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New, stable polyamine-bonded polymer gel column

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ABSTRACT

The Asahipak NH2P-50 column, packed with polyamine-bonded polymer gel, was investigated in terms of mono- and oligosaccharide separation efficiency, reproducibility and column durability. Elution consistently occurred in the order mono-, di- and trisaccharide, and elution volume increased with increasing acetonitrile concentration. Chromatograms obtained over a period of one week of some 170 saccharide analyses on a single NH2P-50 column were virtually indistinguishable, while under the same conditions the efficiency of an amino-bonded silica gel column steadily decreased. The NH2P-50 column was amenable to both acidic and alkaline eluents, with no decrease in column efficiency after extended alternating passage of acidic and alkaline solutions. It also exhibited high separation efficiency with eluent containing tetrapropylammonium hydroxide and acetic acid solution.

INTRODUCTION

High-performance liquid chromatographic (HPLC) analysis of saccharides is commonly performed on amino-bonded silica gel columns using aqueous acetonitrile as eluent. However, the efficiency of such columns declines rather rapidly with use, apparently because of a loss of their amino groups due to hydrolysis [1].

The Asahipak NH2P-50 column was developed to eliminate this problem, and we investigated its use in saccharides analysis with the following results: (a) it matched aminopropyl silica gels in chromatographic resolution; (b) it fully retained column efficiency in prolonged use; (c) it permitted the use of eluent over a wide pH range; and (d) it underwent little shrinkage or swelling related to eluent polarity.

EXPERIMENTAL

Gel synthesis

NH2P gel was obtained by activating the hydroxyl groups of a vinyl alcohol copolymer gel (average particle diameter, 4.8 μm; pore size, approximately 200 Å; hydroxyl groups, 2.5 mequiv. per g of dry gel) with epichlorohydrin and then reacting the gel with pentaethylenehexamine of 72.3% purity obtained from Wako (Osaka, Japan). The extent of polyamine bonding was determined by washing the NH2P gel with dilute aqueous sodium hydroxide and then with acetone, dispersing the gel in aqueous 0.5 *M* potassium chloride, titrating the suspension with 0.1 *M* hydrochloric acid and calculating the quantity of polyamine groups present from the titration curve.

Equipment and material

The equipment consisted of a degasser (GL Sciences, Tokyo, Japan), an 880-PU pump (Japan Spectroscopic, Tokyo, Japan), an AS-2000 autosampler (Hitachi, Tokyo, Japan) and a Shodex SE-61 refractive index detector (Showa Denko, Tokyo, Japan).

The NH2P-50 column was obtained by packing the above NH2P gel in a steel cylinder of 250 mm inner length × 4.6 mm I.D. The amino-bonded silica gel columns, all 250 mm × 4.6 mm I.D. and having a gel particle size of 5 μm, were the YMC pack PA-03 (pore size, 120 Å), Cosmosil 5NH_2 (pore size, 110 Å) and Nucleosil 5NH_2 (pore size, 100 Å).

Pure mono- and oligosaccharides were purchased from Wako or Sigma (St. Louis, MO, USA). Sam-

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ples were dissolved in acetonitrile–water (50:50) except where otherwise noted. Acetonitrile was HPLC grade.

Solvent regain

The solvent regain (S_R) of the NH2P gel was determined for water and acetonitrile. The dry gel was first dispersed in the solvent and left at room temperature for 16 h or more. The gel dispersion was then centrifuged at 3000 rpm (1318 *g*) and 0°C for 1 h, or in the case of water at 20°C for 90 min, to remove interstitial solvent. The weight (W_1) of the resulting wet gel was measured. The gel was then dried under vacuum at 60°C for 16 h or more and again weighed to obtain W_2. The solvent regain of the gel was calculated using the equation

$$S_R \text{ (ml/g gel)} = [(W_1 - W_2)/\text{sp.gr.} - 0.036W_2/d]W_2$$

where sp.gr. and d are the specific gravities of the solvent and the gel, respectively [2].

Equilibration of NH2P-50

The amino bases of the gel in the NH2P-50 column were equilibrated prior to its initial use by passing 100 m*M* ammonium acetate (pH 9.4) through the column at 0.5 ml/min for 2 h at room temperature. As noted below, the column was also equilibrated following the passage of 0.005 *M* sodium hydroxide.

Recovery of saccharides

Saccharide recovery was measured as the ratio of the eluted peak area obtained with the column by isocratic elution at 1.0 ml/min at 30°C to that obtained when the same amount (50 μg) of the saccharide was injected into approximately 10 m of tightly coiled PTFE tubing of 0.5 mm inner diameter and eluted under the same conditions.

Stability in acidic and alkaline eluents

The ability of the NH2P-50 column to resist a reduction in its efficiency by acidic and alkaline eluents was determined by separation of fructose, glucose, sucrose and maltose using water–acetonitrile (25:75) as eluent before and after passage of acidic and alkaline solutions.

The acidic solution 0.005 *M* sulphuric acid was passed through the NH2P-50 column at room temperature and 0.1 ml/min for 63 h. Before analysis, it was necessary to obtain free amino bases by passing 0.1 *M* sodium hydroxide through the column at 0.5 ml/min for 2 h at room temperature.

The alkaline solution 0.005 *M* sodium hydroxide (pH 11.4)–acetonitrile (25:75) was passed through the column at 1.0 ml/min for 160 h at room temperature. Before analysis, it was necessary to equilibrate the amino bases by passing 100 m*M* ammonium acetate (pH 9.4) through the column at 0.5 ml/min for 2 h at room temperature.

RESULTS AND DISCUSSION

NH2P gel characteristics

The chemical structure of the NH2P gel, having an average particle size of 4.8 μm and a pore size of approximately 200 Å, is $X\text{-NH}(CH_2CH_2NH)_n\text{-}CH_2CH_2NH_2$, where X represents the vinyl alcohol copolymer. Titration showed the bonded amino group content of the gel to be 1.38 mequiv. per g of dry gel. Its solvent regain was measured as 0.70 ml/g for water and 0.69 ml/g for acetonitrile. The maximum allowable flow-rate of the packed column was 1.5 ml/min.

Capacity factor, number of theoretical plates and saccharide recovery

Table I shows the capacity factor (k'), number of theoretical plates (N_{TP}) and asymmetry (A_S) as determined for saccharide analysis on the NH2P-50 column and three commercial amino-bonded silica columns with water–acetonitrile (25:75) as eluent. The NH2P-50 exhibited high N_{TP} values for all of the saccharides, including galactose, k' values similar to those of the silica columns, and the same elution order except for a reversal of glucose and galactose and of maltose and lactose. These results suggest that the chromatographic behaviour of saccharides is mainly determined by the amino groups of the gel, although some influence of its base material may be evident in the above reversal of retention order and in the relatively higher N_{TP} for galactose and the generally greater peak symmetry observed with the NH2P-50 column.

The elution curves obtained with the NH2P-50 were generally more symmetrical than those obtained with the silica-based columns, as indicated in Table I by the proximity of its A_s values to 1.0, in

TABLE I

CAPACITY FACTORS, N_{TP} AND A_s VALUES OF SACCHARIDES ON NH2P-50 AND AMINO-BONDED SILICA COLUMNS

Eluent, water–acetonitrile (25:75); flow-rate, 1.0 ml/min; sample concentration, 5 mg/ml; sample volume, 10 μl; detection, refractive index temperature, 30°C. Samples were dissolved in water.

Saccharide	NH2P-50			YMC pack PA-03			Cosmosil 5NH_2			Nucleosil 5NH_2		
	k'	N_{TP}	A_s	k'	N_{TP}	A_s	k'	N_{TP}	A_s	k'	N_{TP}	A_s
(1) Xylose	1.73	6500	1.2	1.02	5700	0.8	0.93	7700	17.4	1.17	7000	33.0
(2) Rhamnose	1.24	7300	1.4	0.74	4800	11.4	0.64	7700	2.1	0.87	6100	2.1
(3) Fructose	1.90	7900	1.6	1.44	8200	0.7	1.18	9000	2.0	1.65	9900	3.4
(4) Mannose	2.35	8200	2.2	1.69	4700	10.2	1.47	8400	9.7	1.92	6900	13.3
(5) Galactose	2.56	6200	1.8	2.01	2600	25.0	1.70	5100	7.7	2.28	3000	47.6
(6) Glucose	2.70	6200	1.0	1.82	5000	0.5	1.64	6700	1.7	2.05	6200	2.1
(7) Sucrose	4.17	8200	1.2	2.93	9000	0.7	2.62	9300	1.6	3.26	10 800	2.1
(8) Lactose	4.97	6900	1.7	4.45	5900	1.4	3.64	7600	2.4	4.98	7300	5.1
(9) Maltose	5.22	6400	0.9	3.78	5600	0.6	3.46	7400	1.7	4.18	6600	4.3
(10) Raffinose	8.56	8700	1.1	7.42	9100	0.7	6.21	9400	2.7	8.25	10 400	3.3

contrast to the much higher $A_s$values exhibited by the other columns as a result of peak tailing.

As shown in Fig. 1, the k' value for saccharides increased with increasing acetonitrile concentration on the NH2P-50 column in essentially the same manner as on the silica-based YMC pack PA-03 column. This observation, and the above similarities in chromatographic behaviour, indicate that the mechanism of saccharide separation on the NH2P-50 is predominantly that of normal-phase chromatography, and thus essentially the same as that of amino-bonded silica-based columns.

The results show the NH2P-50 column to equal or exceed silica-based columns in terms of peak res-

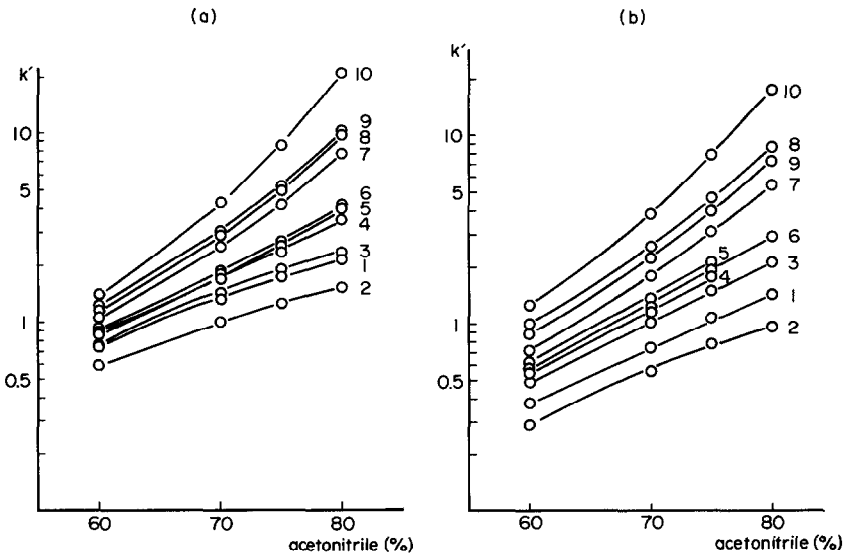

Fig. 1. Capacity factors (k') for mono- and oligosaccharides *vs.* water–acetonitrile ratio of eluent. Numbers indicate saccharides as listed in Table I. (a) Asahipak NH2P-50; (b) YMC pack PA-03. Conditions as in Table I except eluent.

TABLE II

RECOVERY OF SACCHARIDES

Conditions as in Table I.

Saccharide	Recovery (%)			
	NH2P-50	PA-03	Cosmosil $5NH_2$	Nucleosil $5NH_2$
Xylose	40	45	35	64
Rhamnose	58	58	57	64
Fructose	100	97	104	102
Mannose	22	31	12	25
Galactose	22	20	11	44
Glucose	88	74	85	91
Sucrose	107	98	106	106
Lactose	65	53	62	70
Maltose	82	53	70	88
Raffinose	111	113	107	121

olution and symmetry for saccharides, in clear contrast to the common perception of polymer-based columns for reversed-phase HPLC as being inferior to the corresponding silica-based columns in separation efficiency.

As indicated in Table II by the saccharide recoveries observed using water–acetonitrile (25:75) as eluent, the NH2P-50 column was found to be quite similar to amino-bonded silica columns in terms of both the recovery values for individual saccharides and their variation among saccharides.

Stability in aqueous acetonitrile solution

Table III and Figs. 2 and 3 show the N_{TP}, elution times and chromatograms, respectively, for fructose, glucose, sucrose and maltose on the NH2P-50 column and a silica-based column in a series of 170 or more hourly analyses with water–acetonitrile (25:75) as eluent. On the silica-based column, even with this neutral eluent, the elution times decreased steadily, and a clear difference was observed between initial and final N_{TP} values and chromatograms, all indicative of a relatively short service life for such columns.

On the NH2P-50 column, the elution times, N_{TP} and chromatograms remained unchanged throughout the week-long series of analyses, thus indicating excellent stability in aqueous acetonitrile solution.

Stability in acidic and alkaline eluents

The NH2P gel was found to be stable in eluents ranging in pH from 2 to 11, and apparently up to pH 13.

Table IV shows the elution volumes and N_{TP} obtained with the NH2P-50 column before and after exposure to acidic solution flow (0.005 *M* sulphuric acid) for 63 h. Table V shows those obtained before and after alkaline solution flow [0.005 *M* sodium hydroxide (pH 11.4)–acetonitrile (25:75)] for 160 h. No decrease in column efficiency occurred in either instance. The elution volumes and NTP were stable despite the extended exposure to pH 11.4.

As indicated in Table VI, the NH2P-50 column also showed no reduction in column efficiency following exposure to a solution of even higher alkalinity [0.1 *M* sodium hydroxide (pH 13)–acetonitrile (80:20)] for 6 h.

Chemically bonded silica gels are generally known to lack chemical stability because of the in-

TABLE III

ELUTION VOLUME (V_R) AND N_{TP} ON NH2P-50 AND PA-03 COLUMNS

Eluent, water–acetonitrile (25:75); flow-rate, 1.0 ml/min; sample concentration, 5 mg/ml each; sample volume, 10 µl; detection, refractive index; temperature, 30°C.

Saccharide	NH2P-50				PA-03			
	Initial		At 170 h		Initial		At 170 h	
	V_R (ml)	N_{TP}	V_R (ml)	N_{TP}	V_R (ml)	N_{TP}	V_R (ml)	N_{TP}
Fructose	7.43	11 000	7.43	11 200	9.36	10 000	8.60	8500
Glucose	9.57	6700	9.54	6400	11.00	6200	9.87	5000
Sucrose	13.48	10 500	13.40	10 600	15.58	10 600	13.63	8100
Maltose	16.41	7700	16.29	7100	19.16	7800	16.32	5700

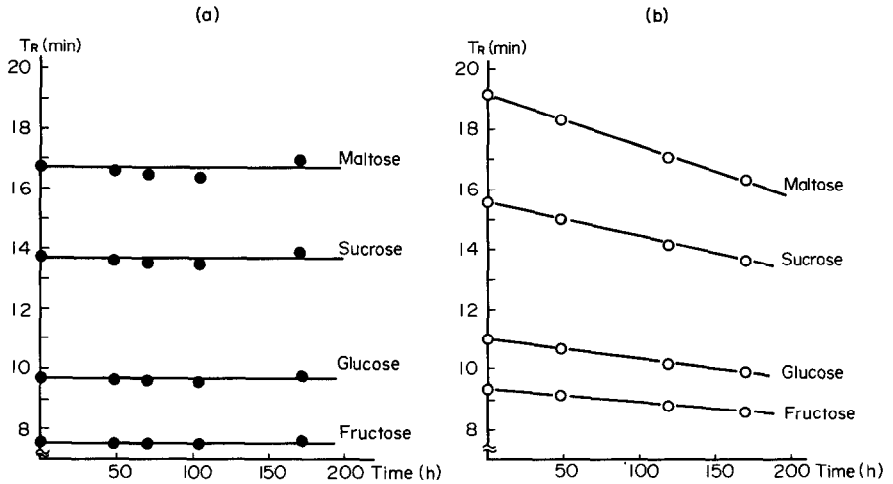

Fig. 2. Elution time (T_R) in repeated usage. (a) Asahipak NH2P-50; (b) YMC pack PA-03. Conditions as in Table III.

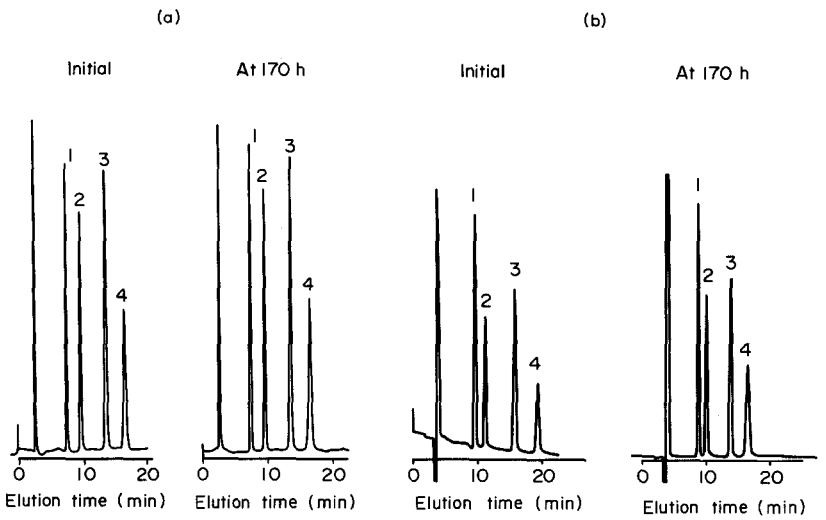

Fig. 3. Chromatograms of saccharides. (a) Asahipak NH2P-50; (b) YMC pack PA-03. Conditions as in Table III.

TABLE IV

COLUMN EFFICIENCY OF NH2P-50 COLUMN BEFORE AND AFTER PASSAGE OF ACIDIC SOLUTION

Conditions as in Table III.

Saccharide	Before		After	
	V_R (ml)	N_{TP}	V_R (ml)	N_{TP}
Fructose	6.73	9600	6.70	8400
Glucose	8.59	7900	8.58	8500
Sucrose	11.88	9600	11.93	9300
Maltose	14.24	8500	13.90	8600

TABLE V

COLUMN EFFICIENCY OF NH2P-50 COLUMN BEFORE AND AFTER PASSAGE OF ALKALINE SOLUTION

Conditions as in Table III.

Saccharide	Before		After	
	V_R (ml)	N_{TP}	V_R (ml)	N_{TP}
Fructose	7.52	7100	7.62	8100
Glucose	9.63	6100	9.68	7700
Sucrose	13.41	8400	13.40	9700
Maltose	16.64	7200	16.08	7400

TABLE VI

COLUMN EFFICIENCY OF NH2P-50 COLUMN BEFORE AND AFTER PASSAGE OF HIGHLY ALKALINE SOLUTION

Conditions as in Table III.

Saccharide	Before		After	
	V_R (ml)	N_{TP}	V_R (ml)	N_{TP}
Fructose	6.80	10 200	6.73	9600
Glucose	8.69	7100	8.59	7900
Sucrose	12.06	9800	11.88	9600
Maltose	14.47	7800	14.24	8500

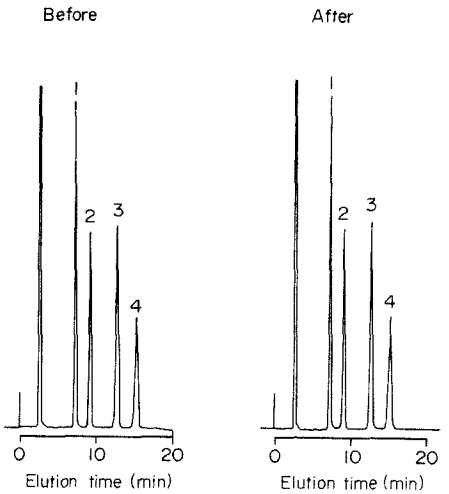

Fig. 4. Chromatograms obtained with the NH2P-50 column before and after repeated passage of water and acetonitrile alternately. Conditions as in Fig. 3.

stability of the silica base. This limitation does not apply to the NH2P gel, largely because of the excellent chemical stability of its base vinyl alcohol copolymer.

Stability with eluents of differing polarities

The NH2P-50 column was found to retain its efficiency after repeated exposure to eluents of differing polarities, as indicated by the column efficiency and chromatograms of sugar analyses shown in Table VII and Fig. 4. The column was subjected to five cycles of distilled water and then acetonitrile passages each at 1.0 ml/min for 1 h at room temperature, and showed no difference in elution times, N_{TP} or A_s before and after this exposure.

The results thus indicate that the NH2P-50 column undergoes no reduction in efficiency attributable to gel shrinkage and swelling under the influence of eluents of different polarities, as may be expected from the nearly equal solvent regain values (0.70 and 0.69) of the NH2P gel for water and acetonitrile.

Applications

The above results indicate the NH2P-50 column to be superior to other columns for the analysis of saccharides, as it equals or surpasses silica-based columns in terms of separation efficiency and peak symmetry while exhibiting far greater column sta-

TABLE VII

COLUMN EFFICIENCY OF NH2P-50 COLUMN BEFORE AND AFTER ACETONITRILE PASSAGE CYCLE

Water and acetonitrile were passed through the column in five cycles, each consisting of alternating 1-h passages of water and acetonitrile at 1.0 ml/min. Conditions as in Table III.

Saccharide	Before			After		
	V_R (ml)	N_{TP}	A_s	V_R (ml)	N_{TP}	A_s
Fructose	7.31	10 800	1.8	7.29	10 600	1.7
Glucose	9.29	7800	0.9	9.24	7500	0.9
Sucrose	12.91	11 200	1.0	12.84	10 900	1.0
Maltose	15.46	8500	0.9	15.41	8100	0.9

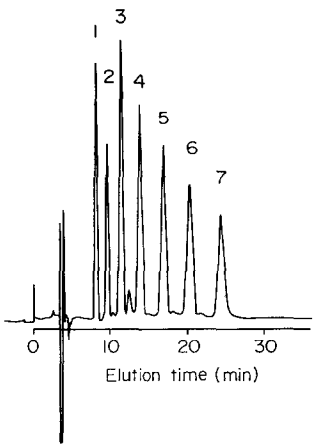

Fig. 5. Separation of maltooligosaccharides. Column, Asahipak NH2P-50; eluent, water–acetonitrile (40:60). Peaks: 1 = glucose; 2 = maltose; 3 = maltotriose; 4 = maltotetraose; 5 = maltopentaose; 6 = maltohexaose; 7 = maltoheptaose; all in water at a concentration of 0.71 mg/ml (1 and 2) or 1.43 mg/ml (3–7). Other conditions as in Table III.

Fig. 6. Effective separation of dextran hydrolysates on Asahipak NH2P-50. Peaks: 1 = glucose; 2 = isomaltose; 3 = isomaltotriose; 4 = isomaltotetraose; 5 = isomaltopentaose; 6 = isomaltohexaose; 7 = isomaltoheptaose; 8 = isomaltooctaose; all in water at a concentration of 10 mg/ml. Other conditions as in Fig. 5.

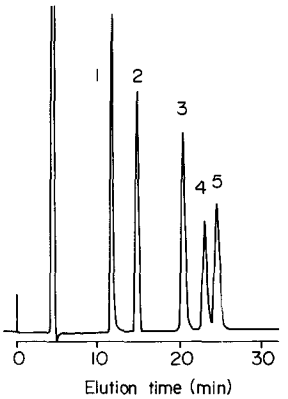

Fig. 7. Separation of saccharides on Asahipak NH2P-50 using an alkaline eluent. Eluent: 10 m*M* tetrapropyl ammonium hydroxide/acetic acid (pH 10)–acetonitrile (25:75). Peaks: 1 = fructose; 2 = glucose; 3 = sucrose; 4 = lactose; 5 = maltose; all in 10 m*M* tetrapropyl ammonium hydroxide/acetic acid (pH 10)–acetonitrile (50:50). Other conditions as in Table III.

bility and durability and providing the opportunity for a far wider range of chromatographic conditions. This is supported by the results of the following analyses.

Fig. 5 shows the separation of maltooligosaccharides. Separation was complete, with elution occurring in the order of increasing chain length.

Fig. 6 shows the effective separation of dextran hydrolysates, identified as sugars ranging from glucose to isomaltooctaose.

It is generally preferable to maintain a constant, small proportion of free amino bases in amino-bonded columns, and buffers are often added to the eluent for this purpose. Under acidic conditions, however, a small proportion of free amino bases often tends to result in separation of anomers [3]. With the use of a highly alkaline buffer, and thus an alkaline eluent, it is possible in principle to both maintain the proportion of constant free amino base and avoid anomer separation. The NH2P-50 column, because of its high stability in alkaline eluents, is the first amino-bonded gel column to make such applications practical, as shown in Fig. 7.

CONCLUSIONS

Investigation of the Asahipak NH2P-50 column, packed with polyamine-bonded polymer gel, for the

separation of saccharides showed k' values to be similar to those obtained of amino-bonded silica columns, and N_{TP} and A_s values to be equal or superior. The chemical stability of its polymer gel effectively eliminates the problems of declining elution times and short service life commonly associated with silica-based columns. It is also free from the problems of gel shrinkage and swelling associated with conventional polymeric gels exposed to changes in eluent polarity. It is, moreover, stable over a wide pH range. Its stability in alkaline eluents, in particular, may be expected to broaden the range of analysis conditions and applications for saccharide separation by normal-phase HPLC.

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