CHROMATOGRAPHY OF OLIGOSACCHARIDES FROM XYLAN BY VARIOUS TECHNIQUES

JAROSLAV HAVLICEK AND OLOF SAMUELSON
Department of Engineering Chemistry, Chalmers Tekniska Högskola, Göteborg (Sweden)
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ABSTRACT

Chromatographic studies of the oligomeric sugars obtained from birch xylan by acid hydrolysis revealed oligosaccharides containing 2–18 D-xylose residues. The results indicate that the 4-*O*-methyl-D-glucuronic acid groups are randomly distributed in the xylan. The nonasaccharide and the lower homologues were isolated by chromatography on charcoal–Celite and by partition chromatography on ion-exchange resins using aqueous ethanol. At a high concentration of ethanol, the sugars are eluted in order of increasing molecular weight, whereas the order is reversed at a low concentration of ethanol. At one critical concentration, which depends upon the type of resin, there is no separation at all. The validity of Martin's rule is demonstrated for partition chromatography on ion-exchange resins, as well as for permeation chromatography on ion exchangers and polyacrylamide gel.

INTRODUCTION

The oligomeric sugars of the β-(1→4)-linked D-xylose series have previously been studied by adsorption chromatography on charcoal–Celite[1], paper chromatography[2], and most recently by permeation chromatography on polyacrylamide gel[3]. The present paper deals with their separation by means of ion-exchange resins by both partition chromatography in aqueous ethanol and by gel-permeation chromatography in water. A comparison is made with the results obtained in experiments with the other column chromatographic techniques.

EXPERIMENTAL

Preparation of oligosaccharides. — A solution of birch xylan[4] (50 g) in 98% trifluoroacetic acid (1 litre) was kept at room temperature for 12 days. Most of the acid was then removed by evaporation to a small volume at 35°, and the remaining acid was taken up by a strongly basic anion-exchanger in its hydrogen carbonate form. An excess of resin was used so that acidic hydrolysis products, such as uronic acids, were also removed. After filtration and washing, the solution was evaporated to dryness.

The oligosaccharides were separated on a charcoal–Celite column[5] (2:3, 50 × 580 mm). To avoid over-loading of the column, four separate runs were made, each with 25% of the saccharide mixture. Despite this precaution, the individual fractions contained appreciable amounts of neighbouring oligomers besides the desired sugar. The fractions were therefore purified by partition chromatography in aqueous ethanol (70–82%) on Dowex 1-x8 resin[6] (17–24 μm, SO_4^{2-}). Clear-cut separations were obtained at 75° on a column having dimensions 10 × 110 mm, provided that ≯250 mg of oligosaccharides were used. The fractions were evaporated to dryness and the sugars recrystallized from 60–85% aqueous ethanol.

The equivalent weights, determined by hypoiodite oxidation[7], were in good agreement with theory. D-Xylose was the only monosaccharide obtained after acid hydrolysis. The di-{m.p. 184–186°; $[\alpha]_D$ −26.8° ($c \sim 1$, water)}, tri-(209–211°; −47.8°), tetra- (225–227°; −58.2°), penta- (236–238°; −66.4°), hexa- (232–234°; −73.6°), and hepta-saccharide (241–243°; −77.2°) were obtained crystalline. The tetrasaccharide and the higher oligomers decomposed at their melting points. The octasaccharide and nonasaccharide were isolated as amorphous solids. The fractions were chromatographically pure.

In the kinetic experiments referred to in Fig. 3, the xylan (1 g) was dissolved in trifluoroacetic acid (20 ml). Samples (2 ml) were withdrawn and diluted with water, and the acids were removed by an anion-exchange resin (HCO_3^-). After filtration, washing, and evaporation to dryness, the saccharide mixture was dissolved in water and subjected to permeation chromatography.

Chromatographic analysis. — The separations by partition chromatography on ion-exchange resins were carried out under conditions similar to those applied previously[8], and the eluate was analyzed automatically by the orcinol method[6]. The photometer was equipped with two flow-cells, coupled in series, with light paths of 1 and 10 mm, respectively. The separations on polyacrylamide gel were performed as described by John *et al.*[9]. Experimental details are given in Figs. 1, 4, and 5.

The volume distribution coefficients (D_V) were calculated from the peak elution volumes ($\overline{V}$) by use of the equation $D_V = \overline{V}/X - \varepsilon_I$, where X is the volume of the chromatographic bed and ε_I is the relative interstitial volume[10]. Since the swelling of the ion-exchange resin decreases for an increased concentration of ethanol, the exchange capacity per unit volume of the bed increases with an increased concentration of ethanol. To avoid chanelling in the resin bed and re-packing of the column, the experiments at different concentrations of ethanol were carried out as far as possible in the order of increasing concentration. In those cases where it was necessary to change to an eluent of a lower concentration of ethanol, the column was repacked. The particle size of the resin was determined at room temperature in 85% aqueous ethanol. In the calculations, ε_I was taken to be 0.4.

RESULTS AND DISCUSSION

Permeation chromatography on polyacrylamide gel. — The charcoal–Celite method[5] was the least satisfactory of the three chromatographic methods employed

in this work for analytical purposes, as far as separation efficiency and time are concerned. On the other hand, the method is quite useful for preparative purposes in combination with partition chromatography on ion-exchange resins. It has the great advantage that large amounts can be handled and that stepwise elution can be applied without disturbances.

Permeation chromatography on a polyacrylamide gel in water is of great interest for analytical purposes, especially when a large number of oligomers are present. The chromatogram reproduced in Fig. 1, which involved 5 mg of xylan

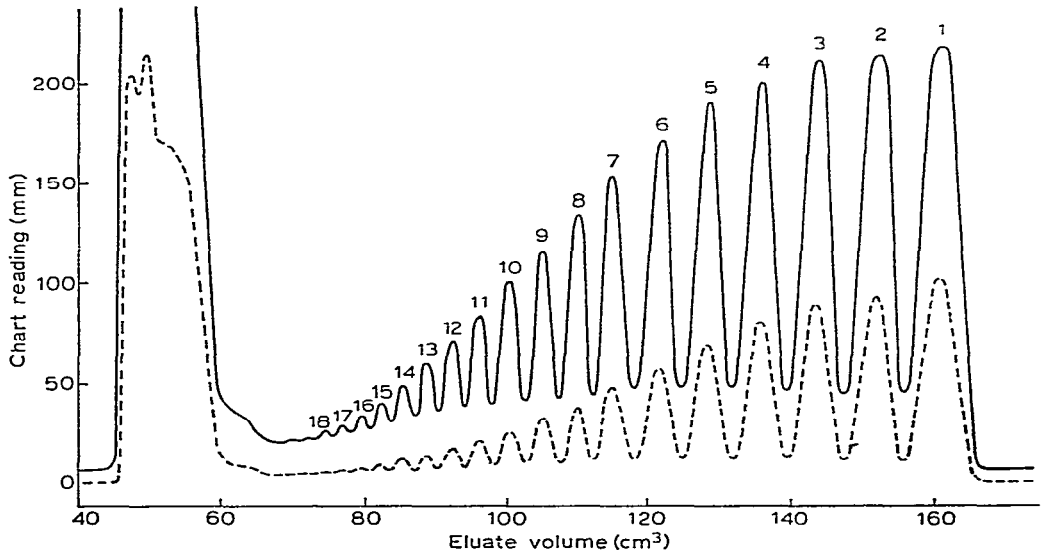

Fig. 1. Permeation chromatography of the hydrolysis products produced from birch-xylan hydrolysis in 98% trifluoroacetic acid. Bio-Gel P-2, 12–18 μm, 10 × 1160 mm; 65°, deionized water. Flow rate: 0.25 cm.min^{-1} (calculated for an unpacked column). 1, D-xylose; 2, disaccharide; 3, trisaccharide; etc.

hydrolysis products (applied to the column in 0.5 ml of water), gives valuable qualitative and quantitative data. The identification of the oligomers containing 2–9 D-xylose residues was made by separate runs with authentic samples, the positions of which differed only slightly from those recorded with mixtures of several compounds. A straight-line relationship (Martin's rule) was found to exist between log D_V and the number of D-xylose residues in the oligomeric series. This permits an identification of the higher oligomers (Fig. 2). The validity of Martin's rule for the lower oligomers has recently been demonstrated[3]. The numerical values for the distribution coefficients differ from those reported[3], probably because of differences in properties of various batches of the gel[11] and in the working temperature. A quantitative estimate was obtained by comparison of the peak areas with those obtained in calibration runs.

The method was applied to determine the formation of D-xylose and its oligomers during the acid hydrolysis of birch xylan. The results given in Fig. 3 show

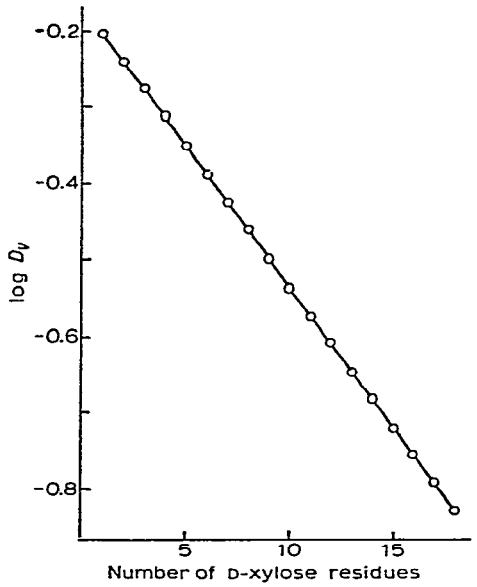

Fig. 2. Relationship between log D_V and number of D-xylose residues in the oligosaccharides. Bio-Gel P-2, 65°, deionized water.

that the amounts of all oligosaccharides studied increased during the first period of the hydrolysis and that, after passing through a maximum, the yields decreased slowly upon prolonged reaction. The shape of the curve corresponding to D-xylose indicates extensive decomposition[12]. As expected, the relative amounts of the higher oligomers were much higher after a short time of hydrolysis than after prolonged reaction. An inspection of the original chromatograms (*cf.* Fig. 1) showed that appreciable amounts of oligomers having a d.p. of 18 were present after a hydrolysis time of 4–12 days, whereas after 20 days, only oligomers having a d.p. of 2–10 could be identified with certainty.

Since the products of acid hydrolysis, mainly 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose and higher D-*xylo* polymer homologues, were removed prior to the separation of the oligosaccharides, it can be concluded that birch xylan contains unsubstituted chains consisting of more than 18 D-xylose residues. It was known previously that birch xylan contains approximately one 4-*O*-methyl-D-glucuronic acid side-chain per ten D-xylose residues, and that blocks containing neighbouring acid side-chains are absent[13]. The results obtained in the present work permit the conclusion that the uronic acid groups do not appear at regular intervals, which means that it is most likely that there is a random distribution of uronic acid groups along the xylan chain.

The time required for the separation by permeation chromatography referred to in Fig. 1 was ~13 h. A disadvantage, from a practical point of view, is that neighbouring oligomers overlap, so that it is difficult to make accurate, quantitative

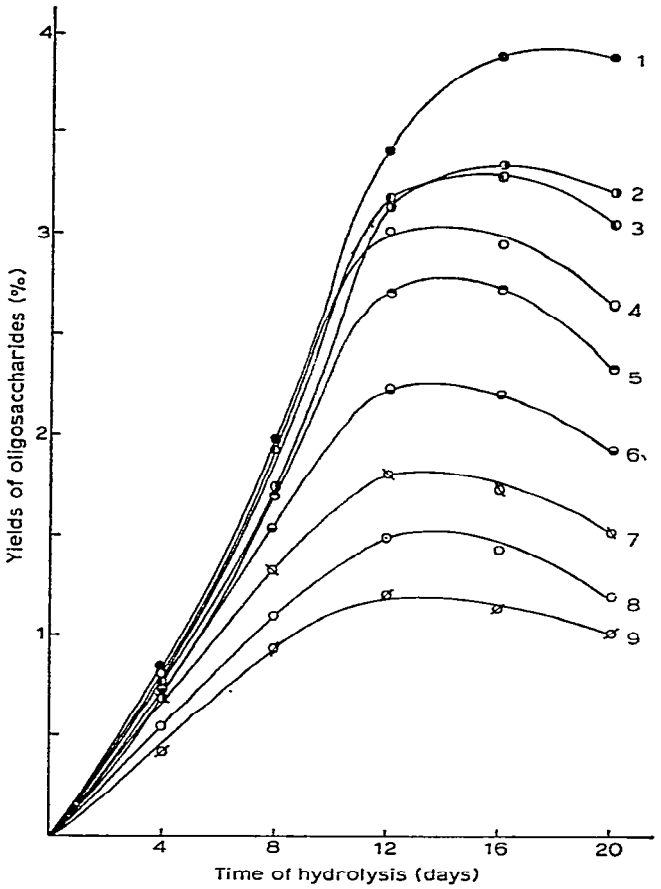

Fig. 3. Rate of hydrolysis of birch xylan. The numbers indicate the d.p. of hydrolysis products. Yields based on xylan.

determinations. In investigations where a precision of approximately ±5% can be tolerated, the method can be used to advantage for the determination of the higher oligomers. For the lowest oligomers, the precision is approximately ±2% in successive runs. These figures obtain only when very small amounts of material are applied to the column. As can be seen from Table I, the resolution decreases markedly with an increased column loading. Moreover, it is seen that the overlapping of neighbouring peaks was more serious with a longer flow-cell than with a shorter one. This effect is more marked when large amounts are chromatographed.

The results show that permeation chromatography on polyacrylamide gel is an excellent tool for analysis of oligomeric sugars. For preparative purposes and when samples have to be isolated for identification purposes, the method is inferior to the charcoal–Celite method and to partition chromatography on ion-exchange resins.

Partition chromatography on ion-exchange resins. — Partition chromatography

TABLE I

PEAK RESOLUTIONS[a] OF NEIGHBOURING COMPOUNDS APPLIED IN EQUAL AMOUNTS TO A POLYACRYLAMIDE GEL

β-(1→4)-Linked D-*xylose saccharides*	*Resolution at different column loadings and lengths of the flow-cell*			
	30 μg		*600 μg*	
	1 mm	*10 mm*	*1 mm*	*10 mm*
Di- and tri-saccharides	1.22	1.15	1.04	0.78
Penta- and hexa-saccharides	1.07	1.01		
Octa- and nona-saccharides	0.86	0.84		

[a]Calculated according to IUPAC-recommendations[18].

on ion-exchange resins in aqueous ethanol has the advantage that the oligomeric sugars can be easily separated as discrete bands and that the broadening of the elution bands is small compared to that observed for charcoal–Celite. This means that the accuracy in the quantitative determination depends only upon the analyzing system which, under favourable conditions, gives a reproducibility within ±1%. Chromatograms illustrating the results obtained on a sulphate column and on a lithium column are given in Figs. 4 and 5. It is seen that the oligomeric sugars are well separated both on the anion exchanger and on the cation-exchange resin.

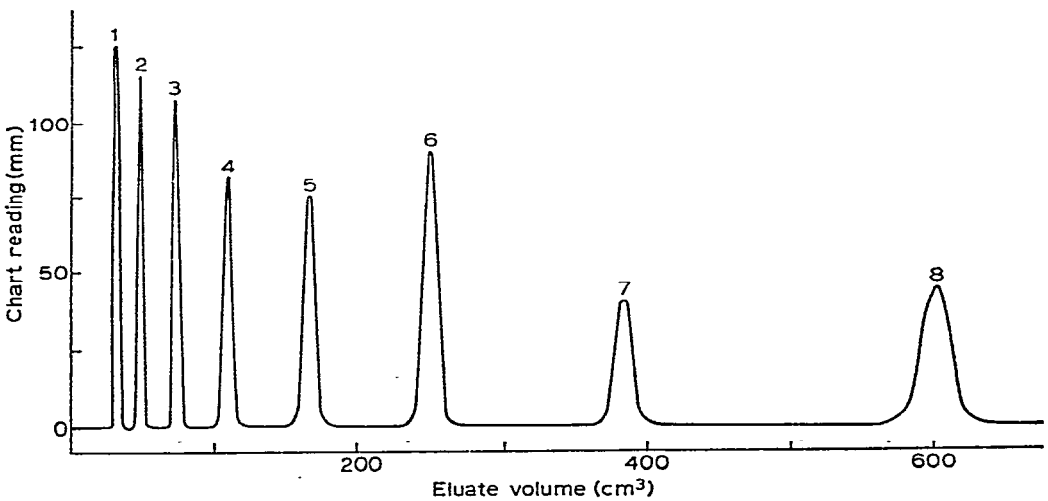

Fig. 4. Partition chromatography of xylan oligosaccharides in 75% aqueous ethanol at 75°. Resin bed: 4×600 mm, Technicon T5C, SO_4^{2-}, 14–17 μm. Flow rate: 2.8 cm.min^{-1}. 1, D-xylose (5 μg); 2, di- (5 μg); 3, tri- (6.5 μg); 4, tetra- (9 μg); 5, penta- (13 μg); 6, hexa- (25 μg); 7, hepta- (18 μg); and 8, octa-saccharide (25 μg).

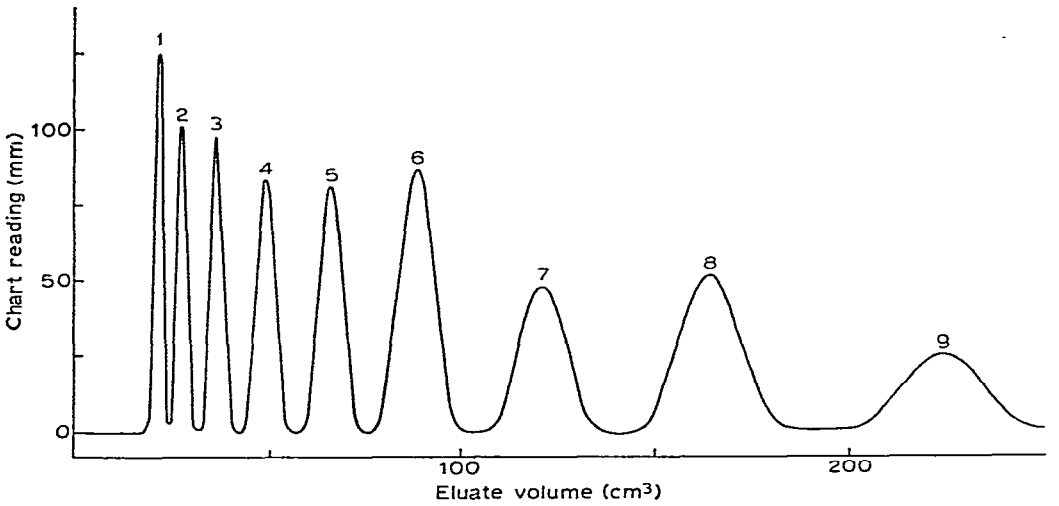

Fig. 5. Partition chromatography of xylan oligosaccharides in 80% aqueous ethanol at 75°. Resin bed: 4×910 mm, Dowex 50W-x8, Li^+, 17–21 μm. Flow rate: 3.0 cm.min^{-1}. Peaks 1–8, see Fig. 4. Peak 9, nonasaccharide (18 μg).

In the run on the sulphate column (Fig. 4), the ethanol concentration was chosen so that D-xylose and the disaccharide were well separated, *i.e.* the distances between the peaks corresponding to the higher oligomers became excessively large with

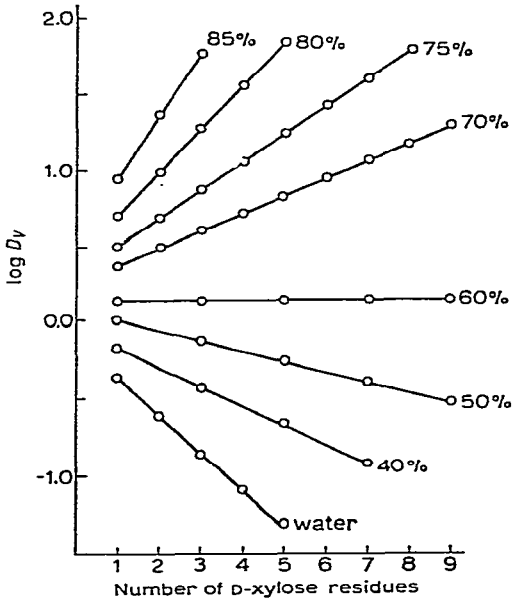

Fig. 6. Relationship between log D_V and number of D-xylose residues in the oligosaccharides at various concentrations of ethanol. Sulphate resin (T5C, 14–17 μm), 75°.

correspondingly high retention times. The last component in this run was eluted after 22 h. In agreement with observations made with other sugars[14], the optimal concentration of ethanol is higher on the lithium column than on a sulphate column. The chromatogram from the lithium column [Fig. 5] was recorded in a run at an ethanol concentration slightly lower than that which gave a complete separation of the first oligomers. In this run, the nonasaccharide appeared after 8 h.

In an earlier study[15] of the cellobiose oligomeric series, it was found that a straight-line relationship exists between the logarithm of the distribution coefficient and the number of monomeric units (n) in the oligosaccharides. In the present study of D-xylose oligomers, the validity of this rule was studied for a larger number of oligomers and over a wide range of eluent composition.

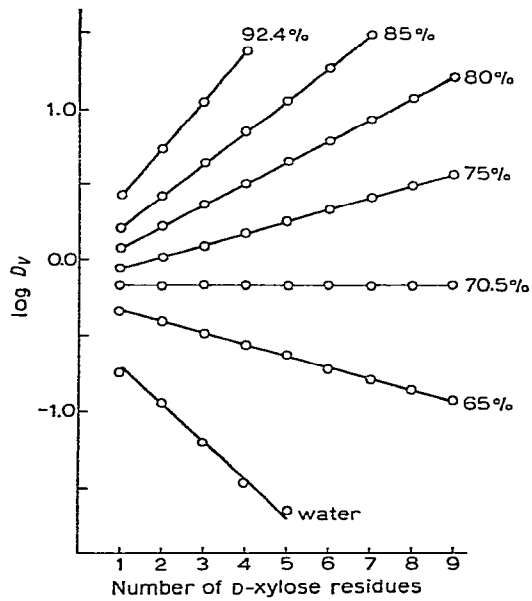

Fig. 7. Relationship between log D_V and number of D-xylose residues in the oligosaccharides at various concentrations of ethanol. Lithium resin (Dowex 50W-x8, 17–21 μm), 75°.

The results reproduced in Figs. 6 and 7 show that Martin's rule holds true at both high concentrations of ethanol, where the sugars are strongly held by the resin, and at low concentrations of ethanol, *i.e.* under conditions where the concentration of the sugar inside the resin is lower than in the external solution. The rule holds true even with water as eluent.

In agreement with earlier observations[16], the sugars are eluted in the order of increasing molecular size at a high concentration of ethanol, whereas at a low concentration of ethanol the elution order is reversed. Since the swelling of the resin is higher in water than at a high concentration of ethanol, the exclusion effects observed

in water are not due to steric exclusion or restricted diffusion. Instead, the exclusion effects are due mainly to the high swelling-pressure of the resin and, as found by Mattisson and Samuelson[17], the main factor which determines the distribution coefficient is the product of the swelling pressure and the partial, molal volume of the non-electrolyte.

A consequence of the fact that log D_V is proportional to n, at both high D_V values and when the separations are governed by exclusion effects ($D_V < 1 - \varepsilon$), is that a critical eluent composition should exist at which all oligomers exhibit the same distribution coefficient, *i.e.* where no separation occurs.

With the applied resins and working temperature, the critical concentration of ethanol was 60% for the sulphate resin. At this concentration, all oligomers were eluted at $D_V = 1.3$. For the lithium resin, the critical point corresponded to a higher concentration (70.5%). The D_V value of all the species was 0.7. This means that the concentration distribution ratio, defined as the ratio of the molar sugar concentration inside the resin to the concentration in the external solution, was ~ 2.2 for the sulphate resin at the critical eluent concentration, whereas the corresponding value for the lithium resin was ~ 1.1.

The results given in Figs. 6 and 7 can serve as a guide in the choice of working conditions for analytical as well as preparative purposes. Obviously, it is advantageous, for most practical purposes, to carry out the separation at an ethanol concentration higher than the critical concentration, instead of applying the exclusion technique. As can be seen, the choice of ethanol concentration is extremely important. A concentration which is too low gives unfavourable separation factors, whereas an excessively high concentration gives rise to high retention times and a corresponding broadening of the peaks which can be disturbing, especially for the higher oligomers. Gradient elution with a decreasing concentration of ethanol can be applied to speed up the elution of the higher oligosaccharides, but it should be emphasized that only small changes in eluent concentration can be made without disturbances occurring in the resin bed due to the swelling changes[16].

In large-scale separations for preparative purposes of the whole series of oligomers, it is preferable to elute the lower members as a group of overlapping bands at a low concentration of ethanol (*e.g.* 72% on a sulphate column), and to re-chromatograph the lower oligomers in a separate run at a somewhat higher concentration (*e.g.* 80%).

The results obtained in the present work show that gel-permeation chromatography on ion-exchange resins in water can be applied in separations of the lower homologues of the β-(1→4)-linked D-xylose series, but that very long columns are required to obtain quantitative separations. This technique is inferior both to permeation chromatography on polyacrylamide gel and to partition chromatography on an ion-exchange resin in aqueous ethanol.

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REFERENCES

1 R. L. WHISTLER AND C. C. TU, *J. Amer. Chem. Soc.*, 74 (1952) 3609.
2 C. T. BISHOP, *Can. J. Chem.*, 33 (1955) 1073.
3 W. BROWN AND Ö. ANDERSSON, *J. Chromatogr.*, 57 (1971) 255.
4 C. P. J. GLAUDEMANS AND T. E. TIMELL, *J. Amer. Chem. Soc.*, 80 (1958) 941.
5 R. L. WHISTLER AND D. F. DURSO, *J. Amer. Chem. Soc.*, 72 (1950) 677.
6 B. ARWIDI AND O. SAMUELSON, *Sv. Papperstidn.*, 68 (1965) 330.
7 A. I. VOGEL, *Elementary Practical Organic Chemistry*, Part 3, Longmans Green, London, 1958, p. 740.
8 P. JONSSON AND O. SAMUELSON, *Science Tools*, 13 (1966) 17.
9 M. JOHN, G. TRÉNEL, AND H. DELLWEG, *J. Chromatogr.*, 42 (1969) 476.
10 O. SAMUELSON, *Ion Exchange Separations in Analytical Chemistry*, Almqvist and Wiksell, Stockholm; Wiley, New York, 1963.
11 M. JOHN, G. TRÉNEL, AND H. DELLWEG, *Chromatographie von Kohlenhydraten und Nucleotiden an Polyacrylamid-Gel-Säulen*, Technicon Symposium, Bad Homburg, 1970.
12 P. ALBERSHEIM, D. J. NEVINS, P. D. ENGLISH, AND A. KARR, *Carbohyd. Res.*, 5 (1967) 340.
13 T. E. TIMELL, *Advan. Carbohyd. Chem.*, 19 (1964) 247.
14 O. SAMUELSON AND H. STRÖMBERG, *Acta Chem. Scand.*, 22 (1968) 1252.
15 E. MARTINSSON AND O. SAMUELSON, *J. Chromatogr.*, 50 (1970) 429.
16 O. SAMUELSON, in J. MARINSKY (Ed.), *Ion Exchange II*, Dekker, New York, 1969.
17 M. MATTISSON AND O. SAMUELSON, *Acta Chem. Scand.*, 12 (1958) 1386.
18 IUPAC-recommendation, *Pure Appl. Chem.*, 8 (1964) 553.