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SEPARATION OF CARBOHYDRATES AND POLYOLS BY A RADIALLY COMPRESSED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SILICA COLUMN MODIFIED WITH TETRAETHYLENEPENTAMINE

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SUMMARY

Optimization of separation of carbohydrates and polyhydric alcohols on a silica column modified with tetraethylenepentamine is described. Eluent tetraethylenepentamine concentration, pH, solvent concentration and flow-rate were optimized with respect to compound separation and baseline stability. This method offers advantages over existing techniques, including room temperature operation, low operating pressure, low cost, simplicity, high resolution, relatively lengthy column life, and high linear sample capacity.

INTRODUCTION

The simultaneous analysis of carbohydrates and polyols by high-performance liquid chromatography (HPLC) has received considerable attention in recent years[1–7], including methods employing amine-modified silica as the stationary phase[8–12]. Of various amine-modified systems considered by Wheals and White[12], tetraethylenepentamine (TEPA) was found to provide the best separation of fructose, glucose, sucrose, maltose and lactose as well as long term column stability. The addition of TEPA and amines of similar characteristics increases the retention of carbohydrates by silica columns[1] and thus allows the resolution of carbohydrates of biochemical interest which are not resolvable by a number of HPLC techniques[9]. However, the list of carbohydrates and polyols investigated in such separation systems has been limited and the variables of pH, amine concentration in the eluent, solvent flow-rate and composition have not been optimized previously.

The recently developed radial compression cartridge[13,14] offers a number of advantages over previously available HPLC techniques. The relatively short, large-

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diameter column provides a closer approximation to "infinite diameter[15]." Further, the pressurized flexible column walls produce an ability to compensate for packing material non-homogeneity[13]. This allows for a wider range of eluent pH than with uncompensated columns since compression compensates for silica dissolution at high pH. We have used a single Radial-Pak silica cartridge (Waters Assoc., Milford, MA, U.S.A.) and basic eluent pH (9.2) for several months without noticable degradation of column performance. Further, employment of solvent recirculation and the relatively low cost of the Radial-Pak silica cartridges results in a considerable decrease on cost per assay.

EXPERIMENTAL

Apparatus

A Waters Model R401 differential refractometer was used in conjunction with a Waters Model 6000A pump and WISP 710B automated injection system. The output from this system was recorded on a Waters data module. A Radial-Pak silica cartridge (10 cm × 8 mm I.D.) was employed in a Waters RCM-100 radial compression module to effect separation of these compounds. A Waters guard column filled with AX/Corasil was placed in line with the Radial-Pak B column to protect the column and saturate the eluent with SiO_2.

Chemicals and reagents

Acetonitrile used for solvent preparation was Fisher HPLC grade (Pittsburgh, PA, U.S.A.), technical grade TEPA was obtained from Eastman (Rochester, NY, U.S.A.) and carbohydrates and polyols from Sigma (St. Louis, MO, U.S.A.). Water used in solvent preparation was deionized and glass distilled. The pH of elution solvents was adjusted with glacial acetic acid. Eluents were degassed by vacuum filtration through Millipore 0.2-μm filter (Bedford, MA, U.S.A.).

Column pretreatment

Waters Radial-Pak silica cartridges were initially conditioned by pumping 50 of acetonitrile–water (70:30) containing 0.1% (v/v) TEPA (pH 9.2) through the column. The final elution solvent was then introduced [nominally, pH 8.9 acetonitrile–water (81:19) containing 0.02% TEPA] and the column stabilized by recirculating this solvent overnight before column usage.

Operating conditions

Solvent effluent was returned to a continuously stirred 1-l pump reservoir for recirculation. Columns were nominally operated at *ca.* 26°C and a flow-rate of 2 ml/min which produced a backpressure of approximately 2.75 MPa (400 p.s.i.). Carbohydrate and polyol standards were dissolved in distilled water (5 mg/ml); nominal injection volume was 50 μl.

RESULTS AND DISCUSSION

Separation of polyols, mono-, di-, and trisaccharides

Initial separation of polyol and carbohydrate mixtures employing a pH 9.2

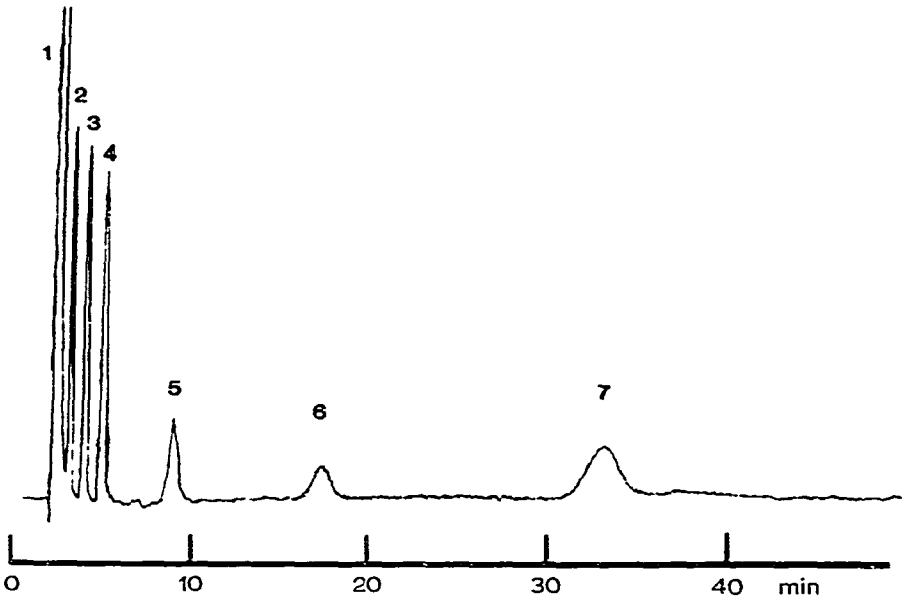

Fig. 1. Chromatogram of elution of polyol–saccharide mixture by a hydraulically compressed Radial-Pak silica column (10 cm × 8 mm I.D.) modified with TEPA. Elution solvent: acetonitrile–water (81:19), pH 8.9, containing 0.02% TEPA. Eluent flow-rate, 2 ml/min, pressure, 2.34 MPa (340 p.s.i.), 26°C. Injected sample contained, in 50 μl, 250 μg of each of the following: 1 = solvent "front"; 2 = ethylene glycol; 3 = glycerol; 4 = erythritol; 5 = glucose; 6 = maltose; and 7 = raffinose.

mobile phase containing acetonitrile–water (81:19) produced results (Fig. 1) similar to those published for other amine-modified silica columns[1,4,10,12] with respect to the order of carbohydrate elution and to produce excellent separation of components in the test mixture. Capacity factors (k') of these compounds were found to be a function of the composition of the mobile phase (Fig. 2), particularly for the di- and trisaccharides. Changing the acetonitrile–water ratio did not change the elution order of these compounds but did have an effect upon the k' of all compounds in the test mixture. Capacity factors were found to be independent of flow-rate over the range of 2 to 10 ml/min (not shown); however, below 2 ml/min, k' was found to decrease with increasing mobile phase velocity.

To test the suitability of this method for the separation of a wide range of carbohydrates and polyols, additional compounds were chromatographed under identical conditions (Table I). Whereas most compounds eluted in the approximate order of molecular size, certain exceptions were noted. For example, sucrose elutes before inositol and sedoheptulose anhydride was found to elute prior to a number of hexoses. Separation in this system may, therefore, be due to factors other than molecular size, such as the number and geometry of hydroxyl groups, as suggested by D'Ambroise *et al.*[9] for a similar system employing LiChrosorb-NH_2 as the stationary phase. This may also explain why ribose, galactose, arabinose and mannose were not detectable with this system, while very similar compounds were detected.

The order of elution and approximate number of theoretical plates calculated (N) for these compounds are consistent with data from similar investigations[1,4,9,12].

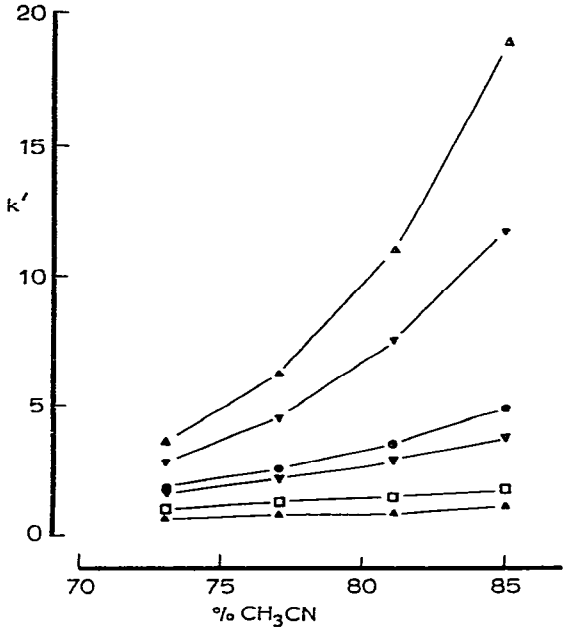

Fig. 2. Effect of eluent acetonitrile content upon k' of a standard polyol–saccharide mixture. △ = Trehalose; ▼ = sucrose; ● = sorbitol; ▽ = fructose; □ = erythritol; ▲ = glycerol. Other conditions as in Fig. 1.

TABLE I

ELUTION OF CARBOHYDRATES AND POLYOLS FROM A RADIAL-PAK SILICA COLUMN
Conditions of elution as in Fig. 1.

Compound	*Molecular weight*	*k'*	*N/10 cm*	R_s*
Ethylene glycol	62.07	0.497	634	1.889
Glycerol	92.09	1.017	657	1.355
Rhamnose	164.16	1.451	903	0.417
Erythritol	122.12	1.594	834	0.136
Threitol	122.12	1.640	1125	0.610
Fucose	164.16	1.880	596	0.515
Xylose	150.13	2.091	1259	0.480
Ribitol (adonitol)	152.15	2.286	812	0.010
Sedoheptulose anhydride	210.20	2.331	924	0.355
Arabitol	152.15	2.497	800	0.321
Fructose	180.16	2.903	837	1.038
Sorbitol	180.16	3.451	1185	0.270
Dulcitol (galactitol)	182.16	3.594	1160	0.035
Mannitol	182.16	3.617	583	0.620
Glucose	182.16	4.046	1066	3.100
Sucrose	342.30	6.520	946	1.680
Inositol	180.16	8.257	1153	0.131
Cellobiose	324.30	8.400	1189	0.100
Maltose	360.31	8.617	1270	0.581
Trehalose	378.33	9.286	1132	0.424
Lactose	342.30	9.829	1049	5.091
Raffinose	594.52	17.640	1470	

* Calculated resolution between adjacent members of the table.

The maximal number of theoretical plates we observed for this column (14,700 plates/m) is high in comparison to similar calculations for other HPLC carbohydrate columns[4,10,12].

If we consider a peak resolution (R_s) of 0.8 to be the value of least acceptable resolution[16], we see that a number of compounds in Table I are acceptably resolved, some are marginally resolved (*e.g.*, R_s glucose:mannitol = 0.62) and a number are too poorly resolved to be separated by this system (*e.g.*, R_s sedoheptulose:ribitol = 0.01). For those showing marginal resolution, increased separation may be achieved by increasing the percentage of acetonitrile in the eluent (Fig. 2). For example, resolution of sorbitol and fructose (*cf.* Fig. 2) is very poor with acetonitrile–water (73:27) (R_s = 0.45) but increases to virtual complete separation (R_s = 1.57) with acetonitrile–water (85:15) as the eluent. Such a strategy would be expected to have a greater chance of success for carbohydrates and polyols with a molecular weight greater than approximately 150, due to the roughly exponential effect of acetonitrile concentration upon k' mentioned earlier (Fig. 2). Since increased resolution gained by increasing acetonitrile concentration in the eluent is achieved at the expense of extended elution times, minimally acceptable component resolution will have to be balanced against maximal allowable elution times.

Maximization of separation and baseline stability

In order to maximize flow-rate for greatest efficiency of separation, data taken from the elution of glucose were evaluated (Fig. 3). A plot of reduced velocity (v) versus reduced plate height (h), a Knox plot[15–17], theoretically passes through a minimum at the reduced velocity of maximal separation efficiency. Data gathered over the flow-rate range of 0.4 to 9.9 ml/min shows an apparent decrease in slope at lowest flow-rates but significant scatter was noted in data corresponding to flow-rates under 2 ml/min. Nonetheless, this column appears to be most efficient at flow-rates of approximately 2 ml/min and diffusion does not interfere with efficiency of separation even at the lowest flow-rates investigated[16,17]. Unlike a similar system employing silica modified with γ-aminopropyltriethyloxysilane[10], we find an almost direct proportionality between plate height and elution velocity (Fig. 3), reinforcing our conclusion that low elution velocities lead to the greatest separation efficiency in this system.

Further, the low back pressure generated by the unusual geometry of this column allows the use of flow-rates as high as 10 ml/min, rates which would create unacceptably high pressures with many other HPLC columns. This low pressure response to flow-rates is reflected in an unusually low flow resistance parameter[16–19] value ($\varnothing$ = 390) for the elution of glucose from this column, which is much less than that for other commercially available HPLC carbohydrate systems. The unusual column geometry is also apparent in the exceptional column sample capacity we find with this system (Fig. 3). Most silica HPLC columns have linear sample capacity values which lie between 10^{-4} and 10^{-3} g/g column packing[18], but we find no significant decrease in k' even with sample loads of $2 \cdot 10^{-3}$ g/g. The response of this system is linear with respect to sample size and it produces symmetrical, reproducible peaks for injected glucose samples ranging in size from 20 to 20,000 μg.

Carbohydrate and polyol separation in this system were found to be a function of eluent pH (Fig. 4). Elution mixtures with acidic pH values strongly decrease k' but not elution order for all compounds tested, by compressing the elution pattern with

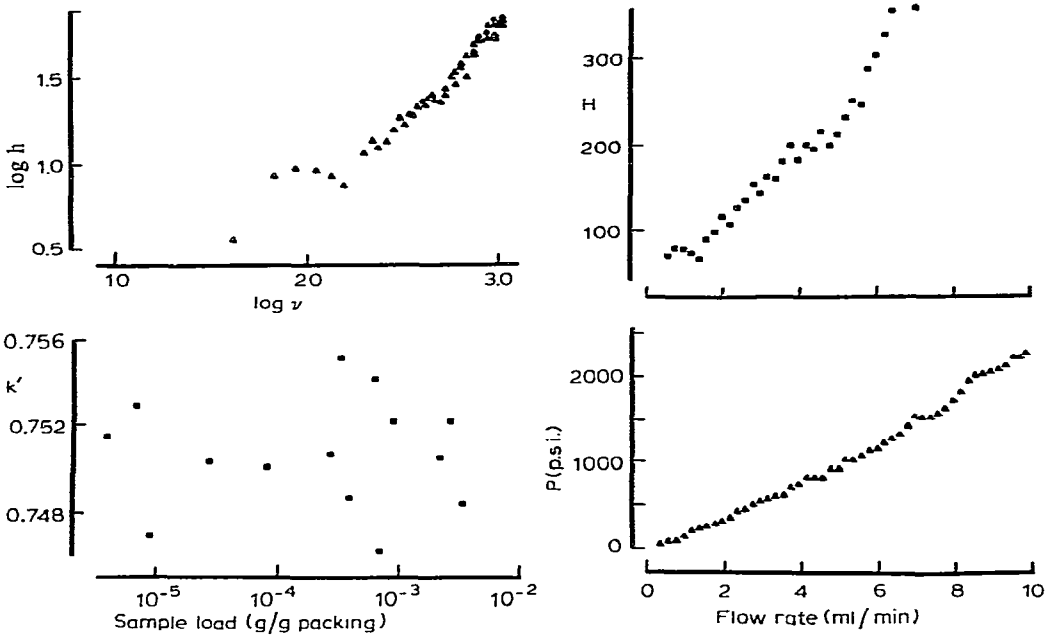

Fig. 3. Effect of eluent velocity upon glucose elution. Theoretical plate height (H) and pressure (P) plotted as a function of flow-rate. Reduced plate height (h) and reduced velocity (ν) plotted as a Knox plot from which, in theory, the least slope represents the elution velocity corresponding to maximal separation efficiency[15–17]. For this calculation, the effective spherical diameter of the packing was taken as 8.5 μm and the diffusivity coefficient of acetonitrile–water (81:19) was taken to be $2.1 \cdot 10^{-9}$ m^2/sec. Linear sample capacity plot (k' *vs.* g/g) is plotted for samples varying from 20 to 20,000 μg glucose. Other conditions as in Fig. 1.

respect to time. However, such acidic eluents introduce a strong negative peak following trisaccharide elution (Fig. 4). In addition, a second (but shorter) baseline deflection was also noted during the elution of the polyol–saccharide test mixture (arrow). The later negative peak decreases the apparent amount of monosaccharides with similar retention times. Both of these baseline deflections were found to decrease with increasing pH (Fig. 4), the larger deviation completely disappearing at pH 8.9. Elution of solvents with basic pH values also increases the k' of the mixture, but does not alter the order of elution of these compounds.

A series of injections of water, TEPA and various acetonitrile–water mixtures was employed to clarify the negative peaks noted in Fig. 5. The concentration of TEPA in the sample was found to have a relatively minor effect upon these negative deflections and no effect upon the k' of components in the test mixture. Of those concentrations tested (Fig. 5), the addition of 0.02% TEPA was found to produce the greatest baseline stability. Higher concentrations of TEPA introduced a perturbation (arrow) approximately 12 min following injection.

The negative peaks were found to be primarily a function of injected sample water (Fig. 6), being proportional in magnitude to injected water mass (Fig. 6). These negative peaks were also determined to be a function of eluent pH. Higher pH values eliminated the major perturbation and decreased the minor dip (arrow) which occurs

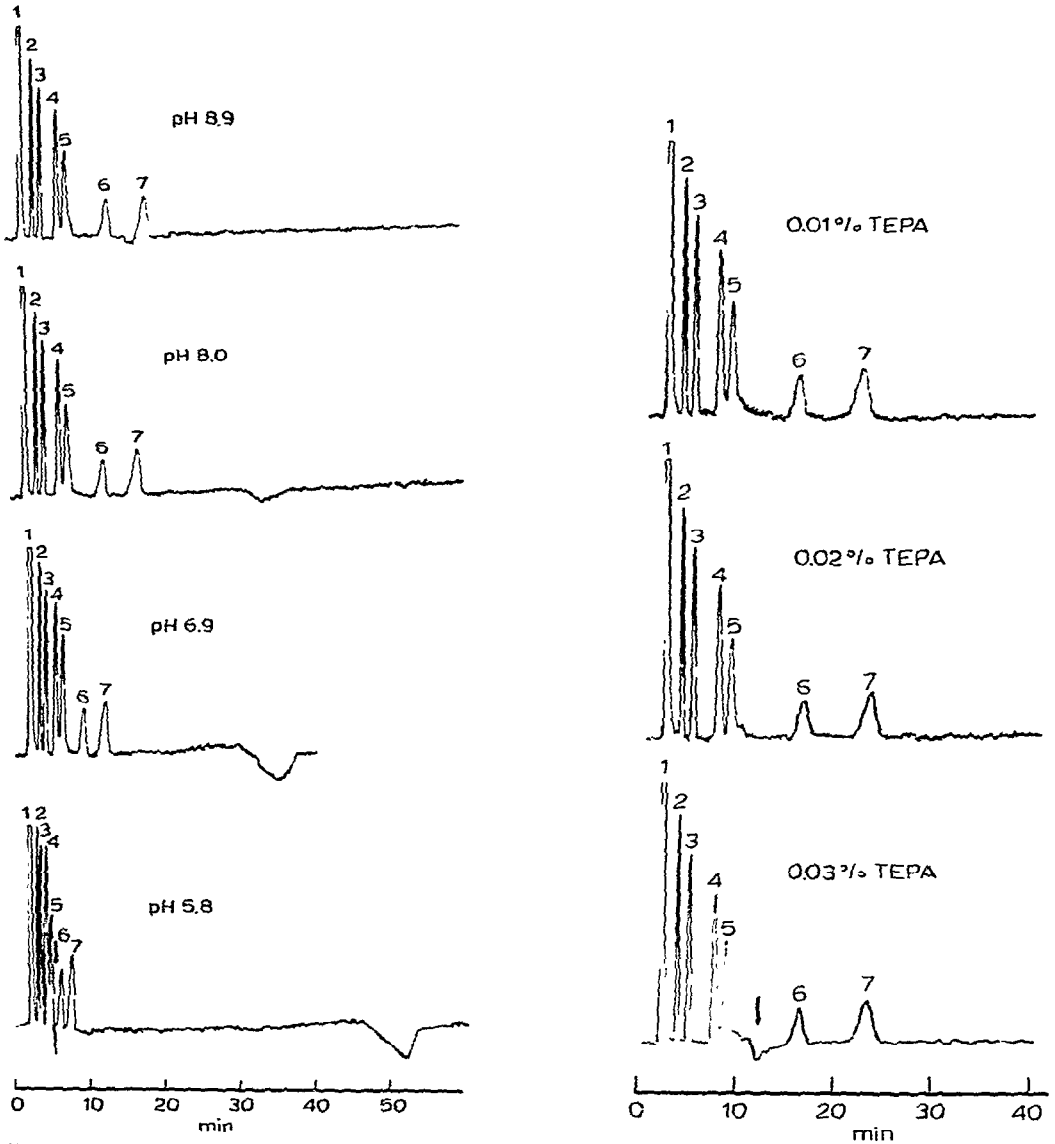

Fig. 4. Effect of pH of eluent upon elution of a polyol–saccharide mixture. Injected sample contained 250 μg of each of the following: 1 = solvent "front"; 2 = glycerol; 3 = erythritol; 4 = fructose; 5 = sorbitol; 6 = sucrose; and 7 = trehalose. Eluent pH from top to bottom: 8.9, 8.0, 6.9, 5.8. Other conditions as in Fig. 1.

Fig. 5. Effect of varying TEPA concentration in eluent upon chromatograms of standard polyol–saccharide mixture. Elution conditions and identification of compounds as in Fig. 4.

during monosaccharide elution. The problems associated with water injection could be eliminated by injection of materials dissolved in the elution mixture, but the superior solubility of many carbohydrates and polyols in water suggests that water would serve best for injection of unknown biological samples. The major negative peak

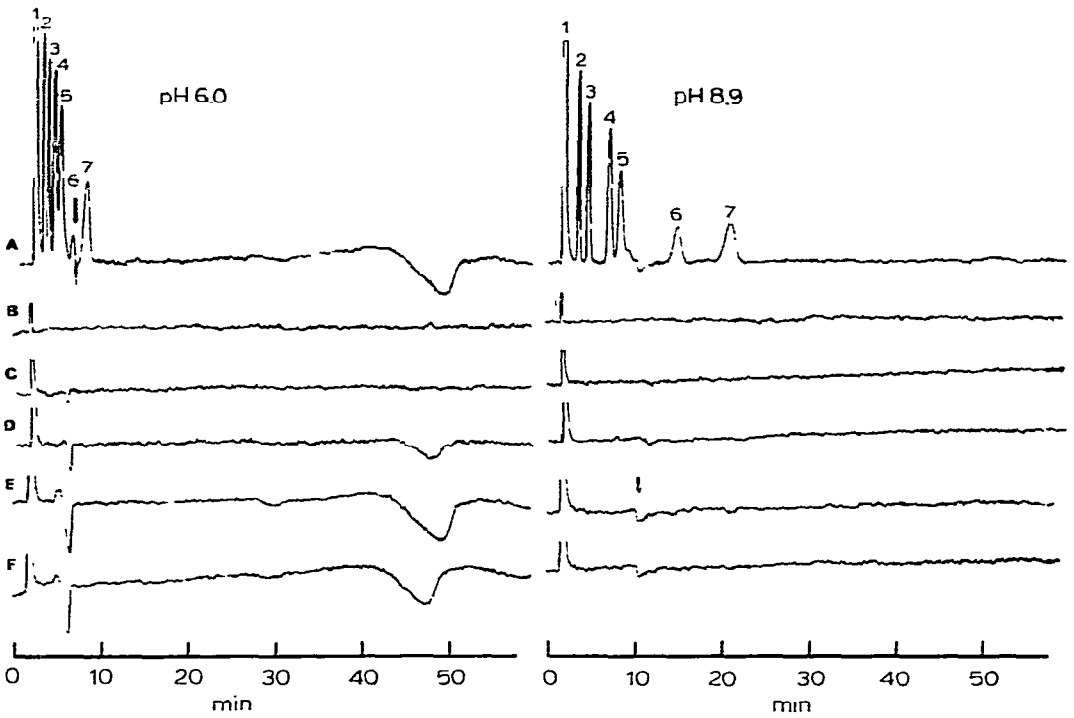

Fig. 6. Effect of sample water content on negative peaks produced in elution solvents at pH 6.0 and 8.9. Elution conditions and identification of compounds as in Fig. 4. B corresponds to an injection of 50 μl of eluent; C, D and E correspond to injections of 10, 25 and 50 μl of water, respectively; F corresponds to an injection of 50 μl 0.1% TEPA in water. Note the shift in size and k' in the smaller water-induced negative peak (arrow) with pH and the elimination of the larger negative peak at pH 8.9.

occurs after the elution of most carbohydrates and polyols, but would prove very unfavorable in automated injection systems or in situations where the number of analysis per time must be maximized since the baseline would require a significant period (nearly an hour) to restabilize before subsequent injections could be made.

CONCLUSION

Hydraulically compressed silica columns modified with TEPA have been shown to provide a simple, rapid and inexpensive method for the analysis of carbohydrates and polyols. Separation of these materials occurs over a wide range of pH, but basic eluents have been found to eliminate negative peaks found with acidic eluents. Both the k' and compound resolution of polyols and carbohydrates was found to be a function of the percentage of acetonitrile in the eluent. The k' of di- and trisaccharides were found to be especially sensitive to the eluent composition.

Unlike other HPLC carbohydrate columns, relatively low elution rates were found to produce the maximal separation efficiency in this system, due to the almost direct proportionality between plate height and flow-rate. The unusual geometry of this column produces a very low flow resistance ($\varnothing = 390$) and an exceptionally high linear sample capacity ($>2 \cdot 10^{-3}$ g/g column packing).

Carbohydrate and polyol separation in this system was found to be a function of eluent pH, but the relation between pH and elution was not as significant as that between elution rate and eluent acetonitrile content.

Several negative peaks were detected which were determined to be largely a function of injected water (in the sample). These peaks were also determined to be, to a lesser extent, a function of eluent pH.

The conditions determined for maximal separation efficiency were found to include a relatively high pH (9), which must normally be avoided with non-compressed silica columns due to shortened column life from silica etching at high pH. The radial compression design employed in the present paper tends to compensate for such silica dissolution and, combined with the addition of TEPA to the eluent[12], provides a relatively long column life, even at pH 9.2.

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