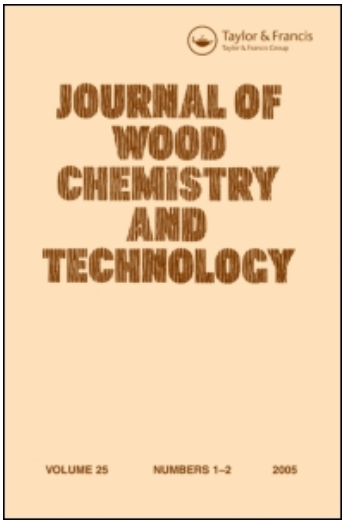

Journal of Wood Chemistry and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597282

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To cite this Article Minor, J. L.(1986) 'Chemical Linkage of Polysaccharides to Residual Lignin in Loblolly Pine Kraft Pulps', Journal of Wood Chemistry and Technology, 6: 2, 185 — 201
To link to this Article: DOI: 10.1080/02773818608085223
URL: http://dx.doi.org/10.1080/02773818608085223

JOURNAL OF WOOD CHEMISTRY AND TECHNOLOGY, 6(2), 185-201 (1986)

CHEMICAL LINKAGE OF POLYSACCHARIDES TO RESIDUAL LIGNIN IN LOBLOLLY PINE KRAFT PULPS

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ABSTRACT

The chemical bonding of polysaccharides to the residual lignin remaining in bleachable grade loblolly pine (Pinus taeda) kraft pulp was examined by methylation analysis. Before analysis, most of the nonbonded polysaccharides were enzymatically hydrolyzed and the sugars were washed from the remaining solid to give a residual lignin that contained about 8% carbohydrates. The analysis indicated that the majority of the remaining sugars were present as oligomeric segments of well-characterized pulp polysaccharides. About one monomeric unit per oligomer was bonded to lignin.

The methylation data indicated that the major bonding was to the primary C-6 position in hexans and the primary C-5 position in arabinan. Xylan was bonded to lignin at C-3 with a small amount at C-2. The overall pattern and quantity of bonding was sufficiently similar to that previously determined for Pinus taeda wood to conclude that formation of alkali-stable, acid-hydrolyzable lignin-carbohydrate bonds is not significant during kraft pulping.

The predominant methylated derivatives obtained from galactose and arabinose indicated 1-4 and 1-5 linkages, respectively. This is characteristic of pectic galactan and arabinan structures. It is especially interesting that these structures, which were previously noted with the wood lignin, were carried through the pulping process. They appear to be intimately associated with the lignin polymer.

Arabinan, galactoglucomannan, and galactan were solubilized when the residual pulp enzyme lignin was oxidized with sodium chlorite. The insoluble cellulose, glucomannan and arabinoxylan oligomers no longer contained units bonded to lignin.

0277-3813/86/0602-0185$3.50/0

INTRODUCTION

Detailed knowledge of the physical and chemical association of lignin with polysaccharides in the wood cell wall is of fundamental importance to pulping and bleaching operations. In both cases the goal is to remove lignin from the wood fiber as selectively as possible to maximize yields or to be able to control the hemicellulose removed with lignin to optimize pulp properties for the desired paper product.

A completely characterized compound containing a lignin-to-carbohydrate chemical bond has never been isolated from wood or pulp. However, circumstantial evidence continues to accumulate in support of covalent linkages. Most studies on lignin-carbohydrate bonds have been conducted on wood lignins or isolated lignin-carbohydrate complexes (LCC). Recent studies on the lignin-carbohydrate bonds in pulps have been based on the changes in the carbohydrate content and composition, as various residual lignins are fractionated or subjected to chemical reactions.[2,3]

There are reasons to believe that the lignin-carbohydrate bonding in unbleached chemical pulps might be different from that in the original wood. New bonds may have formed under the harsh chemical treatment, and the less easily removed "residual" lignin may be chemically different from the total lignin and therefore have different bonding to carbohydrates. The question of lignin uniformity through different morphological regions of wood is still unanswered.

The chemical linkage of loblolly pine wood polysaccharides to lignin has been investigated by methylation analysis.[4] The present paper describes a similar investigation of the polysaccharides remaining with the residual lignin in unbleached loblolly pine kraft pulp after enzymatic removal of most of the pulp polysaccharides. The results of the two studies are compared to see what changes in the lignin-carbohydrate association within the fiber may have taken place during pulping.

RESULTS AND DISCUSSION

Preparation and Analysis of Enzyme Lignin

Unbleached chemical pulps can be attacked directly by polysaccharidase enzymes, unlike wood, which requires preliminary ball-milling. The process requires successive treatments of loblolly pine kraft pulp with fresh enzyme and buffer. As the carbohydrate content of the pulp decreases, the proportion of glucose (cellulose) decreases and mannose and galactose become significant sugar products. This may be partly due to the resistance of certain hemicellulose structures to hydrolysis by the particular enzymes in the crude commercial polysaccharidase mixture. The solid residue usually retains some of the enzymes as indicated by nitrogen analyses of 0.2-1%. The chemical analysis of an enzyme lignin, which was prepared from a 30.4-kappa pulp and subsequently used for methylation analysis, is given in Table I. The lignin was subjected to a preliminary reduction with sodium borohydride for the reducing end unit analysis and to stabilize the carbohydrates during the alkaline methylation.

End Group Analysis

The end groups were estimated by a deuterium labeling procedure similar to that used previously.[4] The end units are represented by the protio-form of the sugar alditols generated in the preliminary reduction with sodium borohydride. After hydrolysis of the oligomeric segments in the enzyme lignin, the free sugars are reduced with sodium borodeuteride. Thus the chain units are represented by alditols with one deuterium atom at carbon one. The ratio of end units to chain units is determined by gas chromatography/mass spectrometry of the alditol acetates. The precision of the mass spectral procedure was improved by using chemical ionization and selectively monitoring masses 303-306 for pentitol acetates and masses 375-378 for hexitol acetates. The

TABLE 1

Chemical Composition of Loblolly Pine Kraft Pulp Enzyme Lignin

	Before reduction	After reduction
	- - - - (%) - - - -	
Yield from pulp	4.4	4.0
Lignin yield[a]	71.3	66.8
Sugar[b]	8.2	8.5
Protein[c]	6.9	6.2
Percent of total sugars[d]		
Glucose	46.3	43.1
Xylose	10.8	9.5
Galactose	17.9	22.4
Arabinose	1.9	2.6
Mannose	23.1	22.4
Uronic acid	0	0

[a]Corrected for sugars and protein.

[b]In enzyme lignin as anhydrides.

[c]Percent N multiplied by 6.25.

[d]As alditol acetates.

results of this analysis are given in Table 2. The data are converted to apparent number average degree of polymerization (DP_n). The values ranging from 4 to 13 are the same order of magnitude as those obtained by similar treatment of loblolly pine wood.[4,5] These values do not represent a real chain length for galactan and arabinan because galactose and arabinose may also be present as side chain substituents of arabinoxylan, galactoglucomannan, and arabinogalactans. However, the values should be indicative of chain lengths of glucomannan, glucan (cellulose), and xylan

TABLE 2

End Group Analysis by Ratio of Protio/Deuterio Alditol Acetates

Corrected mass ratios	Apparent DP_n
Arabinitol - 303/304 = 0.229 ± .016	5.4 ± 0.3
Xylitol - 303/304 = 0.298 ± .012	4.4 ± 0.2
Mannitol - 375/376 = 0.217 ± .012	5.6 ± 0.2
Glucitol - 375/376 = 0.166 ± .012	7.0 ± 0.4
Galactitol - 375/376 = 0.086 ± .016	12.6 ± 1.8

oligomers. Formation of saccharinic acid end groups is possible in kraft pulps. Such acid units may still be present after enzymolysis, but for each sugar, the methylation analysis values for the nonreducing terminal units were smaller or nearly equal to the values obtained for the reducing end units by the deuterium method. This indicates that the amount of saccharinic acid type end units in the enzyme lignin is not significant.

Methylation Analysis

A major difficulty in attempting to permethylate unfractionated wood or pulp enzyme lignin is the poor solubility in any suitable solvent. Because of the general applicability of the methylation analysis, the procedure has been studied in detail and several recent reports have suggested improvements in speed and quantitation.[6,7,8] Previous work on enzyme lignins[4,9] employed repeated methylations by the Hakomori procedure or combinations of Haworth and Hakomori methylations, but the overall yields were low. The best yields have been obtained by a slight modification of the recently reported method of Ciucanu and Kerek,[7] which uses

solid NaOH in dimethyl sulfoxide. Three applications of reagents are required for complete methylation, and even then, a small amount of insoluble cellulose remains underivatized.

Gas chromatographic resolution of all of the partially methylated alditol acetates obtained from methylated wood polysaccharides is a challenging task, but recent improvements in capillary column technology have made the task easier. Nearly complete resolution of the products from methylated pulp enzyme lignin was achieved. The products were identified by mass spectrometric analysis and retention time comparisons. Stereo isomers were identified by comparison of retention times and mass spectra with those obtained from the products of partial methylation of individual sugars.

The identified products and their retention times relative to two standards are listed in Table 3. Values are calculated relative to those for 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol and 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-glucitol by the method of Gavrichev and Berezkin.[10]

The quantitative results of methylation analysis on the pulp enzyme lignin, which are characterized by the data in Tables 1 and 2, are listed in Tables 4 and 5 by individual sugar. The results from previous work[4] on loblolly pine milled wood enzyme lignin (MWEL) are given for comparison. The appearance of some additional minor products is a result of improved resolution and identification.

The penta-O-methyl hexitols that are obtained from the non-bonded reducing end units are very volatile and are easily lost by evaporation after hydrolysis unless special precautions are taken. Higher yields than those reported here were obtained from the sample analyzed in the preliminary report.[9] A better estimate of the amount of reducing end groups present is obtained from the deuterium analysis. Those values, calculated from the data in Table 2, are also reported in Table 4.

Overall, the results from the analysis of kraft pulp enzyme lignin are sufficiently similar to those from wood MWEL to con-

TABLE 3

Retention Times of Partially Methylated Alditol Acetates on the SP 2340 Column

Alditol and position of methylation[a]	Retention time[b] (R)	Relative retention time[c] (RRT)
	(Min)	
1,2,3,5,6-Man	15.11	0.61
1,2,3,5,6-Glu	16.75	0.67
2,3,5-Arab	17.65	0.71
2,3,4-Xyl	21.73	0.87
2,3,4-Arab	21.80	0.88
3,5-Arab	24.40	0.98
2,3,4,6-Glu	24.91	1.00
2,3,4,6-Man	25.09	1.01
2,3,4,6-Gal	26.79	1.07
2,3-Arab	27.52	1.10
2,3-Xyl	28.90	1.16
2,4,6-Glu(man)	30.11	1.21
3,4,6-Man	30.33	1.22
2,4,6-Gal	31.11	1.25
2,3,6-Man	31.24	1.25
2,3,4-Man	31.85	1.28
2,3,4-Glu	32.03	1.28
2,3,6-Gal	32.27	1.29
2,3,6-Glu	32.83	1.32
3-Xyl	33.43	1.34
2-Xyl	33.90	1.36
2,6-Man	34.10	1.37
2,3,4-Gal	34.27	1.37
2,6-Gal	34.95	1.40
2,6-Glu	35.57	1.43
3,6-Man	36.19	1.45
3,6-Gal (Glu)	36.65	1.47
2,3-Man	36.90	1.48
2,4-Glu	37.84	1.52
2,3-Glu	38.95	1.56
2,3-Gal	39.52	1.58
2,4-Gal	39.98	1.60
Glucitol Hexaacetate	51.98	2.08

[a] Arab, Gal, Glu, Man, and Xyl indicate arabinitol, galactitol, glucitol, mannitol, and xylitol respectively.

[b] Programmed temperature 150° C (10 min) to 220° at 3/min. He flow = 30 cc/min.

[c] Calculated using $RRT = k + p[(R_x - R_1)/(R_2 - R_1)]$,[10] where R_1 = R for 2,3,4,6-glu and R_2 = R for 2,3-glu.

TABLE 4

Products of Methylation Analysis of Loblolly Pine Enzyme Lignins from Hexans

Position of methylation in partially methylated alditol acetates	Product quantities from enzyme lignin[a]	Product quantities from loblolly pine milled wood enzyme lignin[4]
	(Mole %)	(Mole %)
Mannitol		
1,2,3,5,6	0.2, 3.56[b]	Present
2,3,4,6	1.9	(2.7)[c]
2,3,6	8.7	11.4
2,3,4	0.6	--
2,4,6	(0.3)	--
3,4,6	0.3	--
2,6	0.2	0.2
3,6	0.2	0.1
2,3	4.0	2.6
Glucitol		
1,2,3,5,6	0.3, 7.75[b]	Present
2,3,4,6	4.1	(3.0)
2,3,6	34.7	(24.8)
2,3,4	1.4	--
2,4,6	(0.4)	0.4
3,6	(0.1)	(0.1)
2,6	0.4	--
2,4	0.6	--
2,3	3.2	2.1
Hexaacetate	0.9	2.9
Galactitol		
1,2,3,5,6	--, 1.71[b]	--
2,3,4,6	0.9	3.2
2,3,6	10.3	(13.8)
2,4,6	4.0	--
2,3,4	0.7	0.9
3,6	(0.3)	0.1
2,6	0.4	--
2,3	1.2	2.1
2,4	1.7	1.2

[a]Area percent of all eluted products after solvent peak, converted to mole basis. A response factor of 1.0 was assumed.

[b]Theoretical methylated reducing end unit calculated from deuterium end group analysis as: 1/DP *(sum of products not derived from the reducing end).

[c]Values in parentheses are estimations for individual stereoisomers that may coelute.

TABLE 5

Products of Methylation Analysis of Loblolly Pine Enzyme Lignins from Pentans

Position of methylation in partially methylated alditol acetates	Product quantities from enzyme lignin[a]	Product quantities from loblolly pine milled wood enzyme lignin[4]
	(Mole %)	(Mole %)
Xylitol		
1,2,3,5	--, 2.91[b]	--
2,3,4	3.3	1.8
2,3	5.2	8.6
3	0.1	0.9[c]
2	2.2	2.4[c]
Arabinitol		
1,2,3,5	--, 0.5[b]	--
2,3,5	0.6	1.7
2,3,4	0.1	0.1
3,5	0.15	--
2,3	1.8	2.8

[a]Area percent of all eluted products after solvent peak, converted to mole basis. A response factor of 1.0 was assumed.

[b]Theoretical methylated reducing end unit calculated from deuterium end group analysis as: 1/DP *(sum of products not derived from the reducing end).

[c]Mixture.

clude that there is little if any lignin-carbohydrate bond formation during pulping as measured by methylation analysis. There is the possibility of bond formation of types not measured by methylation analysis, such as C-C bonds,[11] but separate investigations will be required to determine their significance.

As noted previously, the majority of the methylated products derive from well-known softwood polysaccharide structures, e.g., the 2,3,6-tri-O-methyl glucitol and mannitol from cellulose and glucomannan. Other methylated products are considered to arise from lignin-carbohydrate bonding.

The major lignin bonding to hexans is indicated to be at the monomeric C-6 position. The sum of the proportion of 2,3- and 2,3,4-methylated products from glucose and mannose indicates the extent of C-6 bonding and is in excess of the possible C-6 substitution by galactose in galactoglucomannan (maximum of 0.9% terminal 2,3,4,6-tetra-O-methyl galactitol observed). The quantities of 2,3-di-O-methyl glucitol and mannitol in this sample are slightly larger than the quantities of corresponding products from milled wood enzyme lignin. This could imply bond formation at C-6 in glucomannan during pulping. However, the proportion of 2,3-di-O-methyl derivatives in other samples from pulp is closer to that from wood and thus the differences are considered to be within experimental error.

As was observed with wood, galactose is present primarily in the 1-4 linked form that is characteristic of galactan from compression wood and is associated with the "pectic substances." There is an increasing amount of evidence indicating that the pectic substances are intimately associated with lignin. Meshitsuka and coworkers[12] have recently separated carbohydrates from the lignin in birch and spruce LCC by use of purified pectinases. The association of lignin with pectin was demonstrated, but it is not clear what type of chemical bonding was involved. The well-known presence of some alkali-labile bonds[5,13,14] has suggested ester linkages through uronic acids. However, in the present case, the kraft pulp has been subjected to harsh alkaline treatment and the residual enzyme lignin contained no uronic acid (Table 1). Some 1-3,1-6-linked arabino-galactan appears to be present and some terminal galactose units may be attached to the backbone chain in galactoglucomannan. There is sufficient 2,3-di- and 2,3,4-tri-O-methyl galactitol to show bonding of the 1-4 linked galactan to lignin through at least one C-6 unit per chain.

1-5-linked arabinan is also a "pectic substance" and its presence is indicated by 2,3-di-O-methyl arabinitol. If some of the nonreducing terminal ends (2,3,5-tri-O-methyl) are bonded glycosidically to xylan and galactan as would be expected, bonding to lignin must be primarily at the C-5 hydroxyl function.

Xylan is expected to be a straight-chain 1-4-linked oligomer with some substitution by arabinose at C-3. The data indicate that this is indeed the case. The calculated reducing end is equivalent to the nonreducing ends, so bonding to lignin would be primarily at C-3 with some at C-2.

The yield of methylated sugars from the methylated pulp enzyme lignin, as measured by an internal standard, was essentially quantitative. However, the assumptions necessary to calculate a theoretical yield make it impossible to determine an accurate value. The relative amounts of the individual sugar derivatives correspond reasonably well with the ratios of wood sugars determined from the enzyme lignin, with the exception of mannose. This was also observed previously, although the present difference is much less than that in the preliminary results.[9] It is possible that some fractionation occurs during methylation or hydrolysis.

Chlorite Oxidation of Residual Lignin

A sample of pine kraft pulp enzyme lignin was oxidized with acidified sodium chlorite solution at room temperature. Most of the substrate was solubilized, but a small portion of light-colored solid remained. The insoluble "bleached" material was subjected to a methylation analysis to give the partially methylated alditol acetates as shown in Table 6. The major product was 2,3,6-tri-O-methyl-D-glucitol from residual cellulose and glucomannan. There were also major quantities of 2,3,6-tri-O-methyl-D-mannitol from glucomannan and 2,3-di-O-methyl-D-xylitol from xylan. The relative amount of mono-O-methyl xylitol was much smaller than that observed with the unoxidized enzyme lignin, and mass spectrometry indicated only the C-2 O-methyl derivative. This supports the deduction of lignin bonding at C-3 as well as the smaller amount at C-2. The mass spectrometric results were not conclusive with this sample, however, because of overlap problems with the large quantity of 2,3,6-tri-O-methyl glucitol. The quantity of terminal arabinose observed accounted for the amount of mono-O-methyl xylose derivative.

TABLE 6

Methylation Analysis after Chlorite Oxidation of Residual Lignin

Position of methylation in partially methylated alditol acetates	Mole %
Mannitol	
2,3,4,6	(0.3)[a]
2,3,6	8.6
2,3	0.2
Glucitol	
2,3,4,6	(0.5)[a]
2,3,6	77.9
2,3	0.2
Xylitol	
2,3,4	0.8
2,3	10.9
2	0.4
Arabinitol	
2,3,5	0.8

[a]Peaks not resolved (Durawax 4 column)

Isogai et al.[8] have recently reported the results of methylation of "wood celluloses," which were obtained by alkaline extractions of chlorite holocelluloses from spruce and beech. They noted the presence of several di-O-methyl compounds derived from glucan. The authors suggested that the results implied the presence of glucosyl residues linked at positions 2,3 and 6 to other sugars. The results could also be explained by fragments of lignin remaining attached to the polysaccharides after the chlorite oxidation. The present analysis, however, detected only trace amounts of the di-O-methyl derivatives after chlorite oxidation. The resolution of this point will require further investigation.

Conclusions

The overall similarity of methylation analysis results from wood enzyme lignin compared to those from kraft pulp enzyme lignin indicates that there is no measurable formation of new lignin-carbohydrate bonds during kraft pulping of the type analyzed by methylation. Glycosidic bonds to lignin and to acid-stable bonds are not detected by this procedure. The quantities of reducing end units determined by deuterium analysis compared with those of nonreducing end units argues against glycosidic bonding to lignin.

Most of the methylation products are explainable as arising from oligomers of well-known softwood polysaccharide structures. The most significant finding in that regard is the predominance of methylated galactose and arabinose derivatives from structures characteristic of the so-called pectic group substances, i.e., 1-4-linked galactan and 1-5 linked arabinan. These structures appear to have a unique association with lignin.

Where bonds to lignin are indicated, linkage through the primary oxygen at C-6 of the hexans or the primary C-5 oxygen of arabinan is favored. Smaller quantities of bonds through secondary oxygens of hexans are indicated and xylan appears to be bonded through the C-3 position with a small amount through C-2.

Chlorite oxidation of the residual enzyme lignin releases the polysaccharides. Galactan and arabinan are solubilized. Methylation analysis of the insoluble residue supports major lignin bonding through C-6 of the hexans and C-3 of xylan.

EXPERIMENTAL

Preparation of Pulp Enzyme Lignins

Laboratory-prepared loblolly pine kraft pulp of 30.4 kappa number was washed, pressed to 33.8% solids, and stored at 8° C. A commercially supplied crude mixture of polysaccharidases from Trichoderma viride, "Cellulysin" (Calbiochem-Behring Corp., La Jolla, CA), 5 g, was dissolved in 100 ml of acetate buffer, pH 4.5. A small amount of insoluble material was removed by centri-

fugation and the centrifugate was diluted to 1 L. Wet pulp (200 g) was suspended in the enzyme solution and 1 ml of Tween 80 and 2 ml of toluene (as preservative) were added. The mixture was stirred at 45° C for 1 week. Toluene was replaced as necessary. The solid was recovered by centrifugation and subjected to two more similar treatments with fresh enzyme solutions. The final product was recovered by centrifuging, washing and freeze drying.

Reductions and End Group Analyses

A 3-g sample of pulp enzyme lignin was suspended in 50 ml 0.1N NaOH, treated with 700 mg $NaBH_4$, and stirred 2 h at room temperature. The reaction was stopped and the product precipitated by acidification with acetic acid. The reduced lignin was washed and freeze-dried. End group and sugar analyses were performed on the same sample (20 mg) by a two-stage hydrolysis with 72% H_2SO_4 (0.1 ml) at 35° C for 1 h followed by dilution with 1.7 ml H_2O and heating at 120° C for 1 h. The hydrolysate was filtered and neutralized. The reducing sugars were reduced with $NaBD_4$ (25 mg) at room temperature for 2 h. The alditols were then acetylated without removal of borate by the method of Harris et al.[6] Erythritol tetraacetate (ca. 1 mg) was added as internal standard. The alditol acetates were separated and analyzed by capillary gas chromatography with flame ionization detection on (A) 60 m Durawax 4 (J&W Scientific, Rancho Cordova, CA) or (B) 30 m SP-2340 (Supelco, Bellefonte, PA) bonded phase fused silica columns. The liquid phase in column B is equivalent to Silar 10CP. Complete resolution of the five wood sugar alditol acetates was achieved. The elution sequence was arabinitol, xylitol, mannitol, galactitol, and glucitol on column B. No rhamnitol was detected.

The enzyme lignin was analyzed for uronic acid by the method of Scott.[15]

Methylation of Enzyme Lignin

The reduced, freeze-dried lignin (200 mg) was suspended in 10 ml of dimethyl sulfoxide. Powdered NaOH (150 mg) was added and

the mixture was stirred overnight at room temperature. The next morning, 1 ml of CH_3I was added and stirring was continued for 2 h. The excess CH_3I was then removed by bubbling nitrogen through the solution for 30 min. The reaction mixture was poured into 80 ml of water, neutralized with H_2SO_4, and filtered through a 100,000 nominal-molecular-weight-cutoff polysulfone ultrafiltration membrane. The collected product was washed and freeze-dried; yield: 96% based on starting material. The methylation procedure was repeated twice more. After the second methylation the IR spectrum of a film from $CHCl_3$ contained no OH band, but traces of unmethylated alditol acetates were detected in a separate sample after hydrolysis, reduction, and acetylation. A third methylation gave a product in 85% overall yield based on the original starting material. Glucitol hexaacetate from cellulose was the only unmethylated alditol derivative detected by gas chromatography after hydrolysis, reduction and acetylation. No mono-O-methylated hexitols were observed.

Analysis of Products

The methylated enzyme lignin was hydrolyzed with 5 ml of 90% formic acid for 1 h at 100° C. The formic acid was then removed by evaporation and a secondary hydrolysis was performed with 4 ml of 4% H_2SO_4 at 120° C for 1 h. After filtration from the insoluble lignin, the mixture of partially methylated sugars was reduced and acetylated by the procedure of Harris et al.[6] The partially methylated alditol acetates were separated by capillary gas chromatography on columns A and B. Column B gave the best overall resolution of products, but erythritol tetraacetate as an internal standard overlapped the tetra-O-methyl glucitol and mannitol acetates. The internal standard was separated from other products on column A. Products were identified by GC/MS on a Finnegan 4510 instrument and by comparison of GC retention times.

ACKNOWLEDGMENTS

The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such

use does not constitute an official endorsement or approval by the U.S. Department of Agriculture of any product or service to the exclusion of others that may be suitable.

Enzyme lignins were prepared by J. D. McSweeney. Capillary gas chromatography/mass spectrometry was performed by R. C. Pettersen. Technical assistance was provided by G. L. Kennedy.

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