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Studies on Lignin *

III. Oxidation of Wood from *Picea abies* (L.) Karst. (Norway Spruce) with Nitrobenzene and Alkali

BENGT LEOPOLD

Organisk-kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

In their important paper on the oxidation of spruce lignin with nitrobenzene and alkali, Freudenberg, Lautsch, and Engler[1] reported the occurrence among the oxidation products of a number of phenolic compounds. Vanillin, vanillic acid, 5-carboxyvanillin and guaiacol were identified, but the remaining substances were present in amounts too small to permit isolation and identification in the usual way.

Since then, however, the method of paper partition chromatography introduced by Consden, Gordon, and Martin [2] has made it possible to detect very small amounts of compounds, and the present paper describes a reinvestigation of the nitrobenzene oxidation products from spruce, utilising chromatographic methods. Similar methods have been used by Bland [3,4] who studied such oxidation products from *Eucalyptus* species and isolated small amounts of *p*-hydroxybenzaldehyde, in addition to vanillin and syringaldehyde.**

As a result of the present work a comparatively large number of phenolic oxidation products have been detected and, in most cases, isolated and identified. Recently Pearl [5] has isolated some of the same compounds from the mixture obtained by oxidation with cupric hydroxide of sulphite spent liquor from spruce.

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* Replacing the general title Studies on the Sulphonation of Lignin — Part II. *Acta Chem. Scand.* 4 (1950) 971.

** Note added in proof: Bland, Ho, and Cohen [19] have recently studied the aldehyde mixtures obtained from some Conifer species, and demonstrated (paper chromatographically) that they contain *p*-hydroxybenzaldehyde and, for some species probably also syringaldehyde (*cf.* following paper).

In Table 1 the compounds found are listed, the yields being calculated on the basis of the lignin content of the wood employed. The yields within brackets were estimated visually from the intensities of the corresponding spots on the paper chromatograms and are consequently only approximate.

Table 1. Nitrobenzene oxidation products from spruce wood.

Compound	Yield %
Vanillin *	27.5
p-Hydroxybenzaldehyde	0.25
Syringaldehyde	0.06
5-Formylvanillin	0.23
Dehydrodivanillin **	0.80
Vanillic acid *	4.8
(*p*-Hydroxybenzoic acid)***	—
Syringic acid	(0.02)
5-Formylvanillic acid	(0.1)
5-Carboxyvanillin *	1.2
Dehydrodivanillic acid **	0.03
Guaiacol *	—
Acetoguaiacone **	0.05

* Previously reported by Freudenberg [1].
** Previously reported by Pearl [5].
*** Observed only in two cases.

METHODS

The oxidation was carried out essentially as described by Freudenberg *et al.*, but reaction conditions more close to optimum [6] were employed (180°, 2 hrs).

After the removal of nitrogenous compounds and material insoluble in alkali, the oxidation mixture was extracted with trichloroethylene and the extract divided into aldehydic, phenolic and acidic fractions as described on p. 44. In addition, an ether extraction was made, yielding 9 % of oxalic and 2 % of fumaric acid, calculated on the lignin content of the wood. The latter acid has not previously been isolated from the oxidation products of spruce wood.

The three fractions soluble in trichloroethylene were examined by means of paper chromatography, employing the procedures outlined below, each fraction being treated separately.

Aldehyde fraction

The use of paper partition chromatography for the separation of phenolic aldehydes has been described previously by Bland [3] who separated a mixture of vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde, using ligroin as the mobile phase. He also separated *p*-hydroxybenzaldehyde from vanillin and syringaldehyde by means of a "chromatopile"[4]. Recently Pearl and Dickey [7] described the quantitative separation of vanillin and syringaldehyde by adsorption chromatography on a column of "Magnesol".

In the case of spruce, Bland's simple procedure had to be modified, owing to the difficulty of detecting small amounts of syringaldehyde, which gives a much more diffuse spot than does *p*-hydroxybenzaldehyde. It was found necessary to use larger amounts of material than is usually employed for a paper chromatogram and this, in turn, resulted in strong trailing effects. Several methods of overcoming this trailing were tried, the most effective being to use papers previously impregnated with a buffer solution of suitable pH. (For details, see experimental part.)

The results of a typical separation experiment using this method are given in Table 2.

The subsequent quantitative separation of the mixture was complicated by the fact that *p*-hydroxybenzaldehyde, syringaldehyde, and 5-formylvanillin constitute only a very small part of the aldehyde mixture (about 2 %), the bulk of which consists of vanillin.

Table 2. Paper chromatographic analysis of aldehyde fraction. Paper: Munktell OB, impregnated with a phosphate buffer of pH 6.8. Solvent: Ligroin (b.p. 85—110°)-benzene (2 : 1). Developer: 2,4-dinitrophenylhydrazine (DNF).

Compound	R_F	Appearance of spot		Remarks
		in UV	with DNF	
Unidentified	0.02	Greenish fluoresc.	Yellow	
p-Hydroxybenzaldehyde	0.06	Dark blue	Red	Spots circular, sharp contours
Unidentified	0.16	Invisible	Yellow	
Syringaldehyde	0.31	Invisible	Brownish	Oblong, diffuse spots, fade rapidly
5-Formylvanillin	0.43	Greenish fluoresc.	Yellow	R_F very sensitive to pH
Vanillin	0.53	Dark blue	Brick red	

To increase the proportion of the desired aldehydes, the crude mixture was recrystallized from aqueous ethanol. The material from the mother liquor from this recrystallization contained less than 20 % of the vanillin and almost all of the other aldehydes, and this mixture, dissolved in benzene, was adsorbed on top of a column of cellulose. A large volume of ligroin-benzene (2:1) eluted most of the vanillin and part of the 5-formylvanillin, leaving all the *p*-hydroxybenzaldehyde and syringaldehyde and a large part of the 5-formylvanillin on the column. The latter aldehydes could then be eluted with ethanol. The final separation of this aldehyde mixture was achieved using a column of "Magnesol" similar to that described by Pearl and Dickey [7], and the "liquid chromatogram" technique with light petroleum-ethanol mixtures. By increasing the percentage of ethanol from 0.8 to 2 when no more vanillin and 5-formylvanillin could be detected in the eluate, a quantitative separation was obtained, the only overlapping fractions being those of vanillin and 5-formylvanillin (*cf.* Fig. 1, p. 45).

From the water-insoluble portion of the aldehyde fraction a further amount of 5-formylvanillin could be isolated. Some dehydrodivanillin was also obtained and this compound is probably the "acid" described by Freudenberg *et al* [1]., of m.p. 301°. The elementary composition reported by them agrees well with that of dehydrodivanillin.

Acid fraction

Methods for separation of phenol carboxylic acid by paper chromatography, using butanol with different admixtures [8,9] or benzene-acetic acid-water [10] as solvents have been described by several authors.

For the present purpose a technique using buffered paper proved to be very effective. With butanol-water as solvent very good results were obtained when the pH of the impregnating buffer solution was a little over 7.

In Table 3, the results of a typical separation experiment using this method are given.

The fraction was then divided into bisulphite-soluble and bisulphite-insoluble portions and each was examined separately. These experiments, combined with the use of 2,4-dinitrophenylhydrazine as a developing agent, confirmed the results given in Table 3 and indicated that the two last mentioned unidentified compounds were aldehydic in nature.

Of the acids detected above, only vanillic acid and 5-carboxyvanillin were isolated.

Table 3. Paper chromatographic analysis of acid fraction. Paper: Munktell OB, impregnated with a phosphate buffer of pH 7.5. Solvent: Butanol. Developer: Bis-diazotized benzidine [11] *(DB).*

Compound	R_F	Appearance of spot		Remarks
		in UV	with DB	
Unidentified	0.06	Blue fluoresc.	Invisible	Possibly dehydrodivanillic acid
5-Formylvanillic acid	0.31	Greenish fluoresc.	Reddish brown	
Syringic acid	0.35	Invisible	Claret red	Colour soon fading to brown
Vanillic acid	0.48	Dark blue	Light brown	
p-Hydroxybenzoic acid	0.53	Invisible	Pale yellow	Colour develops very slowly. Observed only in 2 cases
Unidentified *	0.59	Greenish fluoresc.	Almost invisible	
5-Carboxyvanillin	0.67	Blue fluoresc.	Brown	
Unidentified *	0.81	Greenish fluoresc.	Invisible	

* Present in very small amount.

Phenolic fraction

Several paper chromatographic procedures for the separation of mixtures of this kind have been devised [8,12,13]. However, only a few experiments were carried out with this fraction and the method described above was employed. Guaiacol ($R_F = 0.70$), acetoguaiacone ($R_F = 0.47$) and *p*-hydroxyazobenzene ($R_F = 0.75$) were detected, using ligroin-benzene (2:1) as solvent, and paper buffered to pH 9.

DISCUSSION

Undoubtedly the most interesting compounds isolated are syringaldehyde and *p*-hydroxybenzaldehyde, as this is the first time these substances have

been obtained from spruce. The only analogous compound previously isolated is trimethyl gallic acid obtained in very small yield by permanganate oxidation of methylated spruce wood [14].

The occurrence of *p*-hydroxyphenyl and syringyl elements in spruce lignin is of great interest in relation to the "methoxyl deficiency" of this lignin. Thus, the methoxyl content of spruce lignin is somewhat lower than the theoretical value for a lignin containing only guaiacyl nuclei with three-carbon side-chains, the number of methoxyl groups per ten carbon atoms being about 0.95 instead of the expected 1.0.

This "deficiency" could obviously be explained by the occurrence in spruce lignin of methoxyl-free elements, such as *p*-hydroxyphenyl nuclei. However, *p*-hydroxybenzaldehyde accounts for only about 1 % of the aldehyde mixture which, furthermore, contains about 0.2 % of syringaldehyde which influences the methoxyl value in the opposite direction. The proportion of *p*-hydroxyphenyl elements in the lignin itself would therefore have to be much higher than is indicated by the percentage of *p*-hydroxybenzaldehyde in the aldehyde mixture, *i.e.* the yield of *p*-hydroxybenzaldehyde from the *p*-hydroxyphenyl elements would have to be much lower than the yield of vanillin from the guaiacyl elements. This is, in fact, not at all unlikely, as the *p*-hydroxyphenyl element would be expected to contain a larger proportion of "condensed" elements, *i.e.* containing an extra carbon-carbon linkage in the position *ortho* to the hydroxyl group, than the guaiacyl residues. Completely analogous is the well-known fact that the yield of syringaldehyde from the syringyl elements in hardwood lignin is much higher than the yield of vanillin from the guaiacyl elements.

Whether or not the isolation of dehydrodivanillin indicates the presence of diphenyl elements in spruce lignin is an open question. Freudenberg, Meister and Flickinger [15] found dehydrodiveratric acid among the permanganate oxidation products from methylated spruce and considered it possible that the diphenyl link was formed during the reaction.

Such a dehydrogenation has, however, never been observed to take place during the oxidation of various model compounds, either with nitrobenzene and alkali or with potassium permanganate. Further, since lignin is probably formed in Nature by a dehydrogenative coupling of aromatic precursors it would not be unexpected if diphenyl linkages do occur to a small extent in genuine spruce lignin.

The isolation of 5-formylvanillin does not call for special comment, especially in view of the results previously reported [16] on the oxidation of model compounds.

EXPERIMENTAL

An amount of air-dry powdered spruce wood corresponding to 150 g dry weight (4.92 % OCH_3, 27.5 % Klason lignin) which had previously been extracted with benzene-alcohol (1 : 1) for 24 hours, was mixed with 2 *N* sodium hydroxide (1650 ml) and nitrobenzene (165 ml). The mixture was heated to 180° for two hours in autoclaves rotating in an oil bath, then steam-distilled to remove nitrogenous compounds and, after cooling, centrifugated and the clear centrifugate neutralized with concentrated sulphuric acid. More conc. sulphuric acid (20 ml) was added and the pH of the solution was adjusted to 7.3 by the addition of trisodium phosphate. The pH then remained fairly constant throughout the whole extraction and never exceeded 8.0.

The mixture was continuously extracted with trichloroethylene for about 24 hours, (extract A), then acidified to pH of ca. 2, extracted again with trichloroethylene (extract B) and finally extracted with ether (extract C).

Extract A was shaken with 5 % sodium bisulphite (extract Ab) and then with 2 % sodium hydroxide (extract Ah). The bisulphite solution Ab was acidified with sulphuric acid and the sulphur dioxide was expelled by heating the mixture on the water bath and passing in carbon dioxide. The precipitate (Ab_1) (2.2 g) obtained was collected from the cold solution, and the filtrate was exhaustively extracted with ether. The ether extract was dried (Na_2SO_4) and evaporated, giving a yellowish-red crystalline residue (11.5 g) (Ab_2).

This was recrystallized from dilute ethanol (15 %), yielding yellowish crystals (m.p. 77—79°, 9.55 g) of vanillin contaminated with a small amount of 5-formylvanillin and possibly a trace of syringaldehyde. The mother liquor was extracted with ether and gave a mixture of aldehydes (Ab_2'), (1.83 g), which were further separated on a column of cellulose pretreated with boiling dilute nitric acid as described by Burstall, Davies, and Wells [17].

The aldehyde mixture Ab_2' (1.5 g), dissolved in benzene (25 ml), was applied to the top of the column (4.5 × 50 cm) and elution was commenced with water-saturated ligroin (85—110°)-benzene (2 : 1) at a flow rate of ca. 1 l per hour. When only traces of aldehydic material could be detected in the eluate, the solvent was replaced by ethanol. The first 100 ml of ethanol were collected and evaporated, yielding an oil weighing 282 mg.

The final separation was carried out by means of chromatography on a column (16 × 165 mm) of acid-washed "Magnesol"-"Celite" (5 : 1) (*cf.* Pearl and Dickey [7]).

Light petroleum (b.p. 40—60°)-ethanol (125 : 1) was forced through the column by means of compressed air and 20 ml fractions were collected until no more vanillin or 5-formylvanillin could be detected in the eluate. The ethanol content of the solvent was then increased to 2 %, and elution continued until the eluate was free from aldehydes (Fig. 1).

Evaporation of the solvent from the *p*-hydroxybenzaldehyde fraction, gave a product melting at 107—110° which after recrystallization from light petroleum—benzene formed almost colourless crystals, m.p. 114—115°, undepressed by authentic *p-hydroxybenzaldehyde.*

The crude syringaldehyde was contaminated with ca. 25 % of a coloured impurity, which could, however, be removed by filtration through a small column of acid-washed alumina. Evaporation of the solvent yielded a crystalline residue, which after recrystallization from light petroleum—benzene formed almost colourless needles, m.p. 109—110°, undepressed on admixture with authentic *syringaldehyde.*

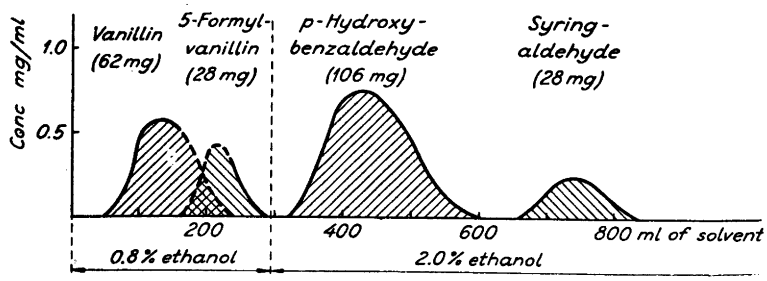

Fig. 1. Chromatographic separation of aldehyde mixture obtained from spruce.

The water-insoluble fraction Ab_1 was repeatedly extracted with boiling water (filtrate Ab_1'), then dissolved in hot nitrobenzene and filtered. The pale brown needles which separated on cooling (m.p. 300°, 0.32 g) were recrystallized from nitrobenzene yielding colourless needles, m.p. 300—301°, undepressed on admixture with authentic *dehydrodivanillin.*

$C_{16}H_{14}O_6$	Calc.	C	63.6	H	4.67	OCH_3	20.6
	Found	»	63.6	»	4.72	»	21.3

On cooling, the aqueous filtrate Ab_1' (200 ml) yielded an amorphous precipitate (1.2 g), from which no crystalline compound could be obtained. The remaining solution was extracted with ether, and the ether extract dried (Na_2SO_4) and evaporated. The oily residue (0.36 g) was extracted with boiling light petroleum and then dissolved in boiling dilute ethanol (15 %) and the solution treated with charcoal and filtered hot. On cooling, a crystalline compound separated. This material, together with the undissolved residue obtained by extraction of the vanillin fraction from the adsorption column with boiling light petroleum, was then recrystallized from dilute ethanol, yielding yellowish crystals (75 mg), m.p. 118—120°, undepressed on admixture with authentic *5-formylvanillin.* From the light petroleum solutions vanillin (0.22 g) could be recovered.

The alkaline fraction Ah was acidified and extracted with ether, and the extract dried (Na_2SO_4) and evaporated. The residue (2.6 g) — a red crystalline mass with a strong smell of guaiacol — was repeatedly extracted with boiling water and the resulting aqueous solution (Ah_2) was filtered hot. The residue was dissolved in very dilute, boiling alcohol, and the solution filtered hot. On cooling, brownish red, crystalline material (1.1 g) precipitated and this, after repeated recrystallization from dilute alcohol, gave brick red crystals of p-hydroxyazobenzene [1] (m.p. 150—151°).

From the aqueous solution Ah_2, there separated a yellow oil which slowly partially crystallised. The crystals were collected, washed with a little dilute methanol and sublimed at 1 mm giving colourless crystals (22 mg), m.p. 114—115°, undepressed on admixture with authentic *acetoguaiacone.*

The oily material probably consisted largely of *guaiacol.*

Of the trichloroethylene solution B, an aliquot was used for paper chromatographic purpose, and the remainder was extracted with 5 % sodium bicarbonate solution. Acidification of the solution gave a brown precipitate (0.8 g) which was filtered off (filtrate B_2)

and repeatedly extracted with hot dioxan which on cooling and dilution with water, deposited 0.4 g of material which was stirred with cold dioxan. Part of the material dissolved, the residue being a crystalline product which after recrystallization from dioxan formed colourless plates, m.p. 252—254°, undepressed by authentic *5-carboxy-vanillin*, although examination by paper chromatography revealed the presence of a small amount of *5-formylvanillic acid*.

The almost colourless material undissolved by the hot dioxan was dissolved in boiling nitrobenzene. On cooling, *dehydrodivanillic acid* (14 mg) separated as fine needles, m.p. 293—295°, undepressed by an authentic sample.

The aqueous solution B_2 was extracted with ether and the extract dried (Na_2SO_4) and evaporated. A sample of the crystalline material obtained was sublimed at 1 mm, yielding *vanillic acid* in amount corresponding to a yield of 1.95 g for the total batch (2.75 g).

For the paper chromatographic experiments a few drops of the aliquot referred to above were used directly, while the remainder was extracted first with 5 % sodium bisulphite and then with 5 % sodium bicarbonate. These two fractions were examined separately as mentioned above.

Evaporation of the ether from extract C (*cf.* p. 44), gave a syrupy residue which soon deposited crystals. On treatment with cold water, the syrup dissolved (C_2), leaving an almost colourless material which was collected, washed with a little water and recrystallized from water (charcoal), giving 0.8 g of crystalline material. The melting point determination was complicated by the volatility of the compound, sublimation commencing at about 230°. The substance was therefore placed in a melting point apparatus (aluminium block type), preheated to about 270° and in this way the melting point was found to be 285—288°. The melting point and sublimation were indicative of *fumaric acid* but a mixed melting point would not be very reliable under these conditions. Consequently the fumaric acid was identified by conversion to N-phenylasparaginanil [18], m.p. 210—211°, undepressed by an authentic specimen.

The aqueous solution C_2 was buffered with sodium acetate and treated with calcium acetate. The cream-coloured precipitate (6.6 g) which formed was collected, washed thoroughly with water and dissolved in dilute hydrochloric acid. The resulting solution was continuously extracted with ether and the ether evaporated to give a crystalline residue (5.8 g) which after repeated recrystallization from xylene, yielded anhydrous *oxalic acid* (3.6 g), m.p. and mixed m.p. 189—190°.

Paper chromatography

For all the experiments the descending method was used. The paper used was Munktell OB (Whatman no. 1 paper was tried but gave rise to serious trailing effects). The paper was cut in strips 10 × 48 cm and the strips were soaked in a 0.1 *N* phosphate buffer solution of the desired pH and then dried at 105° for about 15 minutes before use.

The examination of the aldehyde fraction offered some special problems, owing to the low concentration of, in particular, syringaldehyde and the relative difficulty of detecting the corresponding spots after development. Thus, ten drops of a 5 % alcoholic solution of the aldehyde mixture had to be applied to the paper successively. Several solvent mixtures were tried, using benzene and ligroin as well as butanol. A mixture of 1 part (by volume) of benzene and 2 parts of ligroin (b.p. 85—110°) proved to be very effective. The pH of the impregnating buffer solution was also varied, the most distinct syring-

aldehyde spot being obtained with a buffer of pH ca. 7. The R_F values of the aldehydes other than 5-formylvanillin were virtually independent of pH in the range 6—8 but with 5-formylvanillin, the R_F varied from 0.60 on unimpregnated paper to 0.19 at pH 7.8. A pH-value of 6.8 was chosen, as at this pH the formylvanillin spot appeared in a position where it did not interfere with other spots.

Of the developers tried, only 2,4-dinitrophenylhydrazine proved sufficiently sensitive. The sensitivity seems to increase if the solution used contains more hydrochloric acid than usually. The most satisfactory reagent was prepared by dissolving dinitrophenylhydrazine (1 g) in concentrated hydrochloric acid (300 ml) and diluting to one litre.

Owing to the slow formation of a yellow background, the weaker spots gradually became indistinguishable. Attempts to prevent this formation of a background by rinsing the paper in water after spraying only resulted in blurred spots. The spots were therefore always inspected within a few minutes of spraying.

For the chromatography of the acid fraction, smaller amounts of material gave better results and usually one or two drops of a 1 % alcoholic solution were applied to the paper. The influence of the pH of the buffer solution was studied also with this fraction, and it was found that the best separation was obtained at a pH a little over 7. The only solvent tried was water-saturated butanol. A satisfactory method of detection was inspection in UV light and subsequent spraying with bis-diazotized benzidine solution [11]. Attempts to separate the acids as their ammonium salts, using propanol-ammonia-water as solvent and ninhydrin as developer were not successful.

SUMMARY

The oxidation of spruce wood with nitrobenzene and alkali has been thoroughly studied by means of chromatographic methods.

In addition to vanillin, dehydrodivanillin, vanillic acid, 5-carboxyvanillin, dehydrodivanillic acid, acetoguaiacone, oxalic acid and *p*-hydroxyazobenzene, which have been isolated previously by other authors, the following compounds were detected on paper chromatograms or isolated:

Syringaldehyde, *p*-hydroxybenzaldehyde, 5-formylvanillin, syringic acid, *p*-hydroxybenzoic acid, 5-formylvanillic acid, and fumaric acid.

The significance of the presence of *p*-hydroxybenzaldehyde, syringaldehyde and dehydrodivanillin in connection with the structure of spruce lignin is discussed.

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