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HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF CARBOHYDRATES

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SUMMARY

A procedure is described for the separation of water-soluble wood polysaccharides on Bio-Glas (granular porous glass) and Bio-Gel P (polyacrylamide) packed columns using high-pressure liquid chromatography (HPLC). An example of HPLC employing non-aqueous solvent systems and EM Gel OR-PVA (vinyl acetate copolymer) type packing materials is also discussed.

INTRODUCTION

A technique is described for the separation and isolation of some water-soluble wood polysaccharides and low-molecular-weight carbohydrates using high-pressure liquid chromatography (HPLC). The polysaccharides surveyed include arobinogalactans from western larch (*Larix occidentalis*), water-soluble polysaccharides from loblolly pine (*Pinus taeda*) and a synthetic polymer formed by thermal polymerization of methyl-α-D-glucopyranoside.

EXPERIMENTAL

Apparatus and materials

A Waters Ass. (Framingham, Mass., U.S.A.) liquid chromatograph (Model 202) equipped with a 1000 p.s.i. pumping system and both ultraviolet and differential refractometer detectors was employed. All chromatograms were obtained at room temperature.

Bio-Gel P-2 and P-60 (polyacrylamide gel) and Bio-Glas 500 (granular porous glass) packing materials were obtained from Bio-Rad Labs., Rockville Center, N.Y., U.S.A. EM Gel OR-PVA 500 (vinyl acetate copolymer) packing material was obtained from EM Labs., Elmsford, N.Y., U.S.A.

Molecular weight standards (dextran polymers) were purchased from Pharmacia, Uppsala, Sweden.

Column preparation

Stainless-steel columns, 4 ft. × 3/8 in., were used for both Bio-Gel and Bio-Glas materials while a column 2 ft. × 1/8 in. was employed for the EM Gel packing.

Granular Bio-Gel was suspended in water and allowed to swell overnight. The water was then decanted off in order to remove any fine particles. Sufficient fresh water was added to form a thick slurry, which was then poured slowly into the column. Subsequently, the column was attached to a solvent output line on the liquid chromatograph and allowed to purge with the solvent for ½ h. The process of adding packing material and purging was repeated until the column was completely packed.

Porous Bio-Glas was suspended in water in a stoppered Büchner funnel, and a vacuum was applied. When all bubbling of the slurry had ceased, the column was packed in an analogous manner to the Bio-Gel column.

As EM Gel OR-PVA materials can be used only with non-aqueous solvents, this packing was allowed to swell in methanol overnight. After clamping the 2 ft. × 1/8 in. column in a vertical position, a vacuum was applied at the lower end while a slurry of the packing material was slowly introduced at the top. Final pressure packing of the column was accomplished by using a high-pressure pump. Furthermore, to prevent foreign matter from being introduced into the UV or refractometer cells, all newly packed columns were allowed to purge with appropriate solvents for 3 h before being connected to the inlet lines of the detectors.

Analysis of the carbohydrate materials

The polysaccharides were hydrolyzed and the hydrolyzate was silylated using the procedure of Sweeley *et al.*[1]. The silylated monosaccharides were separated and identified by gas–liquid chromatography (GLC) and quantitatively determined using an electronic integrator as previously described by Laver *et al.*[2]. The value of each sugar was corrected for any loss due to decomposition and for addition of a water molecule during hydrolysis.

Preparation of samples

Water-soluble polysaccharides from loblolly pine[3]. Boards, cut from the sapwood of a green loblolly pine (*Pinus taeda*) log, were ground into small chips and passed through a Wiley mill equipped with a 60-mesh screen. The sapwood meal (*ca.* 1500 g) was then placed in two large containers and extracted with distilled water (4 l) for 24 h at room temperature. The two solutions were filtered and the aqueous filtrates combined. A rotary evaporator was employed to concentrate the aqueous filtrate to a thick, dark brown syrup. This material was labeled total, cold-water-soluble fraction. Fehling's reagent was then added to an aqueous solution of this syrup, causing a light green material to precipitate. The precipitate was collected and washed with a large volume of distilled water and then allowed to air dry. Decomposition of this polysaccharide–copper complex was performed by adding it to ice-cold (0) ethanol containing hydrochloric acid (5%, v/v). The precipitate was collected by filtration and washed successively with ethanol, acetone and diethyl ether. This procedure was repeated three times to yield a purified galactoglucomannan.

The Fehling's-soluble fraction from above was deionized with MB-3 ion exchanger and filtered. The filtrate was then concentrated to a small volume and added to an excess of ethanol. The white precipitate which formed was washed successively with ethanol, acetone and diethyl ether. This material was labeled Fehling's-soluble fraction.

Water-soluble polysaccharides from western larch[4]. Small blocks were cut from

a dry sample board of larch. These blocks were then planed into chips and passed through a Wiley mill. The resultant meal was placed in a Soxhlet unit and exhaustively extracted successively with benzene, benzene–ethanol (1:1) and ethanol. After the sample had been air dried, it was likewise extracted with distilled water (24 h, 20°). The aqueous extract was filtered, concentrated to a small volume and added dropwise to a four-fold excess of denatured ethanol. The fluffy white precipitate which formed was collected, washed sequentially with ethanol, acetone and diethyl ether and dried in a vacuum oven.

Stachyose hydrate (lupeose; α-D-galactosyl-α-D-glucosyl-β-D-fructose), D(+)-cellobiose and D(−)-ribose samples. These materials were obtained in a highly pure grade from Sigma, St. Louis, Mo., U.S.A.

Products from thermal polymerization of methyl-α-D-glucopyranoside. A small sample of methyl-α-D-glucopyranoside was introduced into the heating chamber (initial temperature 310°) of a Chromalytics MP-3 thermal analyzer while the chamber was being purged with nitrogen. The reaction was allowed to proceed isothermally at 310° for 10 min, at which time the sample was removed and dissolved in a small amount of water.

The above temperature and reaction time were chosen because earlier studies had shown that methyl-α-D-glucopyranoside would polymerize under these conditions[5].

RESULTS AND DISCUSSION

Fig. 1 shows the separation of the two water-soluble arabinogalactans from larch on a Bio-Glas 500 packed column. This packing material withstood high pressures well; hence larger flow-rates were employed. Sharp, well separated peaks were obtained for the polymers on Bio-Glas, which illustrated that a random molecular weight distribution did not exist. Subsequently, each component was isolated from the liquid chromatograph and characterized by GLC. The results, which are shown in Table I, agree well with previously reported values for these compounds[6–8].

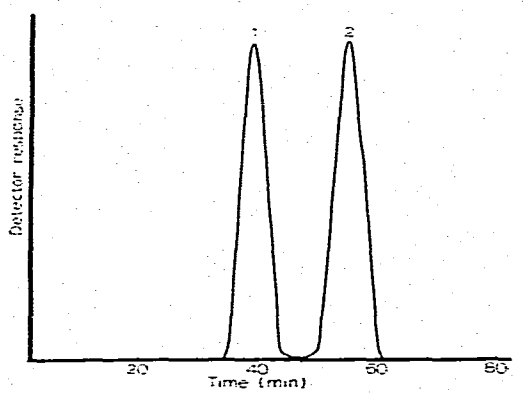

Fig. 1. HPLC refractive index chromatogram of larch arabinogalactans. Column: Bio-Glas 500 (500 Å pore diameter), 4 ft. × 3/8 in., stainless steel. Solvent: water. Flow-rate: 0.9 ml/min. Pressure: 275 p.s.i. (1) Arabinogalactan, M_w = 100,000; (2) arabinogalactan, M_w = 18,195.

TABLE I

ANALYSIS OF LARCH ARABINOGALACTANS

Polymer	*Molar ratio (Arab/Galac)*		*Molecular weight*	
	Reported[7,8]	*Found*	*Reported*[6]	*Found*
Component 1	4.3	4.14	100,000	> 100,000
Component 2	3.8	3.38	16,000	18,195

Molecular weights ($\bar{M}_w$ and $\bar{M}_n$ values) for the two water-soluble arabinogalactans were obtained by making standard runs of dextran polymers on the Bio-Glas 500 column. Apparently, owing to the relative inertness of the porous glass beads, little if any affinity interaction took place between the column and dextran standard. Hence, molecular weights were determined accurately on this column except in the region near the void volume (molecular weight operating range of column = 10,000–100,000). The high-molecular-weight component eluted very close to the void volume of the column; therefore, little fundamental information about its polydispersity or molecular weight could be obtained. However, the other component had a much lower molecular weight, so characterization was easy. This species possessed a polydispersity of 2.16 and $\bar{M}_n$ and $\bar{M}_w$ values of 8405 and 18,195, respectively.

Fig. 2 shows the separation of the total, cold-water-soluble polysaccharides on a Bio-Gel P-60 packed column. The Bio-Gel materials were found to compress very easily under high pressure so a low flow-rate had to be employed. The total elution time was slightly over 4 h.

After introducing large samples of total, cold-water-soluble material via the 2-ml loop injector, fractions were isolated from the total run (see Fig. 2) and analyzed by GLC. The results indicated the presence of an arabinose-, mannose- and galactose-containing polymer, a galactoglucomannan and large amounts of glycerol. One frac-

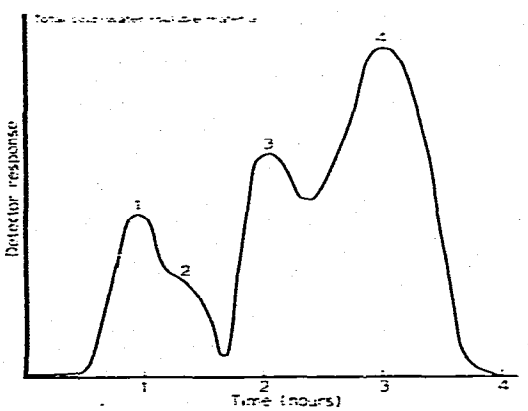

Fig. 2. HPLC refractive index chromatogram of total, cold-water-soluble material. Column: 4 ft. × 3/8 in., stainless steel. Bio-Gel P-60 packing material. Solvent: water. Flow-rate: 0.3 ml/min. (1) Unknown; (2) galactoglucomannan; (3) arabinose-, mannose- and galactose-containing polymer; (4) glycerol.

tion (peak 1) appeared to be a mixture and, because its elution volume was so near the void volume of the column, it could not be resolved any further.

As stated previously, the total, cold-water-soluble material was chemically fractionated using Fehling's solution. The Fehling's-insoluble fraction (copper complex precipitate) yielded a galactoglucomannan similar to that reported by Jones and Painter[9] and by Timell[10] ($[\alpha]_D^{25°} = -19.6°$). Acid hydrolysis and GLC analysis confirmed the presence of galactose, glucose and mannose in the ratio 1.1:1.0:3.8. The periodate consumption was determined spectrophotometrically[11] and found to be 0.98 mole/anhydro unit, while the formic acid liberation was 0.19 mole/anhydro unit.

The Fehling's-soluble fraction afforded a white material containing arabinose, mannose and galactose in the ratio 1.0:1.0:2.7. This material was homogeneous by HPLC criteria and further chemical purification with Fehling's solution failed to alter its mannose content. This polysaccharide appeared completely different from arabinogalactan material reported from larch[4] and Monterey pine[12] and even loblolly pine[3] total wood. A complete structural analysis, which is beyond the scope of this paper, would be necessary in order to confirm this material as being a true arabinogalactan species.

Fig. 3 shows the Fehling's products as they appeared on a Bio-Gel P-60 column. Scan A and B of the Fehling's-insoluble fraction were run in order to illustrate the utility of liquid chromatography in monitoring a chemical purification. Chromatogram A shows the cold-water-soluble galactoglucomannan contaminated with an unknown component (peak 1). After repeated precipitation with Fehling's solution, the galactoglucomannan was resolved into one peak (peak 2), exclusive of any contaminating material (scan B). Peaks 1 and 2 are analogous to those on the chromatogram of the total, cold-water-soluble material. The Fehling's-soluble fraction contained three compounds corresponding to peaks 1, 3 and 4 on the chromatogram of the total, cold-water-soluble material. Components generating peaks 3 and 4 were easily characterized, but peak 1, which was caused by a high-molecular-weight species,

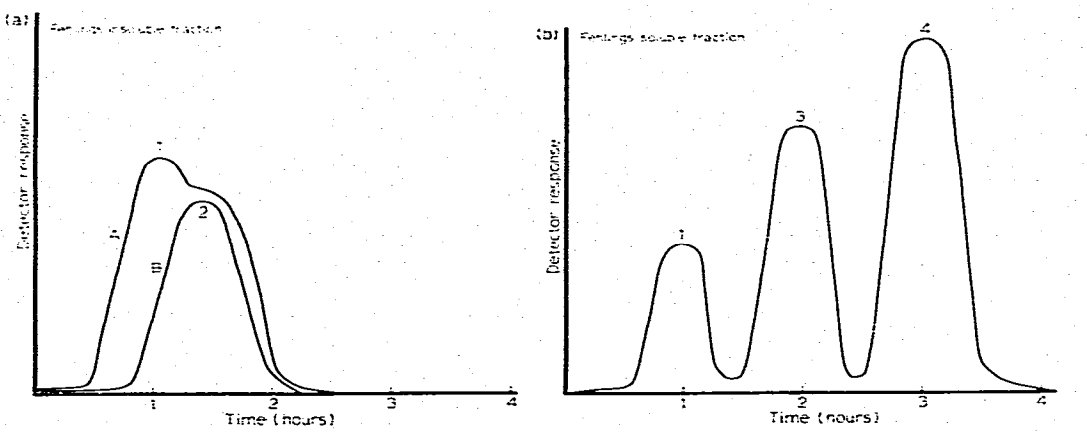

Fig. 3. HPLC refractive index chromatograms of Fehling's-insoluble (a) and Fehling's-soluble (b) materials. Column: Bio-Gel P-60, 4 ft. × 3/8 in., stainless steel. Solvent: water. Flow-rate: 0.3 ml/min. A, unpurified Fehling's-insoluble fraction; B, purified Fehling's-insoluble fraction. (1) Unknown; (2) galactoglucomannan; (3) arabinose-, mannose- and galactose-containing polymer, (4) glycerol.

could not be further purified. As stated earlier, it eluted at the void volume of the column. A Bio-Gel P series column possessing a higher molecular weight operating range would have resolved this material, however.

Fig. 4 illustrates the separation of some low-molecular-weight carbohydrate materials when a non-aqueous solvent system was employed. Again, the packing material was easily compressed at high pressures (high flow-rates), so a low flow-rate had to be used.

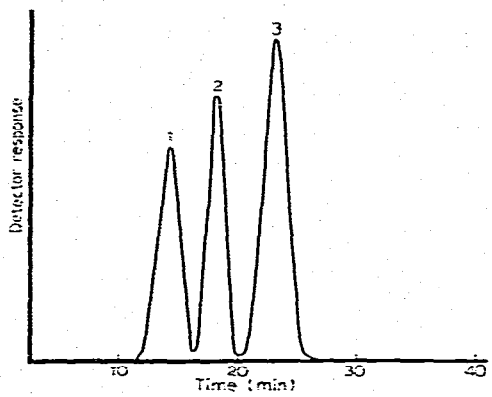

Fig. 4. HPLC refractive index chromatogram of some low-molecular-weight carbohydrates. Column: EM Gel OR-PVA 500, 2 ft. × 1/8 in., stainless steel. Solvent: methanol. Flow-rate: 0.3 ml/min. (1) Stachyose hydrate; (2) D(+)-cellobiose; (3) D(−)-ribose.

Resolution of peaks on the EM Gel column was excellent. And, although a number of compounds were surveyed, the separation of tetrasaccharides from disaccharides and monosaccharides seemed to be one of the greatest assets of the column.

In order to study further the utility of the Bio-Gel packing materials, polymerization products from a thermal decomposition reaction of methyl-α-D-glucopyranoside were investigated. Fig. 5 shows the initial and final products as well as the intermediate compounds formed in the polymerization process.

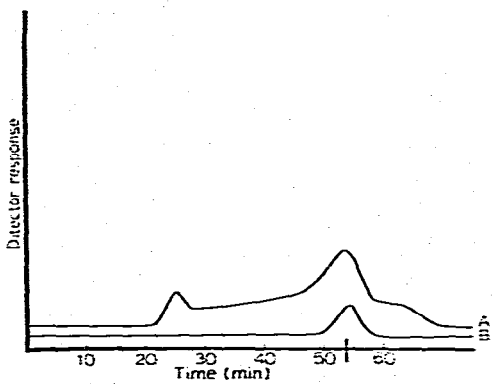

Fig. 5. HPLC refractive index chromatogram of products from thermal polymerization of methyl-α-D-glucopyranoside. Column: Bio-Gel P-2, 4 ft. × 3/8 in. Solvent: water. Flow-rate: 0.9 ml/min. A, Total reaction mixture; B, methyl-α-D-glucopyranoside.

The first peak eluted from the Bio-Gel P-2 column was the highest-molecular-weight compound or "compounds" in the reaction mixture. For this particular reaction, the first peak of scan A corresponded to mol. wt. $\geqslant$ 1300 while the last peak corresponded to mol. wt. $\leqslant$192. The intermediate area between the first and last peak of scan A can be associated with materials in the reaction mixture possessing random molecular weights from 192 to 1300. Scan B of pure methyl-α-D-glucopyranoside was run for comparison. Perhaps the most important fact to be ascertained from the above illustration is that through the use of HPLC, polymerization processes, and many other types of reactions, can be monitored from initiation to termination. Also, the total reaction mixture can be analyzed directly, eliminating the formation of any derivatives. For example, neutral sugars can be studied in their natural states when using HPLC, while analysis by GLC would require common derivative formation (*e.g.*, trimethylsilyl, acetyl and trifluoroacetyl derivatives).

CONCLUSION

Through the use of HPLC, water-soluble polysaccharides, together with low-molecular-weight carbohydrates and polymerization products, were separated, collected and identified. Bio-Glas packed columns gave the most rapid separation of high-molecular-weight, water-soluble polysaccharides, while Bio-Gel was found most useful for separating complex mixtures of polymers. Lower molecular weight carbohydrates were separated on both Bio-Gel and EM Gel OR-PVA columns, with EM Gel giving the best resolution. The only drawback found with the EM Gel packings was that non-aqueous solvents had to be employed. Consequently, analysis of those carbohydrate oligomers which possess more than four units was somewhat limited owing to solubility problems.

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