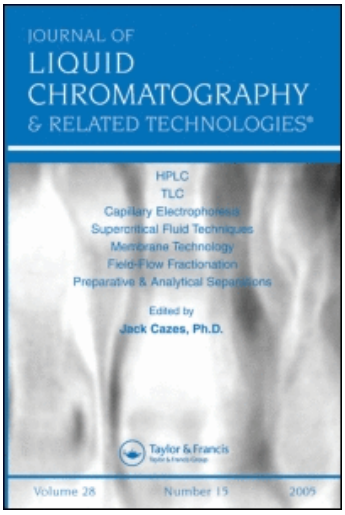

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CARBOHYDRATE ANALYSIS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY USING WATER AS THE ELUENT

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ABSTRACT

A μ-Bondapak C-18 column separates, by reverse phase process, mono, di and oligosaccharides within 40 min when water is used as eluent. The fractionation capabilities of this column are a function of experimental conditions (temperature and flow-rate). Chromatograms showing separations obtained for different carbohydrate mixtures are presented and discussed in terms of solubility parameters.

INTRODUCTION

Until a few years ago, for rapid determination of carbohydrates, mainly thin layer chromatography (T.L.C.) and gas chromatography (G.C.) were used, but these methods are generally complex and/or time consuming [1-3]. Then, the development of liquid chromatography, due largely to improvements in column technology, has allowed to get a fast method for carbohydrate analysis.

Liquid chromatography, in general, requires no derivatization and, in many cases, no sample preparation. The separation of oligosaccharides on Sephadex and polyacrylamide gels has been described in several papers [4-8] but, if these packings have been shown to give good separation of oligomers, the separations take more than 10 hours per sample and monomers which have the same hydrodynamic volume can't be separated. High speed liquid chromatography appears to be the best approach [9] ; three types of liquid chromatographic separation mechanism are mainly used :

- Ion exchange chromatography of carbohydrate complexes
- Partition chromatography on ion exchange resins
- Partition chromatography on chemically bonded phases.

The partitions of carbohydrate complexes on ion exchange resins are performed with potassium borate buffer at 55°C [10,11]. The variability of resolution depends on buffer molarity and on the pH. This method allows the separation of mixture of mono, di- and

trisaccharides in less than two hours and, to shorten the analysis time, it is possible to use a gradient where the concentration of the borate buffer or the pH is continuously changed.

The sugars contain very weakly acidic alcoholic groups and are strongly polar solutes. Therefore this slightly acidic character may account for the sorption on strongly basic anion exchange resins or, as the carbohydrates have a greater affinity for the water , there are interactions between resin in which the water content is higher than in aqueous ethanol solutions used as mobile phase. However, oligomers tend to precipitate at the ethanol concentrations required for efficient separation so, indubitably, it is interesting to work with water which seems one of the best solvent as eluent. Recently, ion exchange resin Aminex Q 15 S (Ca^{2+}) or AG 50 W -X4 (Ca^{2+}) were chosen to separate cellodextrins [12]. These packings give good separation of celloheptaose through glucose within 30 minutes at 85°C with water as eluent, but resin preparation and packing procedure seem to be fastidious.

Various bonded phases as stationary phase have been used for chromatographic carbohydrate analysis and, in particular, "µ-Bondapak carbohydrate" column with acetonitrile - water mixture as eluent[13]. By simply manipulating the ratio of water to acetonitrile in the eluent and/or the flow rate, this column allows to separate mono and disaccharides in less than 15 minutes but as with aqueous ethanol solutions, at acetonitrile concentrations

required, higher oligosaccharides precipitate and the distribution of oligomers is disturbed.

From a general point of view, it seems very important to work in pure water as eluent and this report shows the fractionation of some carbohydrates mixtures by reverse phase chromatography on a hydrophobic C_{18} grafted silicagel eluting with pure water.

EXPERIMENTAL

Equipment and procedures

The liquid chromatograph was a Waters Assoc.M 6,000 with a UK6 high pressure injector and a differential refractometer detector. The detector was connected to a Sefram Servotrace 10 mv full scale.

The column was a Waters Assoc "C_{18} - μ-Bondapak", 30 cm x 3.9 mm ID stainless steel. The column was thermostated with a Haake model F3 - C circulating water-bath. The water used for elution in all experiments was distilled, deionized and then filtered through 0.5 μ Millipore membranes.

Samples :

Sugar standards were commercially available materials. The cellulose hydrolysate was obtained by acetolysis of cellulose (Linters powder Scheicher and Schüll, ref. 124 $\overline{DP} \simeq 2,700$)

Maltodextrins were available from Roussel - France , (ref. Cal. 400) ; Me-galactose series was kindly donated by Dr. G. CHAMBAT and thio-maltose and thio-cellobiose by Drs. H. DRIGUEZ and M. MUESSER (CERMAV - GRENOBLE). Aqueous standard solutions were prepared with concentrations of 2 to 10 mg/ml.

RESULTS

Different parameters play a role on the resolution of the column. In a previous report [14] we have studied the resolution dependence on temperature and flow-rate. The resolution is markedly increased with decrease in temperature corresponding with an increase of solute/support interactions and decreases with increase in flow-rate.

Likewise, it has been shown that the elution volumes increase with increase in DP and depend on the type of oligomer ; a calibration established for quantitative analysis shows that a linear relationship is obtained when the peak area is plotted versus concentration for serveral sugars.

In our experiments, to get good resolution with mono and disaccharides, the flow-rate has been fixed on 0.1 ml/mn and the temperature on 3.5°C.

The elution volumes (Ve) of the most sugars investigated in this paper are given in the table.

a) Separation of monosaccharides

Considering the elution volumes, it is possible to calculate the resolution ($R_S = \frac{Ve_2 - Ve_1}{2(\sigma_1 + \sigma_2)}$ with σ the standard deviation). For monosaccharides, if a constant value is assumed for σ as $\sigma \simeq 0.065$ml, R_S will be higher than 1 and good separation will be obtained if $Ve_2 - Ve_1 \geq 0.26$ ml.

Fig. 1 shows the separation of monosaccharides, eluted at 0.1 ml/mn with H_2O at 3.5°C.

Compared to some other techniques of high performance liquid chromatography applied to sugar separation, the described metnod is not very efficient for separation of monosaccharides [fig.1(a)]. Nevertheless a good resolution is obtained with some modified sugars [Fig. 1(b)] and we obtain an acceptable separation for α-Me-glucoside from β-Me-glucoside [Fig. 1(c)], separation which is critical with"μ-Bondapak Carbohydrate" column, eluted with H_2O : CH_3CN at 5 : 95. More, by coupling two "C-18 μ-Bondapak" columnsin series, the R_S can be markedly increased.

Fig. 2 shows the chromatograms obtained with a Me-galactose series. The 2-3 di-Me-galactose is well separated from a tri-Me and it is possible to see the anomeric equilibrium. With the 2-3-6,

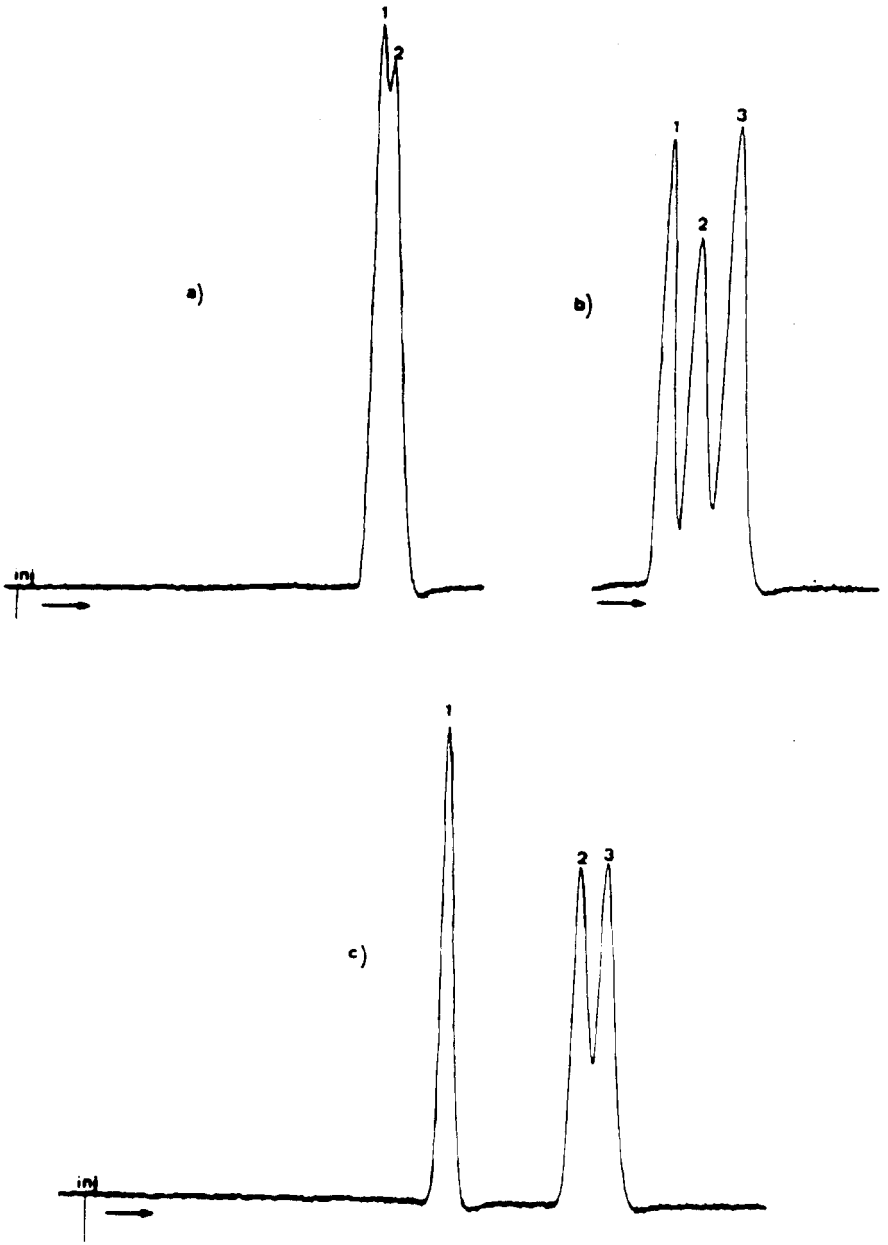
2-4-6, 2-3-4 tri-Me galactose and 2-3-4-6 tetra-Me galactose these anomeric equilibriums don't allow to get a good separation.

b) Separation of disaccharides

With $\sigma \simeq 0.075$ml, $Rs \geq 1$ if $Ve_2 - Ve_1 \geq 0.3$ ml. As with the monosaccharides, the separation of disaccharides is difficult but in fine the fractionation is very acceptable for some of them [Fig. 3 (a)]. Fig. 3(b) shows an interesting separation of maltose from maltitol corresponding to the reduced form of the disaccharide. When a modified disaccharide is used, the separation is easier. Fig. 4(a) shows the fractionation of thio-maltose from thio-cellobiose when maltose is not separated from cellobiose. We can note that the thiomaltose peak is divided into two by the anomeric equilibrium but not the thiocellobiose peak ; the elution volume of thiomaltose being not much different from elution volume of maltose, there is an interference between the maltose peak and the β-form peak of thio-maltose [(Fig. 4-b)]. There is no problem with thio-cellobiose which has an elution volume much larger than cellobiose (Fig. 4-c) ; this difference of behaviour between thio-cellobiose and thio-maltose can perhaps be explained with an important difference of conformation and solubility.

c) Separation of trisaccharides.

Fig. 5 shows the separation of three trisaccharides eluted at 0.2 ml/mn with H_2O at 15°C. The resolution of maltotriose from raffinose



MONOSACCHARIDES		DISACCHARIDES	
Solutes	Ve (ml)	Solutes	Ve (ml)
Xylose	3.50		
Arabinose	3.62	Maltose	4.00
Fructose	3.65	Cellobiose	4.05
Sorbitol	3.52	Gentiobiose	4.28
Glucose	3.48	Isomaltose	3.94
Galactose	3.48	Maltitol	3.78
Mannose	3.56	Cellobitol	3.90
Fucose	4.20	Saccharose	4.46
Rhamnose	3.82	Lactose	3.60
α-Me Glucoside	4.61		
β-Me Glycoside	4.80		

Elution volumes of some sugars eluted at 0.1 ml/mn in H_2O at 3.5°C.

Figure 1 : Separation of monosaccharides

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 : 3.5°C ; detector attenuation x 4 ;concentration : 10mg/ml ; sample volume : 5µl ; chart-speed : 2.5 mm/mn.

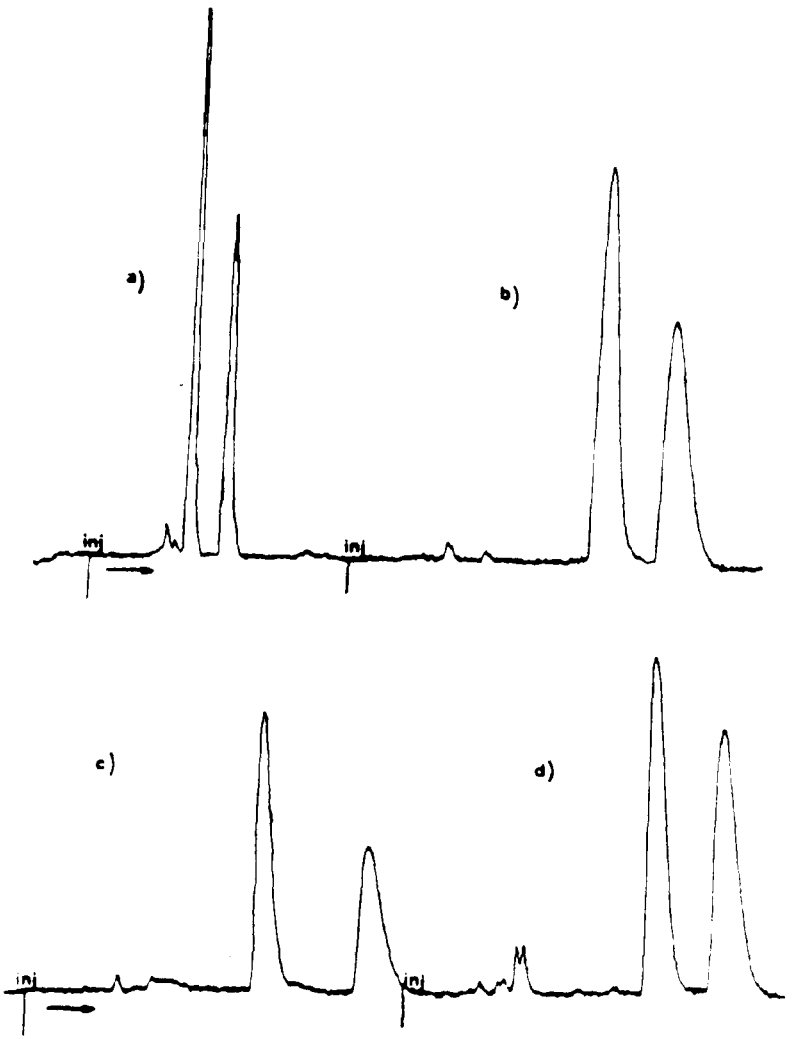


Figure 2 : Chromatograms of Me-Galactose

a) 2-3 di-Me-galactose

b) 2-3-4 tri-Me-galactose

c) 2-3-6 tri-Me-galactose

d) 2-4-6 tri-Me-galactose

Flow-rate : 1ml/mn ; temperature : 5°C ; detector attenuation x 4 ;concentration ~ 5 mg/ml ; sample volume 10µl ; chart-speed : 5 mm/mn.

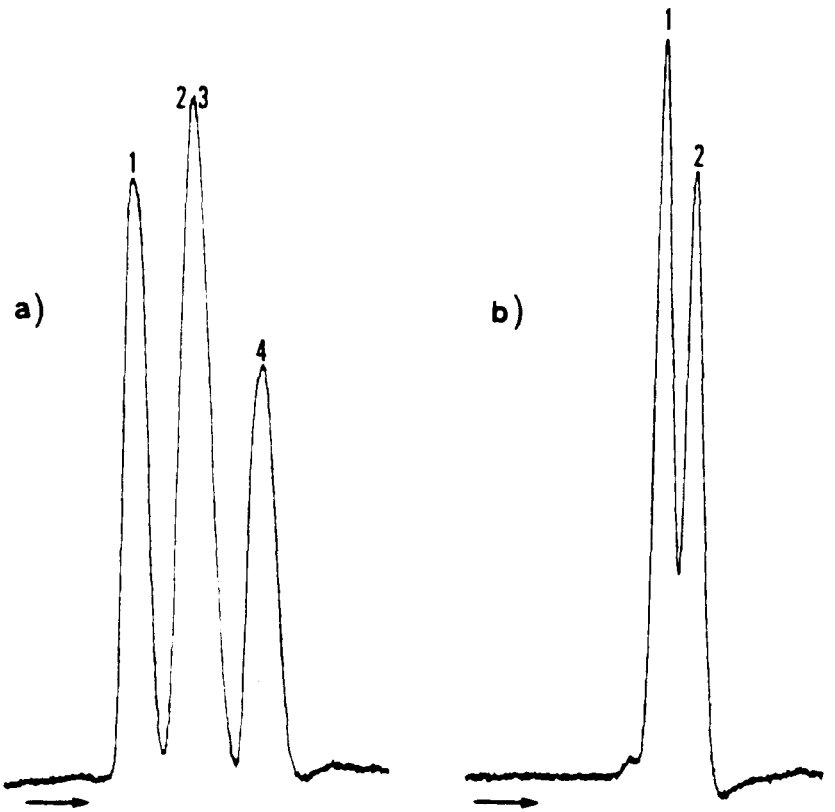


Figure 3 : Separation of disaccharides

a) separation of glucose(1) from isomaltose(2), cellobiose(3) and saccharose(4).

b) separation of maltitol(1) from maltose(2)

Flow-rate : 0.1 ml/mn ; temperature : 3.5°C ; detector attenuation x 4 ; concentration : 10mg/ml ; sample volume : 5 µl ; chart-speed : 2.5 mm/mn

and cellotriose is satisfactory. The affinity for the resin increases with increase in the DP and the structural difference are more marked, so the separation of higher DP will be easier than mono- or disaccharides.

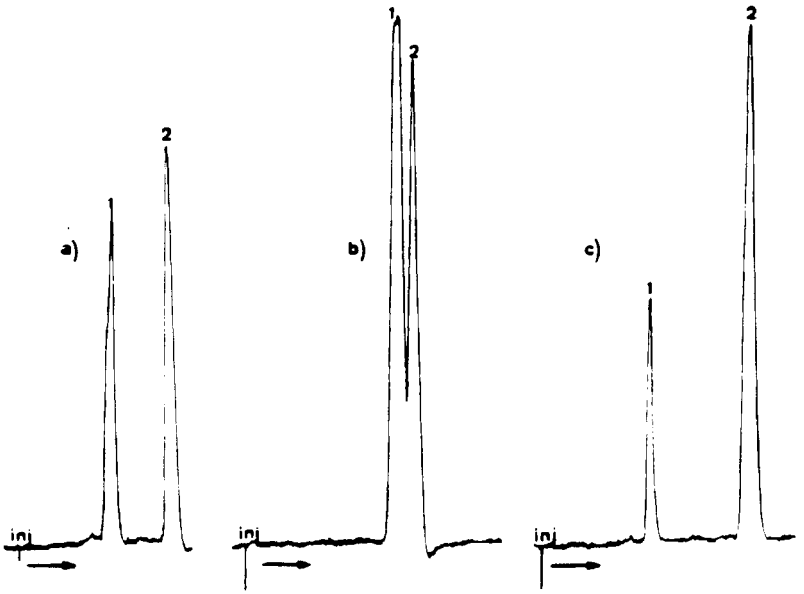


Figure 4 : Separation of thio-disaccharides

a) separation of thio-maltose(1) from thio-cellobiose(2) (flow-rate : 1ml/mn ; temperature : 25°C ; chart-speed : 5 mm/mn.)

b) separation of maltose(1) from thio-maltose(2) (flow-rate : 0.5 ml/mn ; temperature : 5°C ; chart-speed : 5mm/mn.)

c) separation of cellobiose(1) from thio-cellobiose(2) (flow-rate : 1ml/mn ; temperature : 5°C ; chart-speed : 5mm/mn.)

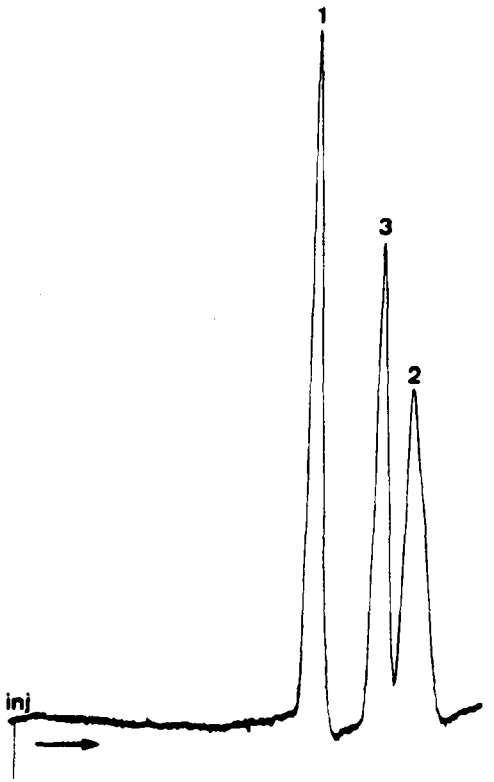


Figure 5 : Separation of trisaccharides : maltotriose(1) from cellotriose(2) and raffinose(3).
Flow-rate : 0.2 ml/mn ; temperature : 15°C ; detector attenuation : x 4 ; concentration : 10 mg/ml ; sample volume : 5µl ; chart-speed : 2.5 mm/mn.

d) Separation of oligosaccharides

We obtained the best results in fractionation of oligosaccharides. Fig. 6 shows the separation of maltodextrins eluted at 0.3 ml/mn with H_2O at 15°C. The "C-18 µ Bondapak" column gives

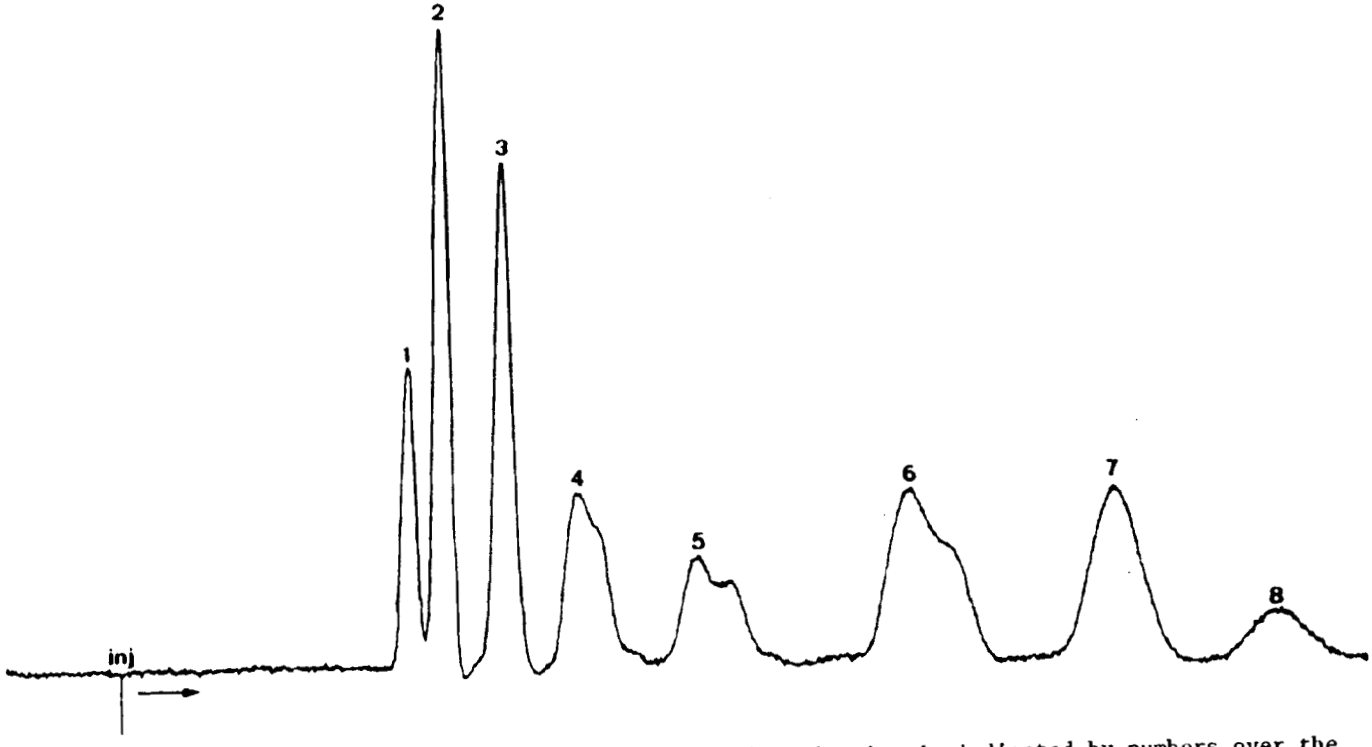


Figure 6: Separation of maltodextrins (the degree of polymerization is indicated by numbers over the peaks). Flow-rate: 0.3 ml/mn; temperature: 15°C; detector attenuation: x 4; chart-speed: 5mm/mn.

good fractionation of maltooctaose through glucose within 35 mn. The increase in resolution with increase in DP allows to show off impurities which have passed unnoticed in gel permeation chromatography on Bio-Gel P2 - particularly due to the decrease of the resolution as the DP increases. With commercial maltodextrins, often obtained by starch hydrolysis, it is well known that α1 → 4 and α1 → 6 osidic bonds must exist such as one obtains homologous series α1 → 4 and oligomers containing both types of bond in the same molecule. With cellodextrins, it is necessary to employ flow program to separate the series of oligomers through DP 5 in 35 min. Fig. 7 shows the separation of cellodextrins obtai-

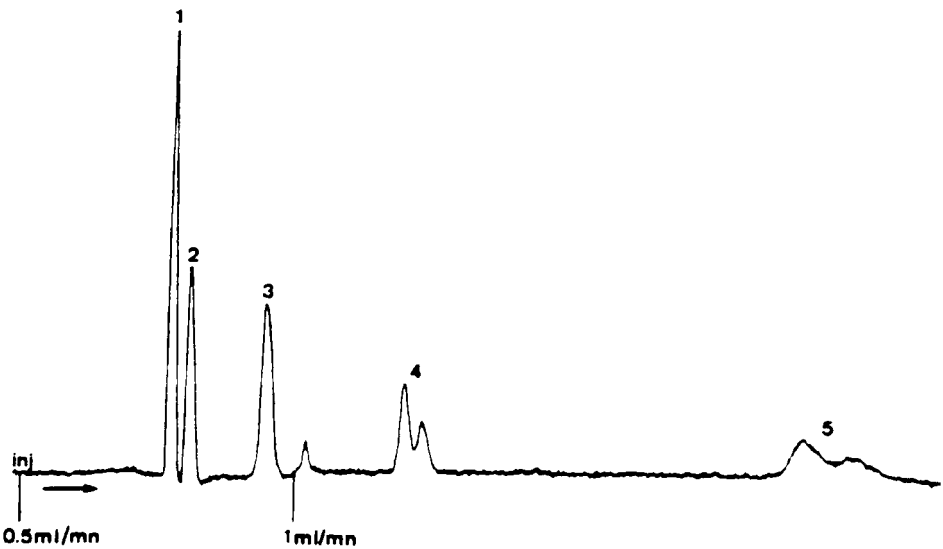


Figure 7 : Separation of cellodextrins (the degree of polymerization is indicated by numbers over the peaks).
Flow-rate program : 0.5ml/mn and 1 ml/mn ; temperature : 15°C ; detector attenuation : x 4 ; chart-speed : 5mm/mn.

ned by acetolysis of cellulose. It is interesting to note that two peaks exist for cellotetraose and cellopentaose corresponding to the α and β form of the anomeric equilibrium.

DISCUSSION

The separation of oligosaccharides in this reverse phase chromatography can be discussed in terms of solubility parameters (δ) of the solvent, stationary phase and solute. The mono and oligosaccharides are polyhydroxylic polar solutes ($\delta_{cellulose}$ = 15.6 $(cal.cm3)^{1/2}$) well solvated by the polar solvent (δ_{H_2O} = 23.4 $(cal.cm3)^{1/2}$). On the contrary, the C-18 stationary pnase has a low polarity ; the polyethylene δ = 8 $(cal.cm3)^{1/2}$ can be assumed as a good approximation .

The solutes interact with the support when water is used as eluent in such a way as the retention volume increases when the polarity of solutes decreases ; the elution volume of the more polar (or more soluble) solute is the lowest. Our experimental results are quite well predicted :

- the elution volume of a free monosaccharide is lower than that of the corresponding glycoside (fig. 1c).
- the same is observed for separation of xylose from methylated pentoses(rhamnose and fucose.) (see. table)

- the reduced forms are eluted before the unmodified oses (cellobitol - cellobiose ; maltitol - maltose).
- the thiooligosaccharides are less polar than the natural oligosaccharides and their elution volumes are increased.
- the maltodextrins are eluted before the cellodextrins for each DP considered even if both dimers are not yet enough differenciate.

CONCLUSION

Though we have a poor resolution with mono and disaccharides, "C-18 μ-Bondapak" column gives a satisfactory separation of oligosaccharides within a short time by employing a flow-program when necessary. This packing has great advantage to separate oligomers in aqueous solutions, at room temperature, filtration being the only pre-treatment given prior to the injection of the samples. This method is very interesting for quantitative rapid estimation of each species during oligosaccharides hydrolysis, acidic hydrolysates being neutralized on ion-exchange resin before injecting.

Pernaps there is a disadvantage of obtaining several anomeric peaks for some carbohydrates and in particular in hydrolysis of methylated polymers. With the most oligomers, nevertheless, the extension to preparative fractionation by HPLC on C-18 μ Bondapak seems

to be an easy process with a degree of purity largely higher than all others the technics available.

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