Correction to

On the composition of dioxane-water extracts of milled spruce wood: Characterization of hydrophilic constituents

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Keywords: Lignins, Carbohydrates, Lignin carbohydrate complexes, Fractionation, Galactose

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Page 112: Discussion section should continue the line "in this region" (line 10 on the left column) with the section which begins with "Lignin carbohydrate complexes with" (the same column, line 2 from the bottom). Discussion section ends with the lines "on the occurrence of and extent of such reactions" (lines 22 and 23 on the left column of page 113) before the head "Carbohydrate analyses."

Corrected Reprint below:

On the composition of dioxane-water extracts of milled spruce wood: Characterization of hydrophilic constituents

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SUMMARY: Dioxane-water extracts of spruce wood milled according to Björkman were examined. Fractionation by liquid-liquid extraction using a system containing water, acetic acid, pyridine and chloroform gave fractions from the organic layer freed from carbohydrates and consisting essentially of lignin with very low carbohydrate content (0.2-0.3%), and fractions from the aqueous layer consisting primarily of carbohydrates and lignin carbohydrate compounds with quite a high carbohydrate content. It could be concluded from experiments with modified extraction procedures that carbohydrate content and, to some extent, molecular mass are of importance for the separation of the constituents of extractives subjected to liquid-liquid extraction. The selectivity of the extraction procedure was examined using model compounds studies. The results suggest that the organic layer is completely freed from constituents consisting solely of carbohydrates. Application of gel chromatographic techniques made it possible to isolate fractions of lignin carbohydrate compounds of low to moderate molecular mass from the aqueous layers. Further examinations (UV, carbohydrate analyses, thin layer chromatography, electrophoresis) corroborated that lignin or lignin-related compounds and carbohydrates were chemically attached to each other in these fractions. In one of the fractions (carbohydrate content, 20.1%; molecular mass about 2000) galactose was a prominent constituent of the carbohydrate moiety.

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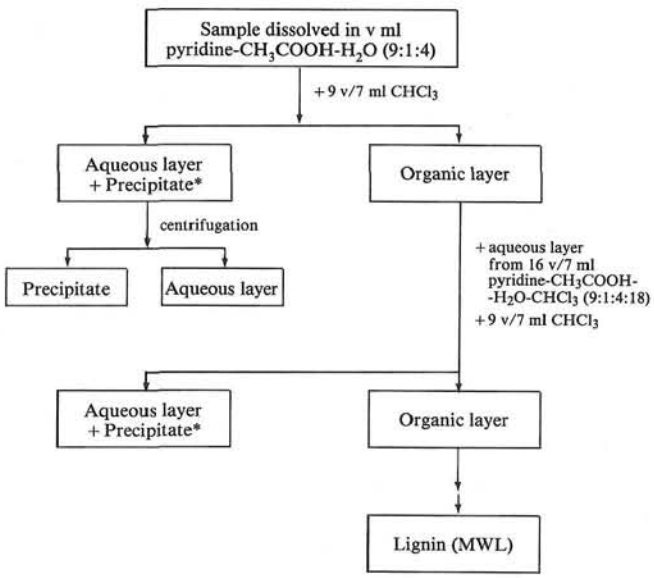
The procedure described by Björkman (1) for the isolation of milled wood lignin includes, as initial steps, disintegration and milling of the wood followed by extraction with dioxane containing 4% water. The materials present in such extracts are referred to in this paper as crude milled wood lignin (crude MWL). Studies on crude MWL from spruce and birch have been reported on in a series of papers (2-5). This work is a continuation of these studies and primarily deals with the hydrophilic constituents extracted from the spruce wood. The separation of the hydrophilic constituents is based on a previously developed liquid-liquid extraction procedure. Some recent results regarding the selectivity of this extraction procedure are therefore included in this paper.

Selectivity of the liquid-liquid extraction procedure shown in scheme 1

The previously developed liquid-liquid extraction procedure [*scheme 1,* see also (2)] aims at a separation of lignin and carbohydrate constituents in crude MWL. We have examined the distribution of some carbohydrates and models for low molecular-mass lignin-carbohydrate compounds in the organic and aqueous layers in order to obtain information about the selectivity of the extraction procedure. The results are summarized in *table 1.* No carbohydrates are found in the final organic layers. Compounds consisting of glucose attached to mononuclear aromatic compounds are primarily found in the aqueous layers and only a small percentage of the compounds are found in the organic layers. This is, however, reversed when a sugar unit (L-rhamnose) is linked to a more complex aromatic compound as is the case in quercitrin (*3*). It should be noted that not only carbohydrate content but also molecular mass seems to be of importance when a polydisperse material like crude MWL is subjected to fractionation using the liquid-liquid extraction procedure [see below and (3, 5)].

Table 1. Distribution of model compounds (in %) on liquid-liquid extraction according to scheme 1.

Sample	First extraction step Aqueous layer	Second extraction step Aqueous layer	Second extraction step Organic layer
Dextran hydrolysis products (see Exp.)	>99	0.3	0
Methyl β-D-glucopyranoside	>99	0.1	0
Arbutin (*1*)	73	21	6
Salicin (*2*)	67	26	7
Quercitrin (*3*)	4	3	93



*In the case of model experiments no precipitate was formed.

Scheme 1. Fractionation procedure applied to crude MWL [cf (2) and (5)] and model compounds (see Experimental).

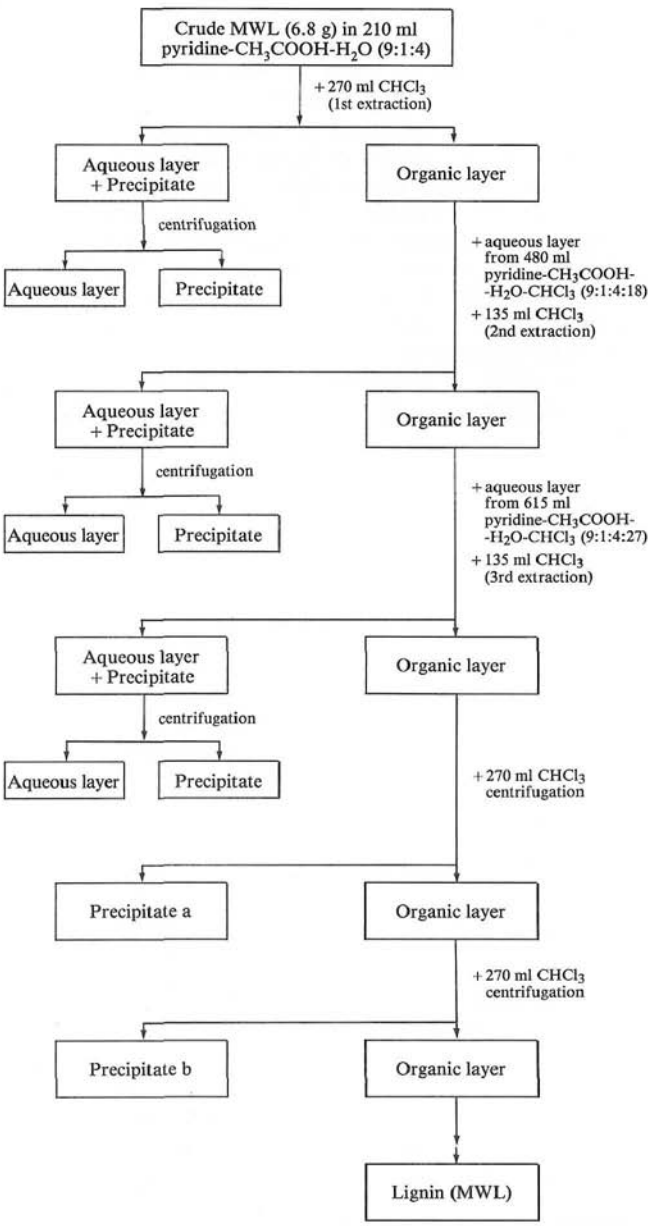
Fractionation of crude MWL from spruce by liquid-liquid extraction

Crude MWL from spruce was fractionated according to *scheme 2*. Yields of the fractions obtained and results from carbohydrate analyses are summarized in *table 2*. It is evident from table 2 that the fractionation results in a successive release of carbohydrate-containing materials from the organic layer.

Some of the fractions obtained were examined by gel permeation chromatography (GPC) using the system Sephadex LH-60/DMF—acetic acid (200:1) (*fig. 1*). It appears in fig. 1 that the precipitates (see scheme 2) contain materials with comparatively high molecular masses. The precipitate obtained in the first extraction step was not included in the GPC studies since it was not completely soluble in DMF.

The number-average molecular-mass of the fractions examined by GPC was determined by vapor pressure osmometry using DMF as solvent. Molecular masses were found to be in the range 3300 (MWL) to 5800 (precipitate from the third extraction step).

For comparison with the GPC results shown in fig. 1, a series of lignin preparations prepared by the use of the procedure shown in scheme 1 were examined by GPC using the system Sephadex LH-60/DMF—acetic acid (200:1) (*fig. 2*). Fig. 2 includes a run with a birch lignin sample.



Scheme 2. Extended fractionation procedure applied to crude MWL (see Experimental).

Table 2. Yields, carbohydrate contents and relative carbohydrate compositions of fractions from liquid-liquid extraction of crude MWL from spruce according to scheme 2.

Fraction	Yield, % of crude MWL	Carbohydrate[a] content, %	Relative carbohydrate composition, %				
			Ara	Xyl	Man	Gal	Glu
First extraction:							
aqueous layer	6.4	15.8[b]	18	21	22	16	23
precipitate	1.0	3.0	31	32	16	14	7
Second extraction:							
aqueous layer	2.0	3.1	18	21	25	14	22
precipitate	1.9	3.9	18	27	24	21	10
Third extraction:							
aqueous layer	1.0	1.6	19	25	25	12	19
precipitate	2.6	1.4	22	26	21	16	15
Precipitate a	6.2	0.4	19	19	23	19	20
Precipitate b	5.5	<0.1					
MWL	48.0	0.1	31	15	20	14	20

[a] Determined using hydrolysis in 0.02 M H_2SO_4 at 130°C for 3 h (sealed glass ampoules).
[b] Analyses of non-hydrolyzed sample showed that one third of the carbohydrates consisted of free monosaccharides with a relative composition of: arabinose 29%, xylose 15%, mannose 15%, galactose 8% and glucose 33%.

Investigation of the aqueous layer from the first extraction step

Materials obtained from the aqueous layer in schemes 1 and 2 (1st extraction step; regarding the composition of the carbohydrate moiety, see table 2) were subjected to GPC using the system Sephadex G-25 Fine/dioxane-water (1:1). The eluate was examined by UV and refraction index measurements and divided into fractions *A*, *B* and *C* (*fig. 3*). Results from carbohydrate analyses of these fractions are summarized in *table 3*. According to model compound

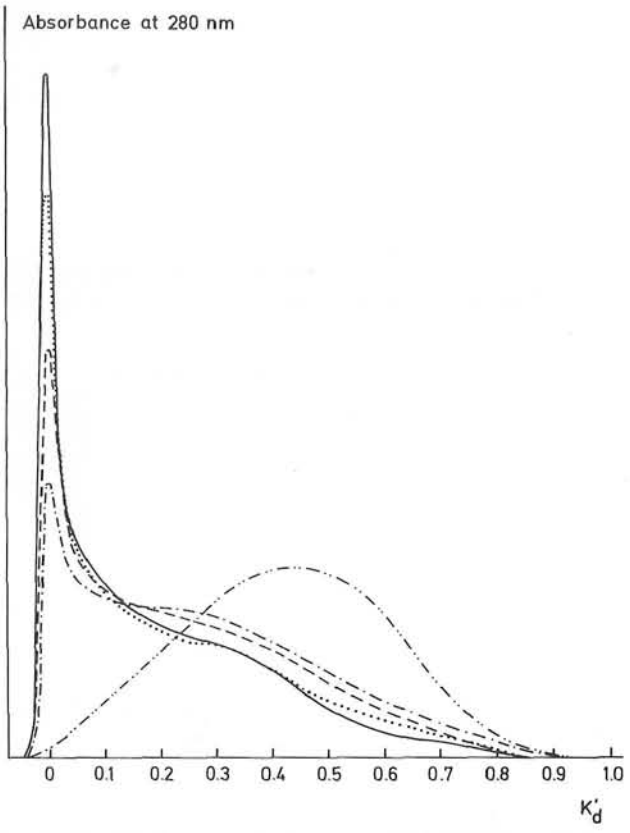


Fig 1. Gel permeation chromatography of some fractions obtained on liquid-liquid extraction of crude MWL from spruce according to scheme 2. System: Sephadex LH-60/DMF-acetic acid (200:1). ····· Precipitate, second extraction. ——— Precipitate, third extraction. - - - - Precipitate a. - · - · - Precipitate b. - ·· - ·· - MWL.

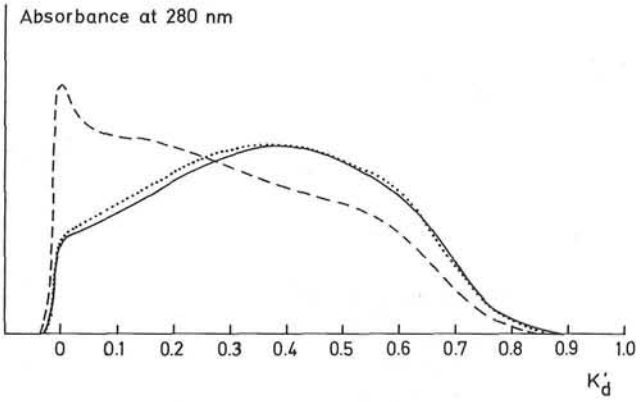


Fig 2. Gel permeation chromatography of a spruce MWL preparation obtained according to scheme 1 (———) and spruce (·····) and birch (- - - -) MWL preparations purified by a second treatment according to scheme 1.

Table 3. Yields (percentage of material in aqueous layer, first extraction step) and carbohydrate analysis of fractions A, B *and* C *(se fig 3).*

Fraction	Yield %	Carbohydrate content, %	Relative carbohydrate composition, %				
			Ara	Xyl	Man	Gal	Glu
A	35	16.5	12	23	27	24	14
B	26	12.0	16	14	49	6	14
C	34	27.0	20	21	13	11	36

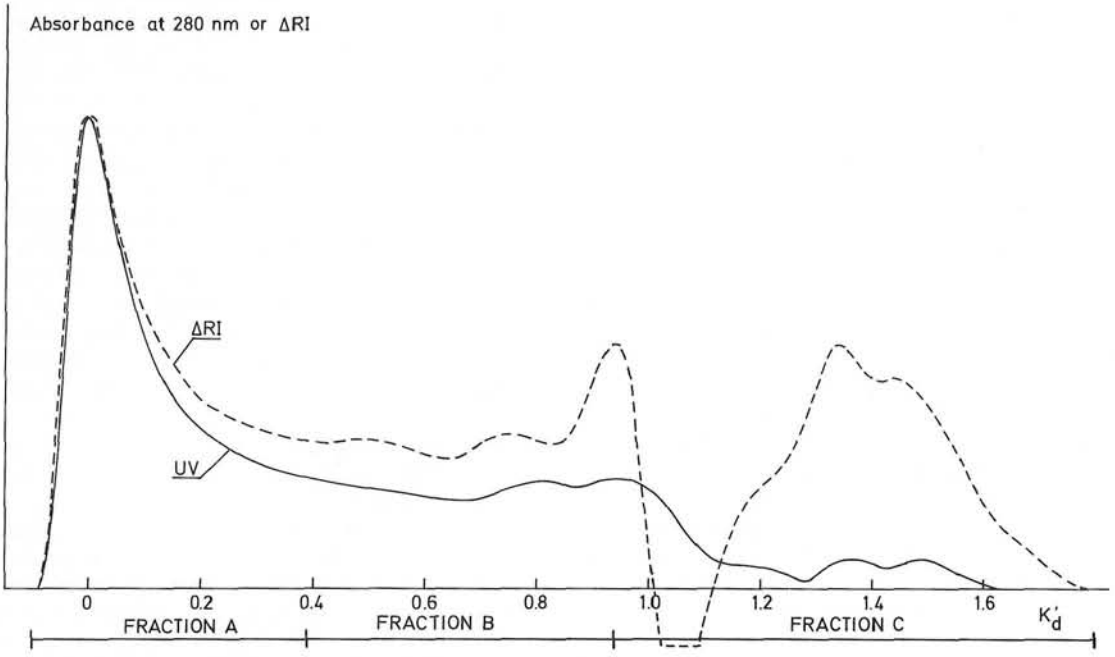
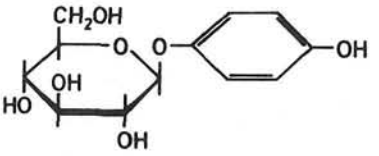


Fig 3. Gel permeation chromatography of materials isolated from the aqueous layer obtained in the first extraction step (scheme 2). System: Sephadex G-25 Fine/dioxane-water (1:1). The effluent was divided into fractions A, B *and* C. *Δ RI is the difference in refraction index between the eluate and the solvent.*



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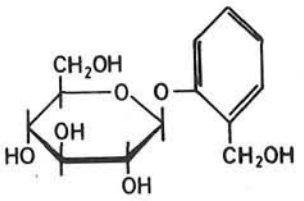

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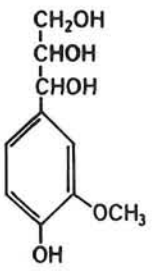

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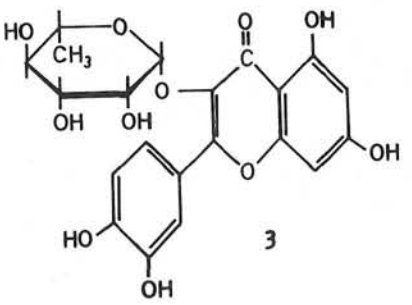

3

studies (6) carbohydrates could be expected to accumulate in fraction *C* while lignin constituents and lignin carbohydrate compounds would be found in fractions *A* and *B*; in connection with the present work the model compound studies were complemented by examination of methyl β-D-glucoside ($K_d' = 1.1$) and quercitrin (formula *3*) ($K_d' = 0.45$). It is, however, possible that carbohydrates of high molecular mass also are present in fractions *A* and *B* (6); the molecular mass of such carbohydrate constituents should be larger than 2500, since constituents of dextran hydrolysates of approximately this molecular mass were eluted at $K_d' > 1$. To eliminate such carbohydrate constituents, the materials in fractions *A* and *B* were subjected to GPC using the Sephadex LH-60/DMF and Sephadex LH-20/DMF systems, respectively.

Table 4. Relative carbohydrate composition in fractions from preparative electrophoresis of "purified fraction A".

Fraction number	Relative carbohydrate composition, % Ara	Xyl	Man	Gal	Glu
1	8	10	13	46	23
2	8	7	14	61	10
3	9	9	14	61	8
4	10	15	30	31	14
5	13	18	36	19	14
6	12	19	40	15	15
7	8	15	45	14	18
8	12	27	32	11	18
9	11	32	28	10	20

The elution curve of fraction *B* showed a peak at $K_d' = 0.68$ attributed to the presence of vanillic acid which was detected by thin layer chromatography (TLC). The eluate in the K_d' range 0.3-0.6 exhibited peaks at K_d' 0.46 and 0.34 which could be due to compounds consisting of a lignin unit and a sugar unit and compounds containing an additional lignin or sugar unit, respectively. In fact, TLC examinations [eluent, 2-butanone saturated with water; compounds *1-4* and a series of sugars (see Experimental) were used as reference compounds] supported the view that the fraction *B* consisted of such compounds, but also revealed that the composition was highly complex. Its relative carbohydrate composition was determined as: arabinose, 15%; xylose, 13%; mannose, 46%; galactose, 8%; glucose, 19%. The fraction was not further investigated.

Fraction *A* gave the elution curve shown in *fig. 4*. The materials eluted in the K_d' range 0.26-0.72 (yield, 27% of the solids in the aqueous layer from the first extraction step; the peak found in the gel chromatogram in the topical range corresponds to a molecular mass of about 2000) was subjected to further investigation. The fraction is denoted "purified fraction *A*" below. It follows from what has been said above that no constituents consisting solely of carbohydrates should be present in this fraction. Carbohydrates found in this fraction could therefore be assumed to be attached to lignin. The carbohydrate content was determined as 20.1% (relative carbohydrate composition; arabinose, 11%; xylose, 21%; mannose, 28%; galactose, 27%; glucose, 13%). The occurrence of lignin carbohydrate interconnections in this fraction was supported by electrophoresis experiments (*fig. 5, table 4*). It appears in fig. 5 that lignin (UV) and carbohydrates (carbohydrate analyses) are distributed in a similar way in the fractions. However, there is considerable variation in the relative carbohydrate composition of the fractions obtained in the electrophoresis experiment (table 4).

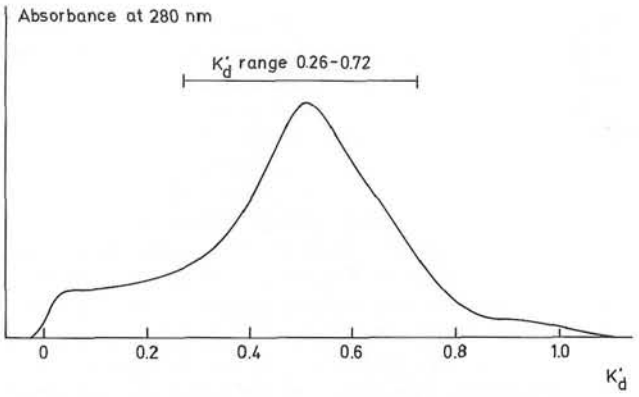

Fig 4. Gel permeation chromatography of fraction A *(se fig. 3) using the Sephadex LH-60/DMF system. The material in the effluent collected between* K_d' *0.26 and 0.72 was further investigated.*

Discussion

Isolation and characterization of constituents of crude MWL from spruce

Carbohydrate analyses have, if not stated otherwise, included hydrolysis of the sample with 0.5 M H_2SO_4 in dioxane-water (1:1). The hydrolysis procedure was designed for the analysis of lignin-rich and water-insoluble samples. No corrections for incomplete hydrolysis, decomposition of monosaccharides liberated and the presence of acetate groups in the samples have been made. (Analyses of standard samples suggest, however, that the losses owing to incomplete hydrolysis and decomposition of monosaccharides are limited.) Moreover, the samples have not been analysed for uronic acids. This implies that the carbohydrate moiety could be substantially larger than it appears from the analytical results which can thus be considered as representing lower limits for the carbohydrate content. We think, however, that the errors in relative carbohydrate composition are only slight and of minor importance.

The experiments with model compounds support the view that the liquid-liquid extraction procedure efficiently separates carbohydrates from lignin and lignin carbohydrate compounds at least in the sense that there are no free carbohydrates in the final organic layer (some lignin carbohydrate compounds with high carbohydrate content will be found in the aqueous layer).

Previous work (3, 5) indicated that constituents were removed from the organic layer not only according to carbohydrate content but also as a result of high molecular mass. In this work we have fractionated crude MWL from spruce according to scheme 2. As is evident in fig. 1 and also from determinations of molecular masses by vapor pressure osmometry, precipitates found in the aqueous layers or formed by addition of chloroform to the organic layer contain fractions with comparatively high molecular mass and have high carbohydrate content. It is of interest to compare the GPC results with those obtained on examination of MWL preparations obtained according to scheme 1 (fig. 2). It is noteworthy that purified lignin from birch contains a larger fraction high molecular mass material than the corresponding spruce lignin (fig. 2). This is in accordance with results from GPC studies of MWL preparations reported in the literature (7).

The aqueous layer obtained in the first extraction step of spruce lignin according to scheme 1 (which is identical with that obtained according to scheme 2) has been examined in some detail. The bulk of the free carbohydrates present in the original extracts are found in this fraction which, as seen in the foot-note of table 2, contains substantial amounts of monosaccharides. Carbohydrates were removed by GPC using a combination of systems. Fractions consisting of lignin carbohydrate compounds of low to moderate molecular mass with rather high carbohydrate content were isolated and subsequently investigated. These fractions should essentially consist of lignin carbohydrate compounds but they also contain minor amounts of lignin constituents (the major part of such materials are found in the organic layer). Interestingly, the dimeric and trimeric fractions isolated contained lignin carbohydrate compounds, as judged from TLC examinations (compounds *1-4* and sugars were used as reference compounds, see experimental). The fractions were, however, very complex and were not further investigated.

The fraction denoted "purified fraction A" (the molecular mass of this fraction was somewhat lower than that of MWL), which constituted 27% of the solids in the aqueous layer, was investigated by preparative zone electrophoresis. All the fractions obtained from the electrophoresis experiment contained substantial amounts of lignin as well as carbohydrates which supports the view that the sample investigated consists of lignin carbohydrate compounds. There is,

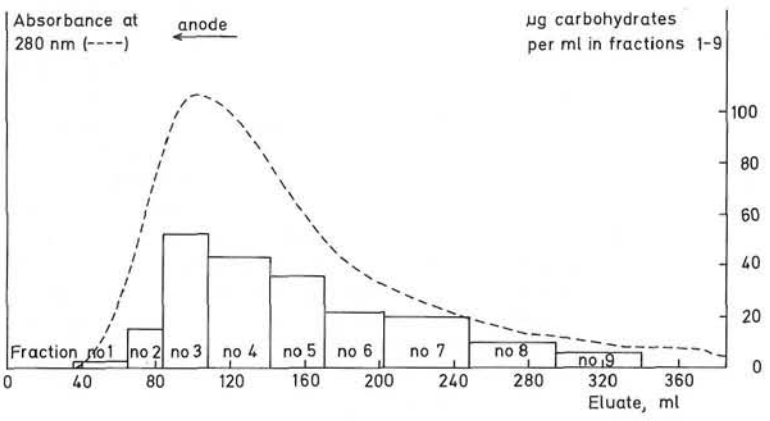

Fig 5. Preparative electrophoresis of "purified fraction A." *No carbohydrates were present in the eluate 0-30 ml.*

however, a considerable difference in relative carbohydrate composition in the different fractions (table 4). The comparatively high galactose content is characteristic of some of these fractions (fractions 1-4, see table 4). It is possible that this reflects a specific morphological origin of the sample. As indicated by the carbohydrate composition, "purified fraction *A*" might originate from the middle lamella region since pectic substances with high galactose contents appear in this region.

Lignin carbohydrate complexes with a high galactose content has been isolated from spruce wood using procedures involving enzymic carbohydrate decomposition (8). It has been suggested that resistance of galactan to enzymic carbohydrate decomposition could play a role in the accumulation of galactan in lignin carbohydrate complexes (9). This explanation for the high galactose content can be excluded in our case, since our procedure for the isolation of the sample does not include enzymic carbohydrate decomposition. At any rate, the carbohydrates in "purified fraction *A*" render it rather hydrophilic since it is soluble in water and not transferred into the organic layer during extraction.

Lignin carbohydrate linkages

MWL from spruce prepared according to scheme 1 contains about 0.3% carbohydrates (4). Repeated purification according to scheme 1 or purification according to scheme 2 lowers the carbohydrate content somewhat, but carbohydrates are still present in detectable amounts. It is assumed that the residual carbohydrates are chemically bound to the lignin. This is strongly supported by the extraction experiments with model compounds reported in this paper. Results from alkaline hydrolysis experiments can be interpreted as indicating that the carbohydrates are linked to the lignin by ester and ether linkages (4, 5). It seems that the former type of linkages are particularly important for the attachment of xylan to the lignin. Thus our studies with MWL from birch suggested that the major part of the xylan in the samples (xylan is the predominating carbohydrate constituent in birch MWL) is linked to the lignin by ester linkages (4). The results of our MWL studies are in line with the observations made by Obst (10) in hydrolysis studies with lignin carbohydrate complexes and woods. In our hydrolysis work with MWL samples we have presupposed the susceptibility of ester groups to alkaline hydrolysis, while the ether groupings (assumed to be of the benzyl ether type on the basis of biosynthetic considerations) are alkali-stable. The alkaline hydrolysis experiments with model compounds of the benzyl ether type made by Kosikova and coworkers (11) provide a justification for this assumption. [Enoki et al. (12) have reported that certain model compounds with p-hydroxy-benzyl ether structures are decomposed on alkaline treatment. However, their results can be explained by reactions of the unprotected sugar units present in the model compounds they have examined rather than cleavage of benzyl ether linkages.] Evidence for the occurrence of lignin carbohydrate linkages of benzyl ether type in lignin carbohydrate complexes from conifers has recently been obtained by the selective oxidative cleavage of such ethers on treatment with dicyanodichloro-*p*-benzoquinone (DDQ) in aqueous dioxane (13). The possibility that certain phenolic acids play a role for the connection of lignin and carbohydrates in grasses has frequently been discussed [see e.g. Scalbert et al. (14)]. Lignin carbohydrate linkages mediated by phenolic acids would hardly be of importance in birch or spruce since such acids are not present in these woods (at least not in appreciable amounts).

The isolation of lignin carbohydrate compounds with relatively high carbohydrate content from extractives obtained from milled spruce wood is described in this paper. As is evident from the discussion of the isolation procedures (GPC in different systems) and further examinations it could be assumed that lignin or lignin related compounds and carbohydrates are chemically linked to each other in these fractions. The nature of the lignin carbohydrate linkages in the fractions has not been elucidated. We have performed some preliminary experiments concerning the presence of end groups in the carbohydrate moiety of "purified fraction *A*" by determining alditols after reduction by borohydride and subsequent hydrolysis [such analysis has frequently been used in studies of lignin carbohydrate complexes, see e.g. Eriksson and Lindgren (15)]. "Purified fraction *A*" gave 2.3% alditols (relative alditol composition: arabinitol, 13%; xylitol, 20%; mannitol, 32%; galactitol, 19%; glucitol, 16%).

Finally it should be pointed out that linkages between lignin and carbohydrates in samples of the types discussed in this paper may be formed in the course of the isolation procedures. It is also possible that such linkages are split during the isolation procedures. The relevance of the conclusions regarding lignin carbohydrate linkages drawn from examinations of the samples isolated in this study is dependent on the occurrence of and extent of such reactions.

Experimental

Methods

Thin layer chromatography (TLC) was performed on silica gel plates (Merck, Kieselgel 60 F_{254}) using benzene-dioxane-acetic acid (90:25:4). (R_F value for vanillic acid, 0.50) and 2-butanone saturated with water (R_F values: cellobiose, 0.00; glucose, 0.02; methyl β-D-glucoside, 0.07; salicin (*2*), 0.21; arbutin (*1*), 0.28; guaiacylglycerol (*4*), 0.37; quercitrin (*3*), 0.44) as eluents. Spots were detected by UV light and by spraying with formalin-H_2SO_4 (1:9) followed by subsequent heating.

Number average molecular masses were measured with a vapor pressure osmometer (Knauer). DMF was used as solvent. Temperature, 120°C.

Gel permeation chromatography (GPC) was performed using the systems Sephadex G-25 Fine/dioxane-water (1:1), Sephadex LH-20/DMF and Sephadex LH-60/DMF-acetic acid (200:1) or DMF (in this case a small amount of acetic was allowed to pass the column before the GPC experiment). The elution was followed by the use of UV detector (280 nm) and/or differential refractometer. The bed height was about 95 cm and the column diameter was 1.3 cm in analytical experiments and 2.5 cm in preparative experiments. The flow rate was about 5 ml/cm^2 • h. To describe the elution properties of a solute, a distribution coefficient, K_d', was used. $K_d' = 1$ corresponds to the elution volume of acetone (representative of small molecules), $K_d' = 0$ corresponds to the elution volume of a polymer excluded from the gel

[e.g. lignin carbohydrate complexes prepared according to Björkman (16)].

Electrophoresis was performed in 0.02 M $Na_2B_4O_7$ on a column filled with polyacrylamide gel (Bio-Gel P-300; Bio Rad.). An apparatus system, LKB 7900 Uniphor, was used. This was constructed for continuous elution during the course of the run. The solute moving towards the anode by the electrophoresis was eluted by a continuous cross flow of buffer solution (flow rate: 4 ml/hour). A UV detector (280 nm) was installed and fractions of about 2 ml were collected. The apparatus was kept at 13°C by circulating thermostatted water. The bed height of the gel was 9 cm and the volume of the gel was 46 ml. The distance between the electrodes was 22 cm and the applied voltage was 440 V, current 16 mA. The amount of sample applied to the column in the runs with "purified fraction *A*" was 50 mg.

Carbohydrate contents of the fractions from electrophoresis experiments were determined by carbohydrate analysis after neutralization with aqueous H_2SO_4, hydrolysis and removal of buffer (cation exchanger, H^+-form; repeated addition and evaporation of methanol to remove boric acid).

Carbohydrate analysis

The samples were hydrolysed by treatment with 0.5 M H_2SO_4 in dioxane-water (1:1) at 100°C for 4 h (sealed glass ampoule) unless otherwise specified. The reaction mixture was neutralized by treatment with an anion exchanger resin in the bicarbonate form, or by aqueous sodium hydroxide. The liberated monosaccharides were separated by partition chromatography on an anion exchanger (Durrum DA-X8 Fine) in the sulfate form, using 85% ethanol as eluent. For the detection of the monosaccharides in the effluent, a colorimetric method described by Mopper and Gindler (17) was used.

Application of the liquid-liquid extraction procedure to model compounds

Model compounds (80-180 mg) were dissolved in 14 ml pyridine-acetic acid-water (9:1:4) and extracted with chloroform according to the procedure described in ref. 2 (cf. Scheme 1). The compounds were isolated by film evaporation of the aqueous and the final organic layers. The yields were determined either by carbohydrate analysis (see above) (i.e. dextran hydrolysis products and methyl β-D-glucoside) or by weighing after drying over P_2O_5 in vacuo. The identity of the isolated products was also confirmed by TLC. Dextran hydrolysate products were obtained by refluxing Dextran T10 (Pharmacia) in 2 M trifluoroacetic acid for 30 min followed by film evaporation (25°C) of the acid to dryness after repeated additions of water. The molecular mass distribution of the hydrolysis products on Sephadex LH-60 (DMF) indicates that the reaction mixture contains about equal amounts of low molecular mass glucoside residues (DP1-5) and higher molecular dextran residues.

Application of the liquid-liquid extraction procedure to crude milled wood lignin

Preextracted (acetone, Soxhlet) wood chips from spruce were milled and subsequently extracted with dioxane containing 4% water, essentially according to the procedures described by Björkman (1). Extracted solids constituted 11% of the milled wood. The extracted product (6.80 g) was dissolved in 210 ml pyridine-acetic acid-water (9:1:4). The solution was extracted according to scheme 2. Separation of the layers was speeded up by centrifugation. Some of the products in the aqueous layers were in the form of precipitates. These were isolated from soluble products by centrifugation. The clear aqueous layers were evaporated to dryness by film evaporation (25°C). In the last steps of the procedure precipitates were formed in the organic layers on addition of chloroform. These precipitates, too, were isolated by centrifugation. The major part of the solvents in the final organic layer was removed by film evaporation. The lignin was precipitated by slowly dripping the residual solution (about 250 ml) into stirred ether (1.6 lit.). The precipitate was washed with 3×325 ml ether. The yield of the different fractions (table 2) were determined after drying over P_2O_5 in vacuo.

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