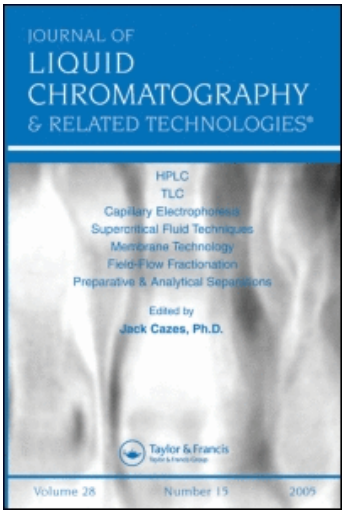

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LIQUID CHROMATOGRAPHY APPLIED TO OLIGOSACCHARIDE FRACTIONATION

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ABSTRACT

This review deals with the separation of monosaccharides and oligosaccharides by liquid chromatography. The principles of the different methods are recalled : successively, gel permeation chromatography, ion exchange chromatography, solid-liquid and liquid-liquid partition chromatographies are described. Then, for each method, the main results obtained in the field of saccharides are listed for natural or chemically modified forms.

The latest technique developed is the high performance chromatography which allows to perform separations often in a matter of a few minutes with better selectivity due to the improvements in column packing technology.

Some consideration of the detectors adapted for saccharide analysis are given at the end with a list of the principal types of applications investigated.

INTRODUCTION

This review is concerned with the application of liquid column chromatography to the separation of monosaccharides and oligosaccharides. Much interest exists now in this problem due to the occurrence of these substrates in various fields such as foods, natural products analysis, structural investigations on polysaccharides. Recently, a new method has appeared, more rapid and more selective

(High Performance Liquid Chromatography) and it seems to be interesting to review this more efficient method of analysis as published in the literature up to date.

The main chromatographic techniques for oligosaccharides fractionation are :

- paper chromatography, corresponding to a liquid-liquid partition ; the stationary phase is adsorbed on cellulose. It was a useful method but it presents disadvantages : long time for elution, difficulties of detection and quantitative determination.

- thin layer chromatography (CCM) using cellulose, silica, Kieselguhr as sorbents. Mechanisms for fractionation are adsorption, partition or ion exchange. Just as in the previous method, it is difficult to perform a quantitative determination.

- gas chromatography (CPG) is a rapid method which allows quantitative determination. Nevertheless, it needs derivatization to volatilize derivatives. Trimethylsilyl derivatives, or better alditol acetates are generally used for monosaccharide analysis.

- liquid column chromatography which, with the recent improvements and especially packings, has been developed under the name of high performance or high pressure liquid chromatography (H.P.L.C.)

This review will list results obtained by this last type of method on oligosaccharides or their derivatives. Successively,
- gel permeation chromatography
- ion exchange chromatography
- adsorption or solid-liquid partition chromatography
- liquid-liquid partition chromatography

will be discussed. Finally, some technical equipment such as detectors will be mentionned and some applications for oligosaccharide separations given.

A/ GENERAL CHARACTERISTICS OF LIQUID CHROMATOGRAPHY

The fractionation is based on the difference in the equilibrium distribution coefficients of the solutes between the mobile and the stationary phases. The solutes pass through a column flowing with a mobile eluent but their rate of mobility depends on their relative affinity for the stationary and the mobile phases.

Retention

Generally, the elution of a pure solute gives a symmetrical Gaussian peak characterized by the elution volume (V_e), retention volume (V_R) or time of retention (T_R) ; T_R is directly related to V_R and depends on the flow rate of the eluent (Figure 1). The characteristics V_e, V_R or T_R are a direct function of the distribution coefficient K between the two phases as follows :

$$V_R = V_m + K V_s$$

V_m is the volume of the mobile phase or "dead" volume of the column,
V_s is the volume of the stationary phase.

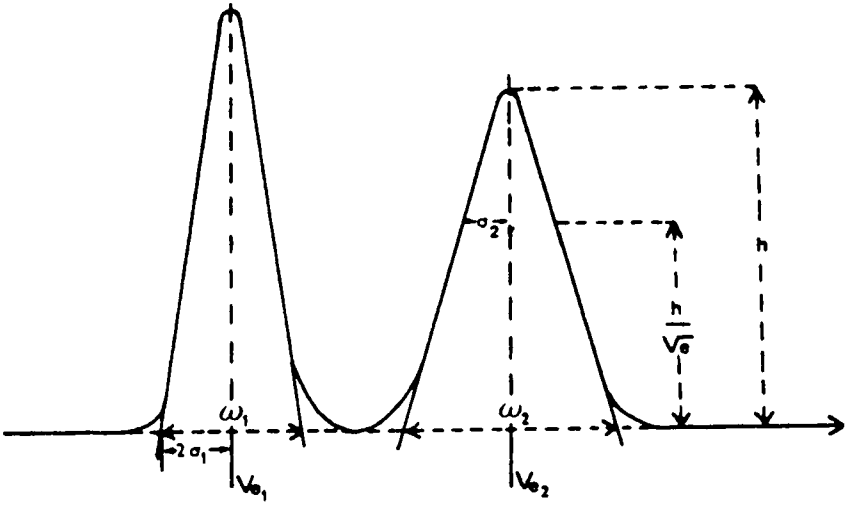


Figure 1. Parameters characterizing the elution of two solutes (1) and (2).

Retention can be expressed in terms of a capacity factor k' :

$$k' = K \frac{V_s}{V_m} = \frac{\text{number of molecules of solute in the stationary phase}}{\text{number of molecules of solute in the mobile phase}}$$

It follows :

$$V_R = V_m (1 + k')$$

or :

$$k' = \frac{T_R - T_o}{T_o} = \frac{V_R - V_m}{V_m}$$

The term k' is directly determined on the chromatogram ; it depends on the experimental conditions (V_m , V_s) and mainly on K.

Resolution

The column resolution R_s characterizes the quality of a separation between two solutes 1 and 2 (see Figure 1) ;it is given by :

$$R_s = \frac{2\,(T_{R1} - T_{R2})}{w1 + w2} = \frac{2\,(V_{R1} - V_{R2})}{w1 + w2}$$

where w is the chromatogram peak width formed by intersection of the tangents to the curve inflection points with the baseline in units of retention volume or time. The subscripts refer to solute 1 and 2, respectively.

R_S may also be written as :

$$R_S = \frac{(T_{R1} - T_{R2})}{4\sigma}$$

where σ is the peak standard deviation (to a first approximation $\sigma_1 = \sigma_2$).

When $R_S < 0.8$, the separation is generally bad. It is possible to relate R_S to the fractionation parameters. For two neighbouring peaks (with $w_1 \simeq w_2$), one proposes :

$$R_S = \frac{1}{4} \left(\frac{\alpha-1}{\alpha}\right) \left(\frac{k'_2}{k'_2+1}\right) (N_2)^{1/2}$$

where α is the relative retention, N the number of theoretical plates. The parameter α is defined as :

$$\alpha = \frac{T_{R_2} - T_o}{T_{R_1} - T_o} = \frac{k'_2}{k'_1} = \frac{K_2}{K_1}$$

and reflects the separation of solutes 1 and 2 due to differences of interactions with the mobile and stationary phases (selectivity of the process).

The number of theoretical plates N is given by :

$$N = 16 \left(\frac{T_R}{w}\right)^2 = \left(\frac{T_R}{\sigma}\right)^2$$

N is related to the efficacity of a column and is proportional to its length.

For simplification one introduces the definition of the number of effective theoretical plates N_{eff} :

$$N_{eff} = \left(\frac{k'}{k'+1}\right)^2 N = 16 \left(\frac{T_R - T_o}{w}\right)^2 = \left(\frac{T_R - T_o}{\sigma}\right)^2$$

and $R_S = \frac{1}{4} \left(\frac{\alpha - 1}{\alpha}\right) N_{eff}^{1/2}$

which express R_S as a function of two quasi-independent parameters. One limits this discussion to the most useful definitions for liquid chromatography needed in the following.

B/ GEL PERMEATION CHROMATOGRAPHY (GPC)

Gel permeation chromatography is based on a steric process of exclusion depending on the molecular weight, or rather the hydrodynamic volume, of the solutes.

The support is a tridimensionnal crosslinked gel, and the elution volume increases when the molecular weight decreases.

1. Principle of the method

GPC is a partition between the liquid mobile phase outside the gel and the solvent inside the pores.

High molecular weight solutes cannot penetrate inside the pores ; they are excluded and are eluted in a volume V_e equal to the dead volume V_o. Very low molecular weight solutes penetrate freely into the pores and are eluted in the total volume $V_T = V_o + V_p$ (V_p is the porous volume ; it is equal to the volume of the stationary phase).

The coefficient of partition K_d in this chromatographic process is defined as the ratio between solute concentration in the mobile phase and that in the stationary phase. It reflects also the fraction of pores accessible to diffusion of the solutes (1)

$$V_e = V_o + K_d V_p$$

$$K_d = \frac{V_e - V_o}{V_p}$$

Fractionation is obtained for $0 < K_d < 1$; when $K_d = 0$, the solute is excluded and when $K_d = 1$, the solute penetrates all the pores. Sometimes K_d is found larger than 1 when some adsorption modifies the process of distribution.

Some authors (2) prefer to introduce another coefficient K_{av} :

$$K_{av} = \frac{V_e - V_o}{V'_T - V_o}$$

where V'_T is the total volume of the column, $V'_T = V_o + V_p + V_g$

(V_g is the volume occupied by the gel itself).

This K_{av} is no longer in use.

Molecular-weight determination. Considering the process of steric exclusion, the volume of elution used to be related to the molecular weights of the solutes and GPC was used to determine molecular weights after calibration of the column. More recently (3) it was demonstrated that the separation of polymers is based on the hydrodynamic volume proportional to the product $[\eta]M$ ($[\eta]$ is the intrinsic viscosity in the same solvent ; M is the molecular weight) (Figure 2).

In Figure 2, the universal calibration curve is schematically drawn ; it is independent of the polymer, eluent ... when the gel is rigid. For each elution volume V_{e_i} , a peak is characterized by a product $[\eta]_i M_i$ and then M_i when $[\eta]_i$ is known in an independent experiment.

When oligomers are fractionated, large resolution is necessary to isolate solutes of an homologous series.

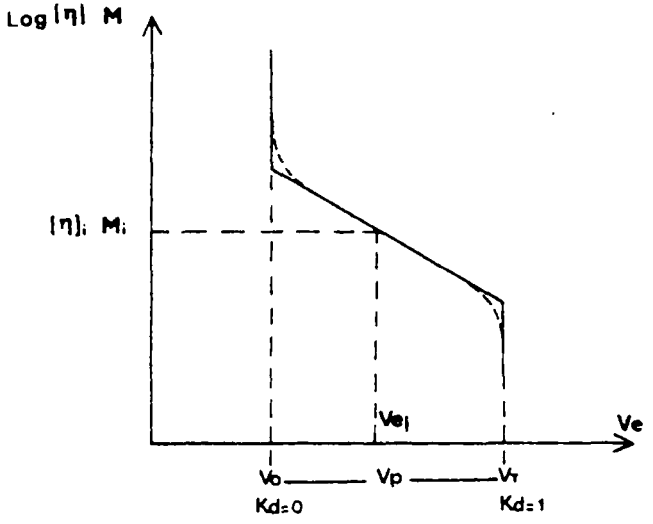


Figure 2. Universal calibration curve in GPC experiment

Generally, it is necessary to increase the length of the column or recycle the effluent to avoid too high column back pressure. Many difficulties appear when charged molecules are chromatographed by comparison with neutral solutes. These effects have been described by Neddermeyer et al. (4, 4 bis) and ourself. An electrostatic exclusion effect is combined with the steric effect : the volume of elution decreases more and more as the solute concentration decreases, the peaks are dissymmetrical ... (5, 5bis, 5 ter, 6, 6 bis). To control the elution volume of an ionic solute it is necessary to add an electrolyte (in a concentration larger than 5.10^{-2}M) to the eluent.

2. The application of gel permeation chromatography to the separation of carbohydrates.

Gel permeation chromatography, known since 1956 (7), was the subject of a previous bibliography by Churms (8). In a gel permeation system, the most important part is the gel itself, so the separation of carbohydrates is investigated with regard to the chromatographic supports. Principal fractionations are given and the mechanisms which modify the filtration are briefly discussed.

a) Gels formed by cross-linked dextrans.

The use of dextran gels, commercially available under the name of Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) was first reported by Porath and Flodin (9, 9bis). This technique, also known as gel filtration, has been employed to fractionate series of cellodextrins (9 ter, 10) and afterwards many homologuous series of neutral (11, 13) or acidic oligosaccharides (14, 17). Brown has reported the separation of xylodextrins (12, 12bis) and mannodextrins (13) on columns of Sephadex G15. With acidic sugars, charge effects have to be taken into account and solution of electrolytes are used as eluents. With 0.05 M phosphate buffer at pH 7 as the eluent, Rexova-Benkova (14) separated, on Sephadex G-25, the first five homologues of D-galacturonic acid obtained by partial acid hydrolysis or by enzymatic digestion of pectic acid. Kohn (15)

resolved mono, di, tri and tetra-saccharides of the galacturonic acid series on Sephadex G-15. Flodin (16) used Sephadex G-25 for separating the oligosaccharides produced by enzymic digestion of hyaluronic acid and chondroïtin 4-sulfate, and Dietrich (17) fractionated the products obtained on enzymic degradation of heparin on Sephadex G-25, with 0.01 M acetic acid as the eluent.

Water is generally used in the fractionation of neutral sugars but there are a few exceptions (18, 19, 20). Helting (18) used Sephadex G-25 with 9/1 (V/V) water-ethanol as the eluent to fractionate the oligosaccharides obtained on acid hydrolysis of chondroïtin 6-sulfate from umbilical cord, and the sialic acid oligosaccharides have been separated by Ohman (19) on Sephadex G-25 with 99/1 water/butyl alcohol. Zelesnick (20) has also reported the separation of Glucose and Rhamnose using Sephadex G-25 with 60/15/25 (V/V) butyl alcohol-M acetic acid-water.

Gel filtration may be also used to fractionate methylated and acetylated oligosaccharides on the lipophilic gel Sephadex LH-20 with methanol as the eluent (21, 22).

The nature of the separation has been extensively investigated by Brown (23, 24). Temperature and solvent dependences of the partition coefficient suggest weak sorption on the dextran gel with the homologuous series of oligosaccharides : cellodextrins, xylodextrins and mannodextrins. The specific solute-gel interactions are related to their relative solubilities. Gel permeation on dextran gels is not a simple sieve effect, adsorption can play a role (25), and the anomalous chromatographic behaviour of Schardinger dextrins on Sephadex G-15 (26) is a good example ; both α and β Schardinger dextrins are retarded and eluted after glucose. This competition between molecular size partition and adsorption has been investigated by Bertoniere (27) on Sephadex G-15 with sugars and sugar derivatives.

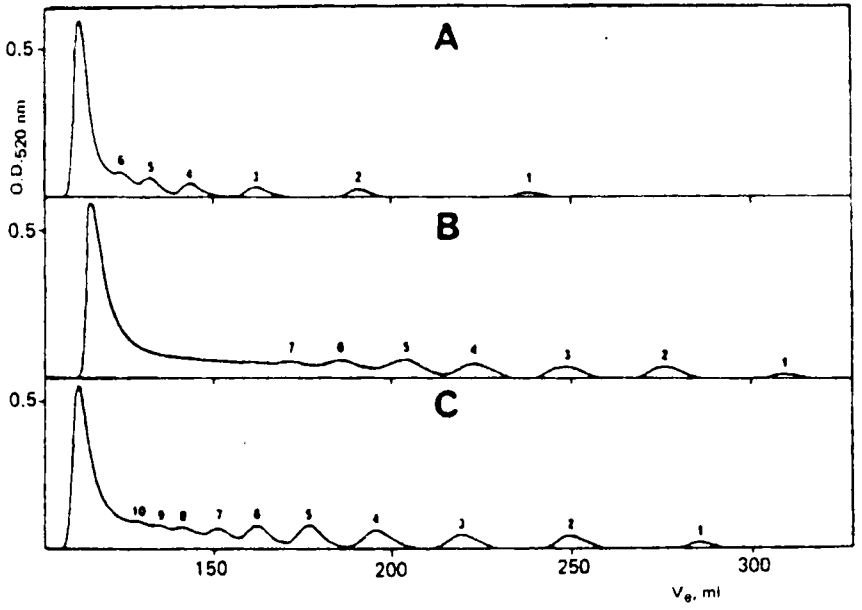
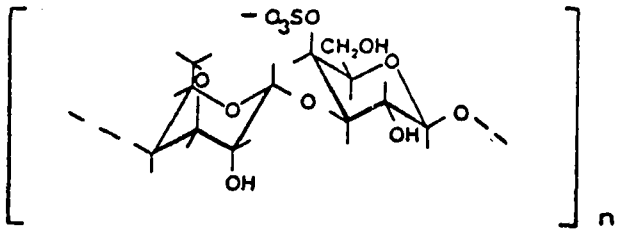


Figure 3. Elution profiles of oligogalacturonic acids on a Bio-Gel P-2 column (203 x 2 cm). A, 0.1 M acetate buffer pH 5 at 65° C ; amount injected, 30 µl of a 20 mg/ml oligomer solution. B, 0.1 M acetate buffer pH 3.6 at 25° C ; amount injected, 50 µl of a 20 mg/ml oligomer solution. C, 0.1 M acetate buffer pH 3.6 at 65° C ; amount injected, 60 µl of a 20 mg/ml oligomer solution. The numbers 1,2,3,...10 refer to the degree of polymerization (40).

b) Polyacrylamide Gel Chromatography.

Many authors have reported the successful use of the polyacrylamide gel Bio-Gel P (Biorad-Laboratories U.S.A.) to separate oligosaccharides. Trenel (28, 31) separated maltodextrins containing up to thirteen glucose units within four to seven hours on a Bio-Gel P2 column. The resolving power was markedly improved by high temperature (28), and maltose and isomaltose, and maltotriose and isomaltotriose were resolved (29). Gel filtration has been used by Enevoldsen to separate series of homologuous oligosaccharides



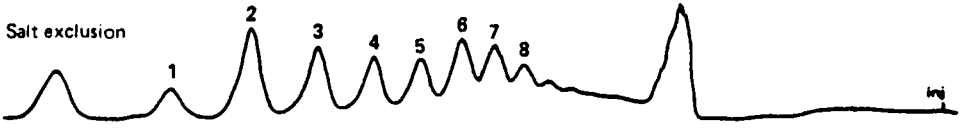


Figure 4. Separation of κ-carrageenan oligomers (n = 1,2...) after enzymic hydrolysis. Biogel P-6 ; eluent : $NaNO_3$ 5.10^{-2} M. T° = 25° C (41).

and their corresponding alditols (32). The fractionation of malto-oligosaccharides of various structures (linear, singly branched, multiply branched and cyclic) on Bio-Gel P2 (33) and P6 (34) or P4 (35) revealed characteristic differences and tne fine structure of the oligosaccharide seems to determine its position in the elution profile. Pontis (36) reported the complete separation of the lower members of the fructosan series (up to DP = 10) using Bio-Gel P2, and the oligosaccharides obtained on acid hydrolysis of chitin have been separated by Raftery (37). As with dextran gels, ion-exclusion of acidic sugars occurs when the column is eluted by water; this interaction is avoided by increasing the ionic strength of the eluent. Kohn (38) used a column of Bio-Gel P4 to separate oligomers of guluronic acid, Van Houdenhoven (39) and Thibault (40) have reported the isolation of oligogalacturonic acids (Figure 3). We fractionated also the oligosaccharides produced by enzymic hydrolysis of K-Carrageenans on Bio-Gel P6 (41) (Figure 4).

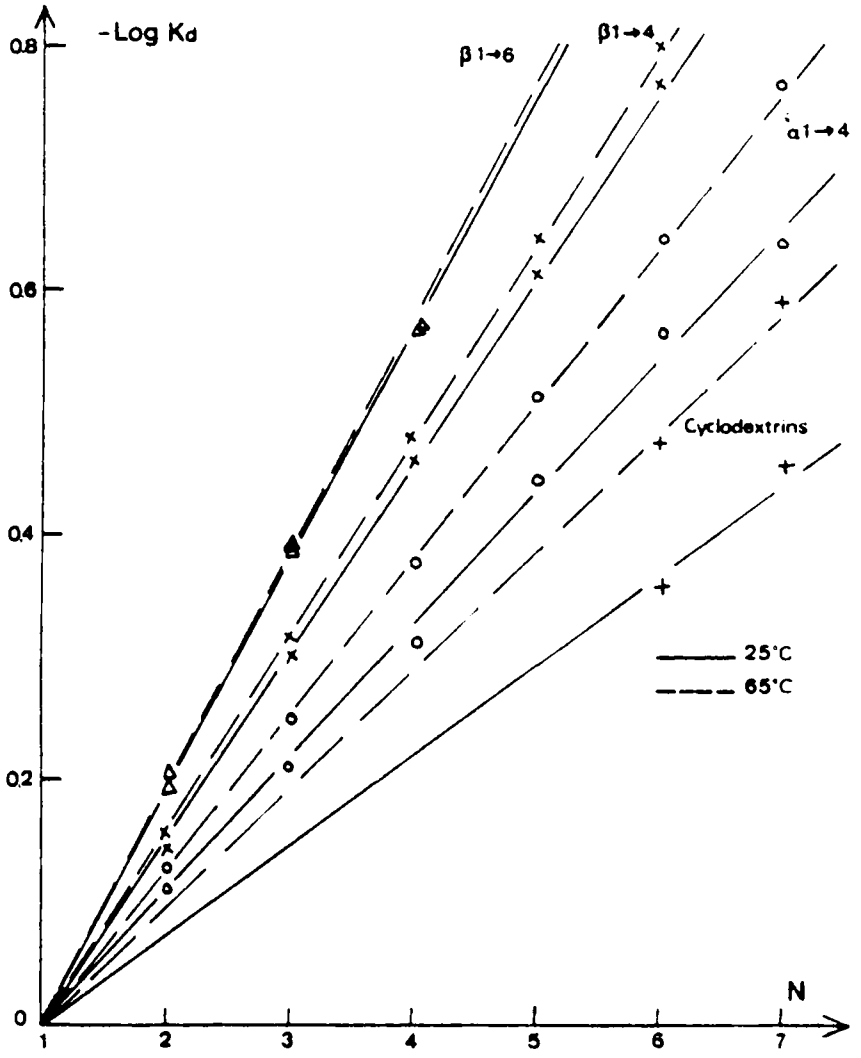


Figure 5. Partition coefficient K_d as a function of the degree of polymerization N for different oligosaccharide series at 25° C (—) and 65° C (.....)

Δ gentiodextrins x cellodextrins

O maltodextrins + cyclodextrins

Biogel P-2 ; eluent H_2O (45).

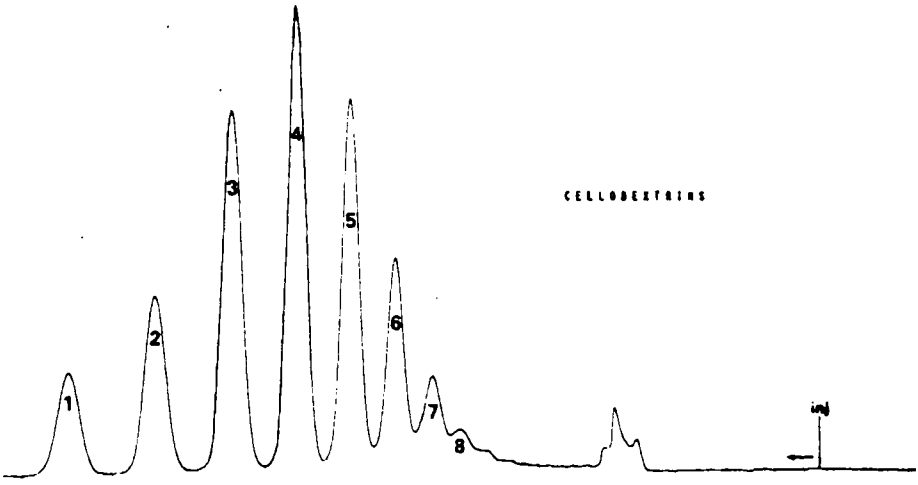


Figure 6. GPC chromatogram obtained on cellodextrins. Biogel P-2; eluent H_2O ; T° = 65° C.

Partially methylated mono- and oligosaccharides can also be characterized by gel filtration on Bio-Gel P2 or P4, and an excellent separation of glucose and its mono-, di-, tri- and tetramethyl ethers was obtained by Grellert (42).

The separation process has been investigated by Brown (10, 12, 12 bis, 13, 23, 24) and the operational parameters (solute concentration, column length, flow-rate and temperature) have been discussed by Sabbagh (43, 43 bis). In view of the principal data obtained on the scope of gel permeation of saccharides (44) and of our results obtained on Bio-Gel P2 (45, 45bis), it seems that adsorption is of less importance on Bio-Gel than on Sephadex. We have tested four series of oligosaccharides whose monomeric unit is D-glucose and have discussed steric exclusion as a result of hydrodynamic investigations. At lower temperature there is a solute-gel interaction characteristic of the oligomer series but at 65°C the fractionation can be interpreted on the basis of an exclusion mechanism taking into account the hydrodynamic volume expressed by the product $[\eta]M$.

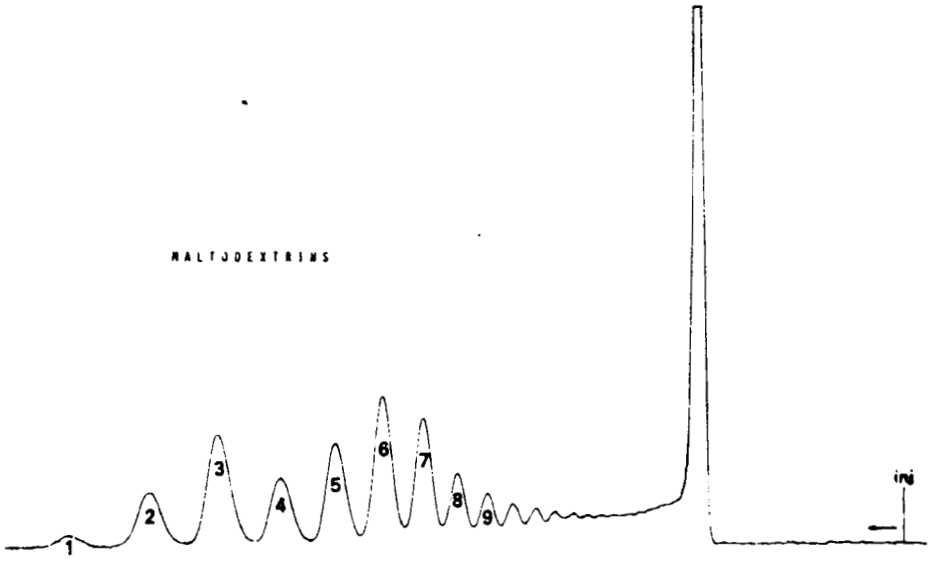


Figure 7. GPC chromatogram obtained on maltodextrins Biogel.P-2 ; eluent H_2O ; T° = 65° C.

The affinity follows the sequence : cyclo-oligosaccharides > malto-oligosaccharides > cello-oligosaccharides > gentio-oligosaccharides (Figure 5).

Typical fractionations of cello- and maltodextrins are given in Figures 6 and 7. From a general point of view, the values of -log K_d and molecular weight (or the degree of polymerization) are in good agreement with a linear function in the temperature range of 25-65° (Figure 8). These results expressed in terms of log $[\eta]M$ lie pratically on the same curve at 65°C and then steric exclusion is proved to be the only mechanism for fractionation (Figure 9).

c) Other supports :

- Decrystallised cotton cellulose has permeation properties comparable to those of cross-linked dextran and polyacrylamide gel (46).

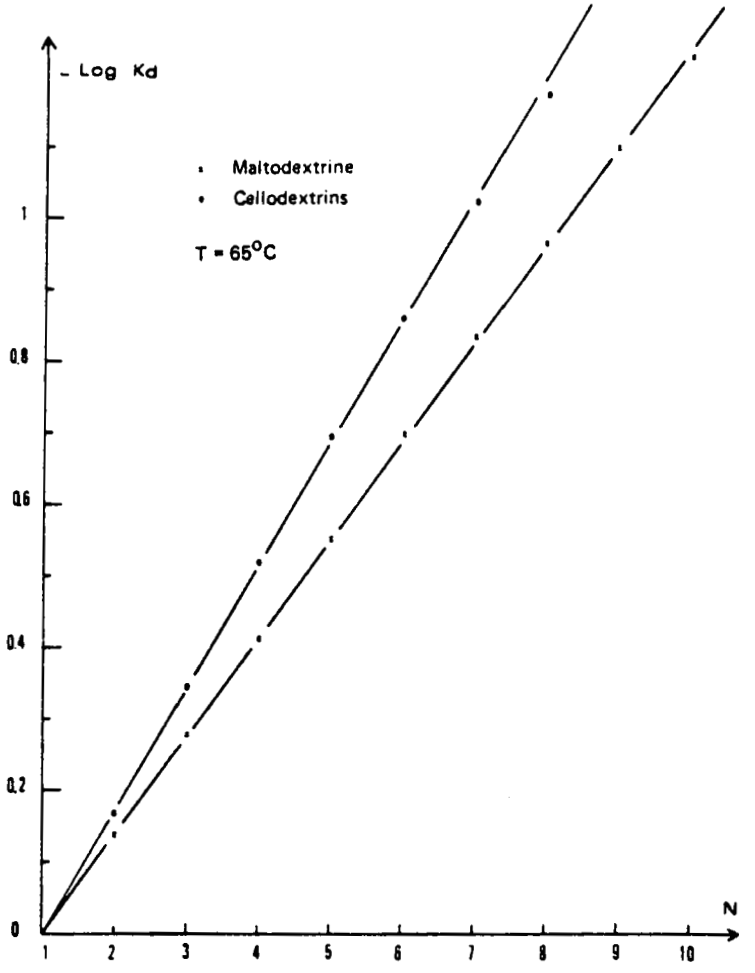


Figure 8. Dependence of the partition coefficient K_d on the degree of polymerization N of maltodextrins and cellodextrins (experimental results Figures 6 and 7).

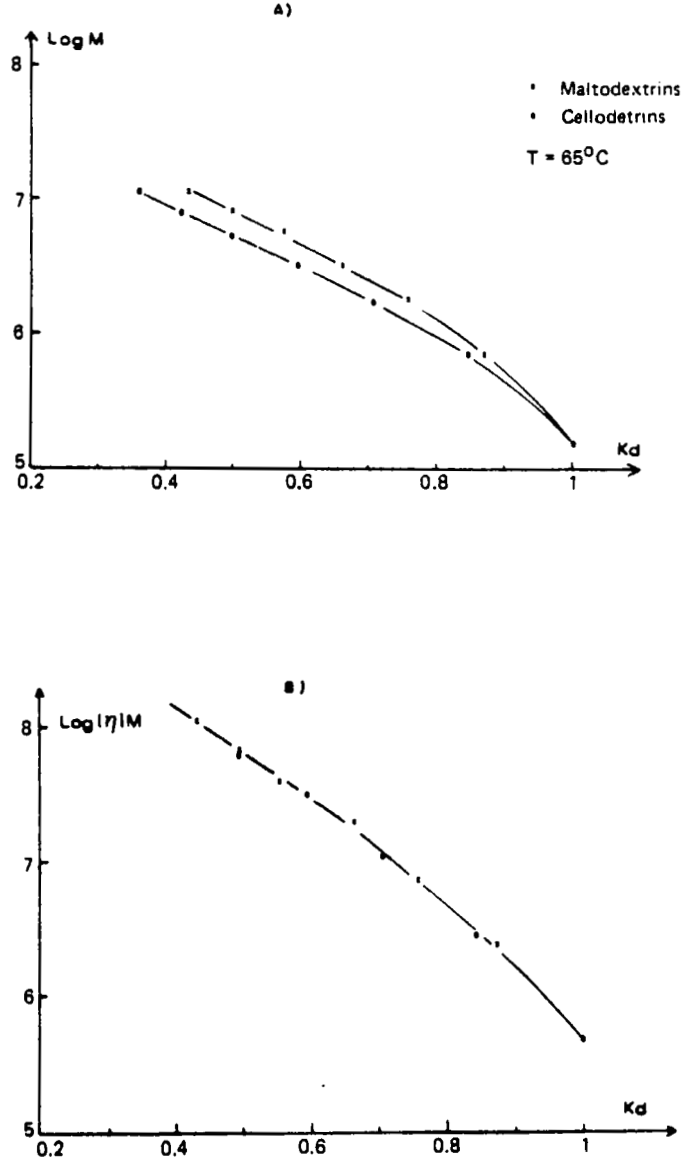


Figure 9. Same experimental results expressed in terms of molecular weight (A) and hydrodynamic volume $[\eta]M$ (B) dependences on the partition coefficient K_d.

It is permeable to compounds of molecular weights below 1500 and a partial separation of raffinose or stachyose from fructose or erythrose has been obtained by Martin. Relative retentions do not differ sufficiently to give efficient separations and cross-linked cellulose has been found to be a more convenient support (47). Xylodextrins, cellodextrins, mannodextrins have been separated by Brown (48) on regenerated cellulose obtained by the cross-linking of viscose with epichlorohydrin. Chemisorption was found with the cellodextrins at chain lengths greater than three units, and weak adsorption for the xylodextrins and mannodextrins. The results suggest that identical conformation of the gel surface and the solute is required for adsorption.

- Starch gel prepared by cross-linking potato starch under alkaline conditions has been used for gel permeation chromatography of oligosaccharides. A mixture of raffinose, cellobiose and glucose has been resolved by Luby (49), using water as the mobile phase ; but the chromatographic properties of this support are not comparable with those of Sephadex or Bio-Gel.

Gel permeation is a convenient method for fractionation of oligosaccharides but a good separation of monosaccharides is very difficult to obtain with water as the eluent, except for glucose and ribose or methyl glycosides. The main results of the literature on the application of gel permeation chromatography to carbohydrates are summarized in Table 1. Dextran gel and polyacrylamide gel are the most widely used supports in gel chromatography. These gels are hydrophilic and swell in water ; water is also used as the mobile phase and there is no problem with water-soluble materials such as oligosaccharides.

However, the long time of the analysis is an substantial disadvantage ; as dextran and polyacrylamide gels have poor mechanical strength, they will not withstand the higher pressure required for faster analysis.

TABLE 1

List of Experimental Oligosaccharide Separations Obtained by Gel Permeation Chromatography.

Gel / Oligosaccharides	SEPHADEX		BIO-GEL		
	G-15	G-25	P2	P4	P6
Cellodextrins	10,12,12bis 13,23,24	9 ter	10,12,12bis,13, 24, 45		
Cyclodextrins	26		34,45		
Fructosan series			36		
Gentiodextrins			45		
Isomaltodextrins			29	32,33 35	
Maltodextrins			28,29,30,31,33 34,45,43,43bis	32, 35,43	
α-1,4/α-1,6 linked oligosaccharides.			33,34	35	34
Mannodextrins	13		13		
Xylodextrins	12,12bis,13 23.		12,12bis,13		
Chitin oligosaccharides.			37		
Maltodextrins alditols				32	
Isomaltodextrins alditols				32	
Oligogalacturonic acids	15	14	39,40	39	
Oligoguluronic acids				38	
Oligohyaluronic acids		16			
Sialyloligosaccharides.		19			
Chondroïtin Sulfate oligosaccharides.		16,18			
Heparin Oligosaccharides.		17			
Carrageenan Oligosaccharides					41

C/ ION EXCHANGE CHROMATOGRAPHY.

Ion exchange chromatography has proved very useful in carbohydrate analysis. This method has been extensively reviewed by Samuelson (50), Jandera (51, 52), Kennedy (53) and Lawrence (54). Our survey is divided into the following sections : anion-exchange resins and cation-exchange resins. The chemical aspects of the separation process are discussed and the main results are summarized.

1. Theoretical aspects of ion-exchange chromatography:

When a non-electrolyte solution is in contact with ion exchangers, the solute can enter the resin phase. This sorption is a reversible phenomenon. If there is no interaction of any kind, it should be expected that the concentrations of the solute inside the resin and in the external solution are equal. This behaviour is rarely found and several effects have to be considered :

- "Salting-out" effects.

The fixed ionic groups and counterions form solvation shells and only a fraction of the total internal solvent is free. As only a part of solvent inside the resin is available for dissolving the solute, the concentration, which refers to the total solvent, is lower in the resin than outside. This effect is most pronounced when the resin is highly cross-linked and the counterions are strongly hydrated.

- Interactions with counterions ; "Salting-in" effect.

The solubilities of a number of organic compounds are increased by the addition of acids. With strong-acid cation exchangers, analoguous effects are observed. This effect is dependent on the resin form ; sorption of a non-polar solute is greatly increased when inorganic counterions are replaced by organic ions such as $N(C_2H_5)_4^+$ but sorption of sugars is reduced. On the other hand, this effect becomes very important when the solute forms strong com-

plexes or even chelates with the counterion. For example neutral saccharides are "salted in" by anion-exchange resins in the sulphite form and in the borate form.

- Swelling Pressure and "sieve action".

The interior of the swollen resin is under a higher pressure than the external solution. The pressure difference is called the swelling pressure. The swelling pressure affects the solute uptake in such a way that the larger molecules are more strongly affected. The relationship describing this phenomenon is :

$$\Pi \, v_N = - RT \ln \frac{\bar{a}_N}{a_N}$$

where $\bar{a}_N/a_N$ is the ratio between the activity coefficients of solute in the resin phase to that in the external solution, Π the swelling pressure, and v_N the partial molal volume of the solute.

This swelling pressure is high if the resin is highly cross-linked, therefore with increasing size of the solute molecule and increasing degree of cross-linking, the effect becomes important.

In addition, sorption of larger molecules can be limited by the purely mechanical sieve action of the matrix. Sorption equilibrium, and in consequence the elution volume, will be dependent on :

- specific interactions between the neutral solute and the matrix, fixed ionic groups, or counterions,
- molecular size of the solute and degree of cross-linking of the ion-exchangers.

With solutions of electrolytes, the effects are more complex. In contrast to non-electrolytes, the electrolytes are excluded by ion-exchangers ; this exclusion is favoured by high capacity and cross-linking of the resin. On the other hand, complex formation, inter-

actions between the ions of the electrolyte and the fixed ionic groups or the matrix can offset the ionic exclusion. The elution volume of ionic solutes depends on the nature of the eluent, buffer composition and pH.

2. Ion exchange chromatography of carbohydrates :

From a pratical point of view three processes can be envisaged :

- The sugar, eluted with water or aqueous solutions of non-complexing agents, interacts by its weakly acidic alcoholic groups with the resin.

- In mixture of water and ethanol, the chromatography of sugars is a partition between the phase inside the resin and the external solution.

- The eluent reacts with sugars to produce negatively charged complexes ; the differences in the stability and the affinity of the various complexes can be used for the separation.

a) Separation of saccharides on anion-exchange resins using water or aqueous solutions of non-complexing agents as the eluent.

In 1960, Hough (55) investigated the use of anion exchange resins in CO_3^{--} and HCO_3^- forms for the separation of carbohydrates. He found that a column of Permutit could be used for the separation of a mixture of raffinose, sucrose, and glucose in 36 hours using water as eluent. On the hydroxide form of Dowex-1 resin, Austin et al. (56) separated : methyl α-D-galactopyranoside, methyl β-D galactopyranoside, methyl α-D galactofuranoside, methyl β-D galactofuranoside in that order. In a similar experiment, a mixture of anomeric D-glucopyranosides and D-glucofuranosides could be separated. This technique is a useful method for the separation of isomeric glucosides ; in general, furanosides are adsorbed more strongly than pyranosides and β-anomers are adsorbed more strongly than the corresponding α-anomers. The free sugars are not eluted from the resin. Evans et al. (57) have shown that on columns

of anion-exchange resin in the chloride and hydroxide forms with water as eluent the order of elution depends on the nature of the resin counterion.

In general, the higher the number of hydroxyl groups in the sugar, the more the aqueous phase is preferred, therefore the sugars are eluted in order of decreasing molecular size. The substitution of a hydroxyl group by an alkoxy group causes a decrease in polarity and the distribution coefficient becomes higher. The degree of cross-linking of the resin is also of importance as steric exclusion may control the sorption process.

Only a few simple separations of neutral saccharides have been effected by this method (Table 2), probably owing to the possibility of inter-conversions of the sugars on the hydroxyl form of anion-exchange resins. In the other hand, there are many examples of the fractionation of carboxylic acids.

In the sorption of hydroxyacids on anion exchangers both ionic sorption and molecular sorption are involved. The ionic adsorption depends on the dissociation of the acid and when this type of adsorption predominates, separation of organic acids with sufficiently different dissociation constants is possible. Larsen (58) separated uronic acids on column of Dowex 1 x 8 with a linear gradient of acetic acid (0.5 N to 0.2 N). Glucuronic and mannuronic acids have been separated from each other and from guluronic and galacturonic acids. In this system, free sugars are not adsorbed and can be isolated from uronic acids. Separation of galacturonic and glucuronic acids in presence of arabinose and galactose has been obtained by Khym (59). Ion exchange chromatography with acetic acid as eluent has been extended to include a large number of other acids (Figure 10) such as aldonic and saccharinic acids (60 - 62 bis) ; the main results are given in Tables 3 and 4. Separation with formic acid has been utilized by Fransson (63) to fractionate uronic acids and aldobiouronic acids but hyaluronic acid oligosaccharides and chondroitin sulfate oligosaccharides were

TABLE 2

List of Experimental Separations Obtained on Neutral Saccharides by Anion Exchange Chromatography.

	Arabinose	Glucose	Lyxose	Raffinose	Sucrose	Ribose	Xylose
Arabinose	-		(55)			(55)	(55)
Glucose		-		(55)	(55)		
Lyxose	(55)						
Raffinose		(55)		-	(55)		
Sucrose		(55)		(55)	-		
Ribose	(55)						
Xylose	(55)						

Resins used : Permutit "De-Acidite" SRA 68 - 3.5 % crosslinked (CO_3^{2-} or HCO_3^-)

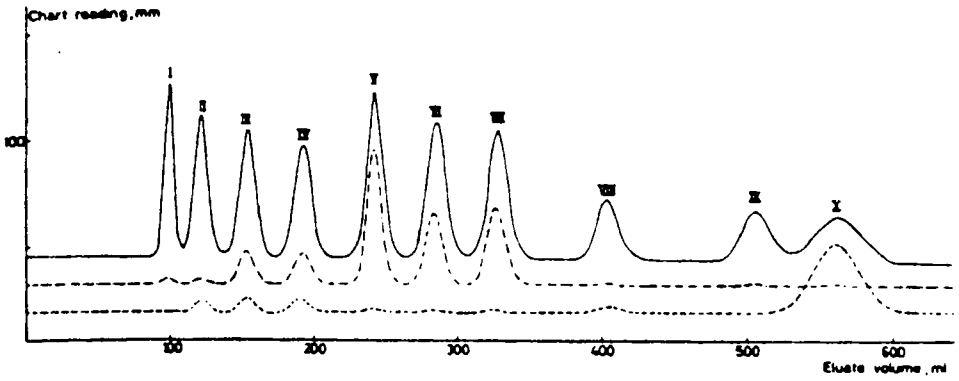


Figure 10. Separation of levulinic (I), melibionic (II), cellobionic (III), maltobionic (IV), D-ribonic (V), D-glycero-D-gulo-heptonic (VI), D-gluconic (VII), lactic (VIII), L-rhamnoic (IX) and D-galacturonic acid (X). Detection methods : chromic acid — ; carbazole ---- ; periodate -·-·-·-. Eluant : 0.5 M acetic acid ; flow rate : 7.7 ml $min^{-1}cm^{-2}$; resin bed : 6 x 760 mm, Dowex I-X8, 24-27 µm(62).

better resolved by elution with LiCl. Anion-exchange chromatography in acetate media is a useful tool for the analysis of mixtures containing several hydroxy acids ; most experiments have been carried out with the sodium acetate system. In this regard, the works of Samuelson (60 - 62 bis, 64-69) have the greatest interest in the separation of various carboxylic acids (Tables 5 and 6). Sodium acetate has also been used by Dirkx (70) for the analysis of mixture of glucose, gluconic acid and glucuronic acid. Sodium formate was found to be a better eluent than sodium acetate, and Nagel (71) isolated a series of oligogalacturonic acids (up to octa-galacturonic acid) and unsatured di- tri- tetra- and penta-galacturonic acids but Samuelson (67 bis) used sodium acetate to separate aldonic acids of the xylonic or gluconic acid series (Figure 11).

TABLE 3

List of Experimental Separations Obtained on Uronic Acids by Anion Exchange Chromatography with Acetic Acid as the Eluent.

	D-Galacturonic acid	D-Glucuronic acid	L-Guluronic acid	L-Iduronic acid	L-Mannuronic acid
D-Galacturonic acid	-	58-59-60	60	60	58,60
D-Glucuronic acid	58-59-60	-	58-60	60	58,60
L-Guluronic acid	60	58,60	-	60	58,60
L-Iduronic acid	60	60	60	-	60
D-Mannuronic acid	58,60	58,60	58-60	60	-

Resins used : Dowex 1 (50μ) (59)
Dowex 1 x 8 (37-74μ) (58)
(13-17μ) (60)

TABLE 4

List of Experimental Separations Obtained on Aldonic and Aldobionic Acids by Anion Exchange Chromatography with Acetic Acid as the Eluent.

	Cel.Ac.	Glu.Ac.	Lact.Ac.	Lyx.Ac.	Malt.Ac.	Mel.Ac.	Rham.Ac.	Rib.Ac.	Tal.Ac.	Xyl.Ac
Cel.Ac.	-	62			62	62	62	62		
Glu.Ac.	62	-		61	61-62	61-62	62	61-62	61	61
Lact.Ac.			-							
Lyx.Ac.		61		-	61	61		61	61	61
Malt.Ac.	62	61-62		61	-	61-62	62	61-62	61	61
Mel.Ac.	62	61-62		61	61-62	-	62 62 bis	61-62	61 62 bis	61
Rham.Ac.	62	62			62	62 62 bis	-	62	62 bis	
Rib.Ac.	62	61-62		61	61-62	61-62	62	-	61	61
Tal. Ac.		61		61	61	61 62 bis	62 bis	61	-	61
Xyl.Ac.		61		61	61	61		61	61	-

Resins used : Dowex 1 x 8 26-32μ (61)
24-27μ (62)-(62 bis)

Cel.Ac. = Cellobionic acid. Glu Ac. = D-Glucuronic acid. Lact. Ac. = Lactobionic acid. Lyx.Ac. = Lyxonic acid. Malt. Ac. = Maltobionic acid. Mel. Ac. = Melibionic acid. Rham. Ac. = L-Rhamnonic acid. Rib. Ac. = D-Ribonic acid. Tal. Ac. = D-talonic acid. Xyl.Ac. = D-Xylonic acid.

TABLE 5

List of Experimental Separations Obtained on Aldonic and Aldobionic Acids by Anion Exchange Chromatography with Sodium Acetate as the Eluent.

	D-allonic acid	Cellobionic acid	Cellotrionic acid	D-Gluconic acid	Melibionic acid	Xylonic acid	Xylobionic acid	Xylotrionic acid	DP 1→14 Xylonic acid.
D-allonic acid.	-	62	67	62	62				
Cellobionic acid	62	-		62 - 67 bis	62	67 bis	67 bis	67bis	
Cellotrionic acid.		67	-	67bis		67 bis	67 bis	67bis	
D-Gluconic acid	62	62-67bis	67	-	62	67 bis	67 bis	67bis	
Melibionic acid	62	62		62	-				
Xylonic acid		67 bis	67 bis	67bis		-	67-67bis	67-67bis	67
Xylobionic acid		67 bis	67 bis	67bis		67-67 bis	-	67-67 bis	67
Xylotrionic acid		67 bis	67 bis	67bis		67-67 bis	67-67 bis	-	67
DP 1→14 Xylonic acid						67	67	67	-
Resins used : Dowex 1 x 2 (20-40μ) (66 bis) Dowex 1 x 8 (25-30μ) (67)									

TABLE 6

List of Experimental Separations Obtained on Uronic Acids by Anion Exchange Chromatography with Sodium Acetate as the Eluent.

	Cellobiouronic acid	D-Galacturonic acid	D-Glucuronic acid	4-O-Me-D-Glucuronic acid	L.Guluronic acid	D-Mannuronic acid
Cellobiouronic acid.	-	60 , 65	60 , 65	65	60	60 , 65
D-Galacturonic acid.	60 , 65	-	60, 62 bis 65	65	60	60 , 65
D-Glucuronic acid	60 , 65	60 , 65 62 bis	-	65	60	60 , 65
4-O-Me-D-Glucuronic acid.	65	65	65	-		65
L-Guluronic acid	60	60	60		-	60
D-Mannuronic acid	60 , 65	60 , 65	60 , 65	65	60	-

Resins used : Dowex 1 x 8 13 - 17μ (60)
40 - 60μ (65)

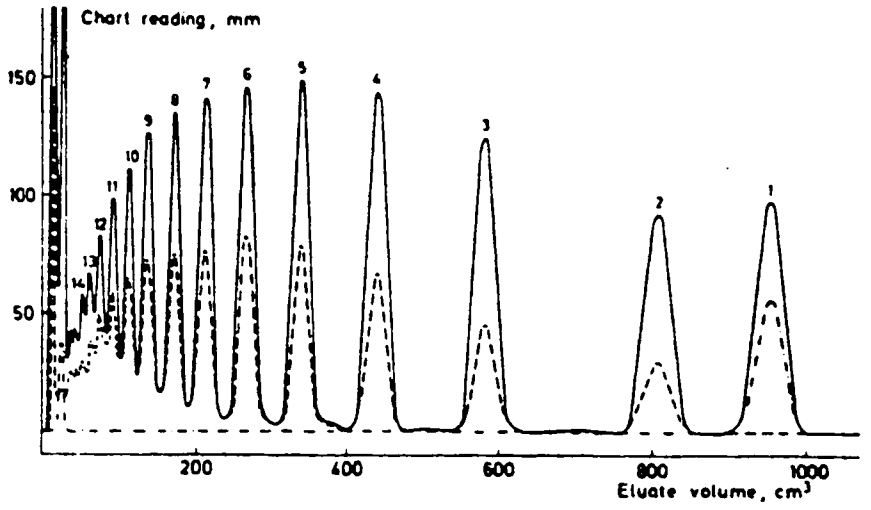


Figure 11. Separation of a mixture (100 mg) of aldonic acids of the xylonic acid series which was prepared from a hydrolyzate of birch xylan. Column (1320 mm x 6 mm I.D.) packed with Dowex 1-X2, 20-40 µm. Eluent 0.02 M sodium acetate ; nominal linear flow-rate, 2.5 cm/min. Detection: —chromic acid ; -·-·-·, periodate-formaldehyde ; ---, carbazole. Peak numbers refer to the numbers of momomeric units (67).

Obviously, a large number of parameters influence the separation process and the distribution coefficients depend on the nature, concentration and pH of the mobile phase, and on the temperature. In elution with salt solutions, the separation factor is determined by the selectivity coefficient of the ionic species to be separated whereas in acid medium the dissociation constants of the acids also have an influence ; with sodium acetate as eluent most hydroxy acids are eluted in the order of decreasing molecular weight (65) and a complex mixture containing both uronic acid biouronic acids can be satisfactorily resolved using gradient elution. Hydroxy acids containing a large number of hydroxyl groups appear before those with a lower number ; this rule holds true

with most of the acids within the aldonic acid series but among organic acids investigated by Samuelson (61) there are two exceptions : mannonic acid and D-glycero-L-manno-heptonic acid.

As the non-ionic interactions with the matrix are small, the above mentioned rule can be explained by the assumption that the hydrated ionic volumes have a predominant influence. Thus the introduction of O-methyl groups increases the hydrated ionic volume and a large decrease in the distribution coefficient value occurs ; in contrast, substitution of a hydroxyl group for an aldehyde or keto group results in a large increase in the distribution coefficient value, attributed to the specific interaction forces between the anions and the resin.

The influence of temperature on the separations was also studied (67 bis). The separation factors were affected only to a slight extent and very little could be gained by working at elevated temperatures. On the other hand, at high temperature the elution peaks are sharper and the counter pressure lower, therefore a more rapid separation can be achieved.

b) Partition chromatography of carbohydrate on anion exchange resins in mixtures of water and ethanol. Anion exchange chromatography with aqueous ethanol solutions has proved very useful in carbohydrate analysis and very good results were obtained. Within the range of solvent composition of interest, the ratio of water to alcohol is higher inside the resin phase than in the external solution, and the sugars which are strongly polar solutes have a greater affinity for the solution inside the resin. In addition, interaction forces between the resin and the polar solute and interactions between sugars and counterions have to be considered.

The sorption behaviour and the possibilities for the separations of sugars have been studied by Samuelson (72, 86) and more recently by Mopper (87, 88). A typical chromatogram obtained with a standard containing 18 sugars is reproduced in Figure 12 (88). The

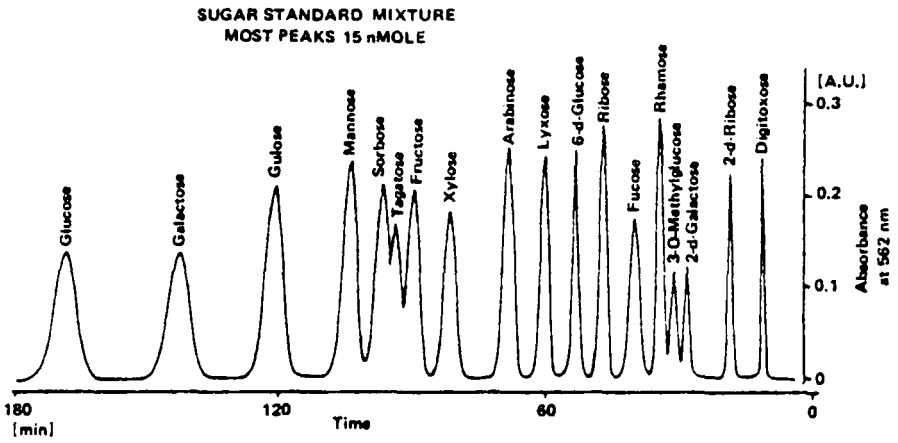


Figure 12. Separation of a standard containing 18 sugars by partition chromatography on an anion-exchange resin in ethanol-water. Most peaks are 15 nmol. 3-O-Methylglucose and 2-deoxygalactose are 7 nmol. The resin was regenerated with NaCl followed by Na_2SO_4 (88).

early investigations of Samuelson (72-76) with resin in the SO_4^{2-} form showed the remarkable capabilities of this system. In 65 % ethanol, it was possible to separate satisfactorily a mixture of the monosaccharide glucose and the pentasaccharide verbascose or a mixture of the monosaccharide glucose, the trisaccharide raffinose and the tetrasaccharide stachyose using a column of Dowex 1 x 8. As distribution coefficients increase when the ethanol concentration is increased, separations of monosaccharides and/or disaccharides can be performed. Therefore, it was possible to resolve a mixture of several mono-, di and trisaccharides using stepwise or gradient elutions. One factor which limits the applicability of this technique is the extremely low rate of diffusion inside the resin particles. In principle it is advantageous to use macroporous resins and small resin particles ; it is not the average porosity which is the rate determining factor but the spectrum of pores, and great differences can be observed among porous resins (73,75). The rate of sorption decreases rapidly with

increased ethanol concentration whereas the distribution coefficients are increased, but with small resin particles and a decrease in the flow rate (74) a complete separation of arabinose, xylose, mannose, galactose and glucose can be achieved in 88 % ethanol. A marked improvement occurs with increasing temperature ; the elution curves are much sharper at elevated temperature but the distribution coefficient is found to decrease and excessively high temperatures should be avoided.

In contrast to ion-exchange chromatography in pure water, distribution coefficients are expected to increase with an increase in the number of hydroxyl groups. Thus, monosaccharides are eluted ahead of the disaccharides and higher oligosaccharides then follow. The distribution coefficients depend on the concentration of ethanol but as a rule the order of elution from individual resins is unaffected within the range of interest in chromatographic work (76,77). Considering the alditols, most of these compounds exhibit lower distribution coefficients than the corresponding aldoses (79). Ribitol and mannitol are exceptions and no simple rule seems to be valid since the partition of the solutes is determined not only by the differences in solubility but also by interaction forces with the resin.

Most separations have been carried out with the sulfate form of styrene-divinylbenzene anion-exchange resins but cross-linked dextrans containing quaternary groups have also been used (81). With a more polar resin matrix, sugars were held more strongly but the order of elution was the same with both resins. Exceptions are the ketoses and mannose ; these monosaccharides are strongly polar solutes and the interaction forces with the resin matrix have a marked influence upon the separation.

The sulfate form of the exchanger is often preferred but the chloride form of the dextran anion exchanger has been used for certain separations. As a rule, the separation factors were less favorable

with the chloride form but the separation xylose and mannose is an interesting exception (81).

Analyses of complex mixtures of sugars are generally performed at elevated temperatures. When the temperature is lowered, a monosaccharide such as glucose can be resolved into two overlapping peaks. At -10°C, in 75 % ethanol a good resolution is obtained and the α and β forms can be separated (86).

Anion exchange chromatography using aqueous ethanol solutions as eluent can be a very interesting method in the fractionation of oligosaccharides. A chromatogram obtained on elution of a sample containing xylan oligosaccharides in a run on Technicon T5C, SO_4^{2-} in 75 % ethanol is given in Figure 13 (84). With a high con-

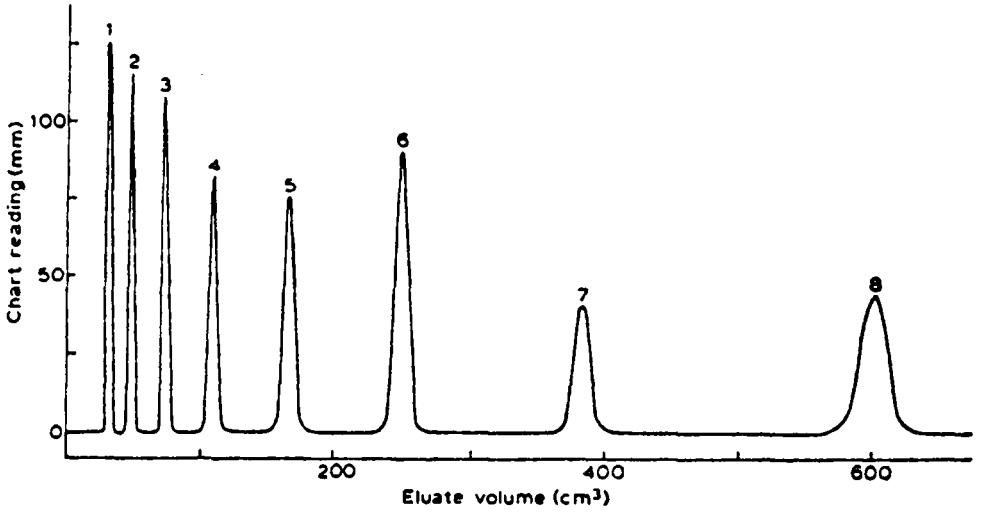


Figure 13. Partition chromatography of xylan oligosaccharides in 75 % aqueous ethanol at 75°. Resin bed : 4 x 600 mm, Technicon T5C, SO_4^{2-}, 14-17 µm. Flow rate : 2.8 cm.min^{-1}. 1, D-xylose (5 µg) ; 2,di-(5 µg) ; 3,tri-(6.5 µg) ; 4,tetra-(9 µg) ; 5, penta- (13 µg) ; 6,hexa- (25 µg) ; 7,hepta- (18 µg) ; and 8,octa-saccharide (25 µg)(84).

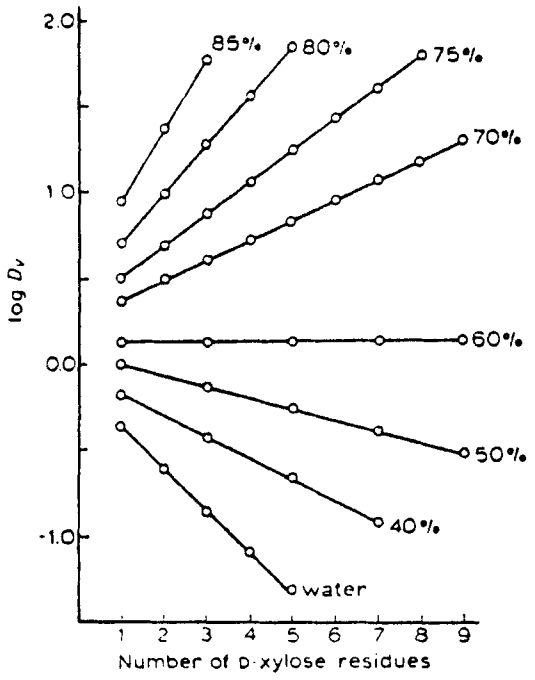


Figure 14. Relationship between log D_v and number of D-xylose residues in the oligosaccharides at various concentrations of ethanol. Sulphate resin (T5C, 14-17 μm), 75°. (84). $D_v = \frac{V_e}{x} - \varepsilon_1$ with x the volume of the chromatographic bed and ε_1 the relative interstitial volume ($\varepsilon_1 = 0.4$).

centration of ethanol the sugars are eluted in order of increasing molecular weight but this order can be reversed at a low concentration. It was found that a straight-line relationship exists between the logarithm of the distribution coefficient and the number of monomeric units (DP) in the oligosaccharides. As shown in Figure 14 (84), this rule holds true at both high concentration of ethanol and with pure water as eluent and a critical eluent composition exists at which all oligomers of the same series exhibit the same distribution coefficient. The dependence of the critical ethanol

concentration on the type of glycosidic linkage can be used to isolate oligomers belonging to different oligosaccharide series (85)

This technique has not been utilized for the separation of acidic sugars and very little with sugar derivatives such as methyl, ethyl benzyl, hydroxyethyl ethers and glycosides. In agreement with the results observed with sugars and polyols, the distribution coefficients decrease with increasing number of non-polar substituents, and all derivatives appear earlier than the sugar from which they are derived (80).The position and the nature of the substituents have, however, a very great influence ; the high distribution coefficient of benzylglucose may be due to strong attractive forces between non-polar solutes and the matrix of the resin. Various separations performed with this technique are given in Table 7.

c) Anion-exchange chromatography based on sugar complexation with the eluent. This technique based on the original experiments of Khym (89 - 92) involves the chromatography of sugar-borate complexes on basic ion-exchange resins in the borate form. Certain polyhydroxy compounds react with borate ion (from boric acid or its salts) to form complexes which are negatively charged ions and may be separated by ion exchangers. A separation of glucose, galactose and fructose was performed by Khym on Dowex-1 with sodium borate solution as the eluent. Afterwards the method has been extended to include other saccharides. In solution three types of complexes are possible but they are in equilibrium and this equilibrium depends on the pH, the ratio of borate to sugar and the absolute concentration of the latter. Moreover, mutarotation, furanose-pyranose interconversion and the position of hydroxyl groups are very important (90, 91). It was found that the furanose was the form most favorable for the formation of a sugar-borate complex ; the elution order of carbohydrates is therefore controlled by the importance of furanose-pyranose interconversion and the ease of mutarotation. Affinity of the borate complex for the resin changes with the type of complex ; the influence of the structure

TABLE 7

Separation of Saccharides on Polycationic Resins with Ethanol/Water Eluent.

RESINS	ELUENT COMPOSITION $EtOH/H_2O$	SACCHARIDES SEPARATED	Ref.
Dowex 1 x 8 (45-75μ)	(65/35-74/26)	Glucose, verbascose -glucose, raffinose, stachyose - xylose, glucose maltose.	72
Amberlyst X N-1001 (45-75μ) Dowex 21 K	(70/30-77/23)	Glucose, raffinose, stachyose- rhamnose, glucose, melibiose	73
Dowex 1 x 8 (45-75μ)	(70/30-82/28)	Glucose,cellobiose - glucose,lactose - glucose,sucrose,raffinose, stachyose, verbascose - glucose,melezitose, raffinose.	74
Dowex 21 K - 15 (45-75μ) (15-40μ)	(74/26-88/12)	2-deoxy-D-glucose,arabinose,glucose,sucrose,melizitose raffinose, rhamnose, ribose,sorbose,galactose,maltose,melibiose,raffinose,stachyose, arabinose, xylose,mannose,galactose, glucose.	75
Macroporous resins	(82/18-92/08)	Arabinose,xylose,mannose,galactose,glucose - 2 deoxyribose,rhamnose, ribose,arabinose,xylose,mannose,galactose,glucose,maltose.	76
Macroporous resins (10-35μ) Dowex 21 K (1-16μ)	(86/14-92/08)	Digitoxose, 2 deoxy-ribose, 2 deoxy-galactose, rhamnose, fucose, ribose, lyxose, arabinose, xylose, mannose, galactose, glucose -2 deoxygalactose, 2 deoxy-glucose, ribose, xylose, fructose, sorbose, mannose, galactose, glucose.	77
Dowex 1 x 8 (10-15μ)	(86/14)	Digitoxose, 2 deoxyribose, 2 deoxygalactose, rhamnose, fucose, ribose, lyxose, arabinose, xylose, fructose, mannose, galactose,glucose.	78
T4 (10-35μ) T5B (3-17μ)	(86/14-89/11)	Digitoxose, glycerol, 2 deoxygalactose,rhamnose, fucose, ribose, arabinitol, xylitol,lyxose, glucitol, mannose, galactitol, mannitol, galactose, glucose, erythritol,xylitol,arabinitol,glucitol, mannitol.	79

T4(10-35μ) T5B (3-17μ)	(94/6)	2,3,4,6 Me-glucose, 1,2,3 Me-glucose, 3,6 Me-glucose,Me-glucose, Me-deoxyglucose, 2-ethylglucose, 3 benzyl-glucose, Me-deoxyglucose, 2 Me-glucose, 3-Me-glucose, 2-deoxyglucose, fucose, 6 Me-glucose, lyxose, arabinose.	80
Crosslinked dextran (10-40μ) (5-30μ)	(95/5)	Ribose,lyxose,arabinose,xylose - rhamnose,ribose,arabinose,xylose,mannose,galactose,glucose - fructose,tagatose - xylose, mannose, sorbose-rhamnose, 2 deoxyglucose.	81
T5B (10-15μ)	(86/14)	Rhamnose,ribose,arabinose,xylose,mannose,galactose,glucose.	82
T5C (14-17μ)	(77/23-88/12)	Threose,6 deoxyglucose,talose,xylose,allose,gulose,galactose,glucose-mycinose,xylose,cellobiose,maltotriose,planteose,nystose, stachyose.	83
T5C (14-17μ)	(75/25-80/20)	Xylo-oligosaccharides (DP 1 - 9)	84
Dowex 1 - x 8 (12-18μ)		Isomaltodextrins Maltodextrins	85
T5C (10-17μ)	(75/25)	Separations of sugars into anomers	86
Technicon type S 20μ	(89/11)	Deoxyribose, rhamnose, fucose,ribose, arabinose, xylose, mannose, galactose, glucose.	87
DA x 8 (8-11μ)	(86.7/13.3)	Digitoxose, 2 deoxy-glucose, 2 deoxygalactose, 3-O-Me-glucose, rhamnose, fucose, ribose, 6-deoxyglucose, lyxose, arabinose, xylose, fructose, tagatose, sorbose, mannose, gulose, galactose, glucose.	88

of the carbohydrates on the formation of theses complexes has been shown (91, 92), and compounds with the greatest number of cis-1, 2 glycols in a furanose system have the highest elution volumes. When elution was performed with dilute borate buffers, the effluent volumes of the monosaccharides were very large (500 ml) and the separation time consuming (up to 60 H.). A modification involving elution at a higher ionic strength has been introduced by Hallen (93) ; mannose, fucose, galactose and glucose can be separated with 150 ml of eluent.

Even with pH modifications and step-changes in borate buffer concentrations, the time required for these analyses was very long ; moreover, the isomerisations of certain reducing disaccharides had to be taken into account ; in borate medium under alkaline conditions, as described by Khym, lactose, maltose and cellobiose were found to undergo chemical changes (94) ; these reactions were negligible when operating at 4°C.

An important disadvantage of the method is the slowness of the separation, and improvements to accelerate the process were introduced. Eluting with a solution of a constant borate concentration (pH 8.0) upon which was superimposed a positive chloride ion concentration gradient, Syamananda (95) reduced the analysis time by a factor of at least three. Similarly, Hough (96) separated in 7 hours trehalose, rhamnose, ribose, mannose, fucose, arabinose, galactose, xylose and glucose with boric acid buffers of pH 7.0 ; the elution times of the various sugars were dependent on the rate of increase of both borate-ion and chloride-ion concentration in the eluent. The use of chloride ion causes shrinkage of the resin, but Davies (97) described a system of neutral borate buffer with sulfate as the eluting counterion. The effect of various parameters such as column temperature and the nature of the resin have been reported. Green (98) and Kesler (99) found that resolution was enhanced by increasing the column temperature and using small particle size resins but compounds exhibited longer elution time

and, at a given flow rate, little advantage was gained between 55° and 70°C. The resolution of sugars has also been shown to be pratically independent on the flow-rate ; little difference was observed by increasing or decreasing the flow-rate by 50 %. A rapid procedure for the analysis of mixtures of glucose, fructose and mannose has been investigated by Verhaar (100). Complete separation can be carried out in 30 min. on a column of Aminex A-27 eluted with boric acid, sodium borate, sodium chloride and acetic acid.

In order to minimize alkaline rearrangement reactions, elutions have been performed at a neutral pH. As the pH is decreased, the ionic strength and consequently the buffering capacity become lower. Introduction of alcohol into the eluent increases the amount of ionizable borate at low pH and a higher capacity buffer is obtained. Walborg (101 - 103) obtained good separations employing boric acid/glycerol or boric acid/2,3-butanediol buffers but the method described requires excessive elution times (Figure 15).

The results of Green (98) and Kesler (99) demonstrated that the use of chloride ions in the eluent was not indispensable. Kesler separated multicomponent mixtures of mono-, di- and tri-saccharides in 4 - 6 H. With a combination of increasing pH (from 7 to 10) and increasing borate concentration (from 0.1 - 0.2 to 0.6 M). This methodology developed by Green has been also utilized by Ohms (104) on a strongly basic anion-exchange resin, Beckman 1-S.

The method reported by Lee, based on this system, for analysis of monosaccharides is faster. The column was eluted with a linear gradient generated with 100 ml each of 0.15 M (pH 7.0) sodium borate and 0.40 M (pH 10.0) sodium borate (105) or with 70 ml each of 0.40 M (pH 8.0) buffer and 0.40 M (pH 10.0) buffer (106). A method for the separation of several mono-,di- and tri-saccharides is described by Floridi (107). The utilization of a three buffer step-wise elution system allowed a good chromatographic resolution

of seventeen sugars in about 10 h. (Figure 16). Separations with borate buffer of fixed borate concentration are more convenient, no re-equilibration being necessary between runs. Bauer (108, 109) separated, using resins of small particles size, in 35 min. sucrose, ribose, mannose, arabinose, galactose, glucose, with H_3BO_3 buffer

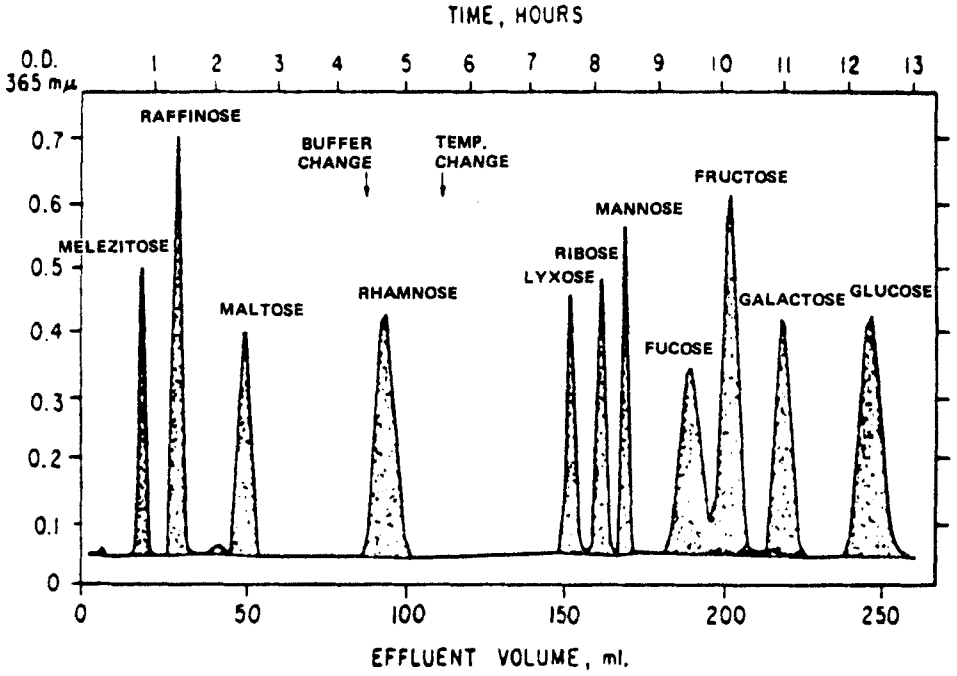


Figure 15. Chromatographic separation of saccharide mixture using a two-borate buffers system on AMINEX-A14 anion exchange resin (103). Saccharides were present in the following quantities : 0.300 μmole melezitose, 0.500 μmole raffinose, 0.800 μmole maltose, 2.0 μmoles rhamnose, 1.5 μmoles lyxose, 1.5 μmoles ribose, 1.0 μmole mannose, 1.5 μmoles fucose, 2.5 μmoles fructose, 2.0 μmoles galactose, and 2.5 μmoles glucose.
Buffer A is followed by buffer B at 4.5 hr (buffers are defined in ref. 102) ; temperature is changed from 40° to 60° C at 5.5 hr.

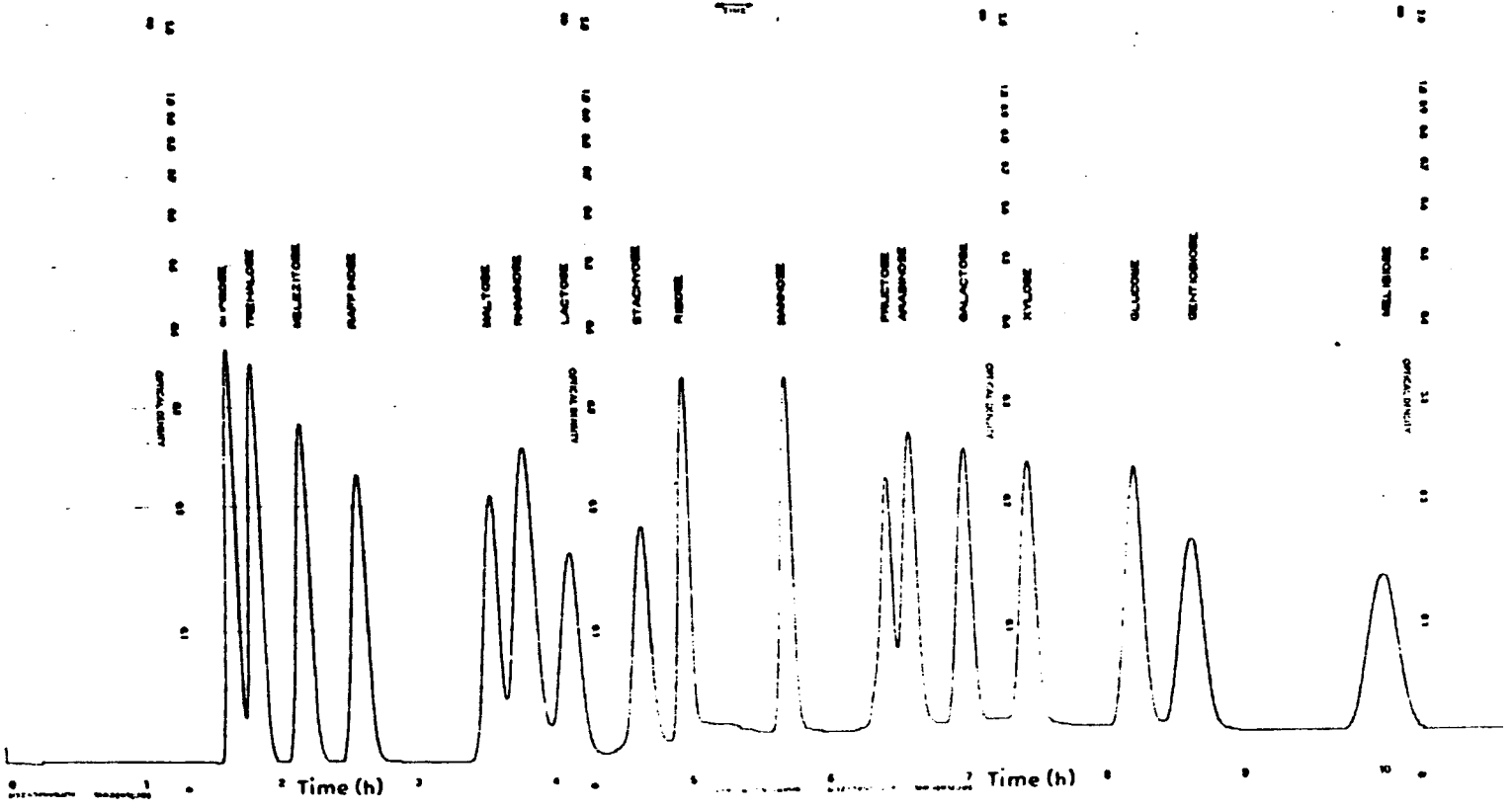


Figure 16. Separation of 17 sugars using a three borate buffer stepwise elution system (107).
Anion exchange resin Dowex 1 x 4, column temperature 55° C.

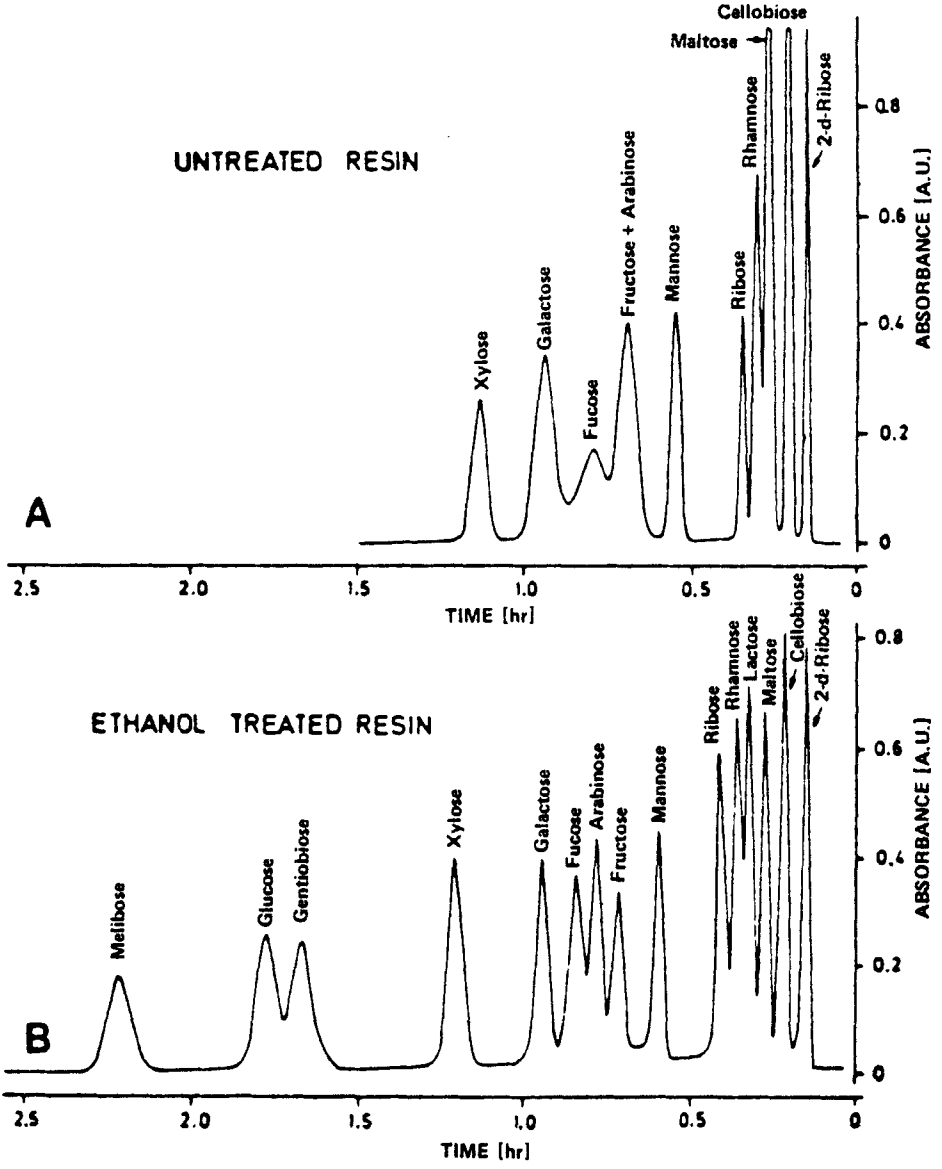


Figure 17. (A) Separation of 11 sugars (15 nmol each) on non-ethanol-treated resin. (B) Separation of 15 sugars (15 nmol each) by borate-complex ion-exchange chromatography. The resin was treated with ethanol prior to packing. Resin DA-X4 (Durrum chem) (111).

0.4 M (pH 9.2), and separations were performed by Sinner (110) with potassium borate buffer of pH 8.8 at various buffer molarity (0.1 to 1.0 M). Ethanol treatment of the resin can increase the separation factor and total analysis time is considerably shorter (Figure 17) On an ethanol treated resin, 15 sugars were well separated by Mopper (111) ; the improved separations appear to be related to an excellent package of the column.

Anion exchange chromatography in borate medium is not limited to analysis of neutral saccharides. This system was found to be well adapted to the separation of hydroxy acids. The principal results have been obtained by Samuelson (112 - 117). Xylonic, arabonic, mannonic, gluconic and galactonic acids could be separated on Dowex 2 or Dowex 1 with a sodium tetraborate solution (112). This method was also used for separation of saccharinic acid from lactic, glycolic, α, β-dihydroxyisobutyric and β, γ - dihydroxiisobutyric acids (113). In borate medium, the separations of aldobionic acids were very difficult. These separations, as well as mutual separations of some simple aldonic acids can be carried out by elution with sodium acetate (114). By contrast, the separation factors of most aldobiouronic acids (Figure 18) are more favorable in sodium tetraborate than in sodium acetate and acetic acid (115). This higher selectivity with borate medium can be attributed to the formation of complexes between borate ions and the polyhydroxicarboxylate anions ; the stability of these complexes tends to increase with the number of vicinal hydroxyl groups and with their distance from the carboxylate group. As with neutral saccharides, there is a correlation between the complex formation and the eluent concentration and between complex formation and temperature (116, 117).

Ion exchange chromatographic separation of sugars based on sugar borate complexation has been extensively employed ; Table 8 summarizes the most important results. Strongly basic anion-exchange columns in the hydrogen sulphite form have also been utilized (118,

TABLE 8

Separation of Saccharides on Polycationic Resins in Presence of Borate.

RESINS	ELUENT SYSTEMS	SACCHARIDES SEPARATED	Ref.
Dowex 1 (200-400 Mesh)	Sodium borate	Fructose, galactose, glucose.	89
Dowex 1 300 Mesh	Potassium borate	Fructose,galactose,glucose-ribose,arabinose,xylose,mannose,fructose-ribose,fructose,galactose,glucose-sucrose,fructose,glucose-sucrose,maltose	90
Dowex 1 (200-400 Mesh)	Potassium borate	Melezitose, turanose, glucose - turanose, fructose - Sorbitol, dulcitol, mannitol.	91
Dowex 1 (10-20μ)	Potassium borate	Deoxyribose,sucrose,raffinose,cellobiose,maltose,lactose,rhamnose and ribose,mannose,fructose,arabinose,galactose,sorbose and xylose,glucose melibiose.	92
Dowex 2 x 8 (200-400 Mesh)	Sodium borate Sodium bicarbonate	Mannose, fucose, galactose, glucose, galacturonate, glucuronate	93
Chromobeads S	Boric acid, sodium chloride	Trehalose,cellobiose,rhamnose,ribose,mannose,arabinose,galactose,xylose, glucose.	96
Technicon 1 S	Boric acid, sodium sulfate.	Maltose, rhamnose, mannose, arabinose, galactose, xylose, glucose.	97
Dowex 1- x 8	Sodium borate and boric acid	2 deoxy-ribose,sucrose,raffinose,cellobiose,maltose,lactose,ribose,rhamnose,mannose,fructose,arabinose,galactose,sorbose,xylose,glucose.	98
Dowex 1 x 8 (200-400 Mesh)	Boric acid	Sucrose,cellotetraose,cellotriose,cellobiose,maltose,rhamnose,lactose,ribose,mannose,fructose,arabinose,galactose,xylose,glucose,gentiobiose.	99
Aminex A-27 (13.5μ)	Boric acid + sodium borate + sodium chloride.	Mannose, fructose, glucose.	100
Dowex 1 x 4 (40 - 100μ)	Boric acid + glycerol.	Sucrose,lactose,rhamnose,mannose,fucose, ribose,galactose,glucose,fructose,sorbose-sucrose,cellobiose,maltose,lactose,rhamnose.	101
Dowex 1 x 4 (- 400 Mesh)	Boric acid + 2,3 butanediol	Sucrose,cellobiose,maltose,rhamnose,lactose - Lyxose,mannose, fucose, arabinose, galactose, glucose.	102

Aminex A-14 (20μ)	Boric acid + 2,3 Butanediol	Melezitose,raffinose,maltose,rhamnose,lyxose,ribose,mannose,fucose, fructose,galactose,glucose.	103
Beckman 1 - S	Boric acid + sodium borate.	Sucrose,raffinose,cellobiose,maltose,lactose,ribose,rhamnose,mannose, fructose, arabinose,galactose,sorbose and xylose,glucose.	104
Chronobeads S	Sodium borate	Mannose,fucose,galactose,xylose - rhamnose,ribose,arabinose,glucose.	105
DA x 4	Sodium borate	Mannose, fucose, galactose, xylose, glucose.	106
Dowex 1 x 4 (200-400 Mesh)	Potassium borate + acid boric.	Sucrose,trehalose,melezitose,raffinose,maltose,rhamnose,lactose,stachyose,ribose,mannose,fructose,arabinose,galactose,xylose,glucose,gentiobiose,melibiose.	107
DA x 4 DA x 4 F	Boric acid	2 deoxyribose,sucrose,trehalose,cellobiose,maltose,rhamnose,lactose,ribose mannose,fructose,arabinose,galactose,xylose,glucose,gentiobiose,melibiose.	108 109
DA x 4	Potassium borate	Sucrose,mannose,fructose,arabinose,galactose,xylose,glucose.	110
DA x 4 (20μ)	Boric acid	2 deoxyribose,cellobiose,maltose,lactose,rhamnose,ribose,mannose,fructose, arabinose,fucose,galactose,xylose,gentiobiose,glucose,melibiose.	111
Dowex 1 (20-37μ) Dowex 2 : 37-45μ, 50-70μ 100-200μ.	Sodium borate	Xylonic acid, gluconic acid, Gluconic acid, galactonic acid, Xylonic acid,arabinonic acid mannonic acid,gluconic and galactonic acids	112
Dowex 1- x 8 (< 400 Mesh)	Sodium borate	Lactic acid, glycolic acid lactic and glycolic acids, α,β dihydroxyisobutyric acid, β γ dihydroxybutyric acid, α-D-isosaccharinic acid.	113
Dowex 1- x 8 (40-60μ)	Sodium borate	Rhamnoic acid, xylonic acid,lactobionic acid, rhamnoic acid, melibionic acid, rhamnoic acid, cellobionic acid, maltobionic acid.	114
Dowex 1- x 8	Sodium borate	Cf. Figure 18	115
Aminex A-25 (17.5 ± 2μ)	Potassium borate	3-Hydroxypropanoic acid, glyceric acid, erythronic acid, ribonic acid, xylonic acid,arabinonic acid, gluconic acid, D-glycero-L-manno heptonic acid.	117

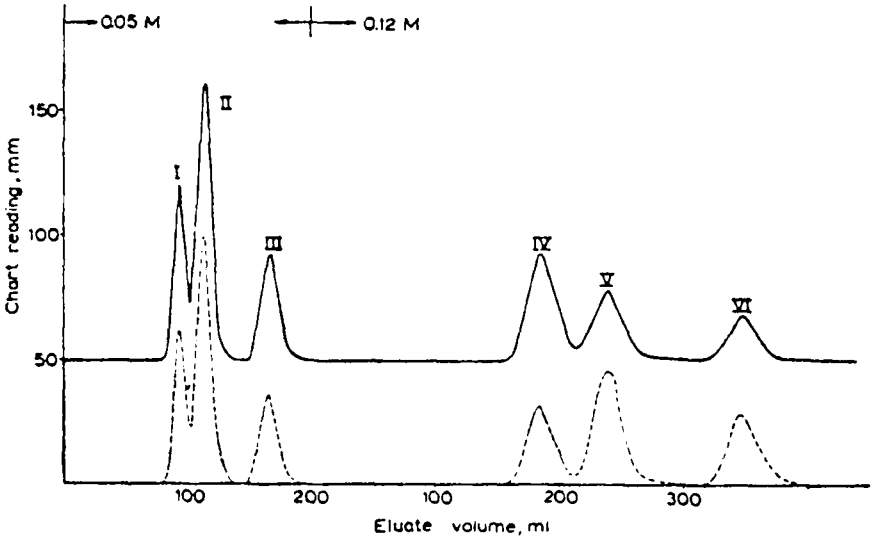


Figure 18. Separation of 2-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-lyxose (I), 2-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-xylose (II), 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose (III), 6-O-(β-D-glucopyranosyluronic acid)-D-galactose (IV), D-galacturonic acid (V), and D-glucuronic acid (VI). Analyzed by the carbazole method (---), and by the dichromate method (——). Stepwise elution with 0.05 M and 0.12 M sodium tetraborate on a Dowex-1, X-8 column, at 30° C (115).

119) with pure water or ethanol-water mixture as eluents. The system is based upon the formation of α-oxysulfonic acids in the resin phase. Ketoses have not tendency to form stable complexes while aldoses yield stable α-oxysulphonic acid, and this method can therefore be employed for ketose-aldose separations. Though sugars are isolated in the pure state, this is a time-consuming process and this type of separation has not been frequently utilized. In the same way, the use of copper or zinc acetate solutions as eluent in the separation of various aldonic and uronic acids can be simply considered as a complement to existing procedures (120).

d) Cation-exchange chromatography of saccharides.

Cation exchange chromatography has been performed for years. Results have been shown to be similar to those obtained with anion-exchange resins but the elution systems are less complex (121). Two elution types can be considered.

- Elution with pure water : in 1960, Jones separated on the lithium (122, 123) or barium (124) forms of Dowex 50 W a mixture of saccharides with water as the eluent.With the lithium form, sucrose, raffinose and glucose were resolved in 24 hours. It has been shown that elution proceeds in order of decreasing molecular size ; with methylated sugars this order was reversed so that the most highly substituted molecules were eluted last. In water, the fractionation of neutral sugars of different size may be regarded as a partition chromatography whereas charged molecules may be excluded from the matrix. Application of these principles to the separation of carbohydrates allowed Barker (125) to separate oligosaccharides on the lithium form of AG-50W x 2 resin, separations being not affected by salts present in the sample. The potassium form of a Dowex 50W x 4 has been used by Mc Cready (126) and Saunders (127). Mc Cready described the preparation of 1-kestose and nystose and Saunders reported the separation of stachyose, raffinose, sucrose, glucose, xylose and fructose.

Several ionic forms have been investigated (125, 128-130). It was found that variation of the counterion alters the chromatographic behaviour and separation of many mixture can be significantly improved by correct choice (Figure 19). The results may be explained by exchanges between the polyol molecule, $R(OH)_n$, and the water molecules held in the hydration sphere of the counterion M^{x+} aq. The stability of the complex will depend on the availability for coordination and the order of elution is in direct relation to the stereochemistry of the sugar molecule, hydrogen bonding playing only a minor role (129). A good example is given by separations of alditols. Bourne (128) used the Cu^{2+} form of a cation-exchange

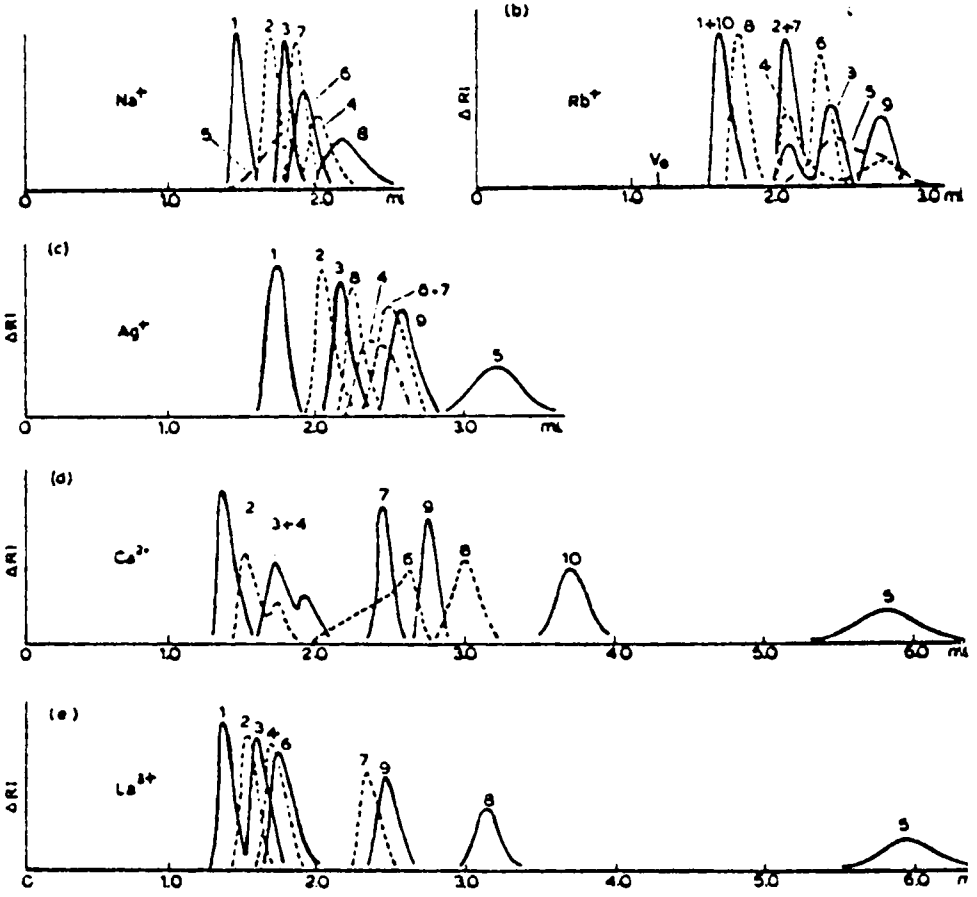


Figure 19. Behaviour of selected polyols on a 50 x 0.28 cm column of Aminex A-5 (M^{x+} aq.), eluting with deionised water at 0.10 ml/mn. Composite chromatograms of sucrose (1), glucose (2), galactose (3), mannose (4), talose (5), fructose (6), glycerol (7), mannitol (8), gulose (9), and galactitol (10). (129).

resin but Petrus (130) has shown that the complexes with rare earth metal ions were more stable and efficient separations were obtained with resins in the lanthanum form.

- Elution with mixture alcohol/water : partition chromatography of anion exchange resins in water-alcohol has been proved to be a useful tool in the separation of various carbohydrates ; Samuelson (131 - 134) applied this process with cation-exchangers for the fractionation of sugar alcohols (Figure 20). The mechanism is similar but nevertheless the alcohol concentrations are higher. The influence of the nature of the counterion upon the chromatographic behaviour is well marked and among the sugars there is in several

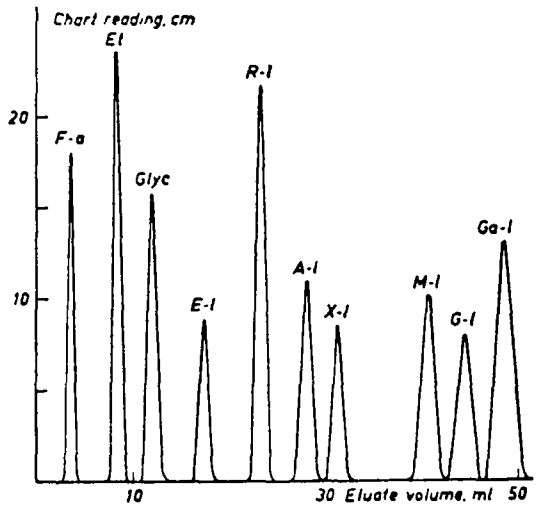


Figure 20. Partition chromatography in 85 % (w/w) ethanol at 75°. Resin bed : 2.6 x 1310 mm, Dowex 50 W-X 8, Li^+, 14-17 µm.Flow rate : 3.0 ml $cm^{-2}min^{-1}$. F-a = Formaldehyde, 20 µg ; Et = Ethylene glycol, 50 µg ; Glyc = Glycerol, 50 µg ; E-l = Erythritol, 20 µg ; R-l = Ribitol, 100 µg ; A-l = Arabinitol, 50 µg ; X-l = Xylitol, 50 µg ; M-l = Mannitol, 60 µg ; G-l = Glucitol, 60 µg ; Ga-l = Galactitol, 100 µg (132).

TABLE 9

Separation of Saccharides on Polyanionic Resins.

RESINS	ELUENT SYSTEM	SACCHARIDES SEPARATED	Ref.
Dowex 50W X 2 (Li^+) (200-400 Mesh)	H_2O	Sucrose, raffinose, glucose	122
Dowex 50W X 2 (Li^+) (200-400 Mesh)	H_2O	Glucose, maltose - Raffinose, melibiose - Lactose, galactose.	123
Dowex 50W x 8 (Ca^{2+} and Ba^{2+}) (200-400 Mesh)	H_2O	Xylose, ribose	124
Dowex 50W x 2, x 4, x 8 (Li^+) (200-400 Mesh)	H_2O	Amylopectine, laminaripentaose, laminaritriose, maltose, glucose.	125
Dowex 50W x 4 (K^+) (200-400 Mesh).	H_2O + 0.2% potassium benzoate (pH=7.3)	1-kestose, nystose	126
Dowex 50W x 4 (K^+) (200-400 Mesh).	H_2O	Stachyose, raffinose, sucrose, glucose, xylose, fructose.	127
Amberlite IR-120 (Cu^{2+})	H_2O + 0.2 to 2.5 % copper acetate.	Glucose-glucitol - Mannose, mannitol - Cellobiitol, maltitol - Maltitol, isomaltitol - Ribitol, arabinitol, xylitol.	128
Aminex A.5 (11 ± 2μ)	H_2O	Sucrose, glucose, galactose, mannose, glycerol, fructose, gulose, mannitol, galactitol, talose.	129

Dowex 50W x 8 (La^{3+}) (200-400 Mesh)	H_2O	Allitol, mannitol, altritol, galactitol, glucitol, iditol -penta-erythritol, talose, glucitol.	130
Amberlite IR-120 (Li^+ or K^+) (3-17μ)	Et-OH/H_2O (92.4/7.6)	Rhamnose, 2 deoxyglucose, xylose, arabinose, tagatose, glucose, galactose - tagatose, sorbose, fructose - digitoxose, 2 deoxyribose, 2 deoxyglucose, 2 deoxygalactose.	131
Dowex 50W x 8 (K^+, Na^+ or Li^+) (14-17μ)	EtOH/H_2O (85/15-95/5)	Erythritol, ribitol, arabinitol, xylitol, mannitol, glucitol, galactitol.	132
Durrum DC-2 (Li^+)	EtOH/H_2O (75/25)	Rhamnose, arabinose, glucose, galactose, maltose, gentiobiose, isomaltose.	133
Aminex A6 (Li^+) (15-19μ)	EtOH/H_2O (70/30)	Separation of various oligomeric sugar alcohols.	134
Aminex A6 (Trimethyl-ammonium (17.5μ)	EtOH/H_2O (85/15)	Tetramethylglucose, rhamnose, ribose, xylose, arabinose, mannose, glucose, galactose, maltose, lactose.	135
M 71 (Li^+)	Propanol^{-1}H_2O EtOH/H_2O (89/11)	2-de-oxyribose, erythrose, fucose, xylose, ribose, arabinose, sorbose, fructose, mannose, glucose, galactose.	136

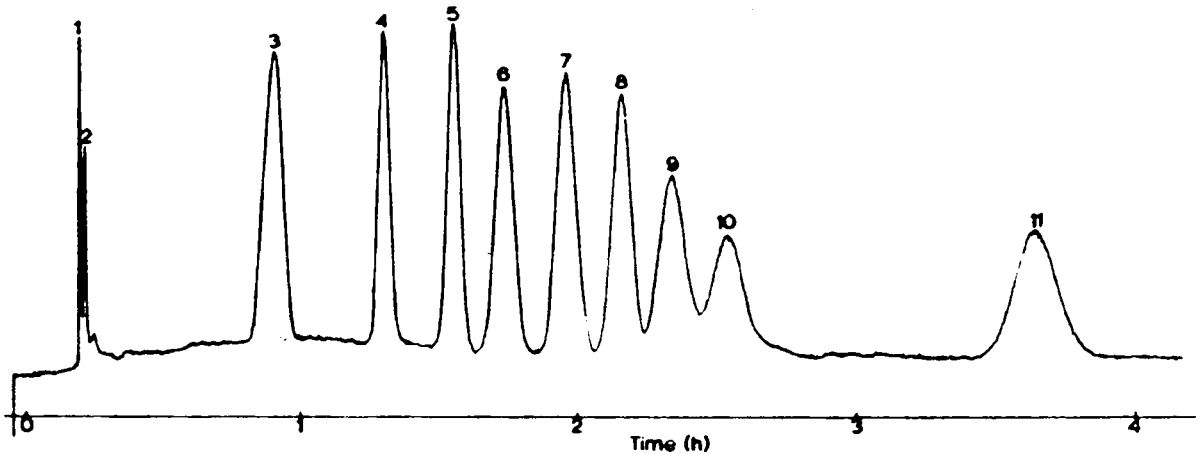


Figure 21. Separation of mono- and disaccharides. Column, 100 x 0.4 cm I.D. Aminex A-6, triméthylammonium form ; eluent, 85 % (w/w) ethanol in water ; temperature, 65° ; flow-rate, 0.266 ml min^{-1}. Each peak corresponds to approximately 150 µg carbohydrate. 1 = Methylpalmitate ; 2 = tetramethylglucose ; 3 = rhamnose ; 4 = ribose ; 5 = xylose ; 6 = arabinose ; 7 = mannose ; 8 = glucose ; 9 = galactose ; 10 = maltose ; 11 = lactose (135).

instances a reversed order of elution when the counterion in the resin is changed (131).

The utilization of organic base counterions has been described by Lawrence (135). In contrast to the inorganic cations effect, an increase in the size of the counterion decreases the capacity ratios for the carbohydrates. Increasing hydrophobic character is possible with substitution of methyl or ethyl groups for the hydrogen, but interactions tend to be reduced. The trimethylammonium form of the resin gives the best separations and a mixture of tetramethylglucose, rhamnose, ribose, xylose, arabinose, mannose, glucose, galactose, maltose, lactose can be separated in 4 hours on a column of Aminex A-6 in 85 % ethanol at 65°C (Figure 21). In fact using n-propanol/water (89/11) as the eluting solvent with the lithium form of resin M71 at 90°C, 11 monosaccharides have been resolved in 140 mn. (136). Complex formation is enhanced by high concentrations of ethanol. Angyal (137), operating on a calcium form column at 0°C, has found that, with 30 % methanol, improvments in the separation of many polyhydroxyl compounds were observed. Main chromatographic separations performed on cation exchangers are given in Table 9. Cation exchangers and anion exchangers have been used successfully in the separation of various mixtures and it is often difficult to give preference to one method. The two methods should be considered as complementary and with mixtures of several mono- and di-saccharides it may be preferable to start with the anion exchanger and re-chromatograph fractions containing disaccharides on the cation exchanger.

D/ ADSORPTION CHROMATOGRAPHY

1) General

The separation is based on affinity of a solute for the support compared with its solubility in the eluent. The adsorbents mainly used are : alumina, silica, carbon.

The general term "polarity" allows the interpretation of the adsorption and solubility process and to explain the partition : a polar solute is preferentially adsorbed on a polar adsorbent and it is necessary to elute with a solvent much more strongly adsorbed on the support than the solute. A consequence is that the polar substances cannot be separated by adsorption. Generally, the better the solvent, the less a solute is adsorbed. The retention volume V_R is given by :

$$V_R = V_m + KW$$

where W is the weight of adsorbent in the column. The partition coefficient K is a function of the solvent, of the adsorbent and of the solute ; in adsorption particularly, the configuration of the solute is an important factor.

In the adsorption process, there is competition for the active sites between the solute and solvent molecules. First the effectiveness in displacing solutes from an adsorbent, corresponding to the elution power of the solvent, was related to its dielectric constant. Then an ordered presentation of solvents, called the eluotropic series, was given by Snyder ; the eluent strength function is defined as the adsorption energy per unit area of the solvent :

$$\log K = \log V_a + E_a (S^\circ - A_s \varepsilon^\circ)$$

where V_a is the adsorbent surface, E_a the adsorbent energy function, which is proportional to the average surface energy of the adsorbent ; S° is the adsorption energy of the solute, A_s is the area of the solid occupied by the adsorbed solute.

When K is determined for the same solute on the same adsorbent with two different mobile phases (1 and 2) the equation becomes :

$$\log K_1/K_2 = E_a A_s (\varepsilon_2^\circ - \varepsilon_1^\circ)$$

TABLE 10

Eluotropic Series in Partition Chromatographies.(D).

	ε° Al_2O_3	δ	P'
n-Pentane	0.00	7.1	0.0
n-Hexane	0.01	7.3	0.0
n-Heptane	0.01	7.4	0.0
Cyclohexane	0.04	8.2	0.0
Carbon disulphide	0.15	10.0	1.0
Carbon tetrachloride	0.18	8.6	1.7
Isopropyl ether	0.28	7.0	2.2
2-Chloropropane	0.29		
Toluene	0.29	8.9	2.3
1-Chloropropane	0.30	8.3	
Chlorobenzene	0.30	9.6	2.7
Benzene	0.32	9.2	3.0
Bromoethane	0.37	8.8	3.1
Ethyl ether	0.38	7.4	2.9
Chloroform	0.40	9.1	4.4
Dichloromethane	0.42	9.6	3.4
Tetrahydrofuran	0.45	9.1	4.2
1,2-Dichloroethane	0.49	9.7	3.7
Methyl ethyl ketone	0.51		4.5
Acetone	0.56	9.4	5.4
Dioxan	0.56	9.8	4.8
Ethyl acetate	0.58		4.3
Methyl acetate	0.60	9.2	
Pentan-1-ol	0.61		
Dimethyl sulphoxide	0.62	12.8	6.5
Aniline	0.62		6.2
Nitromethane	0.64	11.0	6.8
Acetonitrile	0.65	11.8	6.2
Pyridine	0.71	10.4	5.3
Propane-2-ol	0.82	10.2	4.3
Ethanol	0.88	11.2	5.2
Methanol	0.95	12.9	6.6
Ethylene glycol	1.11	14.7	5.4
Acetic acid		12.4	6.2

εAl_2O_3 Snyder's eluent strength function

δ Hildebrand solubility parameter $(cal/cm^3)^{1/2}$

P' Polarity index calculated from Rohrschneider's data (Anal. Chem. 45 1241 (1973))

As a result of this treatment it was possible to give numerical values to the term "polarity" and to arrange the solvent in order: the reference is $\varepsilon^\circ = 0$ on alumina when n-pentane is used as the solvent (Table 10).

The previous relation allows the prediction of the retention volume as a function of the eluent and predicts the separation of solutes of different size by A_s.

2) Application to oligosaccharide fractionation

Adsorption was the first chromatographic system applied to oligosaccharide fractionation. Reich (138) has found in 1939 that a mixture of the p-phenylazobenzoate derivatives of glucose and fructose were separated into two coloured bands on an alumina or silicagel column. Many other separations were then published and new supports appeared (139, 140). The separation on a column is generally performed by elution but was first done by extrusion. Many compounds have been separated on different adsorbents. It is clear that often it is difficult to know if it is a solid-liquid partition or a liquid-liquid partition, especially with cellulose as the support.

Carbon

The method was proposed by Durso (141) in 1950. A mixture of mono-, di- and tri-saccharides in water was adsorbed on carbon ; the monosaccharide was eluted with water and for the di- and tri-saccharides a gradient of ethanol was applied. Then the xylodextrins were separated up to DP = 6 by Whistler (142), the isomaltodextrins (DP $\leq$ 8) and laminaridextrins by Whelan (143), the cellodextrins (DP $\leq$ 7) by Miller (144) and the maltodextrins (DP $\leq$ 12) by French (145) (Figure 22). In this last case, French replaced ethanol by n-butanol or tert-butanol resulting in better selectivity with a lower alcohol content. Finally, mention should be made of the separation of nystose from the fungal α-amylase hydrolysis of sucrose (146).

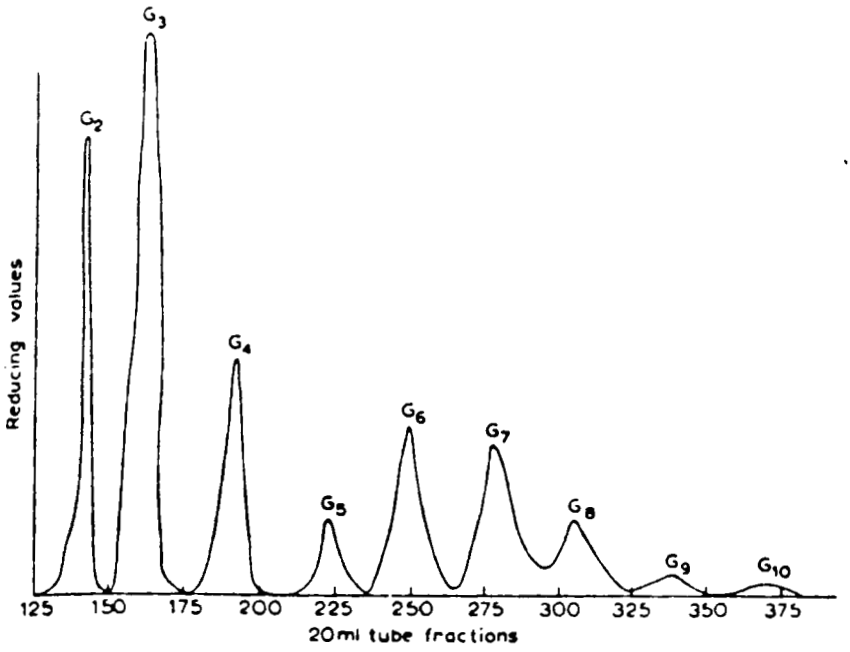


Figure 22. Elution diagram of linear maltodextrins on a charcoal-celite column with n-butyl alcohol gradient elution. Column : 100 g charcoal-75 g celite. Sample : 700 mg maltodextrin (G_2-G_{10}). Elution gradient : 6 l 3 % n-butyl alcohol into 5 l water, followed by 6 l 3.5 % n-butyl alcohol. Flow rate : ca. 2.5 ml/min (145).

Generally, it is necessary to deactivate the carbon by acid treatment ; the adsorption capacity of carbon is low and a high pressure drop is observed, therefore it is usually mixed with celite to accelerate the flow.

Polysaccharide

Powders of starch and cellulose were used, but preferentially cellulose wnich allows a larger flow rate through the column. Hough (147) separated a mixture of L-rhamnose, D-ribose, L-arabinose and D-galactose in n-butanol/water. The technique was also applied in the structural analysis of polysaccharides after methylation and

complete hydrolysis ; the methylated sugars were separated with n-butanol, light petrol ; most of this work was done by Hough around 1950 and was reviewed by Binkley (139).

Starch columns were used for monosaccharides (148) ; the column was prepared in 1-butanol, and L-rhamnose, L-fucose, D-ribose, D-xylose, D-mannose, D-glucose and D-galactose were separated in 1-butanol/1-propanol/H_2O (4/1/1).

Silica

This adsorbent was principally used for extrusion with coloured solutes. Binkley (139) gave some instances eluting with alconol/water, but the capacity of the columns is low. Modified silica gives better results ; on calcium and silicate column (Silene EF) Wolfrom (149) fractionated acetylated oligosaccharides (up to DP 4) from the acetolysis of the cellulose. Similar results are obtained with a column of hydrated magnesium and silicate (Magnesol).

Other adsorbents

Alumina forms complexes with saccharides and the retention depends on the configuration (150). Maltodextrins were separated on hydroxylapatite (151).

A gel of boric acid (152) and a resin of poly(4-vinylbenzeneboronic) acid (1953) were also used ; in both cases, a specific complexe is formed whose stability depends on the pH of the eluent and the position of the hydroxyl groups.

The boric acid gel allowed the separation of L-rhamnose, D-mannose, D-galactose, D-glucose and D-ribose.

This classical adsorption chromatography is no larger used owing to its low selectivity and slow separation.

Modern developments allow much more rapid separations with HPLC and many new supports for this method are now available. However,

in HPLC the mechanism applied is more generally liquid-liquid partition which will be reviewed in the next part.

E/ LIQUID-LIQUID PARTITION CHROMATOGRAPHY

The separation is based on a partition, between two liquid immiscible phases, characterized by an equilibrium constant K ; for a solute X :

$$K = \frac{[X]_s}{[X]_m} = \left[\frac{n_x^s}{n_x^m} \cdot \frac{V_m}{V_s} \right]$$

where $[X]_s$ and $[X]_m$ are the concentrations in the stationary phase (s) and in the mobile phase (m) ; n_x is the number of molecules dissolved in the respective phase of volume V.

The retention volume V_R is :

$$V_R = V_m + K V_s$$

Generally, the capacity factor k' is expressed as :

$$k' = K \frac{V_s}{V_m} = \frac{n_x^s}{n_x^m}$$

The coefficient k' is determined by the relative interactions of X with the two phases ; it depends on the polarity of these phases, and it is usual to vary k' (and R_s) by changing the solvent. Generally K is related to the Hildebrand solubility parameter δ, which is a quantitative measure of the quality of the solvent and can be used to establish the eluotropic series (Table 10). The solubility parameter which indicates the relative position of a solvent helps in the solvent selection.

The eluotropic series following δ is not strictly identical with that obtained in adsorption with $\varepsilon°$, but nearer to that of P' established by reference to solubility measurement (Table 10).

In fact, it is not directly δ which measures the solvent strength but one of the four contributions concerned with specific intermolecular interactions ($\delta = \delta_d + \delta_o + \delta_a + \delta_h$, dispersion, dipole orientation and hydrogen bonding, respectively).

In connection with the solubility parameters of the solute and both phases, a direct relation with the partition coefficient is generally adopted :

$$\log \frac{[X]_s}{[X]_m} = \bar{V}_x \frac{(\delta_x - \delta_m)^2 - (\delta_s - \delta_x)^2}{2.3\ RT}$$

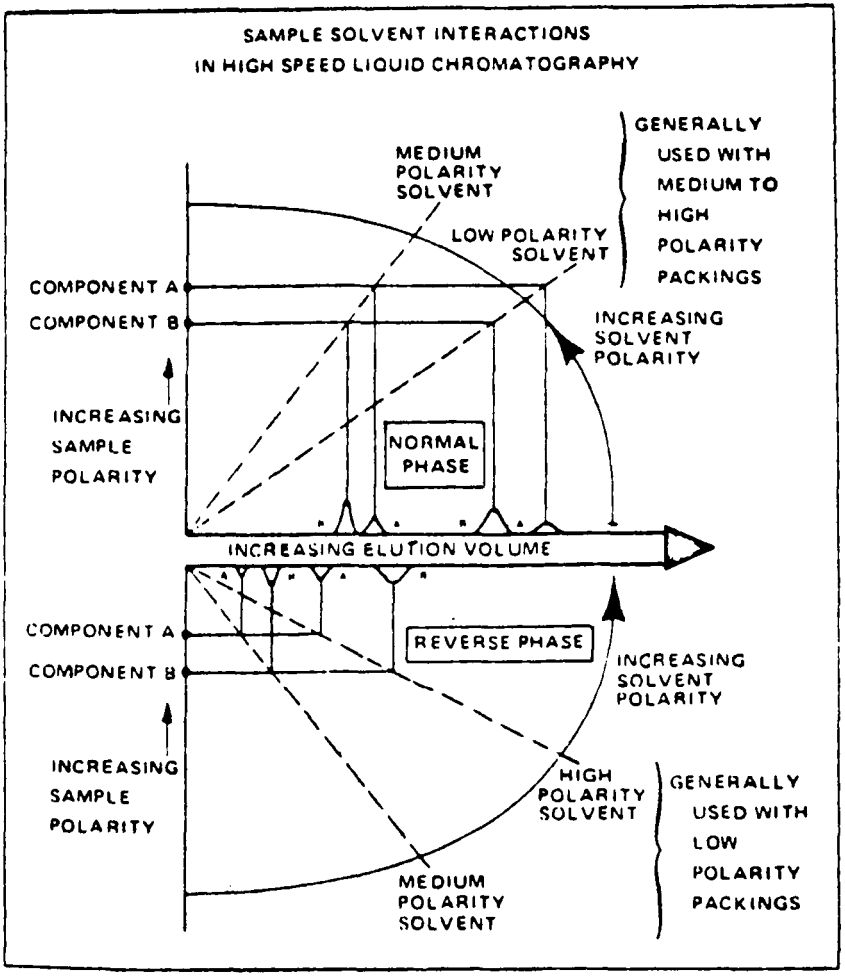
in which $\bar{V}_x$ is the molar volume of X. The δ values for usual solvents are given in the literature ; for a given solute X, it is possible to approximate δ using the Small method based on an additivity rule of individual functions of the solute.

It is assumed that good separations are obtained when $|\delta_s - \delta_m| \geq 4$ and δ_x is located between δ_s and δ_m.

When δ_s and δ_m are too far separated, it is possible to get adsorption. The different types of liquid-liquid partition are the following :

- normal phase. The stationary phase is polar (generally H_2O adsorbed on silica). An organic solvent with medium polarity is used for elution and the less polar solute is eluted first. The retention volume decreases as the polarity of the solvent increases. When the retention is too high, it is necessary to choose a more polar solvent or decrease the polarity of the stationary phase. Generally polar solutes cannot be separated by normal phase partition and it is necessary to adopte the reverse phase (Figure 23).

Figure 23. General interactions between sample and solvent as a function of polarity (Waters Ass.).



	NORMAL PHASE	REVERSE PHASE
PACKING POLARITY	High	Low
SOLVENT POLARITY	Low to Medium	Medium to High
SAMPLE ELUTION ORDER	Least Polar First	Most Polar First
EFFECT OF INCREASING SOLVENT POLARITY	Reduces Elution Time	Increased Elution Time

- reverse phase.Low polarity packings are used and the elution is performed with aqueous solution or medium polarity solvents. The more polar solute is eluted first and the time of retention increases as the polarity of the solvent increases.

- Paired ion partition chromatography (PIC). This is a method used for ionic solute separation ; it seems to be a more general method competitive with ion exchange chromatography. A reverse phase is used and a salt of ion pair type is formed between the ionic solute and a large hydrophobic counterion (with carboxylic acid, elution is performed in presence of a quaternary amine on pH 7-8 ; with basic samples, an alkyl sulphonate is used at pH 3-4). The eluent is a solvent with medium polarity (for example, methanol/water).

Generally, the polarity of a solute decreases when the molecular weight increases and when its functionality decreases ; the solubility is the best when δ_x is equal to the solubility parameter of the solvent. These remarks allow to select the chromatographic method.

Application to saccharide fractionation.

Liquid-liquid partition chromatography was mainly used for paper chromatography in the field of saccharides. Recently it has developed largely as application of HPLC and we will now discuss these results.

F/ HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.

Classical liquid chromatography utilized large diameter particles, porous column packings, large bore columns and low column-head pressures. On the other hand, high speed liquid chromatography is characterized by small particle diameter column packings, narrow-bore columns and high inlet pressure but generally there is no difference of principle and, according to the mechanisms involved, we

will find again the four basic liquid chromatography modes. Carbohydrate analysis by HPLC has been previously reviewed by Schwarzenbach (154) but in this field progress is rapid and the present report deals with the most recent developments.

1) Gel permeation chromatography (G.P.C.)

Gel permeation chromatography has been shown to be well adapted to separation of oligosaccharides but the gels used have poor mechanical strength and cannot withstand high pressures. Meanwhile a procedure for the separation of water-soluble wood polysaccharides on a Bio-Gel P60 packed column was described by Belue (155) ; Bio-Gel P2 column has also been used to study the products formed in the thermal decomposition of methyl α-D-glucopyranoside. Low molecular-weignt carbohydrates are not separated on Bio-Gel P60 but with methanol as the mobile phase and EM Gel OR-PVA 500 (vinyl acetate copolymer), stachyose, cellobiose and ribose can be fractionated in less than 30 min. Utilization of non-aqueous solvents limits the field of application of this process owing to solubility problems. On the other hand, separations of partially and completely substituted monosaccharides were achieved on EM Gel OR-PVA 500 column or a Poragel 60 Å column (polyacrylamide gel) without problems, these compounds being readily soluble in organic solvents (156). The separation by size-exclusion of mono-, di-, tetra-, hexasaccharides, higher oligosaccharides and hyaluronic acid produced by enzymic hydrolysis of glycosaminoglycan hyaluronic acid have also been obtained by Knudsen (156 bis) on a μ-Bondagel E linear column, followed in series by two μ-Porasil GPC 60 Å columns, with 20 m M sodium acetate buffer (pH 4.0) containing 1.5 mg/l of glycosaminoglycan hyaluronic acid as the eluent.

2) Ion exchange chromatography

Anion exchange resins have been used extensively in classical liquid chromatography but only a few examples of separations have been reported in H.P.L.C. These results are summarized in Table 11.

TABLE 11

Separation of Neutral Saccharides by H.P.L.C. on Polycationic Resins

	ARABINOSE	GALACTOSE	FRUCTOSE	FUCOSE	GLUCOSE	LACTOSE
Arabinose	x	157	157	157	157	
Galactose	157	x	157 157 ter	157	157 157 ter	157
Fructose	157	157 157 ter	x	157	157 157 ter	
Fucose	157	157	157	x	157	157
Glucose	157	157 157 ter	157 157 ter	157	x	157
Lactose		157	157	157	157	x
Mannose		157 157 ter	157 157 ter	157	157 157 ter	157
Raffinose		157		157	157 157 bis	157
Rhamnose	157	157	157	157	157	
Ribose	157	157	157	157	157	157
2-deoxy-ribose	157	157	157	157	157	157
Sucrose		157		157	157 157 bis	157
Xylose		157 ter	157 ter		157 ter	

Supports : CDR-10 (5-7μ) sulfate form (Mitsubishi, Tokyo, Japan) -(157)
eluent : ethanol/water (80/10 - 90/10)

Bondapak AX/Corasil (37-50μ) (Waters Ass. Milford Mass. U.S.A. (157 bis)
eluent : water/ethylacetate/isopropanol (25/50/35)

TABLE 11 Continued

MANNOSE	RAFFINOSE	RHAMNOSE	RIBOSE	2-DEOXY-RIBOSE	SUCROSE	XYLOSE
157		157	157	157		
157 157 ter	157	157	157	157	157	157 ter
157 157 ter		157	157	157		157 ter
157	157	157	157	157	157	
157 157 ter	157 157 bis	157	157	157	157 157 bis	157 ter
157	157		157	157	157	
x	157		157	157	157	157 ter
157	x		157	157	157 157 bis	
		x	157	157		
157	157	157	x	157	157	
157	157	157	157	x	157	
157	157 157 bis		157	157	x	
157 ter						x

Hitachi 3013 N (phosphate form) (157 ter)
eluent : acetonitrile/water (80/20)

Oshima (157) obtained interesting separations on the form SO_4^{2-} of a macroreticular anion-exchange resin (CDR-10 Mitsubishi, Tokyo). At 20°C using aqueous ethanol of high ethanol content (80% and 90 % ethanol) as eluent a mixture of 2-deoxy-ribose, rhamnose, fucose, ribose, arabinose, fructose, galactose, glucose, lactose, sucrose was resolved within 60 min. This chromatogram was complicated by the presence of anomers of saccharides. Linden (157 bis) also separated glucose, sucrose, raffinose on a Bondapak AX/Corasil column in water/ethyl acetate/isopropanol (25/50/35). High speed separation of monosaccharides (xylose, fructose, mannose) has been achieved by Noel (157 ter) on a Hitachi 3013 N anion exchange resin in the phosphate form ; the chromatogram is shown in Figure 24.

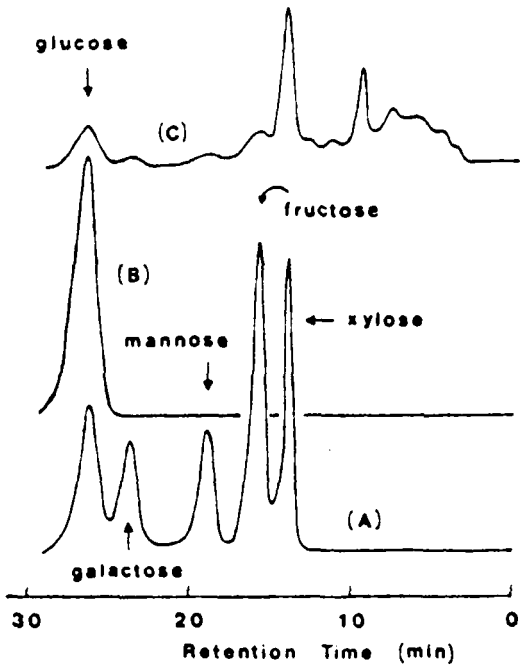


Figure 24. Separation of various monosaccharides on Hitachi 3013N anion exchange resin. Column : 25 cm x 0.43 cm i.d. Elution : isocratic, 80 % acetonitrile in water at 1.0 ml min^{-1} (157 ter).

Cation exchange chromatography has been found to give better results. This method which requires only water as eluent, has been widely used for rapid fractionation of saccharide mixtures. The resins were utilized in the potassium (158), calcium (159-164) and silver (159) forms. As back as 1974, Palmer (158) separated, within 30 min., sucrose, glucose and fructose on a Aminex Q-150S (K^+) column. Separation was carried out at 60°C because the resolution was markedly improved by increasing the temperature. With this system giving rapid and high-resolution separations, liquid chromatography became a powerful tool in the sweetener industry and an automated method has been developed (159). An increase in the possibilities of the process has been obtained by the use of the calcium form of a cation exchanger. Figure 25-A shows the separation of the saccharides present in a medium invert syrup produced from beet sugar on the Aminex Q15 S (Ca^{2+}) column at 85°C ; this column is also capable of separating melezitose, melibiose, dextrose, galactose, arabinose and fructose (159). This procedure has been employed by Ladish (161) with an Aminex 50W-X4 (Ca^{2+}) column in order to study the kinetics of the enzyme-catalyzed degradation of cellulose. A satisfactory separation of cellodextrins (DP 6 through glucose) has been performed within 35 min. (Figure 25-B). The effects of resin cross-linking on both separation and speed of analysis of corn syrup have investigated by Fitt (163). Aminex 50W - X4 at 80°C gives excellent results with linear oligosaccharides but it is also a suitable method for the separation of cyclodextrins (164).

In the use of cation exchange resin for liquid chromatography of sugars, an original procedure has been described by Kumanotani (165,166). On a Hitachi-gel 3019 S in the H^+ form eluted with 0.5% formic acid, Kumanotani separated a mixture of raffinose, lactose, galactose, 2-deoxy-ribose. Using 0.1 % orthophosphoric acid as the eluent on a TSK-gel LS 212 (H^+) column, neutral saccharides such as raffinose, maltose, glucose, galactose, arabinose, ribose were well separated. On these two supports, glucuronic and galacturo-

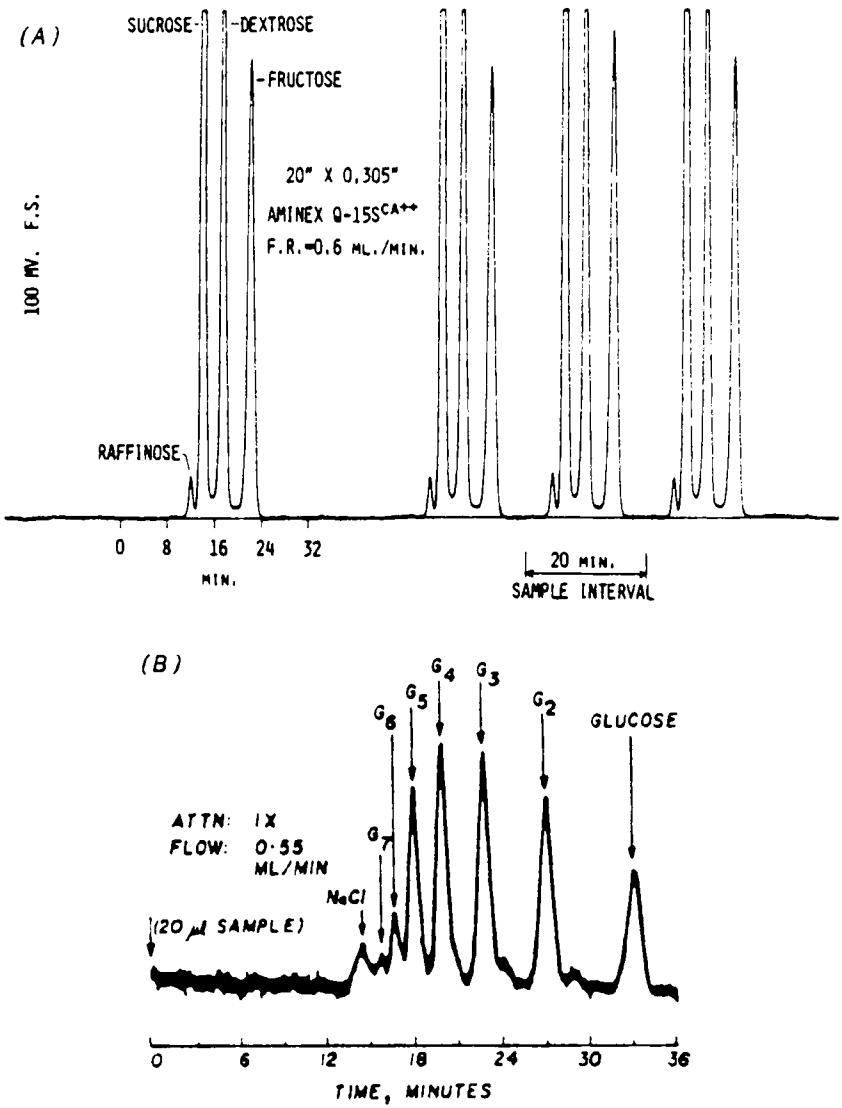


Figure 25. Separation of oligosaccharides on cation exchange resins Ca^{2+} form. Eluent : H_2O

(A) Chromatogram of medium invert sugar syrup on Aminex Q 15S at 85° C (159).

(B) Cellodextrin separation. Chromatogram : G_2 = cellobiose ; G_3 = cellotriose ; G_4 = cellotetraose ; G_5 = cellopentaose ; G_6 = cellohexaose ; G_7 = celloheptaose.
AMINEX AG 50W-X_4 at 85° C (161).

nic acids can also be isolated. The elutions were conducted at room temperature. The separations of neutral compounds are based on differences in molecular size ; with uronic acids, electrostatic repulsion has to be taken into account. Table 12 lists some separations achieved on cation exchange resins.

3) Adsorption chromatography.

Adsorption chromatography, or liquid-solid chromatography (L.S.C.) is not a convenient method for the separation of neutral saccharides. On the other hand it seems well adapted to separations of some sugars derivatives. Lehrfeld (167) analyzed a complex mixture of mono- and disaccharides by conversion into benzoate esters using a Corasil II column with a gradient of diethyl ether in hexane (Figure 26). Nachtmann (168) obtained similar results with nitrobenzoates of sugars and sugar alcohols on silica gel (Lichrosorb SI 60) or alumina (Alox T), eluted isocratically at room temperature with n-hexane/ethyl acetate/dioxane or n-hexane/chloroform/acetonitrile/tetrahydrofuran mixtures. In the same way, the benzyloxime-perbenzoyl derivatives of sugars have been separated by Thompson (169) on a μ-Porasil column with a hexane/dioxane mixture as the eluting solvent.

Another application has been described by Lee (170). Products obtained by the enzymatic degradation of chondroitin sulfates can be resolved on silica gel (Partisil PXS or Lichrosorb SI-100) with the ternary solvent dichloromethane/methanol/0.5 M ammonium formate buffer (pH 4.8). We classify these separations as liquid-solid chromatography but it is often difficult to distinguish between adsorption chromatography and liquid-liquid partition chromatography.

Indeed, n-hexane is saturated with water, the stationary solid surface can be coated by this water and the separation process becomes a partition between the moving phase and the stationary liquid. Such phenomenon seems to occur in the separation of a glu-

TABLE 12
Separation of Neutral Saccharides by H.P.L.C. on Polyanionic Resins

	ARABINOSE	FRUCTOSE	GALACTOSE	GLUCOSE	LACTOSE	MALTOSE
Arabinose	/		159-166	159-161 166		166
Fructose		/		158-159 161		
Galactose	159-166		/	159-161 166	161-165	166
Glucose	159-161 166	158-159 161	159-161 166	/	161	160-166
Lactose			161-165	161	/	
Maltose	166		166	160-166		/
Mannose		161		161		
Melibiose	159		159	159		
Melezitose	159		159	159		
Raffinose	166	159	166-165	159-166	165	166
Ribose	166		166	166		166
2-deoxy-ribose.			165		165	
Sucrose		158-159 161		158-159 161		
Xylose	161			161		

Supports :

Aminex Q 150-S(K^+) 20-35µ (Bio-Rad-Richmond-Calif. U.S.A.) Eluent : water (158)
Aminex Q 15-S(Ca^{2+}) 19-25µ (Bio-Rad-Richmond-Calif. U.S.A.) Eluent: water (159-160)
Aminex A-5 (Ca^{2+}) 11-15µ (Bio-Rad-Richmond-Calif. U.S.A.) Eluent: water (159)

TABLE 12 Continued

MANNOSE	MELIBIOSE	MELE-ZITOSE	RAFFINOSE	RIBOSE	2-DEOXY-RIBOSE	SUCROSE	XYLOSE
	159	159	166	166			161
161			159			158-159 161	
	159	159	166-165	166	165		
161	159	159	159-166	166		158-159 161	161
			165		165		
			166	166			
/							
	/	159					
	159	/					
			/	166	165	159	
			166	/			
			165		/		
			159			/	
							/

AG-50W x 4 (Ca^{2+}) 20-30μ (Bio-Rad-Griffin-Calif. U.S.A.) Eluent : water (161)
TSK-Gel LS 212(H^+) 12μ (Toyo Soda, Tokyo-Japan)
Eluent : orthophosphoric acid 0.1% (166)
Hitachi-gel 3019 S (H^+) 30-40μ
Eluent : formic acid 0.5 % (165)

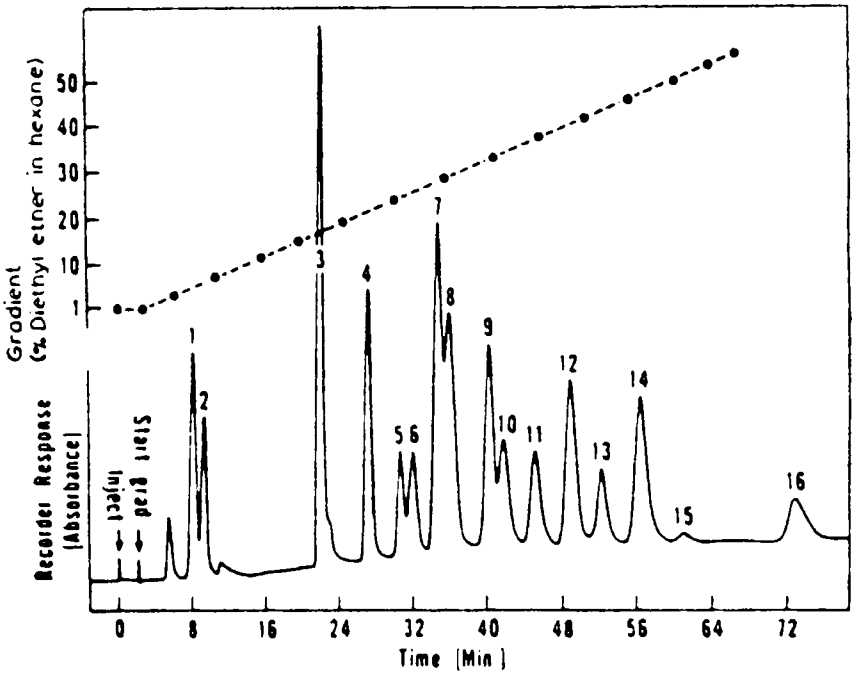


Figure 26. Separation of 16 perbenzoylated hydroxy compounds on Corasil II (37-50 µm ; Waters Assoc.) by HPLC. Temperature, ambient. Benzoates of : 1,1-butanol ; 2,methanol ; 3,ethylene glycol ; 4,glycerol ; 5,α-D-xylose ; 6,β-D-xylose ; 7,α-D-mannose ; 8,α-D-glucose, α-D-galactose ; 9,β-D-glucose,D-galactose ; 10, D-galactose ; 11, β-D-mannose ; 12, sucrose ; 13, α- and β-maltose ; 14, lactose ; 15, maltotriose ; 16, lactose oligosaccharides impurities (167).

cose, fructose, mannose mixture on silica (Lichrosorb SI 60) with acetonitrile containing 0.1 % of water as the mobile phase (170 bis).

4) Partition chromatography.

Partition chromatography, or liquid-liquid chromatography (L.L.C.) is the most frequently used system in analysis of sugars by H.P.L.C.

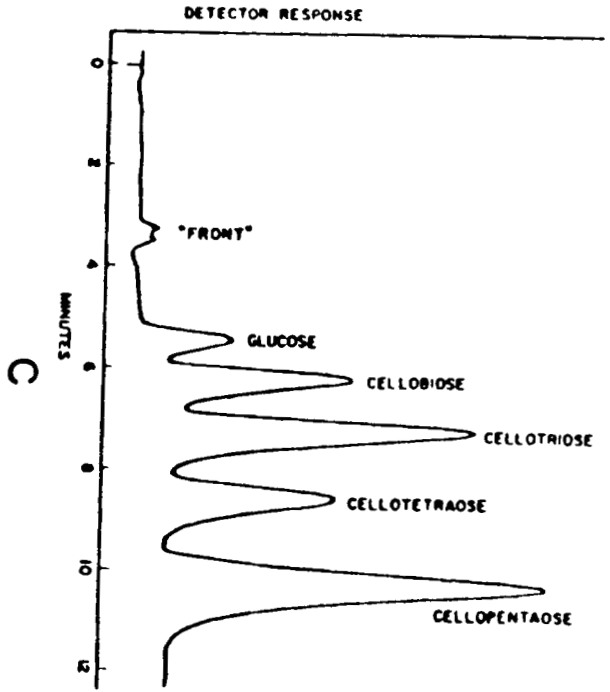
The compounds are separated based on their relative solubilities in the mobile phase and the immiscible phase which is coated or chemically bonded on a solid support. If the stationary phase is polar and the mobile phase non-polar, the technique is termed normal phase ; with a non-polar packing surface and a polar mobile liquid, the affinity mode is termed reverse phase.

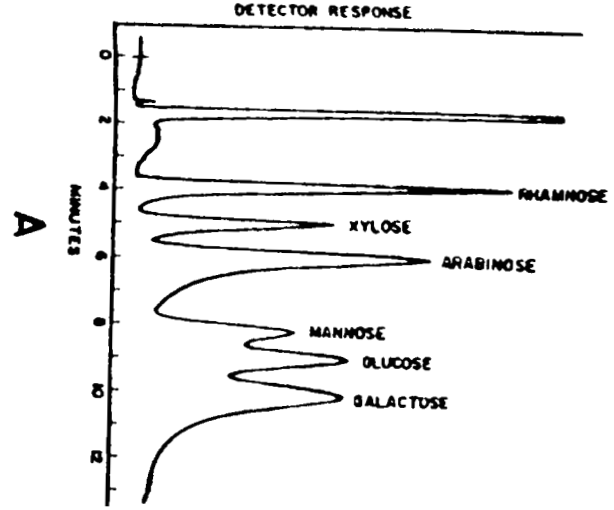
a/ Partition chromatography in the normal phase

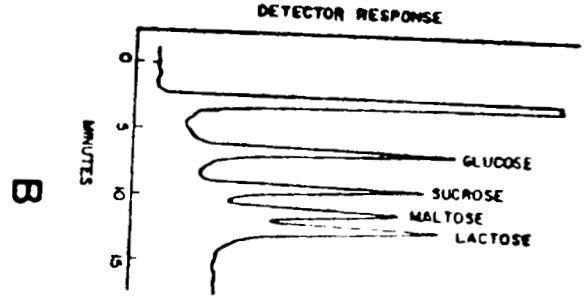
We have to distinguish between covalently bonded supports and supports coated with a component of the eluent.

- Chemically bonded phase

In 1975, Palmer (171) published some very interesting separations on a μ-Bondapak/Carbohydrate column. The support, consisting of μ-silica with bonded amine functional groups, seems able to resolve the most complex mixtures in a short time (Figure 27). The separations are performed with acetonitrile/water as the mobile phase and the retention times of the saccharides depend on their respective concentration. Generally, monosaccharides are separated in acetonitrile/water mixture (85/15) and oligosaccharides with a 65/35 ratio. This system appears a rapid and versatile method for sugar analysis and it has been widely utilized, particularly in the food industry. The μ-Bondapak/Carbohydrate column has been found also suitable for a rapid separation of cyclodextrins (172) and for the analysis of the disaccharides obtained after chondroitinase digestion (173). In the last case, the column was eluted with 0.2 M sodium acetate or 0.02 M sodium sulphate solution containing 0.01 M acetate buffer. A combination of cyano- and amino-bonded phases has been developed. This polar support, available under the name Partisil-10 PAC, has been used successfull by Rabel (174) and Lee (175-176). Rabel separated mono- and oligo-saccharides using acetonitrile/water as the eluent, and to avoid the disadvantage of peak tailing, acids or salts were added. Additions of various anions to the mobile phase and adjustment of pH allow optimization of the separations.







Like the μ-Bondapak/Carbohydrate column, the Partisil-10 PAC column has been used by Lee for separations of unsatured disaccharides derived from chondroitin sulfates (175), heparan sulfate and heparin (176) ; the mobile phase was the ternary solvent system acetonitrile/methanol 0.5 M ammonium acetate (pH 6.5). Similar separations have also been achieved on Altex Lichrosorb NH_2 columns (175). In view of the great interest in chemically bonded phases, certain authors prepared their own supports. By bonding an aminopropyltriethoxysilane group to the surface of a silica gel (Lichrosphere SI 100) Schwarzenbach (177) obtained a support which gave separations similar to those reported by Palmer (171). The factors affecting support bonding have been investigated by Jones (178). He found that with Partisil 5 as chromatographic support a support bonding of 7 % in aminoalkyl substituents led to a packing with excellent fractionation capabilities. This packing can be prepared in 5-10 min. by shaking silica gel and γ-aminopropyltriethoxysilane in hexane at room temperature. According to the specific surface area of silica particles (Silasorb) Kahle (179) adapted the support either for the separation of monosaccharides or of oligosaccharides. Moreover, anomers of monosaccharides could be separated at 0°C on aminopropyl silica gel, converted into the sulphate form, with acetonitrile/water (80/20) as eluent.

Figure 27. Separation of oligosaccharides by HPLC on μ-Bondapak/carbohydrates column. Eluents : water/acetonitrile with different composition (171).

A - Separation of monosaccharides. Flow rate 2.5 $ml.mn^{-1}$ (water/acetonitrile 15/85).

B - Separation of disaccharides. Flow rate 1.5 $ml.mn^{-1}$ (water/acetonitrile 20/80).

C - Separation of ß 1 → 4 linked glucose oligomers. Flow rate $1ml.min^{-1}$ (water/acetonitrile 35/65).

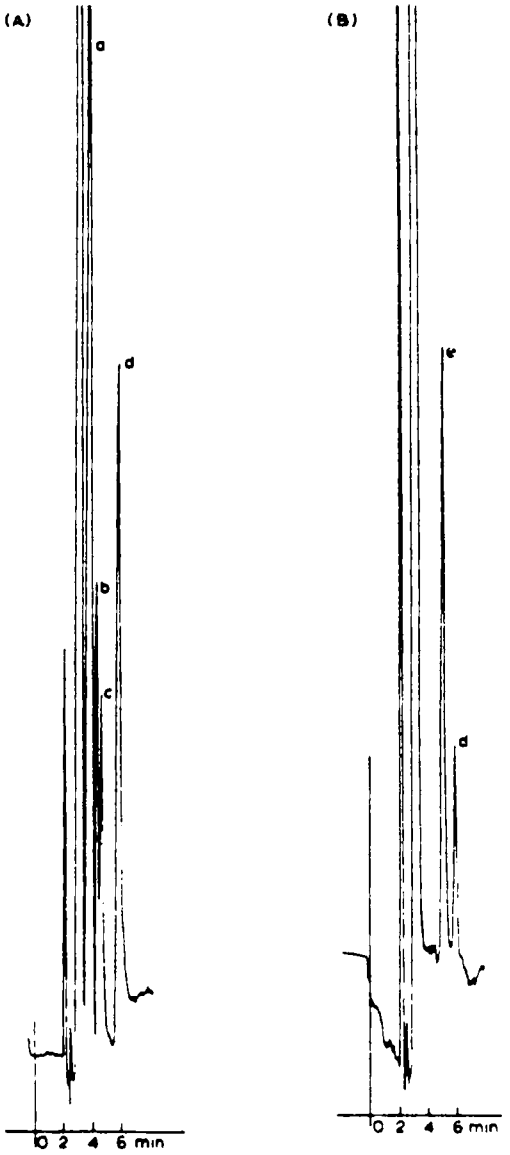


Figure 28. Separation of oligosaccharides on Lichrosorb SI 60 (5 µm) column. Eluent : ethylformate/methanol/water (55:25:10) (180).
(A) a = fructose b = sorbitol c = saccharose d = lactose
(B) e = maltose and cellobiose d = lactose.

- Chromatographic support coated with a component of the eluent.

A typical example of this system was given by Rocca (180) : within 20 min., a fructose, sorbitol, sucrose and lactose mixture was resolved (Figure 28) on a silica (Lichrosorb SI-60 5μ) column eluted with a three components system : ethyl formate/methanol/water (60/20/10). Similar results have been obtained with alumina gel (Alox T - 5μ).

The separation process has a mechanism of partition between the mobile phase and the stationary phase formed by silica impregnated with water. It has been shown that small changes in the methanol concentration has a great influence upon the retention time but that the resolution was little affected. An increased methanol concentration resulted in decreased separation factors ; on the other hand by increasing the water concentration the differences were small. The mechanism was based on a competition between methanol and solute molecules ; methanol stationary phase interactions played a leading part compared to methanol solute interactions. However, monosaccharide mixtures are often not resolved and low water concentrations lead to solubility problem with oligosaccharides. In 1978, Aitzetmuller (181) perfected a rapid and simple method. Polyfunctional amines have a great affinity for the silica, so if a small amount of polyamine (0.01 to 0.1 %) is added to the eluent (acetonitrile/water) it is possible to impregnate a silica column in situ. The separating performances are similar to those of permanently bonded phases. In the presence of the amine modifier the separation factors are greatly increased and a much higher percentage of water can be used. At 30 or 40 % of water, Aitzetmuller obtained good results in such separations as fructose, glucose, sucrose, lactose and raffinose (Figure 29), glucose, maltose, maltotriose etc... and even α, β and γ cyclodextrins (182). Thus an ordinary silica column can be transformed into a powerful column for carbohydrate analysis ; it was interesting therefore to study the influence of the amine modifier structure. Primary, secondary and tertiary butylamine, an homologous

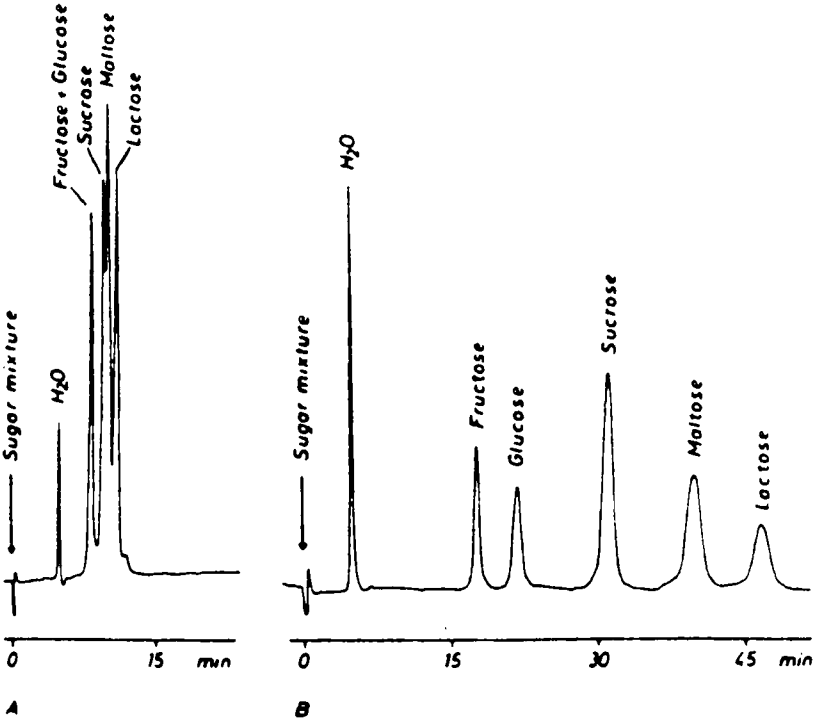


Figure 29. Chromatograms showing increase of retention times after addition of amine modifier to HPLC solvent. Steel column (240 x 7 mm I.D.) slurry-packed with Lichrosorb SI 60 (5 µm), flow rate (2 ml/min ; ca. 30 bar) and an RI detector at X16. Temperature : ambient.

Separation of fructose, glucose, sucrose, maltose and lactose with 25 % of water in acetonitrile as mobile phase : A, without, and B, with, 0.01 % of amine modifier. At this water content, fructose and glucose appear as one peak, and sucrose and maltose as overlapping peaks, on the plain silica column without amine modifier. After in situ impregnation, all are well resolved (181).

series of n-alkylamines, a diamino n-alkyl series and a polyamine series have been investigated by Wheals (183). The best results in the separation of fructose, glucose, sucrose, maltose and lactose have been observed with polyamines. Silica dynamically modified provided a particularly excellent method of separating oligosaccharides. Indeed, 50 % aqueous acetonitrile containing 0.01 % of amine modifier has been found to be a suitable eluent for the separation of maltodextrins up to DP 20 (Figure 30) under 40 min. (184), when a separation up to DP 10 was achieved in acetonitrile/water (65/35) on a μ-Bondapak carbohydrate column.

In general the retentions were lower than those obtained with the chemically bonded amino phase but the amine layer being continually regenerated by the eluent, this coated support proved to be very stable and to give reproducible results.

In the same way, the modification in situ may be carried out on cartridge Radial-Pak Silica (185). The use of this radially compressed cartridge leads to a highly efficient homogeneous column and its principal advantage is to give resolutions comparable with those of traditional columns with an appreciable decrease of the analysis time.

Partition chromatography on bonded or modified silica gel is a rapid and versatile method for analysis of sugars. The most common separations are summarized in Tables 13 and 14. However, aqueous organic eluents are not convenient solvents for oligosaccharide mixtures. Free sugars are readily soluble in water but an increase in the water percentage corresponds to an increase in the polarity of the mobile phase, and higher concentrations of water give no resolution.

Therefore separations of various carbohydrates have been investigated on reverse phase supports.

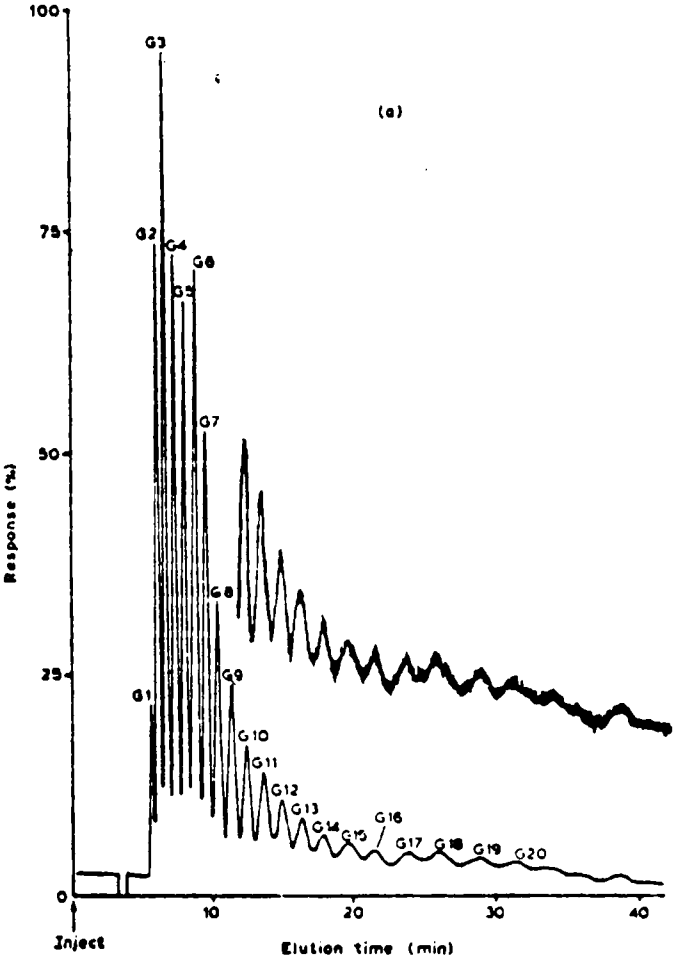


Figure 30a. Fractionation of D-gluco-oligosaccharides present in a sample of hydrolysed starch. Chromatographic conditions : silica (5 µm) column (200 mm x 8 mmi.d.) eluted at 2.0 ml/min with 50 % aqueous acetonitrile containing 0.01 % of polyamine modifier ; the upper trace in shows the signal-to-noise ratio at twice the normal sensitivity (184).

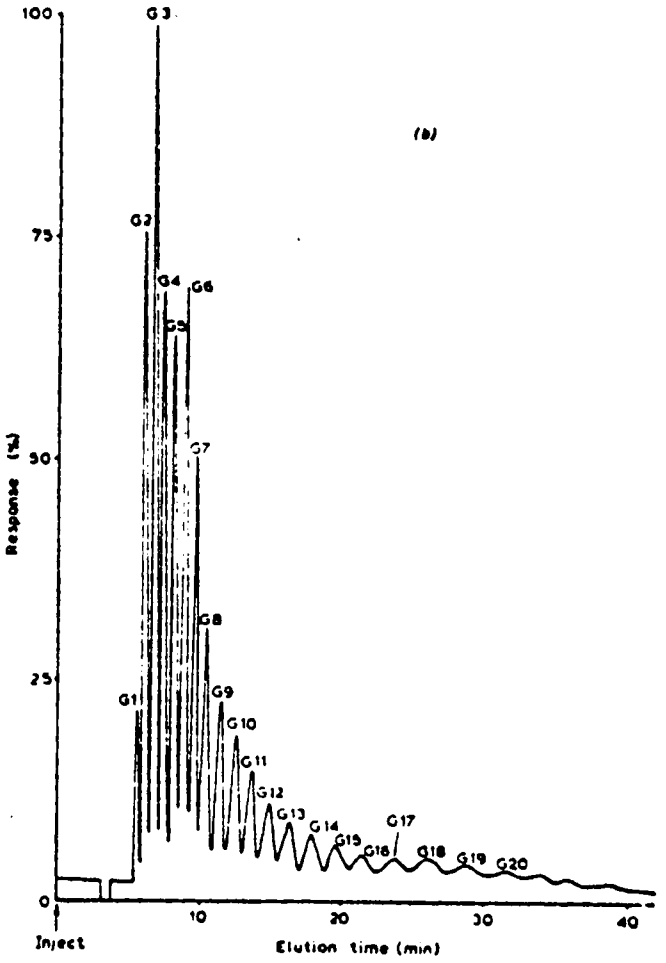


Figure 30b. Fractionation of D-gluco-oligosaccharides present in a sample of hydrolysed starch. Chromatographic conditions: silica (5 μm) column (200 mm x 8 mmi.d.) eluted to 2.0 ml/min with 50% aqueous acetonitrile containing 0.01% of 1,4,-diaminobutane.

TABLE 13

Separation of Neutral Saccharides by Liquid-liquid Partition H.P.L.C. on Bonded Supports.

	Arabinose.	Cellobiose.	Fructose	Galactose.	Glucose	Lactose	Maltose	Maltotriose.
Arabinose	/			171	171-177	177	177	177
Cellobiose		/			171			
Fructose			/		171-157b, 174-177-178-179	174-178	178	
Galactose	171			/	171			
Glucose	171 177	171	171-157b, 174-177-178-179	171	/	171-174 177-178	171-157b, 177-178	177
Lactose	177		174-178		171-174 177-178	/	171 177-178	177
Maltose	177		178		171-157b, 177-178	171-177 178	/	177
Maltotriose.	177				177	177	177	/
Mannose	171			171	171			
Melibiose								
Raffinose								
Rhamnose	171		177	171	171-177			
Ribose	177		177		177	177	177	177
Stachyose								
Sucrose	177		171-157b, 174-178		171-157b, 174-177-178	171-174 177-178	171-177 178	177
Xylose	171	171	174-177 179	171	171-174 177-179	174		

Supports :

μ-Bondapak/Carbohydrate (Waters Ass.) Eluent:acetonitrile/water (171-157 bis)

Partisil 10-PAC PXS 10/25 (Whatman) Eluent:acetonitrile/water pH = 5.0 with H_3PO_4 (174)

Lichrosphère SI 100 5μ (Merck)+3-aminopropyltriethoxysilane(Aldrich) Eluent : acetonitrile/water (177).

TABLE 13 Continued

Mannose	Melibiose	Raffinose	Rhamnose	Ribose	Stachyose	Sucrose	Xylose
171			171	177		177	171
							171
			177	177		171-157bis 174-178	174-177-179
171			171				171
171			171-177	177		171-157bis 174-177-178	171-174 177-179
				177		171-174 177-178	174
				177		171-177 178	
				177		177	
/			171				171
	/					157bis	
		/			174		
171			/	177		174	171-177
			177	/		177	177
		174			/	174	
	157bis	174		177	174	/	174
171			171-177	177		174	/

Partisil 5 (Whatman) + γ-aminopropyltriethoxysilane (Aldrich)
Eluent : acetonitrile/ water (178)

Silasorb (Lachema, Brno, Czechoslovakia) 10u+3-aminopropyltriethoxysilane
Eluent : acetonitrile/water (179)

TABLE 14
Separation of Neutral Saccharides by Liquid-liquid Partition H.P.L.C. on Coated Supports

	CELLOBIOSE	FRUCTOSE	GALACTOSE	GLUCOSE	LACTOSE
Cellobiose					180
Fructose			180	180-181 182-183 185	180-181 182-183 185
Galactose		180		180	
Glucose		180-181 182-183 185	180		181-182 183-185
Lactose	180	180-181 182-183 185		181-182 183-185	
Maltose		181-182 183-185		181-182 183-185	180-181 182-183 185
Raffinose		181-182		181-182	181-182
Ribose		180			
Sorbose					
Stachyose		182		182	182
Sucrose		180-181 182-183 185		181-182 183-185	181-182-180 183-185
Xylose		180	180	180	

Supports :

Lichrosorb SI 60-5μ (Merck)
Alox T 5μ (Merck) eluent : ethyle formate/methanol/water (180)

Lichrosorb SI 60-5μ eluent : acetonitrile/water + 0.01% amine (181-182)

TABLE 14 Continued

MALTOSE	RAFFINOSE	RIBOSE	SORBOSE	STACHYOSE	SUCROSE	XYLOSE
181-182 183-185	181 182	180		182	180-181 182-183 185	180
						180
181-182 183-185	181 182			182	181-182 183-185	180
180-181 182-183 185	181 182			182	180-181 182-183 185	
	182			182	181-182 183-185	
182				182	181-182	
			180		180	
		180			180	
182	182				182	
181-182 183-185	181 182	180	180	182		

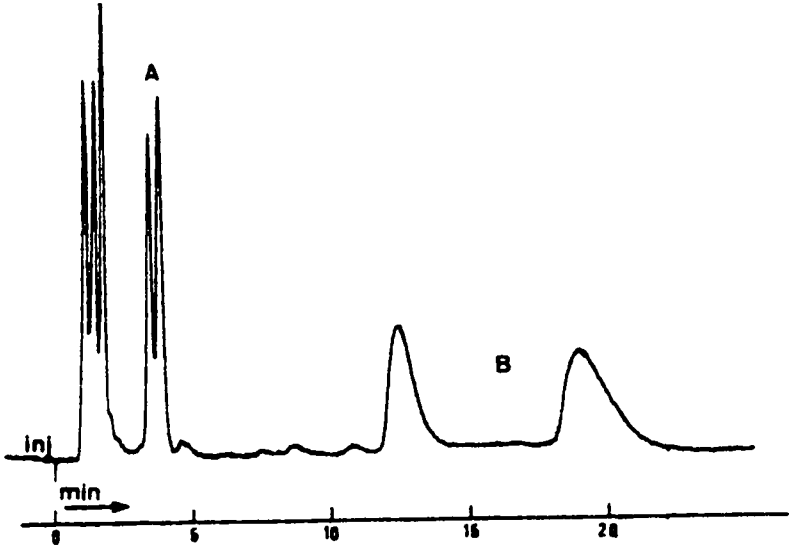
Radial PAK B (Waters Ass.) eluent : acetonitrile/water • 0.1% tetraethylenepentamine (185)

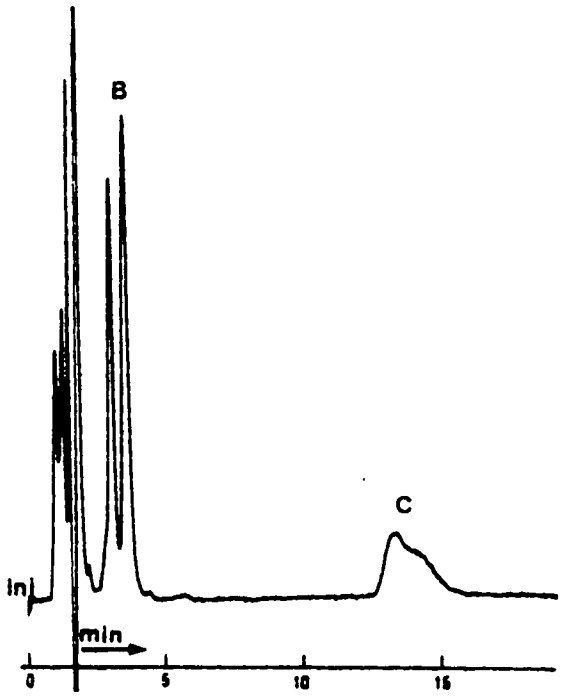
b/ Reverse phase liquid chromatography.

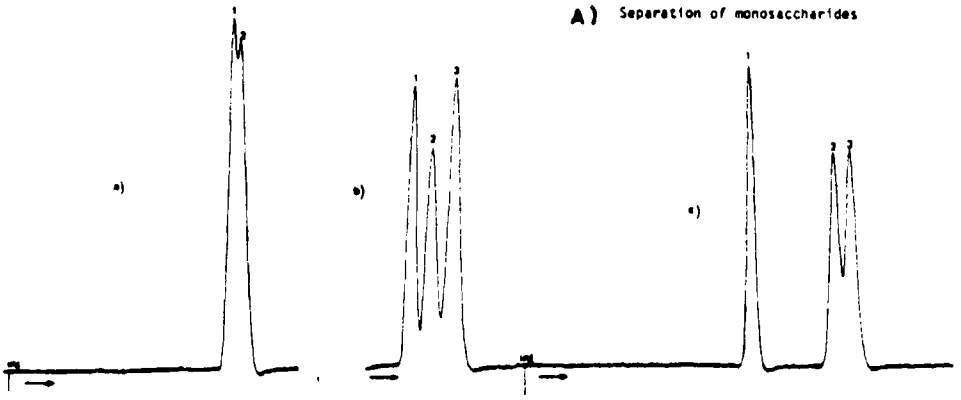
One of the first separations was reported by Noel (157 ter) on a Chromosorb LC9 column. On this reverse phase support, eluted with acetonitrile/water on linear gradient (70 % to 62.5 % acetonitrile in water), oligosaccharides contained in a wood extract were fractionated up to DP 30 in 30 min. Under these conditions no separations of DP lower than 5 can be considered and moreover there is the solubility problem. Recently, it has been found that a reverse phase column with pure water as the eluent provided a high performance system for the separation of certain neutral free saccharides or methylated sugars. Cheetham (186) separated mono-, di- and tri-O-methyl-glucose on a μ-Bondapak C18 column using water with 1 % ammonium acetate as the mobile phase. Addition of salt has little effect on the chromatographic behaviour of the sugar and similar results are obtained in pure water. The more highly methylated compounds are only eluted in the presence of alcohol (5 to 30 %). Indeed the retention time of all saccharides is decreased by increasing the alcohol concentration. The capabilities of the column in separating partially methylated sugars have been adapted with good results (Figure 31) to structural polysaccharide investigations (187).

By eluting water, several separations of mono- and oligo-saccharides are possible (188) ; the elution volumes increase with increase in DP and depend on the type of oligomer. Likewise, the resolution is temperature dependent (189) and is markedly increased with decrease in temperature. The method is not very efficient with mono-

Fig. 31. Structural analysis of a scleroglucan-HPLC of methylglucose derivatives after total hydrolysis on C_{18} μ-Bondpak column. A = 2,4 dimethylglucose. B = 2,4,6 trimethylglucose. C = 2,3,4,6-tetramethylglucose (upper chromatogram = eluent H_2O; flow rate 2 ml/min; lower chromatogram = eluent H_2O/methanol (85/15); flow rate 2 ml/ min (181).





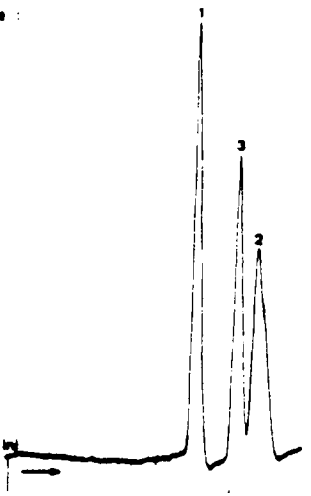


a) separation of glucose(1) from arabinose(2)

b) separation of glucose(1) from rhamnose(2) and fucose(3)

c) separation of glucose(1) from α(2) and β(3) Me-glucoside.

Flow-rate : 0.1 ml/mn ; temperature : 35°C ; detector attenuation x 4 ; concentration : 10mg/ml ; sample volume : 5µl ; chart-speed : 2.5 mm/mn.



B) Separation of trisaccharides : maltotriose(1) from cellotriose(2) and raffinose(3).

Flow-rate : 0.2ml/mn ; temperature : 15°C ; detector attenuation : x 4 ; concentration : 10 mg/ml , sample volume : 5µl ; chart-speed : 2.5 mm/mn.

Figure 32. Separation of monosaccharides (A) and trisaccharides (B) by HPLC on C-18 µ-bondapak (Waters). Eluent : H_2O (188).

and di-saccharides but a glucose, rhamnose, fucose mixture as well as a cellobiose, sucrose mixture may be resolved (Figure 32). On the other hand separations of trisaccharides or series of homologous oligosaccharides are more attractive. Within 35 min., cellodextrins have been fractionated up to DP 5 and maltodextrins up to DP 8.

As found by Cheetham (186) with methylated sugars, certain carbohydrates yielded a chromatogram with two peaks corresponding to the α and β anomeric forms. Obviously the reduced form gives one peak and is readily distinguished from the corresponding free sugar.

The separation process can be discussed in terms of solubility parameters (δ) of the solvent, stationary phase and solute : with monosaccharides bearing five hydroxyl groups, the solute-eluent interactions are prevalent and no separation occurs. If methyl or O-methyl groups are substituted for hydroxyl groups or if the DP increases, the polarity and solubility of the solute decrease and solute-stationary phase interactions increase, resulting in an increased retention time.

The μ-Bondapak C-18 is now available in cartridge for the radial compression system called the radial PAK A cartridge. The results are similar but with better resolution. From this support, Waters Associates recently developed a new cartridge, the "Dextropak", aimed at providing a rapid analysis of oligosaccharides (190). Maltodextrins are separated up to DP 10 in less than 20 min. (Figure 33). Various separations performed in pure water on μ-Bondapak C18 column or C18 radial PAK A cartridge have been listed on Table 15 but most of them can be obtained on the Dextropak cartridge. It is interesting to note that the resolution of monosaccharides is improved on a C8 radial PAK cartridge by increasing the polarity of the stationary phase.

Another application of the reverse phase chromatography has been given by Wells (191). Acetylated oligosaccharides of amylose have

TABLE 15

Separation of Neutral Oligosaccharides by Reverse Phase H.P.L.C. (C-18 μ-Bondapak, Dextropak, Radial Pack A from Waters ; eluent : H_2O)

	Aldo-pentoses	Aldo-hexoses	Keto-hexoses	6-deoxyaldo-hexoses	Cello-biose	Gentio-biose
Aldopentoses	/	/	/	o	o	o
Aldohexoses	/	/	/	o	o	o
Ketohexoses	/	/	/	o	o	o
6-deoxyaldo-hexoses.	o	o	o	/	/	/
Cellobiose	o	o	o	/	/	/
Gentiobiose	o	o	o	/	/	/
Isomaltose	o	o	o	/	/	/
Lactose	/	o	Sorbose	Fucose	o	o
Maltose	o	o	o	/	/	/
Sucrose	o	o	o	Rhamnose	o	o
Trehalose	o	o	o	/	/	/
Cellotriose	o	o	o	o	o	o
Maltotriose	o	o	o	o	o	o
Raffinose	o	o	o	o	o	o

Aldopentoses : Arabinose, ribose, xylose.

Aldohexoses : Galactose, glucose, mannose.

o = separated / = impossible separation

TABLE 15 Continued

Isomal-tose	Lactose	Maltose	Sucrose	Tre-halose.	Cello-triose	Malto-triose	Raffinose
o	/	o	o	o	o	o	o
o	o	o	o	o	o	o	o
o	Sorbose	o	o	o	o	o	o
/	Fucose	/	Rhamnose	/	o	o	o
/	o	/	o	/	o	o	o
/	o	/	o	/	o	o	o
/	o	/	o	/	o	o	o
o	/	o	o	/	o	o	o
/	o	/	o	/	o	o	o
o	o	o	/	o	o	o	o
/	/	/	o	/	o	o	o
o	o	o	o	o	/	o	o
o	o	o	o	o	o	/	o
o	o	o	o	o	o	o	/

Ketohexoses : Fructose, sorbose.

6-deoxyaldohexoses : fucose, rhamnose.

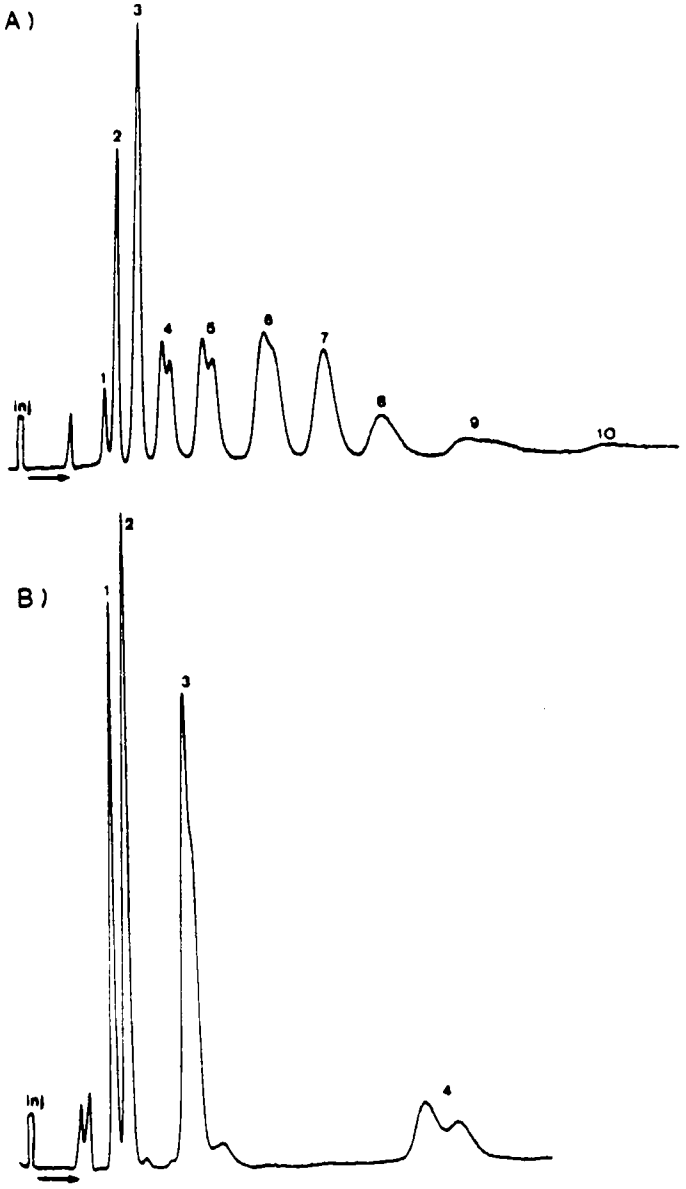


Figure 33. Separation of maltodextrins (A) and cellodextrins (B) by HPLC on Dextropak cartridge (waters). Eluent : H_2O.

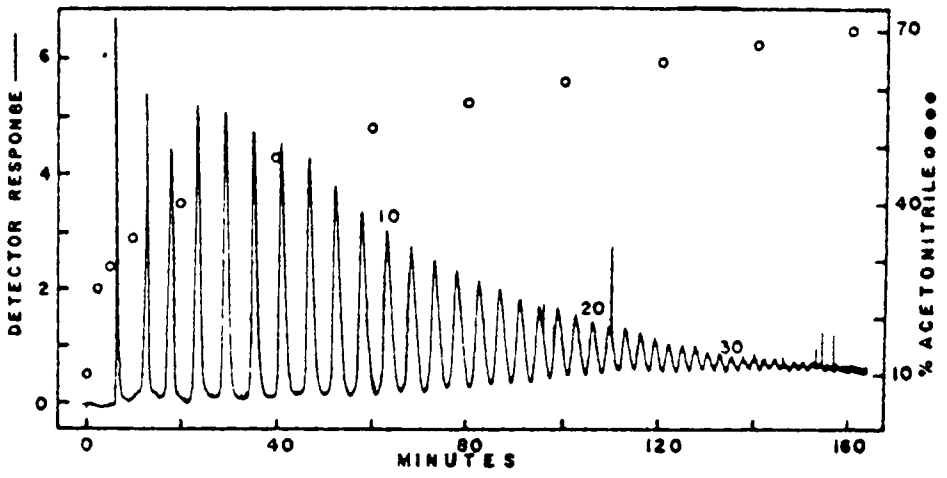


Figure 34. Chromatography of partially hydrolyzed amylose. Oligosaccharides from a 15-min acid hydrolysate of amylose were acetylated and chromatographed. The degree of polymerization is indicated by numbers over the peaks. About 3.5 µeq of hexose was injected in 60 µl (191).

been separated up to DP 30 on two columns connected in series and packed with Vydac reverse phase octadecyl support (Figure 34). The experiment was carried out at 65°C and an exponential gradient of acetonitrile in water (10-70 % acetonitrile) has been required. Supports for reverse phase chromatography have been found of great interest in the separation of hydroxy acids ; this procedure has been designed as an ion-pair reversed-phase method.

c/ Paired-ion chromatography (P.I.C.)

Acid or basic compounds are dissociated in water solution. Owing to the equilibrium

$$\text{(non-ionic form)} \rightleftharpoons \text{ionic form} \qquad (1)$$

in the mobile phase, the solutes are eluted in one broad peak and no separation occurs. With pH adjustments the equilibrium can be

modified towards the non-ionic form but the stationary phase has to be used within a solvent pH range of 2-8. In gel permeation system hydroxy acids were well separated with 5.10^{-2}M any buffer as the mobile phase ;a sharp peak is also obtained (on a reverse phase support) but the separation process is not based upon interactions stationary phase-solute but on a simply sieve effect and the differences in the elution volumes do not enable to obtain even partial separations.

In the paired-ion chromatography method (192), the ionic form of the solute is suppressed by addition of an appropriate counterion to the eluent following the equilibria :

$$\text{sugar} \rightleftharpoons \text{sugar}^- \qquad (2)$$

$$\text{Counterion} \rightleftharpoons \text{Counterion}^+ \qquad (3)$$

$$\text{Sugar}^- + \text{counterion}^+ \rightleftharpoons \text{complex} \qquad (4)$$

With an adequate pH of the mobile phase to provide maximum ionization of both sugar acid and counterion and an excess of this counterion, the equilibrium (4) lies to the right. Hydroxy acids will be separated as neutral complexes. It is obvious that complex stability and thus the retention time depend on the pH of the mobile phase and the nature and the concentration of the counterion used.

Actually, this method has not been frequently utilized for separations of sugar acids. Ototani (193) isolated disaccharides produced by the enzymic degradation chondroitin sulfate on a C-18 μ-Bondapak column eluted with 0.035 M tetrabutylammonium phosphate (pH 7.54). More recently, Lee (176), using a Partisil 10 ODS column, separated sulfated sugars too. This separation of unsaturated disaccharides derived from heparan sulfate and heparin has been performed with a methanol/water mixture (10/90) containing 0.005 M tetrabutylammonium (pH 7.0) as the eluent.

G/ CARBOHYDRATE DETECTION IN LIQUID CHROMATOGRAPHY.

A few years ago, when the separation of saccharides was performed on a carbon-celite column, the eluent was collected in several fractions and the optical rotating power of each was measured. Then, the monitoring of the carbohydrate content of the effluent has been automated by using various continuous recording system.

1) Detection of sugar after post-column reactions

In these systems, separated constituents are identified by continuously adding chemical reagents to the column effluent to produce a color or an electrochemical reaction that can be detected and quantified.

- Colorimetric method :

Many processes are available ; the produced color is detected with a continuous flow colorimeter that measures the absorbance of the reaction mixture at a given wavelength. Anthrone colorimetry offers carbohydrate specificity (194-195) but absorption maxima vary somewhat for each sugar and maximal color development times also depend on the nature of the sugar. In contrast, phenol-sulfuric acid reagent is more suitable and has been extensively used in various carbohydrate analyzers (196-198). The absorbance of the reaction mixture is monitored at 480 and/or 490 nm. The reactions with orcinol (199-201) or cysteine (202), with an absorption maximum at 420 nm, give also satisfactory results. Another interesting system consists of mixing the carbohydrate eluted from the column only with sulfuric acid to produce UV - absorbing chromophores in the 290-310 nm region (203). The aniline/ acetic, orthophosphoric acid reagent can also be used (204). However, these systems are corrosive, so other procedures have been investigated. Non-corrosive dye reagents for detection of reducing sugars based on the formation of a deep lavender copper (I) complex of 2, 2' bicinchoninate (205-205 bis) or the reaction with an alkaline solution of tetrazolium-blue (87) have been applied. The absorbances are measured at 562 nm and 520 nm, respectively. These methods are well adapted for the

analysis of neutral sugars but for the colorimetric determination of sugar alcohols a periodate oxidation of the eluate is necessary (79). The oxidized polyols give rise to formaldehyde which is determined by the color reaction with pentane -2, 4 dione at 420 nm. As for acid sugars, the carbazole method has proved to be very efficient (206).

- Electrochemical reaction :

A method based on constant potential coulometry has also been developed (206). Sugars in the effluent are oxidized by basic ferricyanide and the produced ferrocyanide is detected by electrochemical oxidation.

- Fluorescence monitoring by an oxidative detector :

The reagent, cerium (IV), can be reduced to the fluorescent cerium (III) by reducing carbohydrates. The fluorescence generated is detected by a miniature flow fluorometer (208-209) ; the sensivity is better and the resolution is more effective than with a flow colorimeter but the detection is less specific.

All these methods are very sensitive but the post-column derivatization results in peak broadening and the reactions are time consuming. So, except for the reaction using blue tetrazolium which has been improved (210), colorimetric methods are unsuitable for high pressure liquid chromatography.

2) Flame ionization detector (F.I.D.)

The detection is based on a direct combustion of sugar. The solute continuously wets a transport system, the solvent is evaporated and the sugar passes into the F.I.D. where it is burned and ionized (211-212). This system possesses high sensitivity, universal response, independence from the chromatographic process and a wide linear range, but, due to degradation of noble metal carriers by caramelized and carbonized sugars, it is not recommended for the continuous detection of carbohydrates.

Another system of sugar combustion has been investigated, a carbon analyzer (213), but it has never been used in a continuous process.

These previously described systems although often very sensitive are destructive methods and in most cases are not convenient for high-speed liquid chromatography. So continuous monitoring methods including the use of a differential refractometer and an ultraviolet detection system have been tried.

3) Differential refractometers.

This detector is the most widely used in H.P.L.C. of carbohydrates. The process is based on continuously monitoring the difference in refractive index between the pure mobile phase and the eluent plus solute. This method, although less sensitive than colorimetric detection, is well adapted to analysis of sugar chromatography (214) and has the advantage of being reproducible and easy to run. However, important disadvantage of refractive index detectors are that they are difficult to use with gradient elution and they suffer from a total lack of specificity for sugars

4) Ultraviolet spectrometry

Sugars, sugar alcohols and sugar acids can be directly detected at $\lambda \sim 192$ nm (215). This system is often more sensitive than detection by refractive index but the intensity depends strongly on the type of eluent and above all on its purity (216). This detection can be markedly improved by a pre-column derivatization (167-169). The derivatives are detected at either 230 or 254 nm with higher sensitivity. However there is the inconvenience of chromatographing the sample in its modified form, and secondary products can cause difficulties.

In conclusion, in modern liquid chromatography systems, the detection requires a continuous sensitive and rapid process. The typical characteristics of the most widely used systems are given in Table 16. Derivatization being unsuitable, it is a choice between

TABLE 16

Methods of Detection Used in Saccharide Analysis

DETECTION	COLORIMETRIC	ELECTROCHEMICAL	F.I.D.	R.I.	U.V.	FLUORESCENCE
Response	Specific	Specific	General	General	Specific	Specific
Use with gradient elution	Yes	Yes	Yes	No	Yes	Yes
Sensitivity to sugar samples (moles)	10^{-6} - 10^{-10}	10^{-6}	10^{-10}	10^{-7}	10-7 5.10^{-12}*	10^{-9}

* With derivatization.

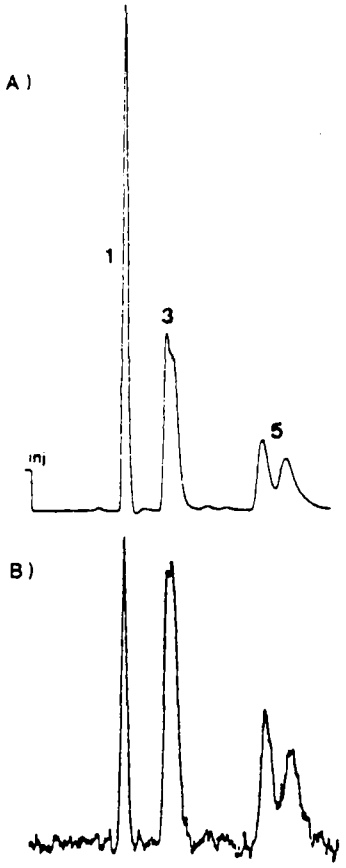


Figure 35. Maltodextrins detection by coupling differential refractometry (A) and optical rotation (B) at 350 nm.

refractometer and ultraviolet spectrometry. The latter is more sensitive but actually refractometry remains the most used method in the analysis of carbohydrates. By coupling two different systems, improvements can be obtained not in sensitivity but in specificity. For example, a refractometer and a polarimeter in series (187) allow the identification of sugar and non-sugar entities and, by addition of an internal standard, ease of recognition of the chromatogramm peaks (Figure 35).

H/ APPLICATIONS

Liquid chromatography has for some years been applied in research work. It is becoming an important analytical tool for following preparative steps and to analyze the purity of the final product. Liquid chromatography can be used to separate, characterize, and identify reactants or products of almost any chemical reaction and is valuable in every area where carbohydrates are present. One recalls some of the main fields investigated.

- Wood chemistry :

Wood, one of the few renewable natural resources, is the chief source of industrial cellulose. Pulping and purification processes produce wood pulp which becomes the raw material for the manufacture of paper and paperboard, regenerated cellulose and cellulose derivatives. Cellulose, in the form of wood pulp, is separated from the digestion liquors, but these liquors contain about 20 % of carbohydrates (xylose, mannose, arabinose, galactose, fructose, aldonic and uronic acids) which are lost.

Chromatography on ion exchange resins has been extensively used in wood industry and the chemistry of pulping processes. Classical applications are the determination of sugars in sulfite waste liquor (217-223) and the determination of the sugar units present in polysaccharides in wood, wood pulp (224-227) and various types of hemicellulose (228-230). Likewise, action of chlorous acid on hydrocellulose (231) and the effect of alkaline hypochlorite in the bleaching of cellulose (232) have been investigated by ion-exchange chromatography.

- Kinetic investigations on carbohydrates:

Cellulose sources produce wood pulp, and wood pulp can be transformed into news papers. An enzymic hydrolysis of newspapers to recover carbohydrates can be performed and the produced sugars have been analyzed by H.P.L.C. on a μ-Bondapak carbohydrate column (233).

The kinetic data are rapidly available and allow improvements to the process for converting cellulosic wastes to usable end products (glucose). So the high pressure liquid chromatography system provides an interesting tool for chemical or enzymic kinetic studies. Hydrolysis of cellodextrins by cellobiohydrolase has been investigated by Tsao (234), and we have studied acid hydrolysis of oligosaccharides by polyelectrolytes and ion exchangers (189-235).

- Food chemistry :

Carbohydrate is the major component of most human diets, therefore the analysis and characterization of food carbohydrates are important, particularly with the increased concern about nutrition and, consequently, nutritional labeling.

This sweetener market is a multi-product and multi-industry complex. Among the applications are the following : beverage industry (soft drinks, malt, malt liquors, distilled liquors, flavouring), baking industry, dairy industry (ice cream, frozen desserts, sweetened condensed milk and sweetened milk products), canning industry (canned food, bottled or frozen foods, jams, jellies, pickles and preserves), confectionery industry (candy, chewing-gum and chocolate). The principal products generally included in this market are : sucrose sweeteners (cane and beet sugar), starch sweeteners (dextrose, conventional corn syrup, high levulose corn syrup), other calorific sweeteners (honey, maple syrup and sugar molasses, sugar cane syrup, refiners syrups) and non-caloric sweeteners (saccharin and others). Classical liquid chromatography, such as ion exchange resin, has been used for estimation of sugars in beet molasses (236) or in wort and beer (237) and gel permeation chromatography has proved to be a useful method for analysis of glucose syrups (238). Meanwhile, the advent of H.P.L.C. in recent years now allows the rapid characterization of sugars in variety of food matrices (239-243). This method has been applied with success in the brewing laboratory (244-245), for the analysis of dairy products (246-248)

and for the determination of simple sugars and/or sugar alcohols in fruit (249-250) or in sugar cane (251-251 bis) ; even the quantitative analysis of the sugar composition of starch hydrolyzates can be performed (252). These separations have been achieved either with μ-Bondapak/carbohydrate column with acetonitrile/water mixture as the eluent or on cation exchange columns at high temperature with water as the mobile phase.

Another field of food chemistry is the cereal chemistry. Leguminous seeds are important potential protein sources for both human consumption and animal feed. These seeds contain considerable amounts of carbohydrates which are the flatus-producing factor. Thus, the carbohydrate composition of various legume seeds has been studied, first by G.P.C. on Bio-Gel (253-254) and now by H.P.L.C. using different supports (255-258).

- Medical chemistry :

The quantities of the constituents of all physiologic fluids represent potentially useful diagnostic information. Therefore, carbohydrates present in blood and urine of humans (259-261) and animals (262) have been investigated. It has been shown that normal and pathological states could be differenciated. Body fluids of normal subjects have a definable normal spectrum of chemical constituents whereas various pathologic states can be associated with abnormal values of one or more of the constituents.

- Polysaccharide structural analysis :

The structure of a polysaccharide is generally established after methylation and complete hydrolysis ; the monosaccharides obtained are di-, tri- or tetra-methylated derivatives depending on its position in the chain and existence of branching position. Up to now, the separation and identification of these derivatives were performed by gas chromatography after derivatization (preparation of alditol acetate for example).

Now, H.P.L.C. allows the direct separation of the monosaccharides partially methylated in water or water/methanol eluent (187). The anomers give two peaks and it is generally convenient to reduce the anomeric position before chromatography (Figure 31). H.P.L.C. is a more rapid technique which can be adapted to every problem of saccharide separation and identification.

- Other applications :

Ion exchange chromatography has been also adapted to analysis of sugars and uronic acids extracted from marine sediment and seawater by various hydrolysis techniques (263). Hydrolysis efficiencies of the acids used can give insights into the sources and the environment of the organic deposit.

Recently, with the shortage of conventional sources of energy, the world turns towards the biomass, and liquid chromatography seems to be an important trumpcard for improvements in the fermentation process using sugar juice and molasses.

CONCLUSION

This work is a review on liquid chromatography applied to mono- and oligo-saccharide separations. The principles and main applications of gel permeation chromatography (G.P.C.), ion exchange chromatography solid-liquid and liquid-liquid partition chromatography have been successively recalled and separations of neutral and ionic saccharides or their derivatives have been described.

Gel permeation is an useful method to fractionate neutral oligosaccharides in water or charged oligosaccharides in presence of an excess of a neutral salt. Ion exchange chromatography, especially in presence of borate, allows the separation of monosaccharides with good selectivity. Partition chromatography neglected a little, becomes again an attractive method with the advent of high performance liquid chromatography (H.P.L.C.). H.P.L.C. provides a fast

method and with the recent improvements in column technology very interesting separations are obtained in a few minutes. The great advantage of this method is the avoidance of derivatization and direct operation with solutions of mono- or oligo-saccharides, especially;reverse phase liquid-liquid partition chromatography has proved to be very suitable for oligosaccharide fractionations using water as eluent, methylated derivative quantitative analysis or/and normal phase for monosaccharide separations in water/acetonitrile mixtures.

In the last part of the review, the methods of detection used are given and fields of application of the liquid chromatography have been summarized. Certainly new column packings and new detectors will further be developed to extend the use of H.P.L.C. in the saccharide field.

This report is not concerned with the analysis of polysaccharides ; the only method appreciable is the gel permeation chromatography under classical or high pressure mode. For water soluble polymers, silicagels or bonded silica may be used but adsorption exists (6 bis). In addition, no support is quite convenient for high pressure chromatography so far. In organic solvents both methods are suitable but often the chromatographic analysis cannot be performed unless a viscosimetric detector is adapted in the absence of available standards of the same polymers to calibrate the columns ; a development in this field is now necessary to complete our tool in saccharide analysis by chromatographic methods.

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