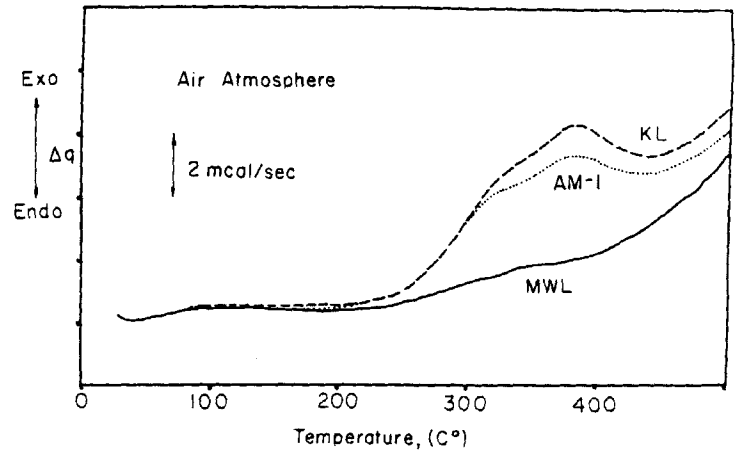


FIGURE 5. DSC analysis of lignin preparations. Air atmosphere. MWL - 1.57 mg, char - 0.72 mg; KL - 1.32 mg, char - 0.31 mg; AM-1 - 1.30 mg, char - 0.33 mg. Rate of heating - 20°C/min. Gas flow rate, 50 ml/min. Y-axis, 2 mcal/sec. inch. Abbreviations as in Figure 3.



runs were inconclusive. In both air and oxygen (Figs. 5 and 6) exotherms appeared at 295-325° (Peak A) and at 342-385°C (Peak B); the third exotherm appearing above 500°C[30] (Peak C) could not be observed due to the 500° cutoff. An appreciable amount of char remained on the pans after the air runs and the MWL oxygen run. Overall, the Klason lignin and CuNEL runs were similar in appearance and left similar amounts of char, while MWL curves were running appreciably below (Fig. 5) with appreciably higher weight of char left; this is in agreement with the results of Arima[30] who observed a strong increase in the 500-600° exotherm and corresponding decrease in the 250-400° exotherms in MWL as compared to Klason lignin.

For quantitative evaluation we expressed the 295-325° and 342-385° exotherms in cm per mg of sample weight used. The total heat (ΔH) produced between 30 and 500° was calculated per 1 mg of sample weight used and per 1 mg of sample weight lost (sample weight minus char weight), and expressed in relation to ΔH Klason lignin set as 1.0 (Table 7).

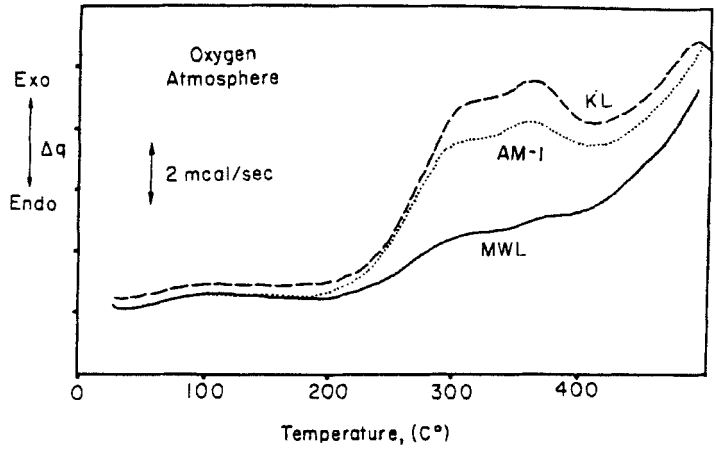


FIGURE 6. DSC analysis of lignin preparations. Oxygen atmosphere. MWL - 1.37 mg, char - 0.49 mg; KL - 1.42 mg, no char; AM-1 - 1.37 mg, no char. Abbreviations and conditions as in Figure 3 and Figure 5.



No significant difference was found in position of Peak A and Peak B between Klason and CuNEL preparations; a small downward shift in Peak A (air and $O_2$) and Peak B (air) could be discerned however with MWL. More important trends were noticed in the ΔH. The CuNEL preparations produced less heat than Klason lignin whether ΔH was expressed per weight of sample used or more realistically, per the weight of sample lost. The difference was not high, however, about 93% of the Klason ΔH for CuNEL preparations. This difference can be attributed to a higher content of carbon plus hydrogen in Klason lignin, since the curves were otherwise similar and the amounts of char left were approximately the same. Expressed on the basis of the weight of the carbon plus hydrogen in the consumed part of the sample, the ΔH value for Klason lignin and CuNEL preparations became comparable (Table 7).

Expressed on the basis of sample weight used MWL produced however, strikingly smaller amounts of heat in all cases, particularly in runs under air. As mentioned above, this is related in the main to the combustion of MWL taking place preferentially

above 500°. For this reason the ΔH results for MWL expressed on the basis of the sample weight lost were in better agreement with ΔH values of Klason lignin and CuNEL preparations. The remaining discrepancies, particularly strong in air runs, could reside in the necessity to determine the weight of char after a 10 min. cool-off period, during which additional lignin loss could have taken place.

Overall, the results of the thermal analysis could be explained by the higher condensation and carbon content of Klason lignin and by the relatively low molecular weight of MWL. The higher degree of condensation of Klason lignin and its higher carbon content explain the slow weight loss in TG as compared with MWL or CuNEL, as well as higher ΔH in combustion as compared to the CuNEL lignin parparations. The low molecular weight of MWL has been stressed time and again. Thus, Chang, et al. in their work on CEL[25] indicated that both MWL and CEL, particularly the former, do not represent well the native lignin in terms of molecular weight which is likely to be much higher. The low molecular weight of MWL as compared to the other lignins is connected in turn with its lower glass transition temperature (Tg), roughly identical with softening temperature.[31] Hatakeyama, et al.[31] and Goring[32] found a nearly linear relationship between Tg and the molecular weight of thiolignin and dioxane lignin, respectively. The lower Tg of MWL as compared with the other lignins has been reported by several investigators.[32-34] It is, thus, to be expected that MWL will soften at a lower temperature than CuNEL or Klason lignin preparations to transform into a more compact material with less surface. About a tenfold drop in surface area has been noted by Stone and Scallan to take place between 120° and 235°C with dioxane lignin.[35] The resulting reduction in the solid/gas interface should also decrease the rate of MWL oxidation by oxygen and result in ΔH shift to higher temperatures. According to TG experiments with CuNEL in air, this softening is probably taking place between 300 and 350°C (Fig. 4).

MWL and CEL are regarded at the present time as closest to the protolignin, although differences in functional groups and in

molecular weight have been noted.[25,36] The differences in functional groups have been generally taken as acceptable and MWL is regarded as the preparation closest to the native protolignin. The differences in molecular weight between MWL and protolignin are likely to be appreciable[25] and result from grinding of wood in a ball mill as well as in preferential extraction of the dioxane soluble, lower molecular weight fractions. This difference has been, in most cases, ignored as influencing little the various studies for which MWL has been used. While it is true that in homogeneous reactions in solvents, as well as in some other cases, the molecular weight of lignin plays generally a less important role, in studies such as those involving heterogenous reactions at higher temperatures the role of molecular weight becomes of primary importance due to its connection to Tg.

At the present moment it is difficult to judge how different the NEL and CuNEL preparations are from protolignin in terms of molecular weight. Little information is available on the influence of fungal decay on the molecular weight of lignin. The GP chromatography of Oki, *et al.*[26] of biodegraded and sound dioxane lignins from *Fagus crenata* allows one to conclude, however, that the changes in molecular weight in NEL could be relatively small. Our purification procedures, particularly ethanol and Cuoxam extractions, could favor, however, the more condensed lignin fractions and increase the molecular weight of CuNEL preparations. While additional studies are in place, it can be concluded that for work with lignin where retention of molecular weight and ease of preparation are primary factors the lignins such NEL and CuNEL should be worth consideration.



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