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Comparison of Sound and White-rotted Sapwood of Sweetgum with Respect to Properties of the Lignin and Composition of Extractives

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Ligninegenskaper och sammansättning av extraherbart material hos splintved från ambraträdet *(Liquidambar styraciflua* L.) före och efter angrepp av vitrötesvampen *Polyporus versicolor* L. ex Fries jämfördes (genomsnittliga viktförlusten hos den angripna veden var 32 %). »Milled wood lignin» framställt från frisk ved och motsvarande preparat från angripen ved visade inga nämnvärda skillnader. Även vedextrakten innehöll en komponent av ligninnatur. I fallet angripen ved hade denna ligninfraktion en relativt kraftig IR-absorption i karbonylområdet (vid 1 730 cm^{-1}); denna saknades i IR-spektrum av extraktivligninet från frisk ved. I övrigt skiljer sig de båda extraktivligninerna i sina spektrala egenskaper ej väsentligt från varandra och ej heller från de båda »milled wood lignin»-preparaten. Små mängder av följande lågmolekylära, ligninet närstående föreningar kunde påvisas i båda extrakten: vanillinsyra (IX), syringasyra (XIV), vanillin (VIII), syringaaldehyd (XIII), koniferylaldehyd (X) och sinapylaldehyd (XV). Båda extrakten innehöll ungefär samma mängder av dessa ämnen, bland vilka föreningarna IX och XIV var de mest framträdande. I extraktet från frisk ved påvisades förutom de sex nämnda fenolerna även gallussyra (XVII), dihydroquercetin (XIX) och spår av *p*-kumaraldehyd (VII). Vidare utgjorde föreningar av garvämnestyp, med galloyl- och 3,4-dihydroxifenylgrupper, en avsevärd del av extraktet från frisk ved. Sådana föreningar fanns ej i extraktet från angripen ved. Extraktet från frisk ved visades innehålla glukos och fruktos, medan glukos, trehalos och arabinitol ingick i extraktet från angripen ved.

Vitrötesvamparnas inverkan på veden diskuteras på grundval av de erhållna resultaten.

Sound sapwood of sweetgum (*Liquidambar styraciflua* L.) and that which had been decayed to an average weight loss of 32 % by the white-rot fungus *Polyporus versicolor* L. ex Fries were compared with respect to properties of milled wood lignin preparations and composition of extractives. The milled wood lignin of the decayed wood did not differ significantly from that of the sound wood. A lignin component was also isolated from the extractives of both of the wood samples. The extractive lignin from the decayed wood exhibited a comparatively strong IR absorption in the carbonyl region (1 730 cm^{-1}), which was absent from the IR spectrum of the sound wood extractive lignin. Apart from this difference, the spectral properties of both extractive lignins were very similar to each other and also very similar to those of the milled wood lignins. Small amounts of the following lignin-related phenolic compounds were present in the extracts of both wood samples: vanillic acid (IX), syringic acid (XIV), vanillin (VIII), syringaldehyde (XIII), coniferaldehyde (X), and sinapaldehyde (XV). Each of these compounds was present in similar amounts in the extractives from the sound and decayed wood, compounds IX and XIV being the most abundant. The extract from the sound wood contained, in addition, gallic acid (XVII), dihydroquercetin (XIX), and traces of p-coumaraldehyde (VII). Substances of a tannin nature, having galloyl residues and 3,4-dihydroxyphenyl moities, were prominent constituents of the material extracted from the sound wood, but were absent from the extractives of the decayed wood. Glucose and fructose were components of the extractives of the sound wood; glucose, trehalose, and arabinitol were components of the extractives of the white-rotted wood.

The differences and similarities found between the decayed and sound woods are discussed in terms of the effects of white-rot fungi on wood.

Splintholz von Sweetgum (*Liquidambar styraciflua* L.), welches durch den Weissfäulepilz *Polyporus versicolor* L. ex Fries bis zu einem durchschnittlichen Gewichtsverlust von 32 % abgebaut worden war, wurde mit gesundem Splintholz der gleichen Art hinsichtlich der Eigenschaften der daraus hergestellten „milled wood lignin"-Präparate sowie der Zusammensetzung der Extraktivstoffe verglichen. Das „milled wood lignin" des abgebauten Holzes unterschied sich nicht wesentlich von dem des gesunden Holzes. Aus den Extrakten von beiden Proben konnte auch eine Ligninkomponente isoliert werden. Nur im Falle des abgebauten Holzes zeigte das IR-Spektrum dieser Komponente eine relativ kräftige Carbonylabsorption bei 1 730 cm^{-1}. Beide Extraktivlignine waren im übrigen in ihren spektralen Eigenschaften den „milled wood lignin"-Präparaten weitgehend ähnlich. Die Extrakte aus beiden Holzproben enthielten ferner folgende, beiden gemeinsame, Verbindungen: Vanillinsäure (IX), Syringasäure (XIV), Vanillin (VIII), Syringaaldehyd (XIII), Coniferylaldehyd (X) und Sinapylaldehyd (XV). Jede dieser Verbindungen war in vergleichbaren Mengen unter den Extraktivstoffen des gesunden und des abgebauten Holzes vorhanden; die Verbindungen IX und XIV überwogen mengenmässig. Der Extrakt des gesunden Holzes enthielt ausserdem Gallussäure (XVII), Dihydroquercetin (XIX) und Spuren von p-Cumaraldehyd (VII). Tanninähnliche Verbindungen mit Galloyl- und 3,4-Dihydroxyphenylresten traten unter den Extraktivstoffen des gesunden Holzes hervor, während sie unter den Inhaltsstoffen des abgebauten Holzes nicht aufgefunden werden konnten. Schliesslich wurden im Extrakt aus gesundem Holz Glucose und Fructose, im Extrakt aus abgebautem Holz Glucose, Trehalose und Arabinitol aufgefunden.

Die Einwirkung der Weissfäulepilze auf das Holz wird an Hand der erhaltenen Ergebnisse diskutiert.

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From the results of the analysis of wood decayed by white-rot fungi it can be deduced that the lignin is decomposed together with the polysaccharide components; usually the percentage of lignin in white-rotted wood is found to be equal to or somewhat less than the percentage in the wood before decay (1—3). In the case of the sapwood of sweetgum (*Liquidambar styraciflua* L.) decayed by the white-rot fungus *Polyporus versicolor* L. ex Fries, the percentage of lignin at any given stage of decay is about equal to the percentage in the original wood (4—6).

The decay of sweetgum sapwood by *P. versicolor* has been studied both by chemical and microscopical means. Chemical studies indicate that at any given stage of decay the bulk of the residual cellulose exhibits little evidence of attack by the fungus (6). Microscopical observations suggest that this is because the cellulose is removed from surfaces exposed to the fungal enzymes within the wood (7); the amount of cellulose thus exposed to attack apparently is small compared to the total amount of cellulose in the wood. The microscopical studies indicate that the lignin-decomposing enzymes of the fungus, in contrast to the cellulose-decomposing enzymes, are able to penetrate and act within the cell walls (7). It is thus conceivable that a substantial amount of the residual lignin in white-rotted sweetgum exhibits signs of decomposition.

In the present investigations we examined that portion of the residual lignin which was removed as milled wood lignin from a sample of sweetgum sapwood decayed by *P. versicolor*. This lignin was compared by chemical and physical methods to milled wood lignin prepared from sound wood. In addition, extractives removed from samples of the sound and white-rotted wood were examined with respect to composition and properties, with emphasis on components related to lignin.

RESULTS AND INTERPRETATION

Comparative Examinations of Milled Wood Lignins from Sound and White-rotted Woods

Milled wood lignin was prepared according to the procedure of Björkman (8) from sapwood of sweetgum decayed to an average weight loss of 32 % by *P. versicolor*, and also from sound wood. The two lignin samples were subjected to comparative examinations, the results of which are given below.

1. Elemental and methoxyl analyses showed no significant dissimilarities between the two lignin preparations (*Table 1*). Analytical values similar to those in *Table 1* have been reported for milled wood lignins from certain other hardwoods (9, 10).
2. The UV-spectra and the $\Delta\varepsilon$-curves for ionization (see ref. 11) were essentially the same for both lignins; (the UV spectra of neutral solutions are included in *Fig. 3*).
3. Infrared spectra disclosed no important dissimilarities between the lignins from the two woods (*Fig. 1*). Slight differences in the carbonyl region were within the limits of normal variation as judged by an investigation of a series of preparations of milled wood lignin from different samples of a second hardwood, *Betula verrucosa* Ehrh.
4. Gel filtration on Sephadex G-50 with dimethyl sulfoxide as eluting solvent revealed no differences in molecular weight distribution of the two lignin preparations. (This experiment was kindly conducted by Mr. Gerhard Miksche.) Under the conditions used lignin of molecular weight greater than about 8 000 was excluded from the gel (12); in the present case, about half of each lignin sample was excluded.
5. The composition of the mixture of ethoxybenzoic acids formed on ethylation-oxidation (13) of the two lignins was the same within experimental error, as judged by gas chromatographic examination of the methyl esters. (The procedure used is given in the Experimental section in connection with investigations of the extractives.) Compounds I—V (*Table 2*) were among the products formed, compounds II and IV being the major products.

Table 1. Elemental and methoxyl analyses of milled wood lignin prepared from sound wood and from white-rotted wood of sweetgum

Sample	% C	% H	% O	% OCH_3
Milled wood lignin from sound wood	59.6	6.0	34.0	21.4
Milled wood lignin from white-rotted wood[1] ..	59.4	6.0	34.2	21.4

[1] Weight loss = 32 %

The results of these several comparisons did not reveal any pronounced differences between the milled wood lignins from the sound and white-rotted woods. It was therefore concluded that the two lignins did not differ more than slightly.

Comparative Examinations of Extractives from Sound and White-rotted Woods

Samples of decayed wood and sound wood were extracted with benzene-ethanol (2: 1) and then with 96 % ethanol (see Experimental). The total amounts of extracted material were about the same from both wood samples (3.6 % of the decayed wood, 3.3 % of the sound wood). The two samples of extractives differed markedly in color; the material obtained from the decayed wood was light brownish-yellow while the material from the sound wood was deep red-brown.

To facilitate their examination, the extractives were subjected to gel filtration on Sephadex G-25, with dioxane-water (1: 1) as solvent. Since lignin-related materials generally show a UV absorption maximum at about 280 nm, the absorbance at 280 nm of the material eluted from the column was determined. A plot of $A_{280\ nm}$ vs. collection tube number is shown in *Fig. 2*. Gel filtration under the conditions used has been shown to fractionate lignin degradation products (14) and lignin model compounds (15) according to molecular weight. Thus lignin-related material present in each of the samples was divided into a high molecular weight fraction, a low molecular weight fraction, and a third

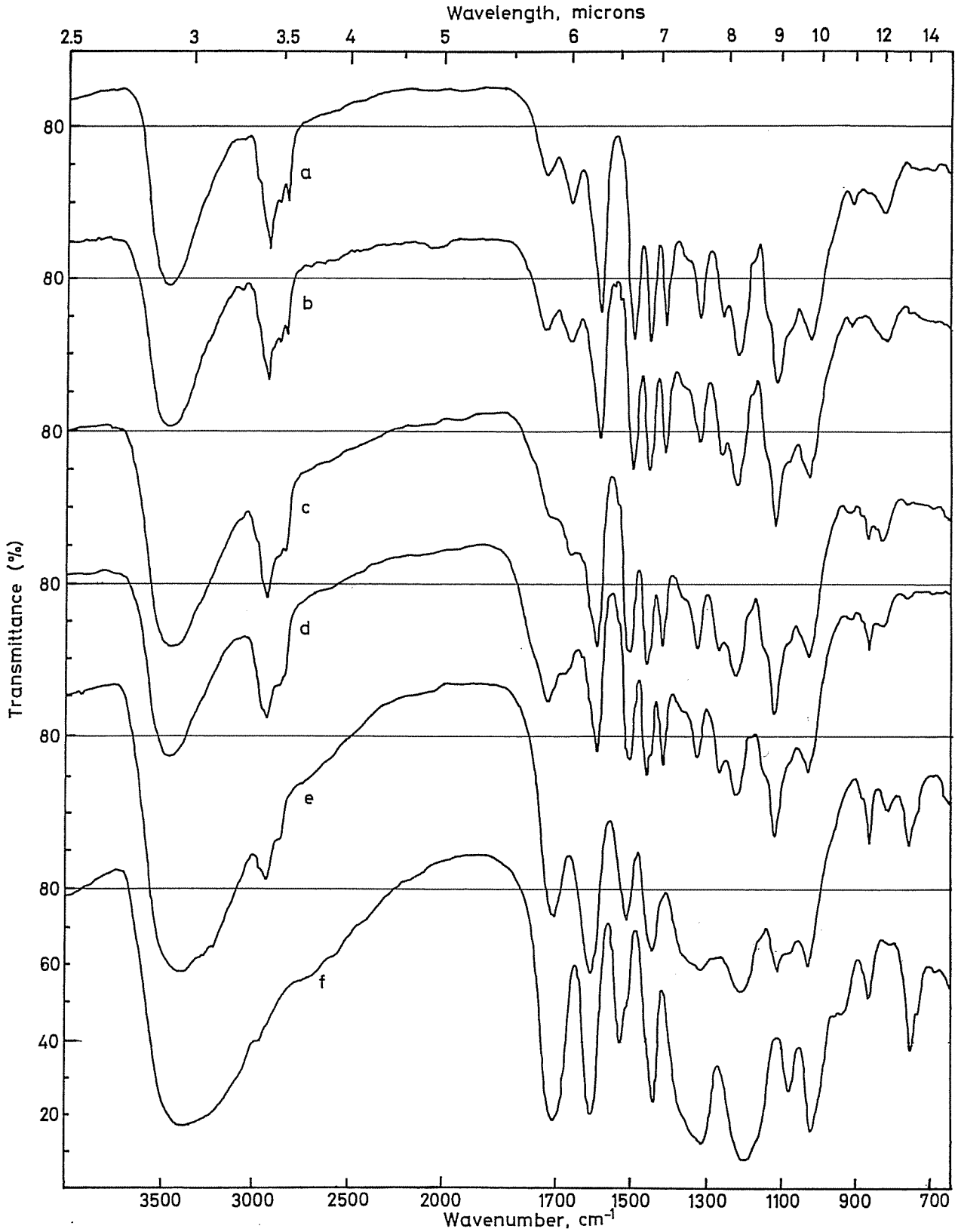


Fig. 1. Infrared spectra: milled wood lignin from a) sound, and b) white-rotted wood; purified extractive lignin from c) sound, and d) white-rotted wood; e) extractive fraction S1, aqueous layer, and f) tannic acid. All spectra were taken with KBr pellets.

fraction which contained the material eluted between these two fractions (see *Fig. 2*). Lignin-related material in the intermediate fraction would have an average molecular weight of approximately 400, based on the known behavior of such materials under the conditions used. It follows that lignin-related materials in the higher molecular weight fraction and the lower molecular weight fraction would have molecular weights greater than 400 and less than 400, respectively. The three fractions from

Table 2. Yields (mg/100 mg original sample) of acids I—VI[1] produced on oxidative degradation of ethylated samples of milled wood lignin and of the materials in the aqueous and organic layers of fraction D1 and fraction S1

Sample	Product I	II	III	IV	V	VI
Milled wood lignin from sound wood[2]	< 0.2	4.7	< 0.1	3.1	< 0.2	not detected
Fraction D1, organic layer	~ 0.2	4.0	~ 0.1	2.9	< 0.3	< 0.1
Fraction D1, aqueous layer	~ 0.2	1.9	~ 0.2	1.4	~ 0.2	~ 0.2
Fraction S1, organic layer	1.0	2.5	0.6	1.5	~ 0.1	0.4
Fraction S1, aqueous layer	1.0	1.5	6.8	0.9	< 0.3	12.5

[1] Determined as methyl esters by gas chromatography
[2] Degradation experiments with milled wood lignin from the decayed wood gave the same result within experimental error

Table 3. Fractionation of extractives from sound and white-rotted wood: weights of fractions

Extractives from sound wood[1] total weight: 399 mg		Weight mg	Extractives from white-rotted wood[1] total weight: 429 mg		Weight mg
Fraction S1	organic layer	47	Fraction D1	organic layer	48
	aqueous layer	53		aqueous layer	25
Fraction S2	organic layer	29	Fraction D2	organic layer	26
	aqueous layer	26		aqueous layer	17
Fraction S3	organic layer	23	Fraction D3	organic layer	30
	aqueous layer	57		aqueous layer	62
Material insoluble in dioxane-water (1:1)		13	Material insoluble in dioxane-water (1:1)		25
Material retarded on gel filtration		151[2]	Material retarded on gel filtration		196[2]

[1] 12.0 g of wood (dry weight)
[2] Determined by difference

the extractives of the decayed wood are designated D1 (MW > 400), D2 (MW ≃ 400), and D3 (MW < 400). The designations for the corresponding fractions obtained from the sound wood are S1, S2, and S3. Each of these six fractions (S1—3 and D1—3) was subdivided by extraction of the dioxane-water solutions with chloroform; the material from each fraction which was transferred on extraction to the organic phase is termed the "organic layer", while the more hydrophilic material which remained in the water phase is termed the "aqueous layer". The weights of the subfractions thus obtained are given in *Table 3*.

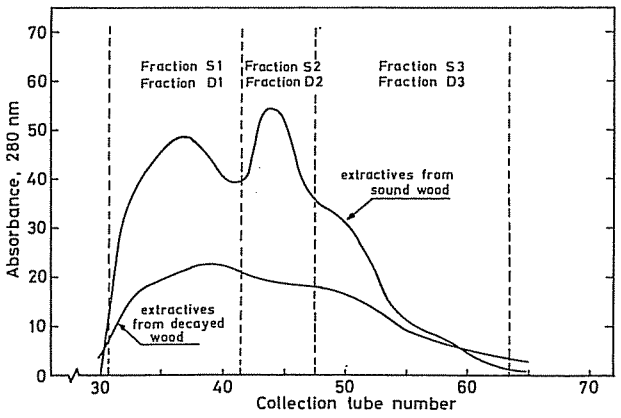


Fig. 2. Plot of $A_{280\,nm}$ vs. collection tube number for fractionation of extractives of sound and white-rotted woods on a column of Sephadex G-25, with dioxane-water (1:1) as solvent. Equal weights (12 g dry weight) of the two wood samples were extracted.

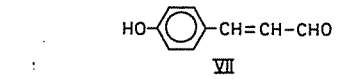
A portion of the total extractives of both woods was not included in fractions D1—3 and S1—3 for reasons described in the following.

Extractive components not included in fractions D1—3 and S1—3

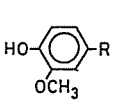
A minor part (see *Table 3*) of the extractives of each wood sample was insoluble in dioxane-water (1:1), and therefore was removed prior to gel filtration. These materials were soluble in chloroform and had a low absorbance at 280 nm compared to lignin-related compounds. They were not investigated in further detail, but were suspected to consist essentially of fatty substances, which commonly occur as components of wood extractives.

Much larger portions of both wood extracts were not included in fractions D1—3 and S1—3 because

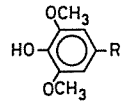
they were retarded by the column during gel filtration, being eluted after the material included in fractions D3 and S3. These retarded materials did not absorb significantly at 280 nm. Preliminary studies indicated that sugars (mono- and disaccharides) were present. In agreement with this finding it was demonstrated that sugars are retarded on gel filtration under the conditions used. Examinations for mono- and disaccharides showed that glucose and fructose were present in the sample from the sound wood, and that glucose was present in the sample from the decayed wood (fructose, if present in this sample, was only in trace amounts). Other monosaccharides appeared to be present in minor amounts, but were not examined. In addition to glucose relatively large amounts of the disaccharide trehalose were found in the sample from the decayed wood. Significant amounts of other disaccharides did not appear to be present in either sample. Further investigations of the retarded materials were made using gas chromatography of trimethylsilyl derivatives; these studies revealed that the extractives of the decayed wood contained substantial amounts of the sugar alcohol arabinitol. Both arabinitol and trehalose were absent from the sample from the sound wood, and are considered to be fungal metabolites (see Discussion).



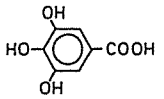
VII



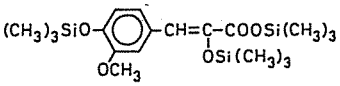
VIII R= -CHO
IX R= -COOH
X R= -CH=CH-CHO
XI R= $-CH_2-CO-COOH$
XII R= $-CH(OH)-CH(OH)-CH_2OH$



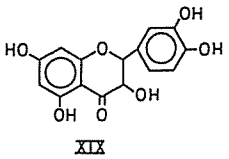
XIII R= -CHO
XIV R= -COOH
XV R= -CH=CH-CHO
XVI R= $-CH(OH)-CH(OH)-CH_2OH$



XVII



XVIII



XIX

Constituents of fractions D3 and S3

As pointed out above, lignin-related materials in fractions D3 and S3 were expected to have molecular weights less than 400, based on the known behavior of such materials under the conditions used to prepare the fractions by gel filtration. In accordance with this, the two fractions were found to contain, *inter alia*, small amounts of the following compounds: vanillic acid (IX), syringic acid (XIV), vanillin (VIII), syringaldehyde (XIII), coniferaldehyde (X), and sinapaldehyde (XV). As judged by the results of paper chromatography, these compounds appeared to be present in comparable amounts in the samples from the sound and white-rotted woods, syringic and vanillic acids clearly being the most abundant. From the sound wood the following additional compounds were found: *p*-coumaraldehyde (VII), gallic acid (XVII), and dihydroquercetin (XIX); compound XVII, in contrast to all of the other above-mentioned compounds, was found primarily in the aqueous layer. Of the last three compounds mentioned, compounds XVII and XIX were present in substantial quantities but the cinnamaldehyde VII was present only in trace amounts. These three compounds were not present in the sample from the white-rotted wood.

Fraction D3 was examined specifically for guaiacylglycerol (XII), syringylglycerol (XVI) and (4-hydroxy-3-methoxyphenyl)-pyruvic acid (XI), which, on the basis of studies with white-rot fungi in liquid culture (16), might be expected to be present in the extractives (see Discussion). Careful examination by means of paper chromatography indicated that compounds XII and XVI were not present. The results with paper chromatography were inconclusive regarding compound XI, but gas chromatographic examination, using the trimethylsilyl derivatives, showed that compound XI also was absent. Similarly, none of these three compounds was detected in fraction S3.

Further investigations revealed the presence of a trace of fructose in the aqueous layer of fraction S3, and small amounts of arabinitol as well as a trace of trehalose in the aqueous layer of fraction D3. As mentioned above, the bulks of these components were found in the portion of each of the extractives that was retarded on gel filtration.

Except for trehalose and arabinitol, no components were detected in fraction D3 which were not also detected in fraction S3; the methods used for the examinations of these fractions were paper chromatography, gas chromatography (after silylation) and, for sugar constituents, ion exchange chromatography.

Constituents of fractions D1 and S1

Lignin-related materials in fractions D1 and S1 were expected to have molecular weights greater than 400, based on the known behavior of such materials under the conditions used to prepare the fractions by gel filtration.

Samples of the organic and aqueous layers of fractions D1 and S1 were ethylated and then oxidatively degraded (13). Products I—VI (as methyl esters) were identified by comparison with authentic samples as to retention times on gas chromatography and as to mass spectra (see Experimental). Quantitative estimations of these products were made by gas chromatography (*Table 2*). The fact that compounds II and IV were formed from each of the four samples (i.e. aqueous and organic layers of fractions S1 and D1) provided some evidence that each consisted to some extent of lignin (cf. results of similar degradation of milled wood lignin, *Table 2*). The acids II and IV were the major products from all of these samples except the aqueous layer of fraction S1, in which case 3,4-diethoxy-

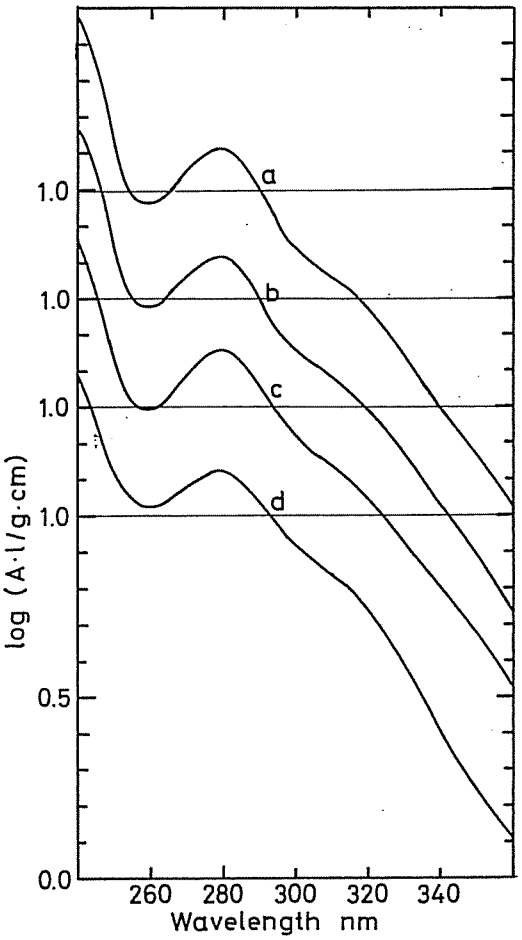


Fig. 3. UV spectra: milled wood lignin from a) sound, and b) white-rotted wood; purified extractive lignin from c) sound, and d) white-rotted wood. Spectra were taken using dioxane-water (1: 1) solutions of the samples.

benzoic acid (III) and 3,4,5,-triethoxybenzoic acid (VI) were the main products (*Table 2*). A smaller but significant amount of 4-ethoxybenzoic acid also was formed from this sample. These results indicated the presence of substantial amounts of 3,4,5-trihydroxyphenyl- and 3,4-dihydroxyphenyl moieties, and lesser amounts of 4-hydroxyphenyl moieties; these structures were either absent, or were present only in very small amounts, in the milled wood lignin (*Table 2*).

The relationship between the gallic acid found in fraction S3 (see previous section) and the product VI suggested that galloyl groups might be present in the material in fraction S1. Consequently a sample of the aqueous layer of fraction S1 was hydrolyzed with acid, and the products examined. It was found that gallic acid had been liberated (yield 14 %), thus indicating that galloyl groups were present. (The possibility also was considered that the 3,4-dihydroxyphenyl- and 4-hydroxyphenyl moieties might similarly be present as esterified acids. This was not the case, however, since the corresponding hydroxybenzoic acids were not liberated on acid hydrolysis.)

The results of these oxidative and hydrolytic degradations were interpreted to indicate that polyphenolic materials, which are termed "tannin" in this paper, were prominent constituents of the aqueous layer of the fraction from the sound wood (fraction S1) (see also description of spectral properties below).

The presence of a small amount of tannin in the organic layer of fraction S1 was indicated by the results of ethylation-oxidation (*Table 2*). However, the composition of the acid mixture obtained on ethylation-oxidation indicated that this sample was primarily of a lignin nature.

Results of ethylation-oxidation indicated that the aqueous and organic layers of the fraction from the decayed wood (fraction D1) similarly consisted essentially of lignin (*Table 2*). The tannin component found in the sample from the sound wood (fraction S1) was absent from fraction D1, as indicated by the fact that significant amounts of products VI, III, and I were not formed.

The absence of important amounts of acid III, as well as the analogous acid 3,4-diethoxy-5-methoxybenzoic acid (V), indicated that significant amounts of *o*-dihydroxyphenyl (catechol) moieties were not present in this extractive lignin sample from the decayed wood (cf. yields of products III and V from the samples of fraction D1 with the yields from the milled wood lignin and from the organic layer of fraction S1, *Table 2*). Catechol moieties have been found recently to be important constituents of lignin isolated from sweetgum wood decayed by the brown-rot fungus *Lenzites trabea*, in which case they obviously arise by fungus-demethylation of guaiacyl and syringyl elements (13). In the present studies, the finding that small amounts of products III and V were formed from the milled wood lignins and also from the extractive samples (*Table 2*) is in line with results of recent investigations of the milled wood lignin of birch, in which case products III and V also are formed in small amounts on ethylation-oxidation (17).

Further comparison of fractions S1 and D1 were made on the basis of spectral properties (IR and UV) of the aqueous and organic layers. The IR and UV properties were in accordance with the results of ethylation-oxidation. Thus it was clear that the aqueous layer of fraction S1 was comprised to a great extent of non-lignin material; the IR spectrum was similar to the IR spectrum of a gallotannin (tannic acid, Fluka "puriss.") (*Fig. 1*) and also to the IR spectra of certain other tannins (18). The UV-spectrum also was similar to that of tannic acid in its general shape (both neutral and alkaline solutions) and in its comparatively high absorptivity. The primary spectral properties of the aqueous and organic layers of fraction D1, and the organic layer of fraction S1, were clearly due to lignin, being similar to the spectral properties of milled wood lignin.

For more definitive examinations of spectral properties the organic layers of fractions S1 and D1 were purified by precipitation into ether. The resulting purified lignin from fraction S1 was very similar to the milled wood lignins in its IR spectrum (*Fig. 1*) and in its UV properties (*Fig. 3*). The UV spectrum of the purified lignin from fraction D1 also was similar to those of the milled wood lignins (*Fig. 3*). The IR spectrum of this sample,

although similar to that of milled wood lignin, differed in having a strong absorbance centered at about 1 730 cm^{-1}. This absorbance, due probably to non-conjugated carboxyl- and/or non-conjugated carbonyl groups, is considered to reflect changes caused by the fungus in the lignin (see Discussion). This strong absorbance at 1 730 cm^{-1} also was a prominent feature of the IR spectrum of the material comprising the aqueous layer of fraction D1.

Constituents of fractions D2 and S2

Lignin-related materials in fractions D2 and S2 were expected to have molecular weights of approximately 400, based on the known behavior of such substances under the conditions used to prepare the fractions by gel filtration.

Spectral properties (IR and UV) of the aqueous and organic layer of the sample from the decayed wood, fraction D2, were very similar to those of the corresponding layers of fraction D1, which, as mentioned above, consisted of lignin which had been modified by the fungus. Similarly, the aqueous layer of fraction S2 corresponded in IR and UV properties to the aqueous layer of fraction S1, the spectral properties of which, as pointed out, were primarily due to tannin materials.

In contrast, however, the organic layer of fraction S2 differed considerably with respect to spectral properties from the corresponding high molecular weight materials (i.e. from the organic layer of fraction S1). It was found that dihydroquercetin (XIX) was a prominent constituent of this sample, and it was the presence of this compound which appeared to a great extent to determine the spectral properties of the total fraction—e.g. a prominent band at 1 640 cm^{-1} in the IR spectrum. As mentioned, dihydroquercetin also was present in fraction S3, in which case its presence also noticeably influenced spectral properties. The presence of a certain amount of materials related to lignin in the organic layer of fraction S2 was indicated by the IR spectral properties.

DISCUSSION

The decayed sweetgum sapwood used in this study appeared to have been bleached in comparison to the sound wood. Such bleaching is commonly caused by white-rot fungi; indeed, it provides the basis for the name "white-rot". It has been shown by Sheffer (5) that the bleaching of sweetgum sapwood by *P. versicolor* is associated with the destruction of colored materials which are located primarily in the lumina of the ray cells. Sheffer showed that these colored materials disappeared concomitantly with the penetration of the hyphae into the lumina of the ray cells, so that at an early stage of decay (weight loss less than 10 %) the bulk of the colored materials had disappeared. Sheffer (5) suggested: "... that this rapid depletion of the chromogenic materials is to be accounted for by the consumption of the original water extractives."

Results of the present study are in harmony with the conclusion of Sheffer. It was observed that the extract from the sound wood was highly colored (a deep red-brown) and that this color was associated with the tannin materials, found primarily in the aqueous layer of fraction S1. The extract from the white-rotted wood had relatively little color (a light brownish-yellow) as a consequence of the absence of these tannin materials. It follows, therefore, that the bleaching of the wood was due at least in part to disappearance of the tannin materials.

In the studies of Sheffer (5) and in the present studies it was noted that extraction of sound wood did not completely "bleach" the wood. This might have reflected an incomplete extraction of tannin materials.

The relationship of these tannin materials to gallotannins was brought out by the similarities of their spectral properties (IR spectra, see *Fig. 1*), the release of gallic acid on acid hydrolysis, and the association with free gallic acid. The presence of 3,4-dihydroxyphenyl moieties in the material in the aqueous layer of fraction S1 is tentatively interpreted to indicate that condensed tannins were present (for a treatment of the structures of condensed tannins, see ref. 19). (It is possible that the 4-ethoxybenzoic acid (I) formed on ethylation-oxidation of this sample (*Table 2*) also was derived from a condensed tannin component.) Thus it is possible that this sample contained a mixture of gallotannins and condensed tannins. A further possibility is that condensed tannins containing galloyl residues (see ref. 19) were present. Because most condensed tannins are considered to originate from flavanoid compounds (19), it is of interest to note that such a compound, viz. dihydroquercetin (XIX), which contains a 3,4-dihydroxyphenyl moiety, was present in the extractives from the sound wood (in fractions S2 and S3).

In addition to the obvious difference between the extractives of the two woods regarding tannin materials, there was also a difference in the low molecular weight sugars, which were present in the material retarded by the column during gel filtration. Whereas the extract of the sound wood contained primarily glucose and fructose and apparently no significant amounts of disaccharides, the extractives of the decayed wood contained glucose and the disaccharide trehalose. Fructose, if present in the latter extract, was only present in trace amounts, and disaccharides other than trehalose were not detected. Trehalose has been shown to be produced by *P. versicolor* (20, 21) and is considered to be a fungal metabolite in the present case. The material retarded during gel filtration of the extractives from the decayed wood contained, in addition to glucose and trehalose, the five-carbon sugar alcohol arabinitol. It is known that arabinitol is produced by a number of Basidiomycetous fungi, and it is thus considered probable that this compound, like trehalose, was a fungal metabolite in the present case. No previous report of the production of arabinitol by *P. versicolor* or other wood-destroying Basidiomycete was found. Interestingly, however, the sugar alcohols threotol and mannitol have been found in extracts of the fruiting bodies of the white-rot fungus *Armillaria mellea*, and xylitol and mannitol have been found in fruiting bodies of the related fungus *Psalliota*

campestris (22). Both arabinitol and trehalose were clearly major components of the extract of the decayed wood, and it is probable that they contributed considerably to the total weight of the material obtained from the decayed wood (see *Table 3*). The significance of these materials to the decay of wood by *P. versicolor* is not apparent.

The reason for the difference between the two wood extractives regarding fructose also is not apparent. It is possible that the free fructose and glucose were removed during decay but that the latter was replenished to some extent through fungal hydrolysis of cellulose or other glucose-containing polysaccharide. The dimeric product of cellulose hydrolysis, cellobiose, was not detected, however.

Results of the present investigations indicated that the fungal attack had not led to an accumulation of low molecular weight phenolic compounds. The weights of fractions S_3 and D_3, in which such compounds occurred, amounted to 0.67 and 0.77 % of the wood samples, respectively. However, all but the most hydrophilic phenolic compounds (e.g. gallic acid, present in fraction S_3), occurred in the organic layers, which amounted to only 0.19 and 0.25 % of the sound and decayed wood samples, respectively. Since spectral properties of the aqueous layer of fraction D_3 indicated that aromatic materials could only be present in negligible amounts, it followed that the total amount of low molecular weight phenolic compounds in the extract of the decayed wood was less than 0.25 % of the weight of the wood.

Small amounts of vanillin (VIII), syringaldehyde (XIII), vanillic acid (IX), syringic acid (XIV), coniferaldehyde (X), and sinapaldehyde (XV) were detected in the extract of the decayed wood, with compounds IX and XIV dominating quantitatively. Higuchi (2) showed that compounds VIII, X, and XIII—and possibly XV—were components of an extract of white-rotted beech wood, whereas Henderson (23) similarly found acids IX and XIV in an extract of white-rotted birch wood. In both of these investigations, and in contrast to the present studies, the woods were pre-extracted before decay, so that these low molecular weight phenolic compounds could be attributed with some confidence to fungal action. In the present study these same six phenolic compounds were found in the extract of the sound wood, and in approximately the same amounts as in the white-rotted wood; thus we could not attribute the compounds to fungal attack.

These six compounds have been identified as products of hydrolysis of hardwoods (24, 25). In addition, their liberation from milled wood lignin of birch on acid hydrolysis has been demonstrated (26). It thus seems reasonable to consider the possibility that the formation of a portion of these compounds on the decomposition of hardwoods by white-rot fungi is due to a hydrolysis during decay rather than to a decomposition of phenylpropane units in lignin. Henderson (27) has previously recognized the possibility that the small amounts of these types of compounds released during decay by white-rot fungi may not arise via decomposition of the "main lignin structure", but rather may come from "side groups".

On the other hand, studies of the effects of white-rot fungi in liquid culture on compounds related to conifer lignin indicated that vanillin (VIII) and vanillic acid (IX) are formed also from the types of structural units that constitute the basic phenylpropane skeleton of conifer lignin (16; see also ref. 28). (Since conifer lignin contains only minor amounts of syringyl units (29), only traces of compounds arising from syringyl structures would be expected to be formed on the degradation of conifer wood.)

Studies with liquid culture techniques indicated further that low molecular weight phenolic compounds other than the six discussed above may be intermediates of the decomposition of lignin by white-rot fungi (16). Thus it has been reported that guaiacylglycerol (XII) and (4-hydroxy-3-methoxyphenyl)-pyruvic acid (XI) are intermediates in the degradation of the lignin model compound guaiacylglycerol-β-(2-methoxyphenyl) ether by white-rot fungi (16). This lends support to an earlier hypothesis (30) that compound XI is an intermediate in lignin decomposition, and led to the suggestion (16) that both compound XI and compound XII are key intermediates in the degradation of the quantitatively important guaiacylglycerol-β-aryl ether structures in conifer lignin. In the present studies compounds XI and XII were not detected in the extractives. Because the present investigations concerned a hardwood in which case the lignin is comprised to a considerable extent by syringyl units (see ref. 29), the extractives also were examined for the syringyl analog of compound XIII, syringylglycerol (XVI). This compound, however, was not detected.

It was mentioned in the Introduction that sweetgum wood decayed by *P. versicolor* contains, at any stage of decay, about the same percentage of lignin as the non-decayed wood, as revealed by Klason analyses (4, 5, 6). In the present case spectrophotometric determinations (31) also indicated that the white-rotted sample had the same lignin content (22 %) as the sound sample. Therefore, since the fungus had destroyed one-third of the original wood substance, it follows that about one-third of the lignin that was present in the wood before decay had been decomposed.

Even so, comparison of milled wood lignins from the white-rotted and sound wood samples revealed no significant differences, and thus showed that a portion of the lignin remaining in the white-rotted wood had not been significantly affected by the lignin-decomposing enzymes of the fungus. Because the yield of milled wood lignin was 27 % it was clear that at least 27 % of the lignin remaining in the white-rotted wood had thus far escaped significant alteration. In connection with this finding it is of value to consider results of microscopical investigations of the decay of hardwoods by *P. versicolor* in relation to wood microstructure.

Microscopial observations have shown that decomposition of the wood substance begins in the cell lumina, in which the fungal hyphae are located, and progresses toward the middle lamella region

(5, 6, 7, 32). Observations with the electron microscope indicate further that the wood substance is removed essentially in a layer-wise manner (32), causing a gradual thinning of the cell walls. However, recent studies (7) indicate that whereas the decomposition of the cellulose is limited to surfaces of the lumen and connecting openings, the lignin-destroying enzymes are able to penetrate and act within the cell walls. Lignin in the secondary wall was observed to be removed considerably in advance of the cellulose but the lignin in the middle lamella and in the cell corners appeared to be relatively resistant to attack (7). The apparent resistance of the middle lamella to degradation has been noted also by Sheffer (5) and by Schmid and Liese (32).

Thus in the present studies, if the milled wood lignin from the decayed wood came primarily from the middle lamella and adjacent regions, it would be expected to be relatively sound. Interestingly, Björkman (33) considered it reasonable to assume that milled wood lignin does come primarily from the middle lamella region; definitive evidence, however, for a specific microstructural origin of milled wood lignin is still lacking.

The actual extent of alteration of the *total* residual lignin in white-rotted wood has not been determined. It is possible that at the time the decay process was interrupted in the present case the amount of altered lignin in the wood was very small compared to the total amount of residual lignin—i.e. in this respect the decay of lignin and cellulose might be similar (cf. Cowling, ref. 6). This would imply that in the decay of sweetgum wood by *P. versicolor* a limited part of the lignin might be attacked and removed before the decay progresses to another part of the lignin. According to this picture, milled wood lignin, irrespective of its microstructural origin in the wood, might be expected to be comprised only to a small extent by altered lignin.

The investigations revealed that the material obtained by solvent extraction of the white-rotted and sound woods consisted in part of lignin; this lignin component amounted to less than 5 % of the total lignin in the wood samples. The major part of these "extractive lignins" was found in the organic layers of the high molecular weight fractions (fractions D1 and S1) of the extractives. They were purified by precipitation into ether, and were then compared with each other and with the milled wood lignins by UV and IR spectroscopic methods (*Figs. 1* and *3*). These comparisons corroborated the lignin nature of the samples, but showed further that the extractive lignin from the white-rotted wood differed from the extractive lignin from the sound wood and from the milled wood lignins in having a prominent IR absorbance centered at 1 730 cm^{-1}. The methoxyl content of this purified extractive lignin was 19.1 % (milled wood lignin 21.4 %), which, together with the results of the oxidative degradation of an ethylated sample (*Table 2*), and the above-mentioned spectral investigations, established the lignin nature of the material. (Slight decreases in the methoxyl content of lignin in wood on attack by white-rot fungi have been reported (see refs. 2 and 28).) No evidence was obtained during the investigations which indicated the presence of constituents other than lignin. Therefore, the strong absorbance at 1 730 cm^{-1} in the IR spectrum is considered to reflect chemical changes caused by the fungus rather than the presence of non-lignin contaminants. This conclusion is supported by the fact that purified lignins, obtained in low yield by solvent extraction, and which display strong absorbance in the carbonyl region of their IR spectra, have been obtained also from spruce woods decayed by white-rot fungi (28, 30, 34). In these cases the absorbance was centered at about 1 720 cm^{-1}, and in all likelihood reflected the same type of fungal alteration(s) as found in the hardwood lignin described in the present case.

Incubation of isolated lignins of pine and spruce with white-rot fungi in liquid culture has also been found to cause an increase in IR absorption in the carbonyl region (35). However, the increase in these cases was centered at about 1 680 cm^{-1}, and thus was not due to the type of structure(s) that gave rise to the absorbance discussed above.

In the present studies, the origin of the IR absorption at 1 730 cm^{-1} in the case of the extractive lignin from the decayed wood has not been clarified. It is considered probable that the absorbance reflected non-conjugated carbonyl groups or non-conjugated carboxyl groups. Previous studies have shown that phenol-oxidizing enzymes, which are produced by *P. versicolor* and by white-rot fungi in general (see refs. 3, 36) can affect phenolic compounds related to lignin in such a way that non-conjugated carboxyl and aldehyde groups are formed (37; see also ref. 38). Such residues in the lignin could conceivably be responsible for some of the absorbance at 1 730 cm^{-1}. However, it has been shown that the action of phenol-oxidizing enzymes on phenolic lignin model compounds also leads to formation of aryl-conjugated carbonyl groups (37, 39) and to *p*-benzoquinone structures (37, 40), neither of which is likely to have been present in more than small amounts in the purified extractive lignin from the decayed wood, based on spectral properties (see below). Further studies are in progress to describe the structure(s) responsible for the IR absorption at 1 730 cm^{-1} in lignin from white-rotted wood.

The fungal effects that were reflected in the IR absorbance at 1 730 cm^{-1} discussed above were not accompanied by effects that caused marked changes in the UV spectral properties of the sample (*Fig. 3*). This result makes it reasonable to suggest that units with chromophores which have spectral properties significantly different from those of the units in the original lignin were not present in more than small amounts. This would include units with aryl-conjugated carboxyl groups such as vanillic or syringic acid residues (cf. ref. 28), and aryl-conjugated carbonyl groups. (For UV spectral properties of these types of chromophores, see ref. 41).

p-Benzoquinone residues would be expected to give rise to an increased absorbance in the long-wave region of the spectrum (see e.g. ref. 42); such an increase was not noted, and thus the presence of

more than small amounts of such residues also is considered unlikely.

Comparison of the $\Delta\varepsilon$-curves for ionization (see ref. 11) for the various lignin samples indicated that the types of phenolic chromophores in the extractive lignin from the decayed wood apparently did not differ significantly from those in the extractive lignin from the sound wood or the milled wood lignins.

EXPERIMENTAL

Wood Samples

The sound and white-rotted woods of sweetgum (*Liquidambar styraciflua* L.) used in the present investigations were provided by Professor Ellis B. Cowling (North Carolina State University at Raleigh, USA). Sapwood of sweetgum had been cut into 2 cm cubical blocks which were treated in "soil-block chambers" (43) with *Polyporus versicolor* (isolate Madison 697) to a weight loss corresponding to about a third of the original weights (the average weight loss of the blocks was 32 %; range: 30.2—33.3 %). Adhering mycelium had been brushed from the blocks immediately upon removal from the decay chambers. Non-decayed blocks from the same lot served as the source of sound wood.

Analyses

Microanalyses were done under the direction of Dr. J. Zak, Mikroanalytisches Laboratorium am Inst. f. Physikalische Chemie der Univ. Wien, Vienna, Austria. Wood samples were analyzed for lignin content by a spectrophotometric method (31) with milled wood lignin prepared according to Björkman (8) as reference substance.

Infrared and Ultraviolet Spectra

IR spectra were recorded on a Beckman IR-9 instrument. They were taken in KBr discs, unless otherwise specified. UV spectra were recorded with a Beckman DK-2A instrument. If not stated otherwise, 80 % ethanol was used as solvent and alkaline solutions were 0.2 *M* with respect to NaOH.

Paper Chromatography

Paper chromatography was performed using the following solvent systems:

System A: The upper layer of ligroin-water-chloroform-methanol (7:5:2:1) was used as moving phase (44). R_f values: *p*-coumaraldehyde (VII), 0.02; sinapaldehyde (XV), 0.20; syringaldehyde (XIII), 0.30; coniferaldehyde (X), 0.33; vanillin (VIII), 0.45.

System B: The upper layer of toluene-acetic acid-water (4:1:5) was used as moving phase. R_f-values: syringic acid (XIV), 0.25; *p*-coumaraldehyde (VII), 0.30; vanillic acid (IX), 0.34; sinapaldehyde (XV), 0.52; syringaldehyde (XII), 0.60; coniferaldehyde (X), 0.68; vanillin (VIII), 0.72.

System C: As eluent butanol-acetic acid-water (4:1:1) was used (45). R_f values: gallic acid (XVII), 0.57; (4-hydroxy-3-methoxyphenyl)-pyruvic acid (XI), 0.74; dihydroquercetin (XIX), 0.78.

System D: Upper layer of butanol-water (1:1) was used as moving phase. R_f values: syringylglycerol (XVI), 0.48; guaiacylglycerol (XII), 0.55.

As detecting agents the following were used: Diazotized sulphanilic acid in 10 % aqueous Na_2CO_3 (IX, orange; XIV, red; XII, orange; XVI, red; XVII, greenish-brown; XI, orange-red; XIX, brownish-yellow), 2,4-dinitrophenylhydrazine in 2 *M* HCl (VIII, orange; XIII, brown-orange), and equal volumes of 0.1 *M* phloroglucinol in 60 % ethanol and 4 *M* HCl (VII, pink; X, violet; XV, purple).

Preparation of Trimethylsilyl Derivatives and their Analysis

Samples (a few mg) were dissolved or suspended in 100 μl of chloroform and 100 μl of N, O-bis-(trimethylsilyl)-acetamide (Pierce Chem. Co., Rockford, Ill.) was added. After about 2 h the reaction mixtures were analyzed by gas chromatography.

A Perkin-Elmer 880 instrument was used for gas chromatography. Column dimensions: 200×0.30 cm o.d. stainless steel tubing. Solid support: Chromosorb G, acid washed and treated with dimethyldichlorosilane, 80—100 mesh. Stationary phase: OV-1, (Applied Sciences Laboratories, State College, Penn.), 5 % by weight of solid support. Injection temperature: 300°. Detector temperature: 230°. Two different column temperatures were used, 205° and 220°. Carrier gas: N_2, 25 ml/min. Detector: Flame ionization.

Mass spectra of the components were taken with a LKB 9 000 gas chromatograph-mass spectrometer unit. For identification of components the retention times on gas chromatography and the mass spectra were compared with those of the corresponding synthetic compounds.

Preparation of (4-hydroxy-3-methoxyphenyl)-pyruvic acid (XI)

The triacetate of *erythro*-α,β,4-trihydroxy-3-methoxyhydrocinnamic acid methyl ester (46, 47) (220 mg) was refluxed in 50 ml 0.2 *M* HCl in dioxane-water (9:1) for 4 h (nitrogen atmosphere). After cooling and subsequent addition of 0.4 *M* $NaHCO_3$ to raise the pH to about 3, the reaction mixture was extracted thoroughly with chloroform. The extract was dried over Na_2SO_4 and the solvent removed by film evaporation. A residual oil, which partly crystallized on standing, was obtained. Recrystallization from acetone-methylene chloride gave 52 mg of nearly colorless crystals of m.p. 158—61°. For compound XI m.p. 161° has been reported (48). The properties of the product on paper chromatography using System C were in agreement with those reported for compound XI in this solvent system (45). The mass spectrum of its trimethylsilyl derivative had a molecular peak at m/e=426; this is in accord with structure XVIII, i.e. the trimethylsilyl derivative of the enol form of compound XI. The above-mentioned properties of the acidolysis product constitutes proof for its identity with (4-hydroxy-3-methoxyphenyl)-pyruvic acid (XI). Yield 43 %.

The NMR spectrum, recorded in deuteroacetone at 35° on a Varian A-60 instrument, indicated that compound XI existed as the enol form in the

measuring solvent. Singlets at $\delta=3.85$ (3 H) and $\delta=6.52$ (1 H) were ascribed to the protons in the methoxyl group and the vinyl proton, respectively.

Extraction of the Woods

Wood samples weighing 12.0 g (dry weight) and ground to pass a 1 mm sieve were extracted in a Soxhlet apparatus successively with benzene-ethanol azeotrope (20 h, about 80 emptyings) and 96 % ethanol (20 h, about 80 emptyings). The extracts were combined and solvent removed by film evaporation. The residues obtained were dried at 20 mm Hg over P_2O_5 and weighed. From sound wood 0.40 g (3.3 % of the wood) of a brown-red oil was obtained. The extract residue from white-rotted wood weighed 0.43 g (3.6 % of the wood). This material, which was brownish-yellow, was considerably lighter in color than the corresponding fraction from sound wood.

Investigation of the Extractives

Fractionation

The extractives were fractionated by gel filtration, using a column prepared with 180 g Sephadex G-25 (medium), and with dioxane-water (1: 1) as eluting solvent. The bed volume of the column (101×3.1 cm) was 760 ml. With this column excluded polymers, e.g. Blue Dextran or milled wood lignin from sweetgum, were eluted at 330 ml, whereas a small molecule such as acetone was eluted at 590 ml. The extractives from each wood were applied to the column in 14 ml of dioxane-water (1: 1). Eluting solvent was applied to the top of the column from a dropping funnel equipped as a Mariotte bottle and fitted to the column by a ground glass joint. The effluent was collected in 10 ml portions in tubes; the flow rate was 20 ml/h.

Minor amounts of the extracted materials (13 mg sound wood; and 25 mg decayed wood) did not dissolve in the solvent. These materials were removed prior to gel filtration. They were soluble in chloroform and showed in comparison to lignin and lignin-related materials only a weak absorption at 280 nm. They were presumably fatty materials, which commonly occur in wood extractives.

The 10 ml fractions obtained from the column on gel filtration of the extractives were examined by UV spectroscopy. An aliquot (80 μl) of each fraction was diluted with ethanol (10 ml) and the spectra in the range 240—360 nm recorded. The fractions which contained materials with absorption in this wavelength range showed a maximum at about 280 nm. *Fig. 2* shows the absorbance at 280 nm versus fraction number for the runs with extractives from sound wood and from decayed wood. On the basis of the behavior of lignin degradation products (14) and lignin model compounds (15) on gel filtration under the conditions used in the present work the eluate fractions obtained from the extractives were divided into three fractions, viz. a fraction containing lignin and lignin-related materials of MW $>$ 400 (tubes 31—41), a fraction containing such materials of MW $\simeq$ 400 (tubes 42—47), and a fraction with lignin-related compounds of low molecular weight (MW $<$ 400) (tubes 48—63). The fractions from decayed wood were designated D1 (MW $>$ 400), D2 (MW $\simeq$ 400), and D3 (MW $<$ 400). The corresponding fractions from sound wood were designated S1, S2, and S3. These fractions were then subdivided by extraction with chloroform (first with 0.5 vol. and then three times with 0.25 vol. of chloroform). The aqueous layers were freeze-dried and the obtained residues weighed (*Table 3*). After drying over Na_2SO_4, solvent was removed from the organic layers by film evaporation. Obtained residues were dried over P_2O_5 at 20 mm Hg and weighed (*Table 3*).

Considerable amounts of the extractives from sound wood as well as decayed wood were eluted after tube 63. On the basis of preliminary chromatographic investigations it was suspected that these retarded materials consisted at least in part of sugars. Separate experiments with some mono- and disaccharides indicated that such compounds were eluted at about tube 70. By examination of the retarded materials from both extractives by ion exchange chromatography (49), a number of sugars were detected. (The examination for sugar was kindly conducted by civ.ing. Eva Martinsson.)

It was also found that the sugar alcohol arabinitol was a prominent constituent of the retarded material from decayed wood. This compound was detected in the form of its trimethylsilyl derivative by gas chromatography. It was identified as a pentitol from its mass spectrum (50) and was distinguishable from the two other isomers, i.e. ribitol and xylitol, by gas chromatograhy. Arabinitol was not present in the extractives from the sound wood.

Ethylation and oxidative degradation of fraction D1 and fraction S1

[Milled wood lignins were also examined by this procedure.]

Samples (15—20 mg) of fraction D1, aqueous and organic layers, and of fraction S1, aqueous and organic layers, were dissolved in 10—15 ml methyl cellosolve-water (2: 1). To the stirred solution diethyl sulfate and 10 *M* NaOH were added alternately and dropwise; the pH was maintained above 8 (nitrogen atmosphere). Addition was continued for about 1 h total time; the temperature was kept at 85—90°. (From determinations of the $\Delta\varepsilon$-curves for ionization (11) it was concluded that essentially no phenolic hydroxyl groups remained in the sample.) The pH of the reaction mixture was then adjusted to 6—7 with 1 *M* H_2SO_4 and solvent removed by film evaporation. The residue obtained was subjected to oxidative degradation with permanganate according to Larsson and Miksche (51) as modified by Kirk and Adler (13). The product mixture was treated with diazomethane, and the mixture of ethoxybenzoic acid methyl esters analyzed by gas chromatography (13). The results are summarized in *Table 2*.

Hydrolysis of the aqueous layer of fraction S1 and subsequent determination of gallic acid (XVIII)

A sealed 2 ml ampoule containing 5.7 mg of the material in the aqueous layer of fraction S1 in 1

ml dioxane-water (1: 1), 5 % in H_2SO_4, was heated at 100° for 24 h. The acid hydrolysis was followed by ethylation with diethyl sulfate according to the procedure described above in connection with the ethylation-oxidation studies. After acidification with 1 *M* H_2SO_4 the reaction mixture was extracted with chloroform. The resulting product was then examined in the same way as the mixture of ethoxybenzoic acids obtained on ethylation-oxidation, i.e. methylated with diazomethane and then subjected to gas chromatography (see above). Gallic acid triethyl ether (VI) was the only detected product present in significant amounts (1.3 mg, corresponding to 14 % gallic acid).

Purification of the "extractive lignin" in the organic layers of fraction S1 and fraction D1

The samples were dissolved in 0.2 ml dioxane-water (9: 1) and the solution was slowly dripped into vigorously stirred ether (14 ml). The precipitated lignin was recovered by centrifugation, the whole procedure repeated, and the product dried over P_2O_5 at 20 mm Hg.

Detection of dihydroquercetin (XIX) in fractions S2 and S3

Fraction S2 was examined by thin layer chromatography on silica gel G, using benzene-dioxane-acetic acid (90: 24: 4) (52) as eluent. Development of the chromatogram by exposure to iodine vapor indicated the presence of a prominent component with R_f 0.19. This component was obtained in a relatively pure fraction by preparative thin layer chromatography. The IR spectrum (NaCl plates) showed a characteristic band at 1 640 cm^{-1}. The UV spectrum showed a maximum at 289 nm. In sodium ethoxide solution (~ 0.01 *M* in ethanol) the UV maximum was shifted to 325 nm. On the basis mainly of the spectral properties it was suspected that the compound was a flavanone (for UV spectral properties of flavanones, see ref. 53). Comparison with an authentic sample of dihydroquercetin (XIX) (thin layer chromatography, paper chromatography in System C, and spectral properties) showed that the compound purified from the fraction was compound XIX. By paper chromatography in System C it was demonstrated that compound XIX also was present in fraction S3.

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