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COMPARATIVE SEPARATION OF LOW-MOLECULAR-WEIGHT CARBOHYDRATES AND POLYOLS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: RADIALLY COMPRESSED AMINE MODIFIED SILICA *VERSUS* ION EXCHANGE

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SUMMARY

A radially compressed silica column (modified with tetraethylenepentamine) was compared to an ion-exchange high-performance liquid chromatographic column for the separation of carbohydrates and polyols. Both columns afford an approximate molecular weight elution sequence but in opposite order. The silica system is characterized by very long life, low cost, high resolution and high linear sample capacity. The ion-exchange column gave greater sensitivity and resolved ethanol from carbohydrates and polyols, but was relatively short-lived, more expensive, had to be run at elevated temperatures for best results, is only partially compatible with automated chromatographic systems and has a somewhat smaller useful sample range than the modified silica system.

INTRODUCTION

The development of high-performance liquid chromatography (HPLC) as an analytical tool has provided a rapid method for the simultaneous determination of non-derivatized low-molecular-weight (<700 Dalton) carbohydrates and polyols. We recently described[1] the optimization of separation of several sugars and polyols in an amine-modified silica system with respect to eluent pH, solvent:amine modifier ratio and flow-rate. Silica columns derivatized with tetraethylenepentamine (TEPA)

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have been found by others[2] to provide better separations of such materials than that provided by related amines. We have also found that the TEPA-modified silica system (AMS) is characterized by exceptional stability over many thousands of injections, even with samples of complex biological material. Further, we have shown that in a radially compressed column these systems are amenable to high eluent pH[1], solvent recycling[3,4] and automated analysis systems.

Ion-exchange (IE) columns provide good resolution of certain polyol and carbohydrate mixtures[5–9]. Columns utilizing calcium-associated ion-exchange resins as the stationary phase and calcium solution in water as a mobile phase have an apparent sensitivity advantage over the AMS system reported here. Further, they can resolve ethanol from other compounds, a highly desirable feature in the analysis of fermentation systems.

With the exception of a small number of studies of obvious commercial interest, the advantages of each system have not been listed in a comparative fashion, nor has a direct comparison of these widely utilized carbohydrate and polyol analysis systems been reported. This paper extends the optimization of analysis conditions of carbohydrates and polyhydric alcohols by AMS and describes characteristics of each method for consideration when chosing an HPLC analysis method for these compounds.

EXPERIMENTAL

Apparatus

An HPLC system consisting of either a Waters 710B sample injector, a Waters Model 201 liquid chromatograph and a Waters 730 data module or a Waters U6K injector and Model 6000A pump were utilized in conjunction with a differential refractometer (Waters R401) for the separation and detection of these compounds. We used a Radial-Pak B silica cartridge (10-μm particles, 10 cm × 8 mm I.D. column) housed in a Waters RCM 100 radial compression module for the AMS column separations[1]. A Waters Sugar-Pak I column in a Waters sugar analyzer system was used for the IE separations.

Column pre-treatment and operating conditions

Ion-exchange columns were initially conditioned by pumping 100 ml of 10^{-3} *M* calcium acetate (90°C) through the column at 0.5 ml/min (4.12 MPa). This treatment was followed by the introduction of the final mobile phase: 10^{-4} *M* calcium acetate (also at 90°C). Approximately 12 h of eluant flow was required for column equilibration in this system.

Silica cartridges were conditioned by pumping 50 ml of acetonitrile–water (70:30) containing 0.1% (v/v) TEPA through the column at 2.0 ml/min (pH 9.2, 23°C, 2.75 MPa). The final mobile phase was then introduced, which consisted of acetonitrile–water–TEPA (either 75:25:0.02 or 81:19:0.02). Column stabilization in the AMS system required approximately 4–6 h in a recirculating mode.

Materials to be analyzed were dissolved in either distilled water or 50% ethanol. The same sample solutions were analyzed by both columns to facilitate a direct comparison.

Chemicals and reagents

Acetonitrile used in the eluent of the AMS system was either Fisher (Pittsburgh, PA, U.S.A.) or MCB Omnisolv (E. Merck, Darmstadt, G.F.R.) HPLC grade. The TEPA employed in the AMS system was technical grade obtained from Eastman Chemicals (Rochester, NY, U.S.A.). Calcium acetate was obtained from Fisher and most carbohydrates and polyhydric alcohols were obtained from Sigma (St. Louis, MO, U.S.A.). Exceptions were gentianose (a gift of Dr. Felix Keller, Zürich, Switzerland) and galactinol (a gift of Dr. Frank Loewus, Pullman, WA, U.S.A.). Filtered deionized water was used in mobile phase preparation. The pH of eluents was adjusted with glacial acetic acid[1]. Solvents were degassed by vacuum filtration through Millipore (Bedford, MA, U.S.A.) Type FGLP 0.22-μm PTFE filters.

RESULTS AND DISCUSSION

Separation of carbohydrates and polyhydric alcohols

The elution characteristics of 63 low-molecular-weight (47–667 Dalton) sugars and sugar alcohols are listed in Table I for both the AMS and IE columns. The AMS system separates these compounds in an approximate sequence of increasing molecular weight. The mean capacity factor (k') for the 58 compounds listed for the AMS system is 2.83 and 48% of these compounds give k' values exceeding 2.0 in the acetonitrile–water–TEPA (75:25:0.02) eluent. The IE system, by comparison, separates the same compounds in approximately the opposite order; greater-molecular-weight materials are eluted first in this method. The mean k' for 62 compounds listed for the IE method is 1.58 with only 24% of the k' values exceeding 2.0. This results from the fact that the IE method produces a narrow elution "window" of k' values between the elution of very large carbohydrates and the solvent front, whereas the AMS method produces a spread of k' values following the appearance of the "solvent front". This distribution may be readily modified by eluent modifications to improve resolution of particular compounds within eluted mixtures (Fig. 1). The k' of stachyose, for instance, can be changed from 2 to 20 by simple eluent adjustment in this system. The IE system does not lend itself to such a strategy since changes in elution will change both ends of the "elution window" simultaneously, negating any elution changes made for increased resolution.

Table I includes the non-carbohydrate compounds tris(hydroxymethyl)aminomethane (Tris buffer) and urea. Both substances are likely in samples of clinical and biochemical interest and could lead to erroneous interpretations of data if their presence was not suspected. Urea does not appear in the IE eluate since it binds to this column. Similarly, plant glycosides (*e.g.*, arbutin, salicin) in extracts of plant material could lead to similar errors in such analysis. Further, high concentrations of anions can cause baseline disturbances in the AMS system, giving a characteristic asymmetric peak in the region of trisaccharide elution (Fig. 2). This points to the need for adequate deionization of analyzed material before injection, a topic we have previously addressed[1,2].

Separations by the two methods differ in other respects than elution order. Ethanol and D-glyceraldehyde move with the solvent front in the AMS system. Idose gives multiple peaks in the AMS but not the IE system. The IE column does not give extremely low k' values and therefore is not as susceptible to the resolution problems

TABLE I

ELUTION OF SUGARS, ALCOHOLS AND UREA FROM AMS AND IE COLUMNS

Substance (synonym)	*Amine modified silica*				*Ion exchange*		
	Mol. wt.	*k'*	*Response/ sucrose*	t_R	*k'*	*Response/ sucrose*	t_R
β-D-Allose (β-D-allopyranose)	180.2	2.33	0.93	4'54"	1.82	0.47	10'44"
D-Altrose (D-altropyranose)	180.2	1.87	0.25	–	1.54	0.96	9'40"
L(+)-Arabinose (pectin sugar)	150.1	1.77	0.19	4'26"	1.77	0.78	10'31"
D-(+)-Arabitol (1,2,3,4,5-pentanepentol)	152.2	1.39	1.75	4'17"	1.88	0.81	11'00"
Arbutin (hydroquinone-β-D-glucopyranoside)	272.3	0.66	1.79	2'35"	2.32	0.25	12'40"
Cellobiose (4-O-β-D-glucopyranosyl-D-glucose)	342.3	4.92	0.54	9'41"	0.83	0.83	6'53"
Dihydroxyacetone (1,3-dihydroxydimethyl ketone)	90.1	0.76	0.33	2'55"	2.55	1.10	13'30"
i-Erythritol (1,2,3,4-butanetetrol)	122.1	1.14	2.34	3'37"	1.82	0.60	10'46"
D-Erythrose [(R)-2,3,4-trihydroxybutanal]	120.1	0.72	0.09	2'49"	2.34	0.47	12'43"
Ethanol (ethyl alcohol)	46.1	*	–	*	2.32	3.42	12'39"
Ethylene glycol (1,2-ethanediol)	60.1	0.47	1.76	2'26"	2.21	1.06	12'14"
β (D)-Fructose (levulose)	180.2	1.80	1.49	4'40"	1.62	0.78	10'02"
L-Fucose (6-deoxygalactose)	164.2	1.33	0.69	3'53"	1.75	0.79	10'28"
Galactitol (dulcitol)	182.2	2.24	0.73	5'15"	**	–	–
D(+)-Galactose (cerebrose)	180.2	2.62	0.16	5'55"	1.55	1.65	9'43"
Galactinol [α-D-galactopyranosyl-(1 → 1)-L-*myo*-inositol]	342.4	8.21	0.43	15'03"	**	–	–
β-Gentiobiose (6-O-β-D-glucopyranosyl-D-glucose)	342.3	5.39	0.32	10'39"	0.79	0.95	6'50"
Gentianose [β-D-glu-(1→6)-sucrose]	504.5	7.21	0.49	13'07"	**	–	–
D-Glucoheptose (D-glycero-D-glucoheptose)	210.2	2.55	0.12	6'24"	1.60	0.43	9'53"
D-Glucose (dextrose)	180.2	2.22	0.83	5'28"	1.36	0.85	9'00"
D-Glyceraldehyde (D-2,3-dihydroxypropanal)	90.1	*	–	*	2.02	0.37	11'30"
Glycerol (1,2,3-propanetriol)	92.1	0.80	1.59	2'59"	2.01	1.25	11'27"
2-Deoxy-D-glucose (D-2-glucodesose)	164.2	0.00	0.04	1'50"	1.38	0.92	9'04"
6-Deoxy-D-glucose (quinovose)	164.2	1.18	0.50	2'10"	1.46	0.77	9'22"
1-O-Methyl-D-glucopyranose	192.4	1.10	2.17	3'28"	1.34	0.84	8'55"
Tris(hydroxymethyl)aminomethane (Tris buffer)	121.1	2.17	2.41	4'35"	2.34	***	12'44"
Idose	180.1	–	–	–	1.73	0.85	10'24"
myo-Inositol (*meso*-inositol, *i*-inositol)	180.2	4.46	0.95	9'01"	1.77	0.86	10'34"
α-Lactose (4-O-β-D-galactopyranosyl-D-glucose)	342.3	5.09	0.43	9'57"	0.93	0.82	7'21"
Lactulose (4-O-β-D-galactopyranosyl-D-fructose)	342.3	4.20	0.83	8'50"	1.03	0.89	7'45"
D-Lyxose	150.1	1.58	0.66	3'45"	**	–	–

Maltose (4-O-α-D-glucopyranosyl-D-glucose)	342.3	4.35	0.61	8'50"	0.88	0.83	7'10"
Maltotriose [α-D-Glu-(1→4)-α-D-Glu-(1→4)-α-D-Glu]	504.4	7.80	0.27	14'32"	0.64	0.73	6'15"
iso-Maltotriose	504.5	10.98	0.10	19'23"	0.61	0.40	6'08"
D-Mannitol (mannite)	182.2	2.15	1.43	5'12"	1.79	0.60	10'37"
D-Mannoheptulose (D-mannoketoheptose)	210.2	2.35	1.36	5'35"	1.41	0.86	9'12"
D(+)-Mannose (D-mannopyranose)	180.2	2.04	0.21	5'04"	1.58	0.64	9'50"
α-Methyl-D-mannoside	194.2	0.88	2.01	3'07"	**	–	–
Melezitose [α-D-Glu-(1→3)-β-D-Fru-(2→1)-α-D-Glu]	504.5	6.11	0.64	11'44"	0.61	1.09	6'08"
α-D(+)-Melibiose (6-O-α-D-galactopyranosyl-D-glucose)	342.3	5.53	0.26	10'47"	0.91	0.70	7'17"
Palatinose (6-O-α-D-glucopyranosyl-D-fructofuranose)	342.3	3.72	0.67	7'48"	0.89	0.65	7'11"
Pentaerythritol [tetrakis(hydroxymethyl)methane]	136.1	1.02	3.33	2'58"	1.72	0.57	10'23"
Perseitol (α-mannoheptitol)	212.2	3.03	1.15	6'39"	1.86	0.49	10'53"
Propylene glycol (1,2-propanediol)	76.1	0.31	1.00	2'10"	2.19	0.75	12'09"
D-(+)-Raffinose [α-D-Gal-(1→6)-α-D-Glu-(1→2)-β-D-Fru]	504.5	7.46	0.42	13'58"	0.61	1.47	6'10"
α-L-Rhamnose (6-deoxy-L-mannose)	164.2	1.21	0.10	3'36"	1.50	0.65	9'31"
Ribitol (adonitol)	152.2	1.54	1.86	4'17"	1.77	0.81	10'33"
D-Ribose	150.1	1.08	0.16	3'31"	2.47	0.23	13'13"
2-Deoxy-D-ribose (α-2-D-ribose)	134.1	1.59	0.19	4'19"	1.75	0.76	10'27"
D-Ribulose (D-erythro-2-ketopentose)	150.1	1.04	1.67	3'18"	2.17	0.35	12'04"
Salicin (salicyl alcohol glucoside)	286.3	0.58	1.28	2'30"	2.46	0.67	13'10"
Sedoheptulose anhydride (2,7-anhydrosedoheptulose)	192.2	1.62	1.89	4'25"	1.68	0.78	10'13"
D-Sorbitol	182.2	2.16	1.53	5'13"	1.97	0.68	11'19"
L(−)-Sorbose	180.2	1.80	1.60	4'40"	1.49	0.82	9'29"
Stachyose [α-D-Gal-(1→6)-raffinose]	666.6	15.59	0.54	27'23"	0.45	0.16	5'31"
Sucrose (α-D-glucopyranosyl-β-D-fructofuranoside)	342.3	3.39	1.00	7'15"	0.87	1.00	7'08"
D-Tagatose (D-*lyxo*-hexulose)	180.2	1.71	0.78	4'20"	1.76	0.70	10'30"
α-D-Talose	180.2	1.56	0.17	4'16"	2.34	***	12'44"
Trehalose [α-D-Glu-(1→1)-α-D-Glu]	342.3	4.45	0.80	9'00"	0.83	0.87	6'58"
DL-Threitol	122.1	1.18	1.98	3'41"	2.02	0.74	11'30"
Turanose (3-O-α-D-glucopyranosyl-D-fructose)	342.3	3.51	0.50	7'38"	0.88	0.97	7'10"
Urea (carbamide)	60.1	0.75	3.56	2'30"	–	–	–
Xylitol	152.2	1.64	1.41	4'17"	**	–	–
D(+)-Xylose (wood sugar)	150.1	1.39	0.52	4'04"	2.32	1.09	12'40"
D-Xylulose (*threo*-pentulose)	150.1	1.08	0.64	3'22"	1.55	0.76	9'44"

* Coelutes with mobile phase.
** Unavailable for test.
*** Coelutes with ethanol.

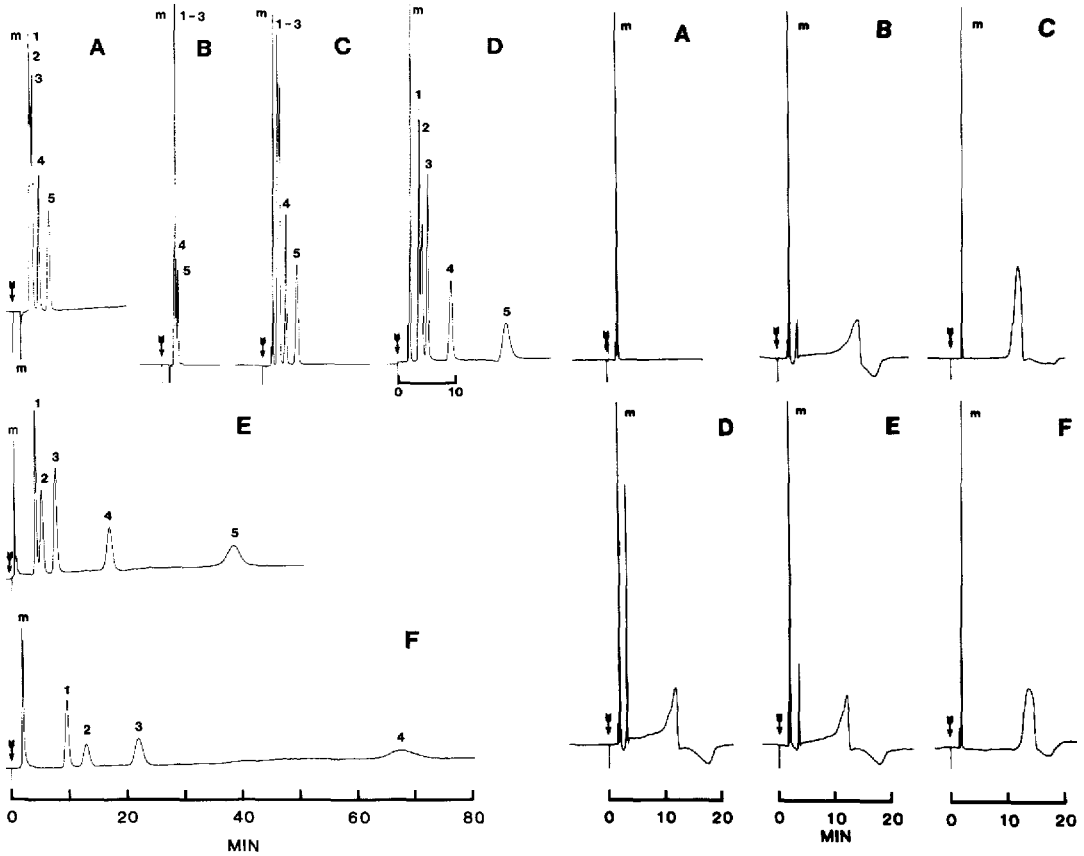

Fig. 1. Effect of eluent modifications upon solute elution from the amine modified silica column. Eluents: A, acetonitrile–water (60:40); B, acetonitrile–water (50:50); C, acetonitrile–water–methanol (50:25:25); D, acetonitrile–water–methanol (60:20:20); E, acetonitrile–water (75:25); F, acetonitrile–water (81:19). Flow-rate: 2.0 ml/min. All eluents were pH 8.9 and contained 0.02% (v/v) TEPA. All 16 × except B (32 ×). Peaks: 1 = fructose; 2 = glucose; 3 = sucrose; 4 = raffinose; 5 = stachyose; m = mobile phase.

Fig. 2. Elution of ions from the amine modified silica column. At arrow, 5 μmoles of the following ionic materials were injected into an eluent consisting of acetonitrile–water–methanol (60:20:20) (*cf.*, Fig. 1D): A, water; B, KCl; C, HCl; D, NaCl; E, sodium formate; F, formic acid. All 16 × .

between the solvent "front" and low-molecular-weight materials as in the AMS method (*cf.*, Fig. 1). Therefore, all the compounds listed in Table I which were available for testing could be detected following elution from the IE column, however, Tris buffer coeluted with ethanol (samples were injected in 50% ethanol). Urea did not elute from the IE system since it bound to the column. The IE column, however, was found to have a 14-min "elution window" corresponding to the time between the elution of the largest carbohydrates and the elution of the solvent (acetate) front (Fig. 3). This elution window precludes practical mobile phase manipulations to enhance resolution of components in a complex mixture but such manipulations can be applied to advantage in the AMS system (Fig. 1). Therefore, the IE column is not practical for the resolution of relatively complex mixtures of carbohydrates or polyols.

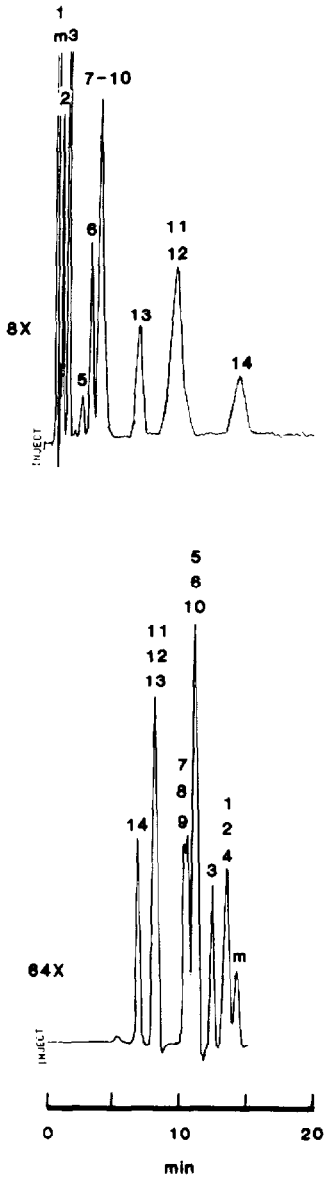

Fig. 3. Comparison of the elution of a carbohydrate–polyol–ethanol mixture from an ion-exchange (bottom) and an amine-modified silica (top) column. Amine eluent acetonitrile–water (81:19) containing 0.02% TEPA (23°C), ion-exchange eluent 10^{-4} *M* calcium acetate (90°C). Peaks: 1 = ethanol; 2 = ethylene glycol; 3 = glycerol; 4 = ribose; 5 = xylose; 6 = fructose; 7 = galactose; 8 = glucose; 9 = mannitol; 10 = sorbitol; 11 = lactose; 12 = maltose; 13 = sucrose; 14 = melezitose; m = mobile phase.

This difficulty with the IE column is further illustrated in Fig. 3. A mixture of fourteen compounds, chosen to represent varied but typical analytical interests, was applied to both columns. With the AMS system, ethanol did not resolve from the "solvent front" but the fourteen component mixture was separated into eight distinct

groups. The IE column resolved these fourteen solutes into only six groups, but did separate ethanol from the "solvent front".

Sensitivity limits and column loading characteristics

The AMS and IE systems differ considerably in apparent sensitivity; the limit of detection in the AMS system being on the order of 5 μg *versus* 500 ng with the IE method. Note that not only the peak heights but also peak areas are significantly different in the two methods. Increasing eluent polarity in the AMS system can somewhat overcome this handicap in sensitivity, but rapidly eluted compounds (low-molecular-weight materials) become increasingly difficult to resolve from the solvent "front" (Fig. 1). The IE method, therefore, is more sensitive if a relatively few substances are to be resolved.

The apparent increase in sensitivity of the IE system compared to the AMS method is not due to the acetonitrile–water mixture employed by the latter *versus* the aqueous system utilized in the former system (Table II). In fact, the refractive index difference between solvent and solvent plus solute is actually greater in the AMS method. A partial explanation of the apparent difference in sensitivity could, however, be due to the difference in operating temperatures employed. A more plausible explanation for the difference in sensitivity would involve complexes formed between eluted compounds and the TEPA incorporated into the AMS eluent, since the reactions of sugars with primary amines are well known[10].

Column temperature maintenance is important in modifier-containing normal phase systems such as the AMS system. For these methods, retention time precision of 1% requires thermal control of ±0.35°C which can be readily obtained with commercially available equipment[11]. The AMS system, therefore, is more sensitive than the IE system to column thermal fluctuations.

Sample loading or column saturation characteristics for both columns are illustrated in Figs. 4 and 5. The peak height response in the AMS system is linear for solute concentrations exceeding 5 mg per injection with column saturation occurring at approximately 25 mg per injection for the compounds tested. The practical range

TABLE II

SUCROSE/SOLVENT REFRACTIVE INDEX DIFFERENCES (RID) IN ACETONITRILE–WATER MIXTURES

Refractive indices determined with a Bausch and Lomb Abbe 3-L refractometer operated at 23.5°C.

Acetonitrile (% v/v)	*Index of refraction*		
	Solvent	*Solvent + 4 mg/ml sucrose*	*RID*
100	1.3426	–*	–*
80	1.3455	1.3465	0.0010
60	1.3462	1.3470	0.0008
40	1.3445	1.3453	0.0008
20	1.3404	1.3410	0.0006
10	1.3374	1.3379	0.0005
0	1.3329	1.3334	0.0005

* Sucrose is insoluble in 100% acetonitrile.

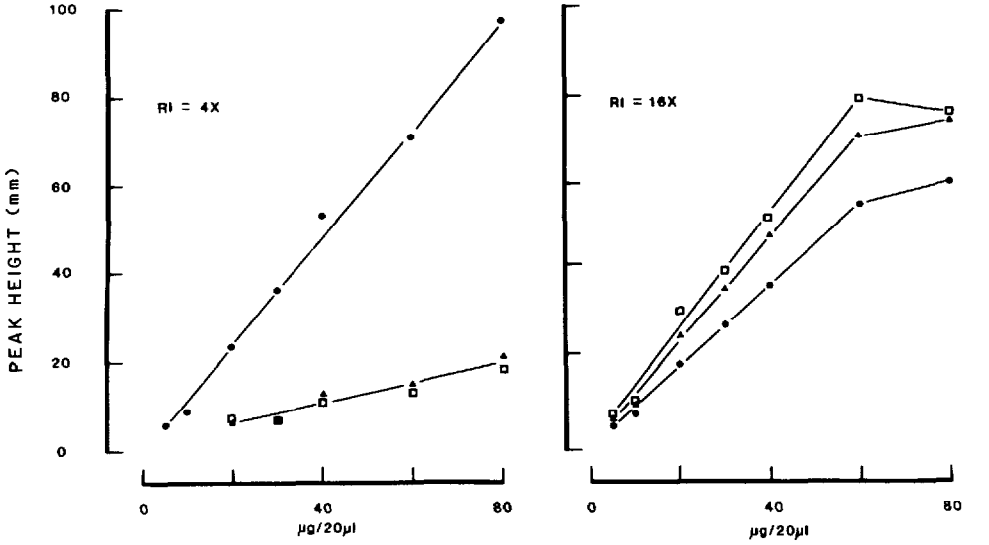

Fig. 4. Column loading characteristics of amine-modified silica (left) and ion-exchange (right) column. Elution conditions as in Fig. 3. Solutes: glycerol (●); glucose (▲); trehalose (□).

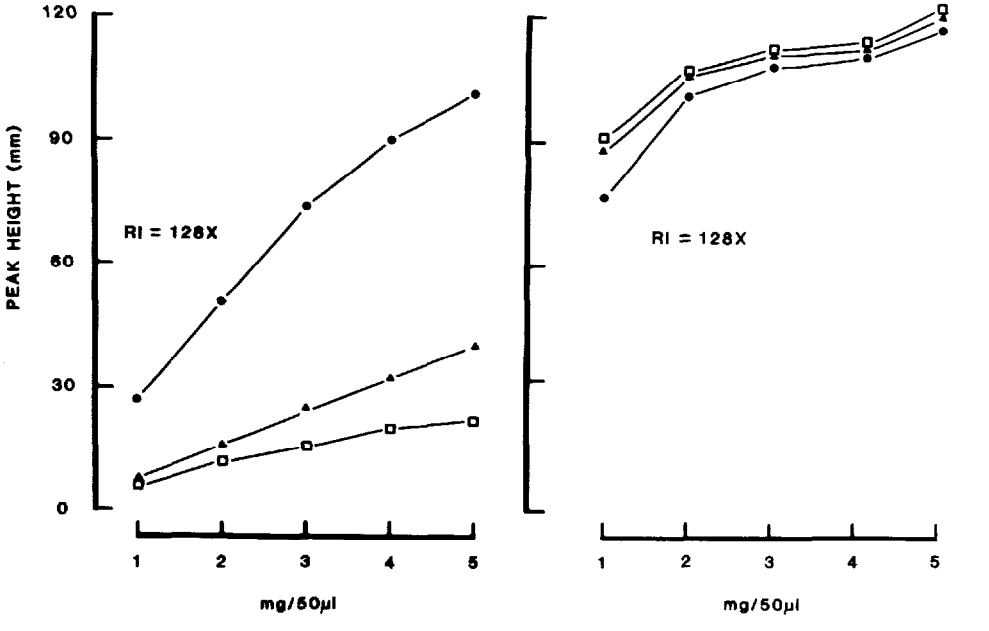

Fig. 5. Column saturation conditions in amine-modified silica (left) and ion-exchange (right) columns. For details see Fig. 4.

of sample load in this system is, therefore, from approximately 20 μg to 20 mg per injection (the upper limit depending upon solubility in the eluent). While the IE system is more sensitive in terms of detection of eluted material, it tends to saturate at lower concentrations of injected solute (approximately 1 mg per injection). The useful "load range" in the IE system is thus from 5 to 500 μg per injection. The IE system is therefore capable of handling sample sizes spread over a 10^2 range but the AMS system could handle sample sizes over a 10^3 range if solubility permitted.

CONCLUSION

Two HPLC systems have been compared for the separation of low-molecular-weight carbohydrates, polyhydric alcohols and related compounds. Each system has certain separation characteristics and advantages. As with most analyses, some degree of compromise must be made in system selection. The IE system is considerably more sensitive and shows good resolution of mixtures of relatively few components. This is especially true for the analysis of very-small-molecular-weight material, particularly ethanol, which the AMS system can not resolve from the "solvent front".

The IE system has a significantly shorter life (approximately 200 *versus* 2000 injections in the AMS system). The IE column also has a lower loading capacity, higher cost per analysis, requires periodic column reversal to insure repeatable separation profiles and needs to be operated at elevated temperatures for optimal results. The necessary column reversals minimize the usefulness of the IE method in continuous automated analytical procedures due to the lengthy (approximately 8 h) periods required daily for restabilization of the column following reversal.

The AMS system is characterized by exceptionally long life (even when used with complex biological material[4]), high stability (no reversal or reequilibration is required), high solute carrying capacity and room temperature operation. Additionally, separation in the AMS system may be performed over a wide range of pH[1] values and solvent ratios (Fig. 1) and these columns are amenable to solvent recirculation and automated analysis systems. However, the AMS columns are not suitable for ethanol analysis and are more sensitive to column thermostating.

Material analyzed by both columns must be properly deionized before analysis. We have shown previously[12] that anion-exchange resins (OH^-) are unacceptable for deionizing carbohydrate or polyol samples. One method which does produce acceptable results in the AMS method is to utilize anion-exchange resins in the formate form[13] followed by freeze drying to remove eluted formic acid.

An additional advantage of the AMS method is the ability to modify the k' values of very large carbohydrates or polyols to increase the sensitivity and decrease analysis times of such materials as tetrasaccharides. In fact, if only oligosaccharides were of interest, the AMS method could provide a very high degree of solute resolution, with the elution time and carbohydrate/polyol separation being readily adjustable by solvent manipulation (Fig. 1).

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