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REVERSED-PHASE CHROMATOGRAPHY OF PEPTIDES

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SUMMARY

The chromatographic behaviour of underivatized peptides on reversed-phase packings has been examined. To study the effects of different amino acids in peptides, simple model substances, such as amino acids and dipeptides, were chosen. The capacity factors of amino acids, L,L-dipeptides and diastereoisomeric dipeptides were measured, and side-effects such as loss by adsorption, double-peak formation and load–retention dependence were studied. Of the four different reversed phases examined, the ODS packings appeared to give both the best selectivity and the best efficiency in the separation of most peptides and diastereoisomers. A marked difference in the retention of basic functions was obtained on two ODS packings of different manufacture.

INTRODUCTION

Chromatography of amino acids and peptides is often performed after derivatization, in order to improve both the separation and the sensitivity[1]. In high-performance liquid chromatography (HPLC), the derivatization can be introduced as either a pre-column or a post-column step[2]. In gas chromatography (GC), derivatives are required in order to obtain volatile and thermally stabile compounds, but low volatility and adsorption problems still make GC less suitable than many other chromatographic techniques[3]. Also in thin-layer (TLC) and paper chromatography derivatives are often preferred[4]. Preparative and analytical separations of underivatized components have traditionally been performed by ion exchange or electrophoresis if high resolution is required. Adsorption chromatography on polystyrene–divinylbenzene resins[5], dextran gels[6–8] and polyacrylamide gels[9] may also be useful, as in separating aliphatics from aromatics or for other specific purposes. With the cumbersome detection process of electrophoresis, the main technique used in peptide separations has been ion-exchange chromatography, either on gravity columns[4] or by HPLC[10].

During the last decade, an increasing number of reports concerned with the isolation of naturally occurring peptides has led to renewed interest in developing additional rapid and sensitive separation methods for peptides. As in previous work on the isolation of peptides from the hypothalamus of the brain[11], reversed-phase HPLC packings were tried, as the traditional methods gave inadequate separations or sensitivity. Even if relatively good separations were obtained on packings such as

Poragel PN, PT and PS and Phenyl Bondapak, problems of tailing, loss of material and double peaks (from one component) occurred. Generally there was a lack of knowledge of which parameters would determine the retention of a peptide. Although reversed-phase packings have been available for several years, to our knowledge only one report has appeared[12] that discusses the usefulness and limitations of these packings for underivatized peptides. A report describing the separation of some nonapeptides on C_{18} and C_8 packings did, however, appear recently[13], as well as a report on the behaviour of amino acids and dipeptides on a bonded peptide stationary phase[14].

In their fundamental work on the reversed-phase (solvophobic) chromatography of ionogenic substances, Horváth and co-workers[15,16] obtained the following theoretical expressions for the capacity factors of monoprotic acids, monoprotic bases and zwitterions:

$$k'_{(\text{acid})} = \frac{k_0 + k_{-1}\dfrac{K_{am}}{[H^+]_m}}{1 + \dfrac{K_{am}}{[H^+]_m}}$$

$$k'_{(\text{base})} = \frac{k_0 + k_1\dfrac{[H^+]_m}{K_{am}}}{1 + \dfrac{[H^+]_m}{K_{am}}}$$

$$k'_{\text{zwitterion}} = \frac{k_0 + k_{-1}\dfrac{K_{a1,m}}{[H^+]_m} + k_1\dfrac{[H^+]_m}{K_{a2,m}}}{1 + \dfrac{K_{a1,m}}{[H^+]_m} + \dfrac{[H^+]_m}{K_{a2,m}}}$$

where k_0, k_{-1} and k_1 are the capacity factors of the zwitterionic, anionic and cationic forms of the ampholyte, respectively, and $K_{a1,m}$ and $K_{a2,m}$ are the corresponding acid dissociation constants.

These expressions are based on the assumption that the equilibrium constant of the reversible association of the solute with the hydrocarbon ligand is determined only by solvophobic interactions. This means that no ionic or hydrogen bonding occurs between the solute and the stationary phase. Chromatography of aromatic acids, amines and amino acids on Partisil ODS[16] verified this by showing a reasonable functional relationship between k' and the hydrocarbonaceous surface area of the solutes, after having corrected for electrostatic effects (as affected by the ionic strength of the eluents). Thus, on the basis of solely solvophobic interactions, controlled ionic strength, controlled pH and calculated hydrocarbonaceous surface areas, the capacity factors of ionogenic substances can be predicted by theoretical means.

If the stationary phase, however, contains functions of higher polarity, these predictions can no longer be expected to be valid. This would be the case with some of the reversed-phase or semi-reversed-phase surface functions, and also for strictly non-polar packings, which still may contain polar sites owing to manufacturing difficulties. The latter is well known with all stationary phases based on silanization of silica, where residual, unreacted silanol groups (or imperfections in the siloxane surface) may prevent the surface from following the "solvophobic theory". On this

basis, amino acids and peptides also would be expected to be useful substances in testing the potential use of packings for the separation of ionogenic solutes and in tests for polar sites on supposedly non-polar packings.

In this paper, the separation of underivatized peptides on four different reversed phases is described. To study the effect of different amino acids in peptides, the simplest model substances, amino acids and dipeptides, were chosen. The L,L-configuration of dipeptides was used, unless otherwise specified. The separation of dipeptides, as such, is also of interest, as they are products of the enzymatic cleavage of larger peptides in sequencing procedures[17,18]. The model peptides had the structures A_2-Ala and Ala-A_1 for the purpose of studying the effect of the amino acids in positions 1 and 2. Other peptides with different structures were also studied in order to obtain additional information. Of the many possible dipeptides, a careful selection among those commercially available was made in order to elucidate the main separation parameters.

EXPERIMENTAL AND RESULTS

Equipment

The HPLC system consisted of a Waters Model 6000 pump, a Waters UK-6 valve-loop injector, a Perkin-Elmer LC-55 variable-wavelength UV detector and/or a Waters R-401 refractive index detector. All runs were performed at ambient temperature.

Columns. Of the columns tested, the following two were pre-packed by the manufacturer: Perkin-Elmer Phenyl-Sil-X-I (13-μm porous particles, 250 mm $\times$ 2.6) and Waters μBondapak NH_2 (10-μm porous particles, 300 $\times$ 4.0 mm). Two columns were slurry packed in methanol–carbon tetrachloride (5:95): Macherey, Nagel & Co. Nucleosil 5 CN (5-μm porous particles, 250 $\times$ 4.6 mm) and H. Reeve Angel Spherisorb S5W-ODS (5-μm porous particles, 250 $\times$ 4.6 mm). One column was packed in methanol (upward packing), Spherisorb S5 Phenyl Bonded (5-μm porous particles, 150 $\times$ 4.6 mm), and one in methanol–water (4:1), Shandon ODS-Hypersil (5-μm porous particles, 250 $\times$ 4.6 mm). The Bondapak NH_2 column had a guaranteed plate height (H) of 0.1 mm. Based upon injection of naphthalene in methanol–water (7:3), the other columns had the following plate heights: Phenyl-Sil-X, 0.25 mm; Nucleosil CN, 0.05 mm; Spherisorb ODS, 0.05 mm; ODS-Hypersil, 0.05 mm; and Spherisorb Phenyl Bonded, 0.06 mm.

Reagents

Methanol was of pro analisi quality from Riedel-de Haën (Seelze-Hannover, G.F.R.) and water was distilled twice. Amino acids and peptides were purchased from Sigma (St. Louis, Mo., U.S.A.).

Loss by "adsorption"

In microscale manipulations with peptides, losses of material by adsorption on glass surfaces may occur, if not counteracted by using silanized glass. On reversed-phase HPLC packings, incomplete reactions or imperfections in the siloxane surface also may cause loss of material due to adsorption on active sites on the packing. The presence of a buffer in the solvent, which can compete with the solute for the active sites, would normally be expected to decrease this adsorption. Such a buffer should

be easy to remove by evaporation, and for HPLC purposes would also be transparent to UV radiation. Ammonium acetate was chosen, and an optimal concentration of 0.01 *M* was determined by injection of 50 μg or less of amino acids and peptides. The presence of adsorption effects was tested by repeated injections of different amino acids on the Phenyl-Sil-X column, and the results were as follows:

(a) Basic amino acids were strongly retained in 0.01 *M* ammonium acetate. Elution with methanol mixtures gave relatively broad peaks, with fairly constant peak heights.

(b) Small aliphatic amino acids and especially the acidic amino acids glutamic acid and aspartic acid showed decreasing peak heights on repeated injections (Fig. 1).

(c) The peak heights of amino acids with aromatic or large aliphatic side-chains were little affected.

(d) The peak heights of dipeptides, including those with glutamic acid and aspartic acid were little affected, also when injected after acidic amino acids.

(e) After several injections an equilibrium was established (Fig. 1), as seen by a constant peak height. Glutamic and aspartic acids had an equilibrium peak height of less than 50% of the first injection.

(f) Increasing time intervals between injections (Fig. 1) resulted in increased peak heights. Extensive washing brought the height of the next injection to approximately the same level as the first injection.

(g) A higher concentration of ammonium acetate (0.1 *M*) did not overcome the peak-decreasing effect, but reduced the rate.

A decreasing peak height showed that an increasing amount of sample was lost on each injection. The contrary would normally be expected, *i.e.*, increasing peak heights with increasing the column saturation. The only explanation of this phenomenon is that the initially adsorbed solute had a stronger adsorbing effect than the packing itself on the next injection, until an equilibrium was reached between adsorbed and desorbed material. This was shown to be the case with ethylamine, which,

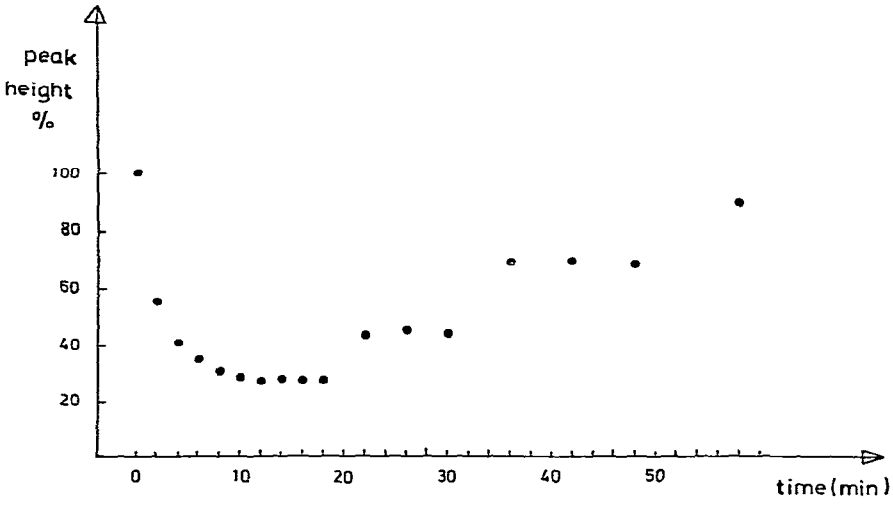

Fig. 1. Relative peak heights as a function of injection intervals from repeated injections of 50 μg of aspartic acid in 0.01 *M* ammonium acetate on Phenyl Sil-X-I.

on repeated injections, gave slowly increasing peak heights until a plateau was reached, and by N-acetyl glutamic acid, which showed no variation in peak height. Hence the initial adsorption must be between the packing and the free amino function of the amino acids, strong interactions between carboxylic groups from the adsorbed solute and amino groups of the freshly injected sample subsequently dominating.

The effects of adsorption were examined on the phenyl, nitrile and ODS columns. The Phenyl-Sil-X column showed the strongest effects by strong adsorption of glutamic and aspartic acids (>50%), less adsorption (<25%) of glycine, alanine, aspartic acid and serine and no noticeable adsorption of phenylalanine, tyrosine, histidine, proline, lysine and dipeptides such as alanylaspartic acid. Mobile phase mixtures containing 50% and 20% of methanol showed the same picture. The nitrile and ODS columns showed the same effect, but less markedly. The conclusion can be drawn that when microgram amounts of peptides are chromatographed together with acidic amino acids on reversed-phase columns, pure fractions may be contaminated by strongly adsorbed material from apparently less retained peaks. So far we have found no solution to this problem, but it is probable that highly efficient silanized surfaces may reduce the effect (this is at present under examination).

Double peaks, pH control

Horváth *et al.*[16] showed that ampholytic solutes may be eluted with different retentions, depending on the distribution of charge. We found that amino acids and dipeptides in the presence of acids could give double peaks on reversed-phase columns. This was apparent also in pure water, where the hydrochlorides of basic amino acids gave one early eluting peak containing chloride and one retained peak without chloride.

However, amino acids and dipeptides in the absence of acids also gave two peaks in water. For small, acidic amino acids and peptides, the first peak was dominant, whereas aromatic and large aliphatic side-chains gave a larger second peak. As the equilibrium forms of ampholytes are not likely to separate chromatographically, we suggest that the first peak is due to the formation of dimeric (or polymeric) complexes formed by ionic or hydrogen bonds. The size of the first peak compared with that of the second peak seemed to be disproportional to the p*I* values of most amino acids, but was also affected by the size of the solvated molecule. Thus a large side-chain seemed to favour the supposedly monomeric structure.

When ammonium acetate was added to the mobile phase, the first peak disappeared almost completely. This pH (6.5) is not sufficiently low for protolysis of most amino acids or peptides, except the basic compounds. Not surprisingly, the front peak was still seen for the basic amino acids arginine and lysine, with p*I* values of 10.76 and 9.47, respectively.

The apparent conclusion is that in order to avoid multiple peaks of peptides on reversed-phase columns, the pH must be controlled with buffers, such as ammonium acetate. A major reason for using a concentration of 0.01 *M* was to allow for maximal sensitivity of the UV detection at low wavelengths. In methanol mixtures, optimal detection of the peptide bond was achieved at 220–230 nm.

Effects of functional groups

Table I gives capacity factors, p*K* and p*I* values for amino acids and Tables II–IV give capacity factors for dipeptides.

TABLE I

CAPACITY FACTORS, k' (AVERAGE OF THREE MEASUREMENTS), FOR AMINO ACIDS IN 0.01 *M* AMMONIUM ACETATE ON THREE REVERSED-PHASE COLUMNS $k' = (V_e - V_0)/V_0$.

Amino acid	pK_1	*pI*	k'		
			Phenyl-Sil-X	*Nucleosil CN*	*Spherisorb ODS*
Asp	2.10	2.98	0.31	0.29	0.48
Glu	2.10	3.08	0.42	0.29	0.52
Tyr	2.20	5.63	0.88	0.69	4.39
Ser	2.21	5.68	0.75	0.31	0.88
Met	2.28	5.74	1.00	0.54	2.18
Trp	2.38	5.88	1.25	2.00	20.82
Phe	2.58	5.91	1.38	1.02	6.91
Val	2.29	6.00	1.00	0.46	1.36
Leu	2.33	6.04	1.25	0.56	2.33
Ile	2.32	6.04	1.31	0.56	2.27
Gly	2.35	6.06	0.75	0.33	0.79
Ala	2.35	6.11	0.78	0.38	0.85
Pro	2.00	6.30	1.38	0.42	1.52
His*	1.77	7.64	2.06	0.40	3.64
Lys*	2.18	9.47	1.25	0.60	3.61
Arg*	2.01	10.76	1.12	0.42	4.88

* Dose dependent; 300-μg doses on ODS.

TABLE II

CAPACITY FACTORS (k') OF L,L-DIPEPTIDES IN 0.01 *M* AMMONIUM ACETATE ON PHENYL-SIL-X, NUCLEOSIL CN AND BONDAPAK NH_2 COLUMNS

Dipeptide	*Column*			*Dipeptide*	*Column*		
	Phenyl	*Nitrile*	*Amino*		*Phenyl*	*Nitrile*	*Amino*
Ala–Ala	1.00	0.40	0.54	His–Ser*	3.50	0.50	2.28
Ala–Asn	0.94	0.40	0.42	His–Tyr*	3.00	0.98	3.20
Ala–Asp	0.38	0.29	7.28	Leu–Leu	2.06	—	1.00
Ala–Glu	0.44	0.29	5.48	Leu–Met	1.94	0.85	1.06
Ala–Gly	0.94	0.40	0.54	Leu–Phe	2.13	2.00	0.80
Ala–His*	3.63	0.52	0.70	Leu–Ser	1.19	0.48	0.78
Ala–Lys*	3.25	0.31	—	Leu–Trp	1.81	3.90	1.78
Ala–Met	1.25	0.63	0.68	Leu–Tyr	1.19	1.13	1.02
Ala–Pro	1.44	0.54	—	Lys–Ala*	3.81	0.38	0.06
Ala–Ser	0.94	0.40	0.54	Met–Ala	1.19	0.48	1.04
Ala–Thr	—	0.40	0.70	Phe–Ala	1.38	0.81	1.38
Ala–Tyr	0.88	0.71	0.90	Phe–Phe	1.88	4.56	2.44
Ala–Val	1.18	0.52	0.35	Pro–Ala	—	0.40	1.00
Glu–Ala	0.44	0.25	6.34	Pro–Gly	1.69	0.43	1.08
Gly–Ala	1.06	0.35	0.56	Pro–Ile	2.50	0.65	1.38
Gly–Gly	0.94	0.35	0.56	Pro–Leu	2.63	0.71	2.46
Gly–Gly-NH_2	2.75	0.52	0	Pro–Met	1.94	0.67	1.26
His–Ala*	3.00	0.42	1.50	Pro–Trp	2.19	2.43	2.42
His–Gly*	2.38	0.50	1.68	Ser–Ala	0.88	0.38	0.92
His–Leu*	6.00	0.79	2.52	Trp–Ala	1.19	1.69	1.66
His–Lys*	—	0.77	0.62	Tyr–Ala	0.88	0.63	1.20
His–Phe*	6.00	1.46	3.16	Tyr–Tyr	0.88	1.58	2.70

* Dose dependent; data for approximately 20-μg doses.

TABLE III

CAPACITY FACTORS (k') OF L,L-DIPEPTIDES ON SPHERISORB ODS IN 0.01 *M* AMMONIUM ACETATE IN 30% METHANOL, 10% METHANOL AND WATER

Dipeptide	*30% MeOH*	*10% MeOH*	*H_2O*	*Dipeptide*	*30% MeOH*	*10% MeOH*	*H_2O*	*Dipeptide*	*30% MeOH*	*10% MeOH*	*H_2O*
Ala-Asp	0.36	0.36	0.36	Gly-Gly-NH_2*	1.85	3.03	–	Phe-Phe	7.36	–	–
Ala-Glu	0.36	0.36	0.36	Gly-Gly	0.73	0.79	0.97	Pro-Gly	0.82	1.21	2.03
Ala-Asn	0.79	0.88	0.94	Gly-His*	2.42	3.61	–	Pro-Leu	1.79	3.42	–
Ala-Thr	0.73	0.94	0.97	His-Gly*	2.64	3.21	–	Pro-Ile	1.61	2.88	–
Ala-Gly	0.73	0.79	0.94	His-Ala*	2.42	3.40	–	Pro-Met	1.27	2.24	–
Ala-Ser	0.73	0.76	0.94	His-Leu*	6.3	11.4	–	Pro-Trp	3.00	12.2	–
Ala-Ala	0.79	0.82	0.91	His-Lys*	5.2	7.5	–	Tyr-Tyr	1.27	4.52	–
Ala-Val	0.82	1.00	1.58	His-Phe*	9.9	17.7	–	Glu-Ala	0.39	0.36	0.36
Ala-Pro	1.06	1.42	2.06	His-Ser*	3.15	3.33	–	Gly-Ala	0.67	0.85	0.94
Ala-Tyr	0.94	1.73	3.15	His-Tyr*	5.72	11.4	–	Ser-Ala	0.67	0.76	0.88
Ala-Leu	1.33	1.76	3.30	Leu-Ala	1.09	1.70	3.45	Pro-Ala	0.85	1.09	1.55
Ala-Ile	1.12	1.58	–	Leu-Leu	2.97	–	–	Met-Ala	0.94	1.21	1.94
Ala-Met	1.18	2.09	4.88	Leu-Met	1.82	–	–	Leu-Ala	1.09	1.76	3.45
Ala-NorVal	1.21	2.00	4.21	Leu-Phe	5.67	–	–	Tyr-Ala	0.94	1.79	3.94
Ala-His*	2.12	3.24	–	Leu-Ser	0.97	1.36	2.30	Phe-Ala	1.48	3.06	–
Ala-Lys*	2.61	3.39	–	Leu-Trp	5.42			Lys-Ala*	3.30	3.48	–
Ala-Phe	1.70	3.45	–	Leu-Tyr	1.61			Trp-Ala	2.03	5.85	–
Ala-Gly-NH_2*	1.91	3.45	–	Leu-Val	1.48	3.24					

* Dose dependent; data for approximately 20-μg doses.

TABLE IV

CAPACITY FACTORS (k') OF L,L-DIPEPTIDES ON ODS-HYPERSIL IN 0.01 *M* AMMONIUM ACETATE IN 30% METHANOL, 10% METHANOL AND WATER

Approximately 20-μg doses were injected.

Dipeptide	*30% MeOH*	*10% MeOH*	*H_2O*	*Dipeptide*	*30% MeOH*	*10% MeOH*	*H_2O*	*Dipeptide*	*30% MeOH*
Ala–Ala	0.44	0.58	0.69	Arg–Asp	0.44	0.58	0.64	Gly–Thr	0.47
Ala–Asn	0.53	0.64	0.67	Glu–Ala	0.38	0.36	0.36	Gly–Trp	1.39
Ala–Asp	0.36	0.36	0.39	*p*-Glu–His	0.47	0.81	1.83	Gly–Tyr	0.64
Ala–Glu	0.39	0.36	0.36	Gly–Ala	0.50	0.58	0.61	Gly–Val	0.64
Ala–Gly	0.47	0.58	0.61	Gly–Asn	0.53	0.58	0.58	His–Ala	0.50
Ala–Gly-NH_2	0.61	0.81	0.92	Gly–Asp	0.38	0.36	0.36	His–Gly	0.53
Ala–His	0.53	0.75	0.86	Gly–Glu	0.36	0.36	0.36	His–Leu	1.08
Ala–Ile	0.83	1.56	2.94	Gly–Gly	0.47	0.58	0.58	His–Lys	0.64
Ala–Leu	0.89	1.78	3.83	Gly–Gly-NH_2	0.58	0.75	1.25	His–Phe	1.56
Ala–Lys	0.53	0.72	0.69	Gly–His	0.58	0.75	0.92	His–Ser	0.50
Ala–Met	0.83	2.14	7.03	Gly–Ile	0.92	1.69	3.44	His–Tyr	0.72
Ala–NorVal	0.81	2.08	6.03	Gly–Leu	0.97	2.03	4.11	Leu–Ala	0.78
Ala–Phe	1.28	4.00	9.0	Gly–Leu-NH_2	1.64	4.94	~16.2	Leu–Gly	0.92
Ala–Ser	0.50	0.58	0.58	Gly–Met	0.69	1.08	2.14	Leu–Leu	3.76
Ala–Pro	0.58	0.69	0.92	Gly–NorLeu	1.11	2.22	4.75	Leu–Met	1.53
Ala–Thr	0.55	0.61	0.58	Gly–NorVal	0.69	1.00	1.61	Leu–Phe	6.97
Ala–Trp	1.39	6.31		Gly–Phe	1.31	3.81	~11.6	Leu–Ser	0.69
Ala–Val	0.64	0.83	1.17	Gly–Pro	0.58	0.69	1.06	Leu–Trp	5.28
Ala–Tyr	0.69	1.42	3.19	Gly–Ser	0.47	0.56	0.67	Leu–Tyr	1.22

From the capacity factors of the model substances A_2-Ala and Ala-A_1 (Tables II–IV), the effect of a particular amino acid in positions 1 and 2 is demonstrated. From these data, a ranking order of the effect of some amino acids on retention has been established (Table V). The main conclusions were as follows.

The elution order of amino acids on the amino column was complex, being determined both by hydrophobic and electrostatic interactions. Acidic amino acids had the strongest effect on retention, in both positions in dipeptides (see p*I* values in Table I).

The nitrile column behaved surprisingly well in accordance with reversed-phase theory, in comparison with the ODS columns.

The Phenyl-Sil-X column gave capacity factors that were strongly influenced by the presence of basic functions or the absence of acidic functions (amides). The 5-μm Spherisorb Phenyl Bonded column gave a similar pattern, but after the major effects had been established this column was not investigated in detail.

The Spherisorb ODS also showed a strong influence of basic functions, but clearly with more reversed-phase behaviour than the phenyl columns. Finally, the ODS-Hypersil column showed an almost perfect reversed-phase fit (determined without any calculations of hydrocarbonaceous surface area).

All columns showed reversed-phase behaviour on variation of the composition of the mobile phase.

The number of peptides run on the more polar columns was less than that on

10% MeOH	*H_2O*	*Dipeptide*	*30% MeOH*	*10% MeOH*	*H_2O*	*Dipeptide*	*30% MeOH*	*10% MeOH*	*H_2O*
0.58	0.72	Leu–Val	1.31	4.47		Pro–Gly	0.56	0.69	1.14
6.14		Lys–Ala	0.64	0.78	0.83	Pro–Ile	0.97	1.86	5.56
1.42	3.69	Lys–Asp	0.39	0.56	0.58	Pro–Leu	1.08	2.31	7.72
0.92	1.50	Lys–Gly	0.56	0.72	0.81	Pro–Met	0.75	1.28	4.00
0.67	1.14	Lys–Leu	1.11	1.86	4.78	Pro–Phe	1.58	5.33	
0.69	1.33	Lys–Lys	0.72	0.78	1.00	Pro–Phe-NH_2	2.92	5.67	
2.97	~11.8	Lys–Phe	1.42	4.11	~14.7	Pro–Trp	1.67	6.89	
0.92	1.58	Met–Ala	0.61	1.06	2.03	Pro–Tyr	0.75	1.47	5.17
7.33		Met–Asn	0.61	0.83	1.58	Pro–Val	0.72	1.06	1.89
0.67	0.92	Met–Glu	0.47	0.58	0.83	Ser–Ala	0.47	0.58	0.67
2.06	~9.5	Met–Gly	0.69	1.19	3.06	Ser–Gly	0.53	0.56	0.64
1.44	4.11	Met–Leu	1.97	9.0		Ser–Leu	0.97	1.61	3.28
2.31	7.11	Met–Met	1.11	4.03	~22	Ser–Phe	1.22	3.14	~10.2
>13		Met–Phe	3.64	>16		Trp–Ala	1.47	5.67	
7.67		Met–Ser	0.56	0.86	1.53	Trp–Gly	1.69	8.4	
		Met–Val	0.92	2.39	~12.9	Tyr–Ala	0.72	1.39	5.11
1.14	2.14	Phe–Ala	1.22	3.94	~15.4	Tyr–Gly	0.81	1.89	8.3
		Phe–Gly	1.39	4.67		Tyr–Tyr	1.00	5.1	
6.56		Phe–Phe	12.7			Val–Ala	0.64	0.78	1.39
		Pro–Ala	0.58	0.64	0.67	Val–Gly	0.69	1.03	2.22

the ODS columns, as the availability of peptides increased during the period of examination.

Separation of diastereoisomers

All columns showed some ability to separate diastereoisomeric dipeptides. The Phenyl-Sil-X column gave little separation, partly owing to its low plate number. The amino and the nitrile columns were slightly better, but by far the best were the ODS columns, which were able to separate L,L- and D,D-isomers from L,D- and D,L-isomers of most dipeptides (Tables VI and VII). The L,L- and D,D-isomers generally had lower retentions than the L,D- and D,L-isomers. Model studies indicated that a better packing of the L,L- and D,D-isomers may partially shield the free amino groups from interactions with the stationary phase. This theory is supported by the result that the same elution order was found on the phenyl column, but not on the amino and the nitrile columns, both of which are less sensitive towards basic groups.

The resolution of diastereoisomers on the ODS columns was impressive. When L-leucyl-L-leucine was chromatographed with 0.01 *M* ammonium acetate in 50% and 30% methanol, the capacity factors were 1.4 and 3.0, respectively. In comparison, the capacity factor of the L,D–D,L mixture increased from 2.7 to 10.6. Thus, for dipeptides containing relatively large aliphatic or aromatic moieties, steric requirements were much more important than any other factor towards retention.

TABLE V

APPROXIMATE EFFECT OF AMINO ACIDS IN POSITIONS 1 AND 2 ON THE RETENTION OF THE DIPEPTIDES A_2-ALA AND ALA-A_1

Column	*Effect of amino acids*	
	Position	*Order*
Phenyl Sil-X	2	Lys > His > Leu > Pro > Phe > Met > Trp > Gly ≈ Ala > Ser ≈ Tyr > Glu
	1	His > Lys > Gly-NH_2 > Pro > NorVal > Leu > Met ≈ Phe > Val > Ala > Asn ≈ Gly ≈ Ser > Tyr > Glu > Asp
Nitrile	2	Trp > Phe > Tyr > Met > His > Ala > Pro > Lys ≈ Ser > Gly > Glu
	1	Phe > Tyr > Met > Leu > NorVal > Pro > His > Val > Gly-NH_2 > Ala > Asn ≈ Gly ≈ Ser ≈ Thr > Lys > Glu ≈ Asp
Amino	2	Glu > Trp > His > Phe > Tyr > Met > Pro > Ser > Leu > Ala ≈ Gly > Lys
	1	Asp > Glu > Tyr > Val > NorVal ≈ Phe > His ≈ Thr ≈ Leu > Met > Ala > Gly ≈ Ser > Asn > Gly-NH_2
Spherisorb ODS	2	Trp > Lys ≈ His > Phe > Tyr ≈ Leu > Met > Pro > Ala ≈ Ser ≈ Gly > Glu
	1	Phe > Gly-NH_2 > Lys ≈ His > NorVal ≈ Met > Leu > Ile > Tyr ≈ Pro > Val > Ala ≈ Ser ≈ Gly ≈ Thr ≈ Asn > Glu ≈ Asp
ODS-Hypersil	2	Trp > Phe > Tyr > Leu > Met > Val > His > Lys > Pro ≈ Ser ≈ Gly ≈ Ala > Glu
	1	Trp > Phe > Met > NorVal > Leu > Tyr > Ile > Val > Pro ≈ Gly-NH_2 ≈ His > Lys ≈ Ala ≈ Asn ≈ Gly ≈ Ser ≈ Thr > Glu ≈ Asp

TABLE VI

SEPARATION OF DIASTEREOISOMERS ON SPHERISORB ODS

Dipeptide	*Configuration*	*Capacity factors (k') with 0.01 M NH_4OAc*				
		50% MeOH	*30% MeOH*	*20% MeOH*	*10% MeOH*	*H_2O*
Ala–Ala	L,L + D,D	0.70	0.70	0.73	0.82	0.91
	L,D + D,L	—	—	—	—	1.24
Ala–Leu	L,L + D,D	0.97	1.33	1.36	1.76	3.30
	L,D + D,L	1.12	1.94	2.36	3.88	9.82
Ala–Val	L,L + D,D	0.82	0.89	0.94	1.00	1.58
	L,D + D,L	—	1.09	1.39	1.88	3.94
Leu–Ala	L,L + D,D	0.85	1.06	1.33	1.80	3.27
	L,D + D,L	1.06	1.79	2.70	4.36	12.64
Leu–Leu	L,L + D,D	1.42	2.97	—	—	—
	L,D + D,L	2.67	10.63	—	—	—
Leu–Phe	L,L + D,D	1.85	5.67	—	—	—
	L,D + D,L	3.06	15.00	—	—	—

TABLE VII

SEPARATION OF DIASTEREOISOMERS ON ODS-HYPERSIL

Dipeptide	*Configuration*	*Capacity factors (k') with 0.01 M NH_4OAc*		
		30% MeOH	*10% MeOH*	*H_2O*
Ala–Ala	L,L + D,D	0.42	0.58	0.67
	L,D + D,L	0.42	0.72	1.08
Ala–Leu	L,L + D,D	0.89	1.78	
	L,D + D,L	1.44	5.28	
Ala–Val	L,L + D,D	0.61	0.83	1.17
	L,D + D,L	0.83	1.86	
Leu–Ala	L,L + D,D	0.78	1.39	4.11
	L,D + D,L	1.47	5.67	
Leu–Leu	L,L + D,D	3.39	>13	
	L,D + D,L	13.8		
Leu–Phe	L,L + D,D	6.97		
	L,D + D,L	>13		

The resolution on the ODS-Hypersil column was slightly better than that on the Spherisorb ODS column for the diastereoisomers tested. This is more difficult to explain by a difference in shielding of the amino groups, as the effect of basic functions was slight on the ODS-Hypersil column. Hence so far we cannot give a satisfactory explanation of the dramatic separation of diastereoisomers on ODS columns.

Mixtures of L,L- and D,D-isomers and of L,D- and D,L-isomers could not be separated on any of the columns examined.

Retention–load dependence

On the Spherisorb-ODS column the retention of basic amino acids and dipeptides containing basic amino acids was found to depend on both the amount applied and on the pH of the solvent. The strongest dependence on load occurred with histidine-containing dipeptides with histidine in a terminal position. Amounts varying from 2 to 200 μg of histidylglycine were injected in 0.01 *M* sodium acetate–acetic acid (9:1) and 0.01 *M* sodium acetate–acetic acid (1:1) in 50% methanol. The pH values of the solvent were 6.65 and 5.73, respectively. In the more basic solvent, a strong retention–load dependence was found (Fig. 2). This was also seen in both 0.01 *M*

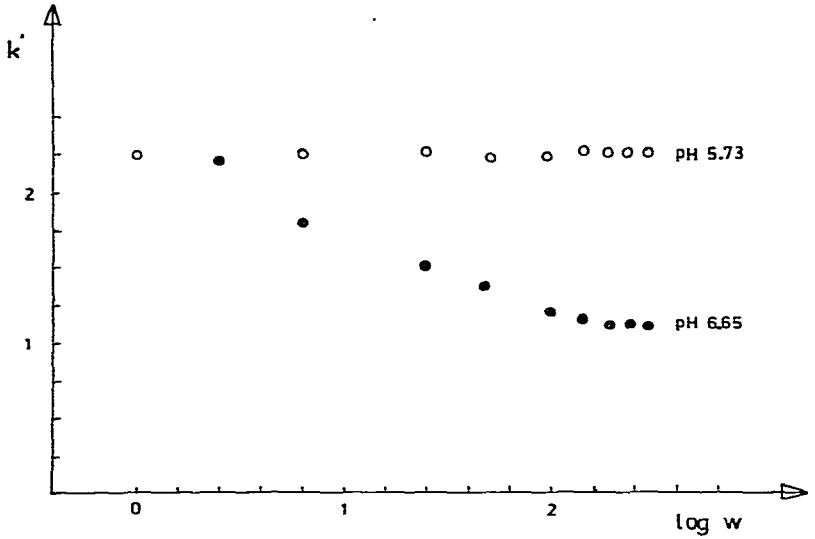

Fig. 2. Variation in the capacity factor (k') of histidylglycine as a function of load, w (μg), in 0.01 *M* sodium acetate–acetic acid (1:1), pH 5.73 (○) and in 0.01 *M* sodium acetate–acetic acid (9:1), pH 6.65 (●) in 50% methanol on Spherisorb ODS.

ammonium acetate (Fig. 3) and 0.1 *M* ammonium acetate. Acidic and neutral dipeptides (including amides) showed no noticeable retention–load dependence in ammonium acetate.

In contrast, the ODS-Hypersil column showed no significant retention–load dependence of basic functions in ammonium acetate, with loads varying from 0.05 to 200 μg (Fig. 3). The same was found in mobile phases containing sodium acetate and sodium acetate–acetic acid (1:1). However, in 0.01 *M* acetic acid in 50% methanol, basic solutes increased their capacity factors by up to 50% when the load was increased by a factor of 10. In the same solvent, acidic and neutral solutes decreased their capacity factors by up to 25% within the same range of application.

Retention–pH dependence

As already shown, a distinct retention–pH dependence was found within the pH range 3.5–7.5, apparently in accordance with the calculations of Horváth *et al.*[16]. No attempt was made to measure k_{-1} and k_1 for different peptides as the pH range

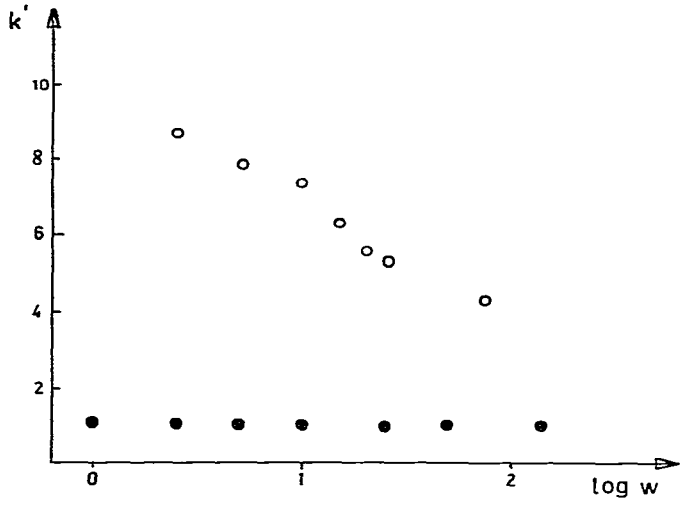

Fig. 3. Variation in the capacity factor (k') of histidylglycine as a function of load, w (μg), in 0.01 M ammonium acetate (pH 6.56) on Spherisorb ODS (○) and on ODS-Hypersil (●).

needed would be beyond that recommended for silica-based packings, but an example of a capacity factor–pH dependence is shown in Fig. 4. Many acidic and neutral dipeptides needed ammonium acetate or sodium acetate to be eluted within a reasonable time, as 0.01 M acetic acid gave too high capacity factors.

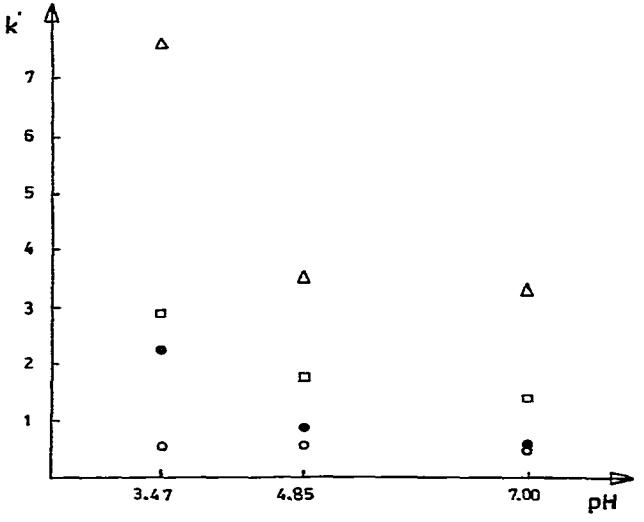

Fig. 4. Capacity factors (k') of 50-μg doses of glycine (○), histidine (●), leucine (□) and phenylalanine (△) at pH 7.00 (0.01 M sodium acetate in 10% methanol), pH 4.85 (0.01 M sodium acetate–acetic acid, 1:1, in 10% methanol) and pH 3.47 (0.01 M acetic acid in 10% methanol) on ODS-Hypersil.

Comparison of packings

The amino column showed large deviations from the reversed-phase mode, as expected.

The nitrile column showed surprisingly constant reversed-phase behaviour, but the column efficiency and the selectivity were inferior to those of the ODS columns.

The phenyl columns gave approximately the same results, but with higher efficiency on the 5-μm Spherisorb. The efficiency and the selectivity were considerably better (for most dipeptides) than those of the nitrile column, but poorer than those of the Spherisorb ODS column. Thus, the general conclusion was that ODS can be recommended as a reversed phase for most peptide separations. Both the phenyl and the Spherisorb ODS columns were strongly affected by basic solutes, in contrast to the ODS-Hypersil column. Hence, for pure reversed-phase separations, ODS-Hypersil columns must be recommended. Peptides with dominating basic functions that otherwise could be totally retained should be purified on such a strictly reversed-phase packing. We found ammonium acetate to be a suitable buffer for use with this packing.

This does not necessarily mean that peptides are generally separated better on the purer reversed phases. For many applications, additional variation of the selectivity (effected by means of differences in silanization) may be useful. However, the problem the user faces is to know what can be expected from a particular column, as this type of information is not easily obtainable. On that basis, peptides may be useful substances for testing the degree of reversed-phase behaviour of these types of packings.

REFERENCES

1 Z. Deyl, *J. Chromatogr.*, 127 (1975) 338.
2 R. W. Frei and W. Santi, *Z. Anal. Chem.*, 277 (1975) 303.
3 H. Lindley and P. C. Davies, *J. Chromatogr.*, 100 (1974) 117.
4 A. Niederwieser, in E. Heftman (Editor), *Chromatography*, Van Nostrand-Reinhold, New York, 1975, p. 393.
5 A. Niederwieser, *J. Chromatogr.*, 61 (1971) 81.
6 D. Eaker and J. Porath, *Separ. Sci.*, 2 (1967) 507.
7 J. C. Janson, *J. Chromatogr.*, 28 (1967) 12.
8 P. Ziska, *J. Chromatogr.*, 60 (1971) 139.
9 D. P. Thornhill, *Biochim. Biophys. Acta*, 279 (1972) 1.
10 R. E. Majors, *Int. Lab.*, Nov./Dec. (1975) 11.
11 T. Greibrokk, J. J. Hansen, R. Knudsen, Y. K. Lam, K. Folkers and C. Y. Bowers, *Biochem. Biophys. Res. Commun.*, 67 (1975) 338.
12 J. J. Hansen, T. Greibrokk, B. L. Currie, K. N. G. Johansson and K. Folkers, *J. Chromatogr.*, 135 (1977) 155.
13 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 27.
14 E. J. Kikta and E. Grushka, *J. Chromatogr.*, 135 (1977) 367.
15 C. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129.
16 C. Horváth, W. Melander and I. Molnár, *Anal. Chem.*, 49 (1977) 142.
17 J. K. McDonald, B. B. Zutman, T. J. Reilly and S. Ellis, *J. Biol. Chem.*, 244 (1969) 2693.
18 H. C. Kruetsch and J. J. Pisano, in R. Walter and J. Meinhofer (Editors), *Peptides, Proceedings of the 4th Annual Peptide Symposium (New York)*, Ann Arbor Sci. Publ., Ann Arbor, Mich., 1975, p. 985.