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RETENTION BEHAVIOUR OF CARBOHYDRATE OLIGOMERS IN REVERSED-PHASE CHROMATOGRAPHY

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SUMMARY

The retention behaviour of starch and cellulose hydrolysates on alkyl-modified silica columns using aqueous eluents has been studied. The influence of eluent pH, addition of sodium chloride or alcohols to the eluent, pore diameter of the silica support and chain length of the alkyl modification were investigated. It was concluded that a neutral aqueous eluent is a better eluent than one of low or high pH; the addition of sodium chloride results in an increased retention time and better resolution; the addition of very small amounts of pentanol-1 ($<0.1\%$, v/v) is a useful means of decreasing of the retention times. Silica supports with a larger pore diameter than the commonly used 60–100 Å and alkyl modifiers between octyl and octadecyl are also useful for decreasing the retention times.

INTRODUCTION

The analysis of carbohydrate syrups obtained by hydrolysis of starch, cellulose and inulin is generally carried out by liquid chromatography. The chromatographic systems used can be distinguished as three types:

(1) a strong cation exchanger as the stationary phase and water as the eluent: separations are carried out at a column temperature of 80–85°C and, owing to the exclusion properties of the resin, elution takes place in order of decreasing molecular weight. The ionic forms applied are Ca^{2+}[1–3] and Ag^{+}[1,4].

(2) A propylaminosilane-modified silica as the stationary phase and acetonitrile–water as the eluent: elution, at ambient temperature, is in the order of increasing molecular weight. By increasing the water content of the eluent a shorter retention time is obtained, indicating a normal-phase chromatographic system[5,6]. The method has been applied for hydrolysates of starch[6–11], cellulose[12,13] and inulin[14].

(3) An octadecylsilane-modified silica as the stationary phase and water as the eluent: elution is in the order of increasing molecular weight[15–18]. The best separations should be obtained at column temperatures below 20°C[15]. However, an increased

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column temperature gives, in addition to a shorter retention time, co-elution of anomeric forms[18].

In comparison with the first two systems, the octadecylsilane-modified silica with pure water as the eluent combines two important advantages, namely a pressure-stable stationary phase and a non-toxic, cheap eluent. These advantages explain the growing interest for the last system. However, there is a lack of information on how the elution can be influenced, except by changes in column temperature. Therefore, we have investigated the retention behaviour of different silica gels with varying pore diameter and aliphatic chain lengths and with different eluent compositions, with respect to carbohydrate oligomers.

EXPERIMENTAL

The experiments were carried out using the chromatographic apparature described previously[19]. The columns (stainless steel, 150 × 4.6 mm I.D.) were packed by a balanced density procedure with the modified silica gels listed in Table I. Moreover, for columns II, III and IV the method used to prepare the silica support and the method of derivatization were the same.

TABLE I

SILICA-BASED COLUMNS USED IN THE EXPERIMENTS

Parameter	*Column No.*				
	I	*II*	*III*	*IV*	*V*
Column material	Polygosil RP-18*	Synchropak R101**	Synchropak R103**	Synchropak R110**	Nucleosil RP-8*
Modification	Octadecylsilane	As I	As I	As I	Octylsilane
Particle Shape	Irregular	As I	As I	As I	Spherical
Particle diameter (μm)	5	6.5	6.5	6.5	5
Pore diameter (Å)	60	100	300	1000	100
Pore volume (ml/g)	0.75	0.7	0.6	0.8	1.0
Specific surface area (m^2/g)	500	170	80	20	300
Density (g/ml)	0.65	0.6	0.6	0.4	0.65

* Obtained from Macherey, Nagel & Co., Düren, F.R.G.
** Obtained from Bètron Scientific, Rotterdam, The Netherlands.

The chromatographic conditions were as follows: column temperature, 20°C; eluent flow-rate, 1.0 ml/min; amount injected, 10 μl of a 5% (w/w) solution of syrup in water; detector, refractive index attenuation, × 32.

Starch syrup was obtained from AVEBE (Veendam, The Netherlands) and cellulose syrup from Gist-Brocades (Delft, The Netherlands). All other chemicals used were commonly available and were of analytical-reagent grade.

With the different columns, the following effects were studied:

column I: the retention behaviour of starch hydrolysate with water of pH 2.0, 6.5 and 10.0 as the eluent, and in neutral water the influence of the addition of sodium chloride; the retention behaviour of starch and cellulose hydrolysate and the influence of the addition of alcohols to the aqueous eluent;

columns II, III and IV: the retention behaviour of both hydrolysates using pure water and water–pentanol-1 as the eluent;

column V: the influence of the chain length of the aliphatic bonded ligand of the stationary phase on the retention behaviour of both hydrolysates.

RESULTS AND DISCUSSION

Influence of the pH of the aqueous eluent on the retention behaviour of starch hydrolysate

The separation of starch syrups on octadecylsilane-modified silica with pure water as the eluent results in doublets of both anomeric forms if the separation is carried out at room temperature. At a higher mutarotation reaction rate, co-elution of the anomeric forms in a single peak can be obtained[20]. An accerelated reaction takes place at higher column temperature or at higher $[H^+]$ or $[OH^-]$.

An increased column temperature results in single peaks but also in decreased retention times[18]. Better resolution could be obtained if the co-elution takes place without shortening of the retention times, so we examined the influence of the eluent pH.

With column I under standard conditions, chromatograms of starch hydrolysate were run with pure water (pH 6.5), 0.005 *M* sulphuric acid in water (pH 2.0, measured at the column exit) and 0.001 *M* triethylamine in water (pH 10.0, measured at the column exit) as the eluent. The results are given in the Fig. 1a, b and c, respectively.

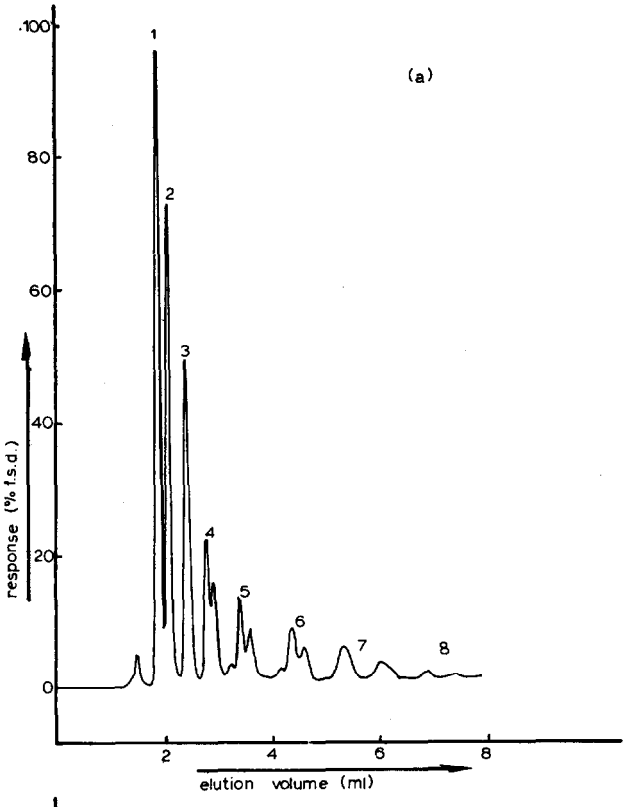
Dilute sulphuric acid does not have a significant effect on the retention times or on the anomeric doublets, in contrast to the amine-containing eluent where anomeric singlets and decreased retention times are obtained, similar to the effect of increased column temperatures. With the amine-containing eluent, however, the improvement in the resolution is not high enough to risk an alkaline-catalysed degradation of the silica support.

Influence of addition of salt to the aqueous eluent on the retention behaviour of starch hydrolysate

With column I and using standard conditions, chromatograms were run of starch hydrolysate with pure water, 0.5 *M* sodium chloride and 1.0 *M* sodium chloride solution as the eluent. The elution volumes of the different components are given in Table II, and Fig. 2 illustrates the effect. Addition of sodium chloride results in an increased retention and resolution in spite of the continued separate elution of the anomeric forms. Injection of a sodium chloride-free sample, however, results in disturbance of the baseline and hence difficult quantification of the dextrose monomer.

Influence of the addition of primary alcohols to the aqueous eluent on the retention behaviour of starch and cellulose hydrolysate

Apart from the possibility of delaying elution, it is also interesting to be able to accelerate the elution, especially for the relatively slowly eluting cellulose hydrolysates. Vratny *et al.*[18] tested a 4% methanol–water mixture and found such a strong decrease in the capacity coefficients of cellulose hydrolysate components that it could not be used to give an acceptable resolution.


(a)
response (% f.s.d.)
elution volume (ml)
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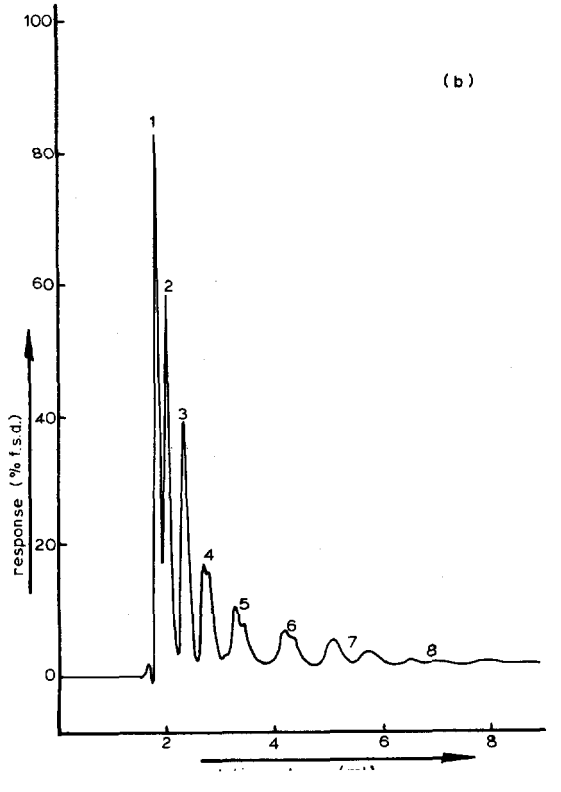
(b)
response (% f.s.d.)
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2
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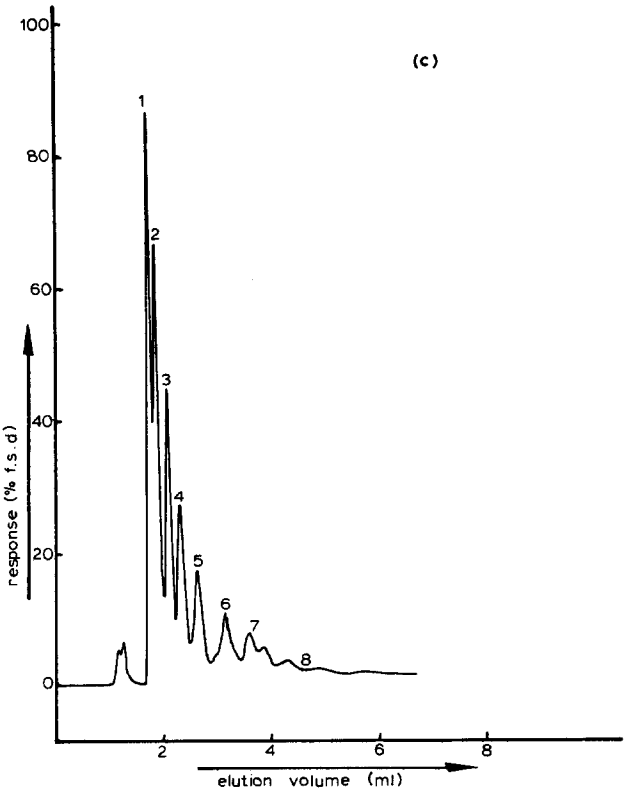

Fig. 1. Chromatogram of starch hydrolysate using column I under standard conditions and as the eluent (a) pure water, (b) 0.005 *M* sulphuric acid in water and (c) 0.001 *M* triethylamine. The peak numbers refer to the number of dextrose units in the carbohydrate oligomer molecule.

We tested the influence of methanol in water at lower contents, using column I and the results are given in Table III. The elution volumes are reduced to an acceptable value at low concentrations of methanol. Injection of a methanol-free sample results in disturbance of the baseline so that the quantification of cellobiose is difficult.

In reversed-phase chromatography, it is to be expected that retention of alcohols will increase with increasing chain length, which means that the "injection peak" can be displaced backwards in the chromatogram when higher alcohols are used. We tested the applicability of pentanol-1 as an elution-accelerating compound and the results are presented in Fig. 3. A considerable decrease in elution volume can be obtained with very low concentrations of pentanol-1. On injection of a pentanol-free sample, no noticeable disturbance of the baseline occurred at the detector sensitivity used. In order to observe the disturbance at higher detector sensitivity, a solution of 1% (v/v) of pentanol-1 in water was injected, using column I under standard conditions and with a 0.05% (v/v) aqueous solution of pentanol-1 as the eluent. The elution volume of pentanol-1 seems to be very high (15 times the empty column volume), so relative to the carbohydrate oligomer peaks a broad disturbance was

TABLE II

ELUTION VOLUMES (ml) OF STARCH HYDROLYSATE OLIGOMERS USING COLUMN I UNDER STANDARD CONDITIONS WITH PURE WATER, 0.5 *M* AND 1.0 *M* SODIUM CHLORIDE SOLUTION AS THE ELUENT

Dextrose units	*Sodium chloride concentration in eluent (M)*		
	0	*0.5*	*1.0*
1	1.71	–*	–*
2	1.91	2.02	2.06
3	2.24	2.44	2.54
4	2.59**	2.89	3.10
	2.72	3.02	3.31
5	3.16	3.65	4.04
	3.34	3.89	4.35
6	4.04	4.85	5.55
	4.25	5.13	5.93
7	4.89	6.08	7.20
	5.54	7.03	8.58
8	6.33		
	6.80		

* The "injection peak" interferes with the dextrose peak.
** Double peaks of the anomeric forms occur, the β-form eluting first.

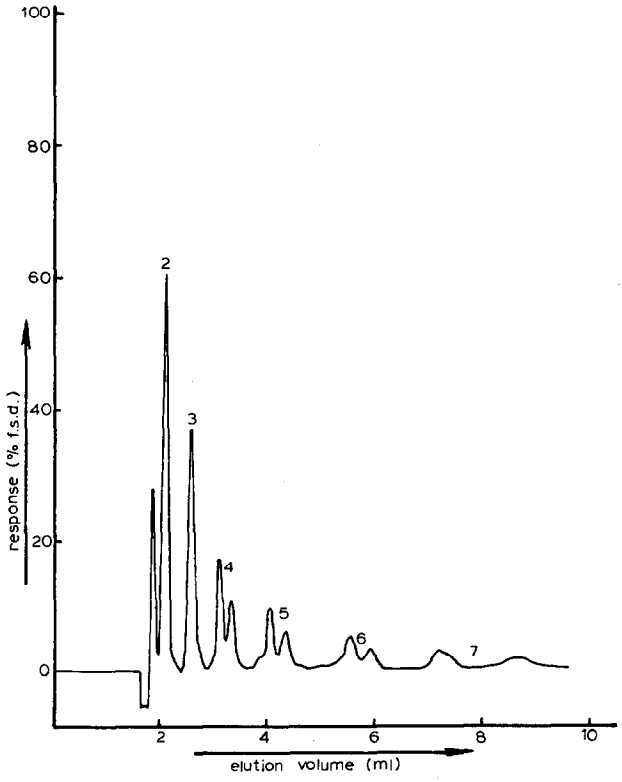

Fig. 2. Chromatogram of starch hydrolysate. Eluent, 1.0 *M* sodium chloride in water. Other conditions as in Fig. 1.

TABLE III

ELUTION VOLUMES (ml) OF CELLULOSE HYDROLYSATE OLIGOMERS USING COLUMN I UNDER STANDARD CONDITIONS WITH PURE WATER AND WATER CONTAINING 2, 3 AND 5% (v/v) OF METHANOL AS THE ELUENT

Dextrose units	*Concentration of methanol in the eluent (%, v/v)*			
	0	*2*	*3*	*5*
1	1.70	1.70	1.70	1.70
2	2.08	–*	–*	–*
3	3.31**	2.45	2.25	2.00
	3.41			
4	7.80	3.93	3.25	2.40
	8.55	4.20	3.41	2.48
5	23.45	7.87	5.66	3.25
	26.20	8.51	6.00	3.35
6		17.95	11.20	4.80
		19.55	12.15	5.10
				7.80
				8.35

* The "injection peak" interferes with the cellobiose peak.
** Double peaks of the anomeric forms occur, the β-form eluting first.

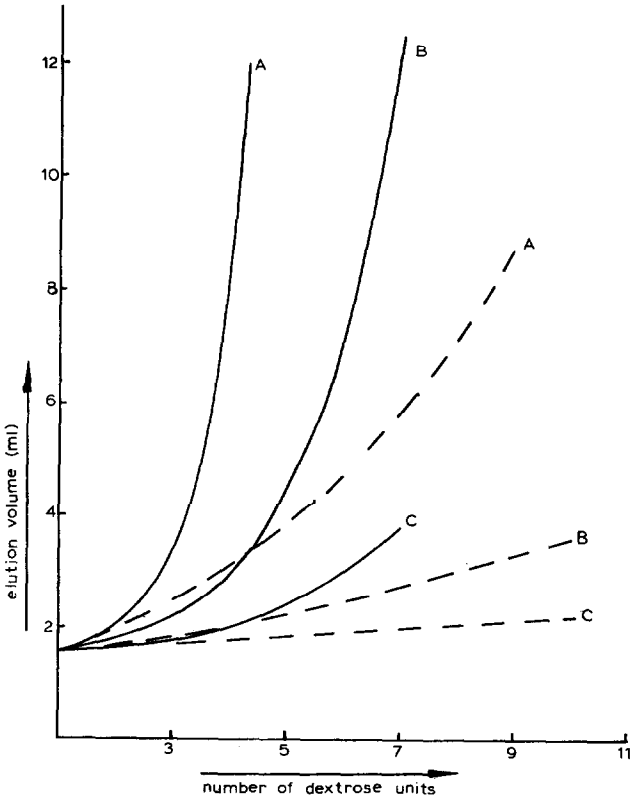

Fig. 3. Dependence of elution volumes (averages, calculated from the elution volumes of both anomeric forms according to the method presented earlier[20]) on the number of dextrose units in the components of starch hydrolysate (broken line) and cellulose hydrolysate (solid line) using column I under standard conditions and as the eluent (A) pure water, (B) 0.05% (v/v) of pentanol-1 and (C) 0.10% (v/v) of pentanol-1.

obtained. In serial analysis this disturbance will appear during the elution of oligomers injected later but can be easily distinguished in width. Injection of a pentanol-free sample into the system mentioned above gives a negative response of 0.2% f.s.d. with refractive index detection at attenuation × 32 as we calculated. Therefore, we conclude that the addition of a small amount of pentanol to water as the eluent is a useful means of reducing the retention times of the hydrolysate components without reducing the accuracy of their quantification.

Influence of the stationary phase in reversed-phase chromatography of carbohydrate oligomers

From literature data and from our research, in which we tested a number of RP-18 columns with different packings, it appeared that the type of silica has no influence on the retention of carbohydrate oligomers. However, all the supports applied had approximately the same pore diameter, 60–100 Å. Because of the increased possibility of exclusion chromatographic effects, which will have an adverse effect on the resolution, smaller pore diameters are hardly useful. Possibly a better and/or different elution pattern would be obtained if octadecyl-modified silica gel of substantially larger pore diameter is used. It is also interesting to study the dependence of the separation on the chain length of the chemically bonded ligand. We therefore carried out some experiments and the results are reporting below.

Variation of pore diameter. We obtained the octadecylsilane-modified silica gels R101, R103 and R110 with pore diameters of 100, 300 and 1000 Å, respectively, from Bètron Scientific (Rotterdam, The Netherlands). For these three silica supports the preparation method was similar to that for the alkylsilane modification. Moreover, columns II, III and IV were packed by an identical method, so that differences in separation properties will be caused only by the pore diameter and as a consequence of the relationship between pore diameter and specific surface area, the number of bonded octadecyl ligands.

For both starch and cellulose hydrolysate we determined the elution patterns on these three columns under standard conditions. The eluent was pure water and water containing 0.1% (v/v) of pentanol-1. The results are given in Fig. 4, where the elution volumes are plotted as a function of the number of dextrose units in the carbohydrate molecules. It can be seen that an increased pore diameter results in a decreased elution volume for both hydrolysate components, and the addition of pentanol-1 causes a strong decrease in the elution volumes, but the curves for cellulose and starch hydrolysate are clearly different. On the other hand, these curves appear similar with respect to pore diameter and eluent composition. The difference in the shape of the curves for the two hydrolysates is most pronounced near the middle. In comparison with the curve for starch hydrolysate, the cellulose hydrolysate curve is flat at a low number and steep at a high number of dextrose units. Thus the cellulose hydrolysate will give a relatively poor resolution of lower molecular weight components, especially when the elution power of the eluent is increased in order to shorten the elution times for the higher oligomers.

For all three columns the amount of pentanol-1 chosen was too high in the starch hydrolysate separation, in contrast to the separation of cellulose hydrolysate, where for column II a reasonable amount was used. A good separation of the last sample can also be obtained when column III is applied with a smaller amount of

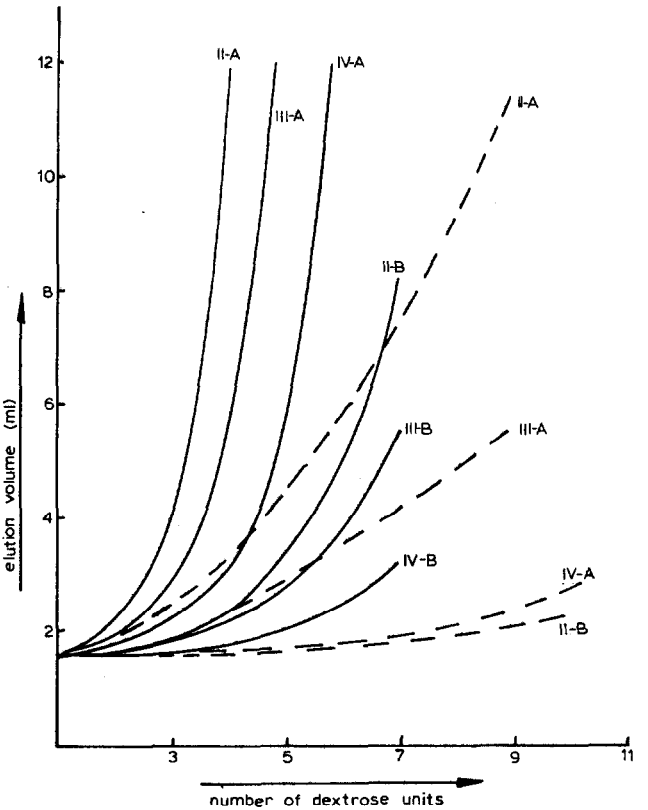

Fig. 4. Dependence of elution volumes (averages; see Fig. 3) on the number of dextrose units in the components of starch hydrolysate (broken line) and cellulose hydrolysate (solid line) using columns II, III and IV under standard conditions and as the eluent (A) pure water and (B) 0.1% (v/v) of pentanol-1 in water.

pentanol-1 in the eluent. The two cellulose hydrolysate chromatograms are given in Fig. 5a and b, respectively. For starch hydrolysate a compact chromatogram can be obtained with the combination of column III and pure water as the eluent, as can be seen in Fig. 5c.

Modification with alkyl groups of shorter chain length. We tested the applicability of octylsilane-modified silica gel with an usual pore diameter of 100 Å to study the influence of the chain length of the organic ligand on the retention of carbohydrate oligomers. Using column V under standard conditions and with pure water as the eluent, starch and cellulose hydrolysates were chromatographed. Starch hydrolysate gave a single, diffuse peak at an elution volume of two thirds of the empty column volume, whereas for cellulose hydrolysate a small retention of the higher molecular weight oligomers was obtained, as can be seen in Fig. 5d.

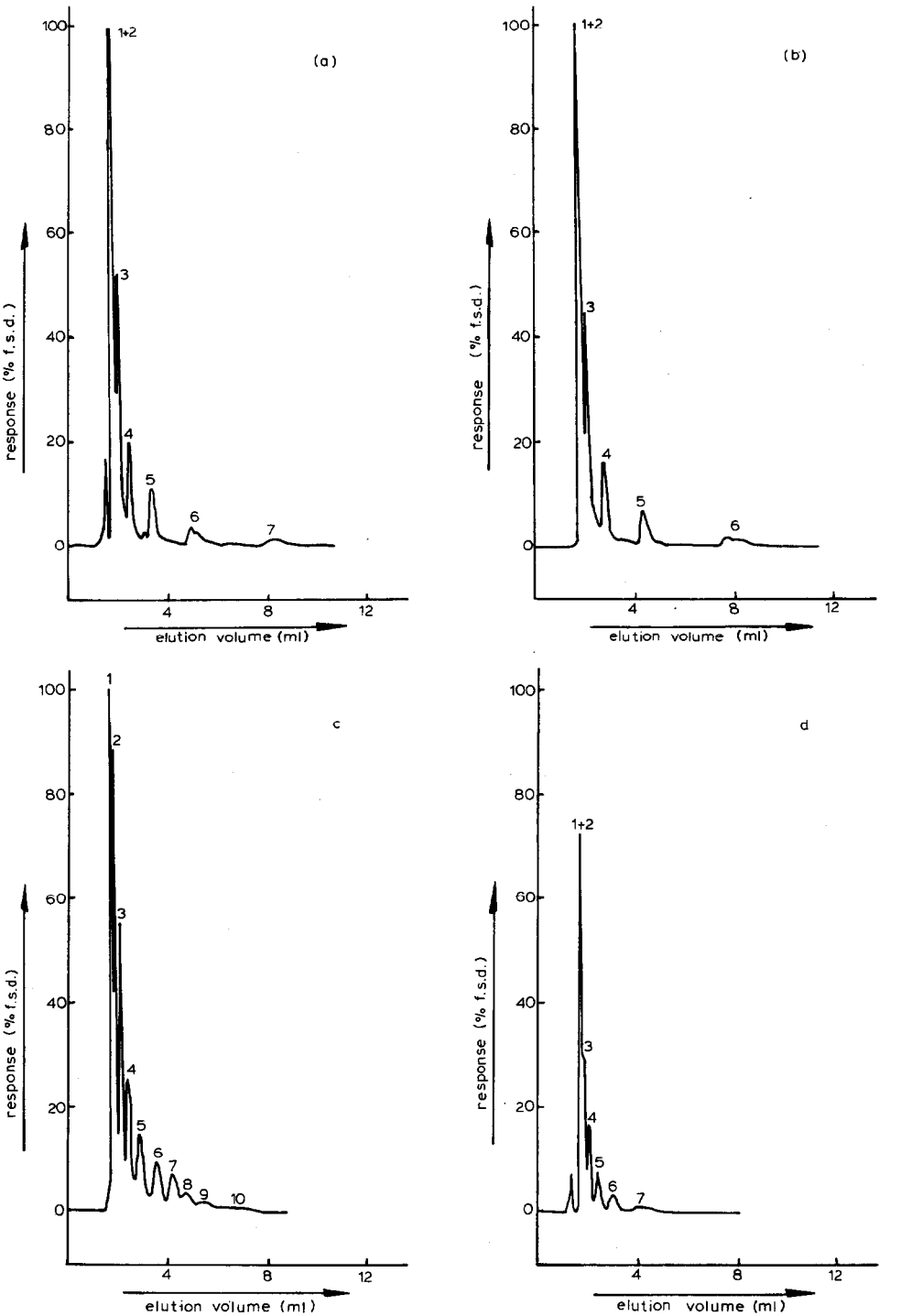

Fig. 5. Chromatogram of cellulose hydrolysate using (a) column II under standard conditions and as the eluent 0.1% (v/v) of pentanol-1 in water, (b) column III under standard conditions and as the eluent 0.05% (v/v) of pentanol-1 in water, (c) column III under standard conditions using pure water as the eluent and injected starch hydrolysate instead of cellulose hydrolysate and (d) cellulose hydrolysate using column V under standard conditions and pure water as the eluent.

CONCLUSION

It appears that there are several ways of optimizing carbohydrate oligomer separations by reversed-phase chromatography. In addition to the application of a pentanol-1-containing eluent, silica gel supports with a larger pore diameter than the commonly used 60–100 Å and with an alkyl modification shorter than the normally used octadecyl chain length clearly offer possibilities.

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