Journal of Chromatography, 397 (1987) 71–80
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1235

EVALUATION OF ADVANCED SILICA PACKINGS FOR THE SEPARATION OF BIOPOLYMERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

III. RETENTION AND SELECTIVITY OF PROTEINS AND PEPTIDES IN GRADIENT ELUTION ON NON-POROUS MONODISPERSE 1.5-μm REVERSED-PHASE SILICAS

G. JILGE, R. JANZEN, H. GIESCHE and K. K. UNGER*
Institut für Anorganische Chemie und Analytische Chemie, Johannes Gutenberg-Universität, 6500 Mainz (F.R.G.)
J. N. KINKEL
Chemical Reagents Division, R & D Chromatography, E. Merck, 6100 Darmstadt (F.R.G.)
and
M. T. W. HEARN
Department of Biochemistry, Monash University, Clayton, Victoria 3168 (Australia)

SUMMARY

Following previous studies of the use of non-porous monodisperse 1.5-μm *n*-octyl- and *n*-octadecyl-bonded silicas in gradient elution of proteins, this work was aimed at elucidating further the properties of this novel column material for peptide and protein separations in comparison with wide-pore silicas.

First, it is demonstrated that with short columns (*e.g.*, 35 × 8 mm I.D.) packed with these non-porous reversed-phase materials, mixtures of small peptides and mixtures of proteins can be very efficiently resolved. When the chain length of the bonded ligand was varied, the retention of a test set of proteins in gradient elution followed the ligand sequence $C_{18} > C_8 \approx C_4 \approx$ phenyl $> C_2$ under constant elution conditions, and the selectivity remained unchanged. Comparison of the S values of these proteins, as determined from evaluation of the log k' *vs.* φ dependences with non-porous silicas and with a LiChrospher Si 1000 C_8 with identical accessible ligand surface areas per unit column volume, indicated lower values for the non-porous materials (k' = capacity factor; φ = molar fraction of organic solvent; S = slope of the plot of log k' *vs.* φ). The origin of this behaviour is discussed.

INTRODUCTION

Reversed-phase gradient high-performance liquid chromatography (HPLC) with acidic aqueous organic eluents of low ionic strength has become a widely used

0021-9673/87/$03.50

technique in the structural elucidation and isolation of peptides[1–24]. Typically, columns 250–5 mm long and of 4 mm I.D. packed with bonded silicas have been employed. *n*-Alkyl ligands with chain lengths betwen C_2 and C_{30} and phenyl and cyanopropyl ligands[12,25–29], chemically bonded to the parent silicas with mean pore sizes between 6 and 30 nm[24] and mean particle sizes between 3 and 10 μm, have been widely used for analytical columns. Peptide retention and selectivity have been observed to be very dependent on the properties of the bonded phase, including the pore size, the type of bonded ligand, the ligand density and topography and the remaining hydroxyl activity of the silica[3,20,22,24].

Under reversed-phase gradient HPLC conditions, the retention behaviour of proteins is characterized by slow diffusion and slow kinetics of desorption and other equilibria, induced by the strong hydrophobic solute surface interaction with the hydrocarbon stationary phase. The consequence of these effects ultimately leads to low column efficiency, conformational changes in the protein structure and loss of enzymatic or biological activity. The rational approach to overcoming these obstacles of slow diffusion and kinetics is to increase further the mean pore size of packings beyond 30 nm and concurrently to reduce the mean particle diameter. Clearly, a situation is reached with this approach in which the stationary phase ceases to have any mechanical strength of acceptable physical characteristics. In a previous study[30], we demonstrated the utility of non-porous, monodisperse 1.5-μm *n*-octyl-bonded silica in the reversed-phase gradient HPLC of proteins, permitting rapid separations with short columns and providing a high column loadability.

This work was aimed at elucidating further the properties of this novel packing material for peptide and protein separations in comparison with wide-pore silicas.

EXPERIMENTAL

Materials

The packings were a non-porous silica of mean particle diameter d_p = 1.5 μm ± 2%, which was converted into its *n*-alkyl- and phenyl-bonded derivatives with *n*-octadecyl-, *n*-octyl-, *n*-butyl-, ethyl- and phenyldimethylchlorosilane, according to a procedure described elsewhere[31]. For comparison with the non-porous particles, the macroporous silica LiChrospher Si 1000 C_8 with a surface area a_m = 20 m^2/g was used. The *n*-alkyl- and phenyl-bonded silicas were slurry-packed into columns (35 × 8 mm I.D.) (Bischoff, Leonberg, F.R.G.) employing tetrachloromethane or tetrachloromethane–paraffin mixtures at a pressure of 100 MPa. The porous silica LiChrospher Si 1000 C_8 (10 μm) was slurry-packed into a 35 × 4.6 mm I.D. column with a pressure of 25 MPa. The end-fittings were made of paper filters, supplied by Schleicher & Schüll (Dassel, F.R.G.), and supported by No. 22800812 metal frits (Bischoff).

Acetonitrile of HPLC grade and trifluoroacetic acid (TFA) of analytical-reagent grade were obtained from E. Merck (Darmstadt, F.R.G.). Water was quartz-distilled and deionized. Pure peptides and proteins from Serva (Heidelberg, F.R.G.) were Tyr–Gly–Phe–Meth, Asp–Arg–Val–Tyr–Ile–His–Pro–Phe, albumin (bovine) and ovalbumin; from Sigma (St. Louis, MO, U.S.A.), ribonuclease A, lysozyme and ovotransferrin; and from Boehringer (Mannheim, F.R.G.), cytochrome *c*, aldolase and catalase.

Apparatus

The S values (S is the slope of the plot of log k' *vs.* φ, where k' is the capacity factor and φ is the molar fraction of organic solvent) of the test substances were measured in a low-pressure gradient HPLC system from LKB (Bromma, Sweden), consisting of a 2150 HPLC pump, a 2152 HPLC controller, a low-pressure mixing unit and a 2155 HPLC column oven. The peptides and proteins were detected with a Biotronik (Maintal, F.R.G.) BT 3030UV detector at 220 nm. All other chromatographic tests were performed on a Merck–Hitachi (Darmstadt, F.R.G.) gradient HPLC system, consisting of two 655A-12 pumps, a dynamic high-pressure gradient mixer, an F 1000 fluorescence detector and 655-61 integrator, fitted with a Model A processor. For all tests, an injection system from Rheodyne (Cotati, CA, U.S.A.) with a 20-μl sample loop was used.

Chromatographic procedures

Solvents and eluents were degassed by helium sparging. The following eluent composition was employed for gradient elution: eluent A, 0.1% TFA; eluent B, acetonitrile–0.1% TFA (67.5:32.5).

The column temperature was 30°C. Sample sizes were varied from 10 to 200 μg. The column dead volume was determined with acetone. The dwell time was obtained by another procedure, based on the baseline inflection method. Because the UV absorbance of the mobile phase increases with increasing volume fraction of eluent B, the dwell time can be measured by determining the extrapolated onset of the gradient. All gradient elution separations used for calculations of S and peak capacity (PC) were carried out using linear solvent gradients. The experimental peak capacities, PC, were obtained from the relationship $PC = t_G/4\sigma_t$, where σ_t is to be band width in time units, and t_G is the gradient time.

The calculated S values of peptides and proteins for short columns were obtained from isocratic separations or gradient elution through plots of log k' *vs.*φ.

RESULTS AND DISCUSSION

Retention, selectivity and resolution of proteins

Previous studies on the effects of the type of *n*-alkyl ligand on the retention and selectivity behaviour of proteins in reversed-phase gradient HPLC indicated that, with the exception of the trimethylsilyl-bonded phase, only minor variations occurred[12,22,26,28,32]. This insensitivity towards significant changes in selectivity, associated with ligand chain length, appears to be related to the large size of the protein molecules, which prevent intensive intercalative contact between hydrophobic residues and the *n*-alkyl chain. As most proteins are probably incapable of being inserted between individual ligand chains at the stationary phase surface in either the collapsed droplet or the extended brush organization, the differences in the *n*-alkyl chain length of the ligand are averaged out when the ligand interacts with the protein via a multi-site attachment. Typical of this behaviour are the retention data for several proteins on various reversed-phase silicas, as illustrated in Fig. 1. For a given protein, retention under constant elution conditions is lowest with the C_2 bonded phase and reaches a plateau for the C_4, phenyl and C_8 phases. A slight increase in retention is seen for the *n*-octadecyl-bonded silica. Little selectivity differences for this group of

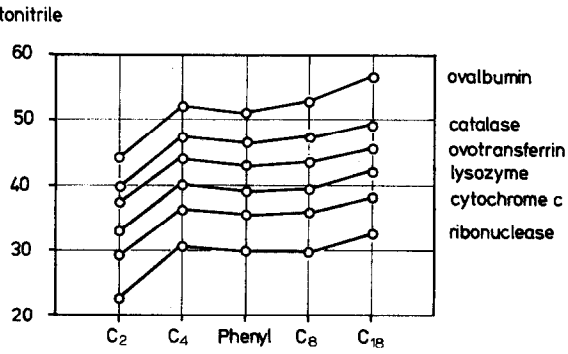

Fig. 1. Retention times of proteins on 36 × 8 mm I.D. non-porous silica columns (d_p = 1.5 μm) with different reversed-phase ligands. Linear gradient: from 0 to 67.5% acetonitrile in 0.1% TFA. The gradient time was constant at 10 min.

proteins occurred with the non-porous reversed-phase silicas, as evidenced by the shape of the curves in Fig. 1. Hence the retention behaviour of non-porous reversed-phase silicas appears to follow that observed with porous packings of the same surface composition[32].

Resolution of solutes in reversed-phase gradient HPLC is proportional to the peak capacity. The peak capacity, *PC*, is a function[33] of the diffusion coefficient, D_m, of solutes, the particle size, d_p, of the packing, the gradient time, t_G, the flow-rate, F, and the column length, L, and can be represented by

$$PC = \alpha\, D_m^{0.5}\, d_p^{-1}\, t_G^{0.5}\, F^0\, L^0 \qquad (1)$$

PC is therefore expected to decrease for solutes of smaller diffusion coefficients, *i.e.*, for large proteins, but to increase on reducing the particle diameter and on increasing the gradient time. According to Snyder *et al.*[33], changes in flow-rate and column length have little effect on peak capacity under the conditions $1 < \bar{k}' < 10$, where $\bar{k}'$ is the mean capacity factor. Table I lists the peak capacities of six proteins as a function of flow-rate, while the gradient time is proportionally adjusted, *i.e.*, by holding the gradient volume ($v_G = ft_G$) constant. In all instances *PC* improved considerably with decreasing flow-rate over the measured range. This behaviour may be attributed to the fact that the maximum plate number (minimum plate height) of the column is approached under very low flow-rate conditions. The optimum flow-rate at the minimum plate height is known to be dependent on the molecular size of the solute via the diffusion coefficient. However, this dependence is not clearly reflected by the data obtained.

In the equation

$$\nu = ud_p/D_m \qquad (2)$$

ν is the reduced linear velocity of the eluent, u the linear velocity, d_p the mean particle diameter of the packing and D_m the diffusion coefficient of the solute in the eluent. When $\nu = 5$, *i.e.*, the minimum of the reduced plate height *vs.* reduced velocity

TABLE I

PEAK CAPACITIES (*PC*) OF PROTEINS AS A FUNCTION OF THE FLOW-RATE AT A CONSTANT GRADIENT VOLUME OF 15 ml

Mobile phase A, 0.1% TFA; mobile phase B, 67.5% acetonitrile in mobile phase A; column, 35 × 8 mm I.D., C_{18} bonded, non-porous 1.5-μm silica gel. Flow-rate, *F*: 1 = 3.0 ml/min (u = 2.5 mm/s, t_G = 5 min); 2 = 2.0 ml/min (u = 1.67 mm/s, t_G = 7.5 min); 3 = 1.5 ml/min (u = 1.25 mm/s, t_G = 10 min); 4 = 1.0 ml/min (u = 0.83 mm/s, t_G = 15 min); 5 = 0.75 ml/min (u = 0.63 mm/s, t_G = 20 min); 6 = 0.5 ml/min (u = 0.42 mm/s, t_G = 30 min).

Protein	*Mol. wt.*	*PC*					
		1	*2*	*3*	*4*	*5*	*6*
Ribonuclease	13700	50	56	59	68	64	65
Cytochrome *c*	12500	56	68	80	80	91	93
Lysozyme	14300	58	61	69	83	95	104
Ovotransferrin	77000	40	48	50	59	62	59
Catalase	240000	58	65	63	83	83	91
Ovalbumin	45000	43	39	41	48	46	46

dependence achieved under isocratic elution conditions, the corresponding linear velocity u = 0.16 and 0.03 mm/s for d_p = 1.5 μm and $D_m = 0.5 \cdot 10^{-10}$ m²/s (10-kilodalton protein)[36] and $D_m = 0.1 \cdot 10^{-10}$ m²/s (100-kilodalton protein), respectively. A comparison with the linear velocities in our experiments (*cf.* Table I) reveals that the values were still above the optimum. It was of interest to assess the effect of the *n*-alkyl chain length of non-porous reversed-phase packings on the peak capacity under otherwise constant chromatographic conditions. The *PC* values shown in Table II indicate that the peak capacity diminishes with decreasing *n*-alkyl chain length of the packing. The phenyl column exhibits peak capacities similar to the *n*-butyl-bonded phase. The reasons for this decline are not yet clear. One plausible explanation relates to the possibility that the plate numbers of the long-chain reversed-phase columns were inherently higher, owing to preferential ligand–ligand solvation interactions and differences in packing densities. However, other reasons might also be considered, *e.g.*, selectivity effects.

TABLE II

PEAK CAPACITY OF PROTEINS CHROMATOGRAPHED ON NON-POROUS REVERSED-PHASE PACKINGS WITH VARIOUS LIGANDS

10-min linear gradient; flow-rate, 1.5 ml/min; for other conditions, see Table I.

Bonded group	*PC*			
	Ribonuclease	*Ovalbumin*	*Catalase*	*Cytochrome c*
C_{18}	49	36	69	64
C_8	42	32	61	59
C_4	38	18	50	42
C_2	19	21	38	44
Phenyl	37	24	45	55

TABLE III

PEAK CAPACTIES OF PROTEINS AND PEPTIDES CHROMATOGRAPHED ON (A) NON-POROUS *n*-OCTYL-BONDED AND (B) *n*-OCTADECYL-BONDED SILICA COLUMNS (35 × 8 mm I.D.) AND (C) A POROUS LICHROSPHER Si 1000 C_8 SILICA COLUMN (35 × 4.6 mm I.D.) AT A CONSTANT FLOW-RATE OF 1.5 ml/min FOR PROTEINS AND 1.0 ml/min FOR PEPTIDES AT DIFFERENT GRADIENT TIMES

Sample	*PC*														
	t_G = 10 min			t_G = 20 min			t_G = 30 min			t_G = 40 min			t_G = 60 min		
	A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
Ribonuclease A	65	94	35	74	121	45	86	136	50	90	168	54	97	173	59
Cytochrome *c*	55	79	25	59	98	45	86	114	37	65	114	40	74	168	45
Lysozyme	59	69	–	67	89	–	70	114	–	72	137	–	77	152	–
Catalase	49	79	33	60	99	37	65	119	44	68	131	46	77	152	52
Peptide 1 (Tyr–Gly–Phe–Meth)	33	–	28	42	–	37	48	–	39	50	–	40	46	–	43
Peptide 2 (Asp–Arg–Val–Tyr–Ile–His–Pro–Phe)	27	98	31	31	137	37	33	154	38	35	174	39	32	172	41

Retention, selectivity and resolution of peptides

As is evident from Table III, the peak capacities for small peptides with the non-porous 1.5-μm *n*-alkyl silicas compare very favourably with those obtained with more conventional porous stationary phases. Consequently, these non-porous stationary phases were expected to exhibit a high resolving power with complex mixtures of peptides, such as those found in tryptic digests of proteins. Figs. 2 and 3 show the chromatograms of the tryptic peptides derived from the digestion of bovine growth hormone and bovine casein[2].

In each instance, sample loads in excess of 300 μg could be employed without a significant loss of resolution or peptide breakthrough. Further, the selectivity and high recoveries enabled individual peptides to be purified to homogeneity rapidly and to be isolated under conditions compatible with direct amino-terminal microsequence determinations with, *e.g.*, commercial gas-phase sequencers. Gradient times of < 10 min can be readily employed with these non-porous *n*-alkyl columns, which exhibit satisfactory pressure-drop characteristics, even at relatively high flow-rates, so that constant V_G conditions can be achieved.

Non-porous vs. macroporous reversed-phase silicas in gradient elution of proteins and peptides

The peak capacity relates the overall quality of a particular separation to several experimental variables, including the band width and relative retention. For a chromatographic system in which the average resolution, R_s, between all adjacent peaks corresponds to unity, the peak capacity can be calculated from the relationship

$$PC = t_G/4\sigma_t \tag{3}$$

where σ_t is the band width in time units. As PC for gradient separations is also related to both N and k' through the expression

$$PC = (2.3/4)\,(S\Delta\varphi)\,N^{0.5}\,[k'\,(1+k')] \tag{4}$$

where S is the slope of the plot of log k' *vs.* molar fraction of organic solvent (φ), $\Delta\varphi$ the change in organic volume fraction during the gradient run and N the column plate number, it was obviously of interest to compare the peak capacities for the non-porous packings with corresponding porous packings, using a variety of peptides and proteins as solutes. For these experiments to have quantitative significance, a stationary phase and column conditions were selected in which the total surface area per unit volume was normalized. Under such conditions, both the relative retention and the chromatographic selectivity would be expected to be constant for gradient elution systems. Consequently, differences in PC would be expected to reflect more precisely changes in N (or σt). The data in Table III summarize the results of these experiments.

For the porous, reversed-phase silicas employed in this study, peak broadening and peak asymmetry were particularly evident as a consequence of the kinetics in the pores. As is evident from eqn. 4, PC is predicted to be proportional to the peptide or protein S value. The S value, which corresponds to the slope (or tangent) of the plots of log k' *versus* φ, can be evaluated from either isocratic or gradient data. In

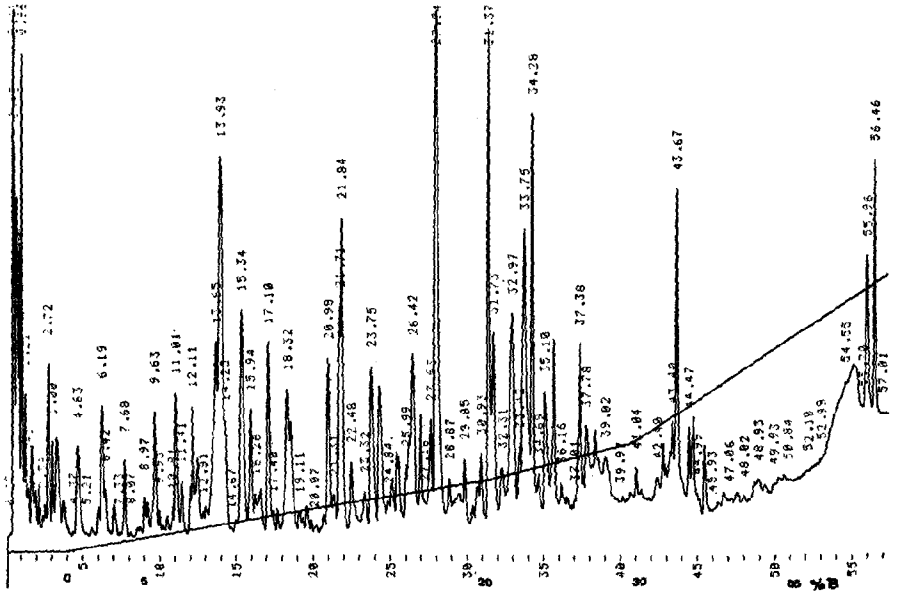

Fig. 2. Elution of a mixture of casein peptides in a tryptic digest on a 35 × 8 mm I.D. *n*-octadecyl-bonded silica column (d_p = 2.1 μm) at a flow-rate of 1.5 ml/min. Gradient, from 0 to 45% acetonitrile in 0.1% TFA (0–60% B) with different gradient steepness; UV detection, 220 nm; injection volume, 20 μl.

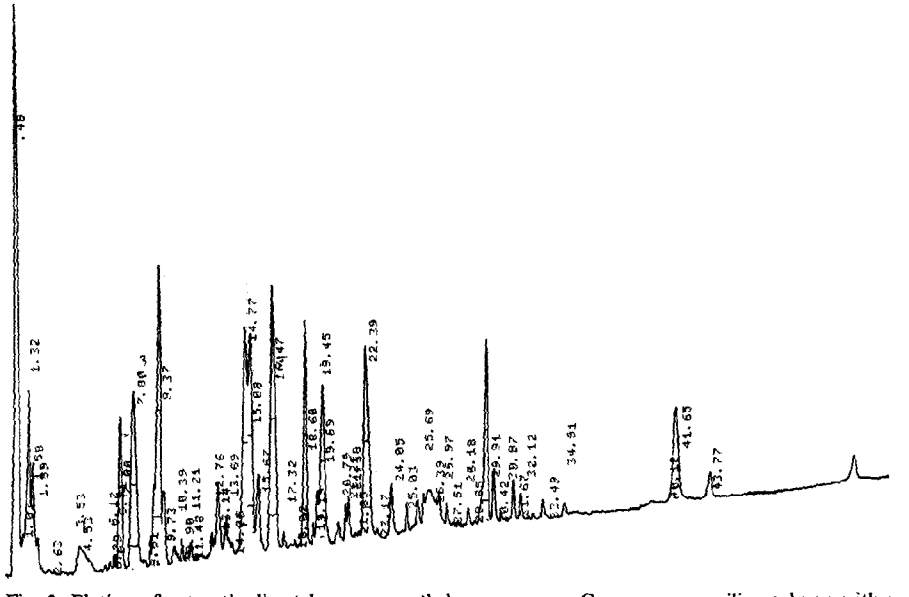

Fig. 3. Elution of a tryptic digest human growth hormone on a C_8 non-porous silica column with a flow-rate of 2.0 ml/min. Gradient, from 0 to 100% acetonitrile in TFA in 60 min 0.4 a.u.f.s.; attenuation. 1×.

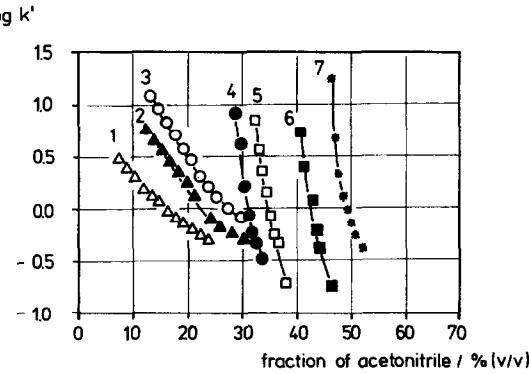

Fig. 4. Plot of log k' *vs.* φ, based on isocratic data at a flow-rate of 1.5 ml/min. S values were calculated as follows: (1) phenylalanine, 5.27; (2) di(phenylalanine), 5.88; (3) tri(phenylalanine), 6.14; (4) cytochrome *c*, 30.2; (5) lysozyme, 34.5; (6) albumin (bovine), 42.7; and (7) catalase, 44.0.

order to exclude the possibility that gradient effects may be manifested in different ways for non-porous and porous media, retention data for various peptides and proteins were obtained under isocratic conditions with mobile phases of different φ values. Representative data from these experiments are shown in Fig. 4. The low S value for catalase (44) might be due to its cleavage into subunits of 60 kdaltons under reversed-phase conditions.

CONCLUSIONS

We have examined the use of non-porous reversed-phase silicas for the separation of proteins and peptides. Based on the results, several conclusions can be drawn. First, compared with porous, reversed-phse silicas, the non-porous columns generate a much higher peak capacity for proteins. The observed effects of flow-rate and *n*-alkyl chain length on *PC* should be explored in more detail in future experiments. Second, contrary to expectation, the non-porous silica column provided sufficient retention capacity to resolve peptide mixtures in a similar manner to that observed with porous reversed-phase silicas. Third, the S values of proteins derived from isocratic elution measurements on the non-porous silica columns were scarcely dependent on the molecular weight of the proteins, indicating that the hydrophobic contact surface between the solute and the stationary phase surface remains nearly the same.

REFERENCES

1 M. T. W. Hearn, F. E. Regnier and C. T. Wehr, *Am. Lab.*, 14 (1982) 18.
2 W. S. Hancock and J. T. Sparrow, in Cs. Horvath (Editor), *High-Performance Liquid Chromatography, Advances and Perspectives*, Vol. 3, Academic Press, New York, 1983, p. 49.
3 M. T. W. Hearn, in Cs. Horvath (Editor), *High-Performance Liquid Chromatography, Advances and Perspectives*, Vol. 3, Academic Press, New York, 1983, p. 87.
4 J. M. Di Bussolo, *Am. Biotechnol. Lab.*, June (1984) 20.
5 J. A. Smith and M. J. O'Hare, *J. Chromatogr.*, 299 (1984) 13.
6 L. A. Witting, D. J. Gisch, R.Ludwig and R. Eksteen, *J. Chromatogr.*, 296 (1984) 97.

7 K. A. Cohen, J. Chazaud and G. Calley, *J. Chromatogr.*, 282 (1983) 423.
8 J. D. Pearson, N. T. Lin and F. E. Regnier, *Anal. Biochem.*, 124 (1982) 217.
9 M. J. O'Hare, M. W. Capp, E. C. Nice, N. H. C. Cooke and B. G. Archer, *Anal. Biochem.*, 126 (1982) 17.
10 N. H. C. Cooke, B. G. Archer, M. J. O'Hare, E. C. Nice and M. Capp, *J. Chromatogr.*, 255 (1983) 115.
11 F. E. Regnier and R. Noel, *J. Chromatogr. Sci.*, 14 (1976) 32.
12 J. L. Meek and Z. L. Rossetti, *J. Chromatogr.*, 211 (1981) 15.
13 E. C. Nice, M. W. Capp, N. Cooke and M. J. O'Hare, *J. Chromatogr.*, 218 (1981) 569.
14 R. V. Lewis, A. Fallon, S. Stein, K. D. Gibson and S.Udenfried, *Anal. Biochem.*, 104 (1980) 153.
15 E. C. Nice, M. Capp and M. J. O'Hare, *J. Chromatogr.*, 185 (1979) 413.
16 J. D. Pearson, W. C. Mahoney, M. A. Hermodson and F. E. Regnier, *J. Chromatogr.*, 207 (1981) 325.
17 K. J. Wilson, E. van Wieringen, S. Klauser, M. W. Berchtold and G. J. Hughes, *J. Chromatogr.*, 237 (1982) 407.
18 J. D. Pearson and F. E. Regnier, *J. Liq. Chromatogr.*, 6 (1983) 497.
19 R. V. Lewis and D. De Wald, *J. Liq. Chromatogr.*, 5 (1982) 1367.
20 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 296 (1984) 61.
21 G. Lindgren, B. Lundström, I. Källman and K.-A. Hansson, *J. Chromatogr.*, 296 (1984) 83.
22 D. W. Armstrong and R. W. Boehm, *J. Chromatogr. Sci.*, 22 (1984) 378.
23 M. A. Stadalius, H. S. Gold and L. R. Snyder, *J. Chromatogr.*, 327 (1985) 27.
24 M. A. Stadalius, M. A. Quarry and L. R. Snyder, *J. Chromatogr.*, 327 (1985) 93.
25 B. Grego and M. T. W. Hearn, *J. Chromatogr.*, 218 (1981) 497.
26 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 282 (1983) 541.
27 M. T. W. Hearn, A. N. Hodder and M.-I. Aquilar, *J. Chromatogr.*, 327 (1985) 47.
28 H. Engelhardt and H. Müller, *Chromatographia*, 19 (1984) 77.
29 C. Dewaele and M. Verzele, *J. Chromatogr.*, 282 (1983) 341.
30 K. K. Unger, G. Jilge, J. N. Kinkel and M. T. W. Hearn, *J. Chromatogr.*, 359 (1986) 61.
31 J. N. Kinkel and K. K. Unger, *J. Chromatogr.*, 316 (1984) 193.
32 K. A. Cohen, S. A. Grillo and J. W. Dolan, *LC Mag.*, 3 (198) 37.
33 L. R. Snyder, M. A. Stadalius and M. A. Quarry, *Anal. Chem.*, 55 (1983) 1412A.
34 G. Guiochon and M. Martin, *J. Chromatogr.*, 326 (1985) 3.