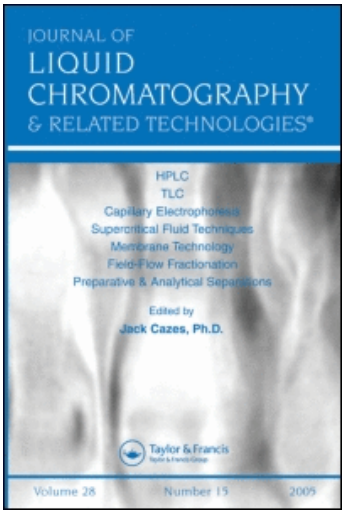

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ANALYSIS OF GROUP RETENTION CONTRIBUTIONS FOR PEPTIDES SEPARATED BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY*

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ABSTRACT

Within the framework provided by solvophobic theory, selectivities for unprotected peptides separated on fully porous, microparticulate, chemically bonded alkylsilicas can be ascribed to differences between the effective hydrophobic contact areas of the solutes. Furthermore, this theoretical treatment predicts that retention behaviour differences can be evaluated from topological parameters which accomodate the influence of amino acid side chain and end group contributions in the retention process. With data obtained for 57 peptides, including a variety of peptide hormones, eluted under the same conditions from a μBondapak C_{18} column, these predictions have been rigorously tested using two methods of numerical analysis. The results provide further evidence that the hydrophobic group retention contributions of the amino acid residues in small peptides have an essentially additive effect on peptide retention with alkylsilicas. Divergences in retention

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behaviour are interpreted in terms of specific silanophilic and solvation interactions.

INTRODUCTION

Reversed phase high performance liquid chromatography (RP-HPLC) has become firmly established as a powerful technique for the analysis and isolation of underivatised peptides [2,3]. This technique predominantly depends upon the hydrophobic expulsion of ionised peptidic solutes from polar mobile phases with the concommitant adsorption onto the surface of a non-polar stationary phase. Under these chromatographic conditions, peptides are retarded to different extents depending on their intrinsic hydrophobicities, the elutropicity of the mobile phase and the nature of the hydrocarbonaceous stationary phase. Because of their favourable mechanical and chemical characteristics, the fully porous, microparticulate chemically bonded alkylsilica supports have attracted most attention as chromatographic packings in RP-HPLC separations of peptides. In order to accomodate the great structural diversity which peptides can exhibit, a large variety of mobile phase combinations have been developed. By suitable manipulation of the mobile phase conditions, precise control over the chromatographic distribution processes can be achieved. The effect of organic solvent modifiers and the participation of secondary chemical equilibria including ionisation, pairing ion and solvation effects, on peptide retention to alkylsilica supports have received much detailed attention [2,3]. It has become apparent from these studies that the nature and relationship of the amino acid side chains to ionised centres have dominant influences on the retention behaviour of peptides with alkylsilicas under elution conditions which involve aquo-organic solvent mobile phases of high to intermediate water content covering the range pH 2.0-7.0.

Considerable success has been achieved with the prediction of retention behaviour of neutral and polar solutes, such as benzene derivatives and weak organic acids and bases, on reversed phases from quantitative estimates of solute hydrophobicities. In many cases, these estimates have been based on such topological indices as the Hansch π constants derived from classical n-octanol/water partition coefficients and related parameters. With homologous peptides it has been noted [2,4-6] that the retention behaviour appears to follow that predicted on the basis of the summated hydrophobic contribution from each amino acid side chain. For example, Molnar and Horvath have demonstrated [4] a linear dependency exists between $\ell n\ k'$ and the number of residues for alanine oligomers. With most other peptides, such approaches have met with more limited successes presumably due to the participation of secondary conditional effects in the retention process. It is apparent from data presented [2,4-11] from several laboratories that with some structurally unrelated peptides the retention order can predominantly be equated with the amino acid composition. However with peptide positional isomers and analogues, subtle selectivity deviations have been described which cannot simply be accomodated in terms of the summated hydrophobic contribution of each amino acid residue. Anomalies of this type have been attributed to polar interactions between the peptide and the stationary phase, competing protic or pairing ion dissociation equilibria, hydrogen bonding interactions and conformation effects. Despite their obvious limitations, tables of retention coefficients and group contributions have recently been used [5,7,9] to predict peptide retention, in some cases with surprisingly high correlation between the actual and the predicted retention orders for peptides up to ca 20 residues. In most earlier studies, the procedures used to derive individual amino acid group retention contribution values have been based [6,12,13]

on repetitive regression analysis associated with forcing routines. The basis of these calculations assumes that peptide retention can be described solely in terms of ideal reversed phase behaviour. The purpose of this paper was to examine more closely some of the assumptions used in the compilation of amino acid retention coefficients from RP-HPLC data for peptides. To this end, we have applied two methods of numerical analysis, using chromatographic data accumulated for various peptides, to assess the reliability of such approaches in the prediction of retention behaviour of peptides on silica-bonded non-polar stationary phases.

MATERIALS AND METHODS

The HPLC system was assembled from modular components and consisted of two Model 600A solvent delivery pumps, a M660 solvent programmer, a U6K universal chromatographic injector and a Model 450 variable wavelength UV detector, all from Waters Assoc., (Milford,Mass, U.S.A.) and a Rikadenki dual channel recorder. Sample injections were made with Microliter #810 Syringes from Hamilton Co., (Reno, Nev., U.S.A). The sources and characterisation of the peptides used in this study have been given previously [5]. All amino acids except glycine were of the L-configuration. All solvents and chemicals were AnalaR grade, water was de-ionised by reverse osmosis (Milli-Q) and double distilled. All chromatograms were carried out at ambient temperature (ca 18^{o}). The peptides were chromatographed with linear gradinets of acetonitrile (0.83% per min.) commencing with 50mM sodium dihydrogen phosphate-15mM orthophosphoric acid (pH 2.65) at 0 min. after injection. The final elution condition was 50% acetonitrile-50% water-50mM sodium dihydrogen phosphate-15mM orthophosphoric acid. The μBondapak C_{18} column was equilibrated to initial conditions for at least 30min. following a gradient elution experiment. The flow rate was

1.0ml/min. Sample sizes varied between 5 and 10µg peptide material injected in volumes of 5-10µl. The relative capacity factors for gradient elution experiments were calculated in the usual way using $NaNO_3$ to calibrate the column void time.

A Burroughs 6700 computer was used to analyse retention coefficients of amino acids. Programme 1, written in Pascal language, was used to perform repetitive regression analysis via a forcing routine. Programme 3, written in Algol language, was used to perform a mathematical analysis by solving linear equations. A subroutine (S/LINEANIMPRV, Burroughs 6700 numerical analysis programme library) written in Fortran was included in Programme 3 to solve linear equations by Gaussian elimination with partial pivoting. Programme 2, written in Pascal was used to convert the input file of programme 1 to the input file for programme 3. Therefore only one input file is needed for these two different methods of analysis.

RESULTS AND DISCUSSION

Theoretical Considerations.

In most previous RP-HPLC studies, the chromatographic process has been viewed as a series of reversible associations between the solute molecules, S_1, S_2 ... S_n and the hydrocarbonaceous ligand, L. In the absence of electrostatic or hydrogen bonding effects, the solute-stationary phase interactions will thus be characteristed by a set of equilibrium association constants, K_1, K_2, ... K_n with solute retention determined solely be the nature of the solvophobic solute-ligand associations. Solute retention in RP-HPLC is usually expressed in terms of the capacity factor, k', which is proportional to the equilibrium association constant, such that in the general case the capacity factor for the solute, S_i, is given by

$$k_i' = \psi \cdot K_i \qquad \ldots.(1)$$

$$\text{where} \quad K_i = \frac{[S_i L]}{[S_i][L]} \qquad \ldots.(2)$$

and ψ is the phase ratio, (volume stationary phase)/(volume mobile phase).

Under conditions of ideal linear liquid-liquid chromatography, column selectivity between two peptides, S_i and S_j, can be expressed as

$$\ell n\ \alpha_{i,j} = \ell n\ k'_i/k'_j \qquad \ldots.(3)$$

Since the separation of peptides by classical liquid-liquid partition chromatography and RP-HPLC has a similar physico-chemical basis, it is thus possible to relate selectivities to both the partition and the association coefficients, i.e.

$$\ell n\ \alpha_{i,j} = \ell n\ P_i/P_j = \ell n\ K_i/K_j \qquad \ldots.(4)$$

where P_i and P_j are the partition coefficients and K_i and K_j the association coefficients of the peptides, S_i and S_j, respectively for a particular mobile phase-stationary phase combination. The liquid-liquid partition model presupposes that the bonded hydrocarbonaceous ligand acts as a bulk liquid. As has been pointed out in several studies [14-16], bonded monolayers of octyl- or octadecyl-phases differ from ideal liquids due to the relatively ordered nature of the alkyl chains. However, determination of the Langmuir adsorption isotherms for a number of organic solvent-water systems in contact with hydrocarbonaceous phases has shown [17-19] that the organic solvent is distributed between the mobile and stationary phases. The non-polar phase thus takes on the characteristics of a dynamically coated support which bears a close surface similarity to nonbonded, physically coated classical liquid-liquid partition chromatographic systems.

If we consider two peptides of similar sequence differing only by one amino acid residue, then the group retention contribution τ due to the different amino acid can be defined as

$$\tau = \ell n\ \alpha_{i,j} \qquad \ldots.(5)$$

where α is the selectivity coefficient of the two peptides, S_i and S_j. The τ contribution is thus a function of the differences in the overall standard unitary free energy changes associated with the transfer of the peptide solutes from the mobile to the stationary phase. Solvophobic theory, as elaborated by Sinanoglu and coworkers [20], has been successfully adapted by Horvath et al. [21] to permit an evaluation of these free energy terms. According to this approach, the surface area, ΔA_s, of the solute molecule in contact with the non-polar stationary phase plays a significant role in determining the magnitude of the hydrophobic interactions. Since linear free energy relationships are also anticipated between bulk phase partition parameters and functional group contributions, linear relationships should exist between retention behaviour, as expressed by $\ell n\ k'$ values, and the hydrocarbonaceous surface areas of the solutes. Under a defined set of chromatographic conditions selectivity can be evaluated from an analysis of the differences in effective hydrophobic contact areas, i.e. from an analysis of the $\Delta(\Delta A_s)$ terms. Experimental studies with non-polar solutes, weak acids and weak bases have generally been in good agreement with these theoretical considerations. For example, RP-HPLC studies with benzoic acids have revealed [22] a linear dependence of selectivity parameters on topological indices, the general form of this relationship being

$$\ell n\ \alpha_{i,j} = a\rho_{i,j} + b \qquad \ldots.(6)$$

where $\rho_{i,j}$ is an appropriate functional group contribution such as the Hansch π terms, the Rekker hydrophobicity fragmental constants or analogous terms. The retention of amino acids and small peptides appears [23] to follow similar dependencies when eluted isocratically from alkylsilicas with low pH mobile phases.

In many cases, the separation of complex mixtures of peptides by RP-HPLC requires gradient elution conditions. Over the range 0-100% organic solvent modifier, binodal dependencies of $\ell n\ k'$ values for peptides and polypeptides on the volume percentage of the organic solvent have been observed [24-26]. Over the operational limits commonly employed in gradient elution of peptides from alkylsilicas with such solvent modifiers as acetonitrile or 2-propanol, i.e. up to ca 50% organic solvent content, the dependence of $\ell n\ k'$ on the volume fraction, ψ, can however be approximated to a linear relationship [2,5,9]. As a consequence, under linear solvent strength gradient elution conditions encompassing this restricted range of mobile phase compositions, the solvophobic model anticipates linear increases in k' (apparent) as amino acid residues are added in an ordered manner to a homologous peptide series, i.e. the k' (apparent) value of a peptide can be expressed in terms of summated group contributions such that

$$k'_{i,app} = \Sigma^{n} c_n \chi_n + d \qquad \ldots(7)$$

where c_n is a numerical factor indicating the incidence of a given fragment in the structure and χ_n represents the group retention contribution due to amino acid, n. In the ideal circumstance when only solvophobic interactions mediate the retention process the intercept term, d, should have the value zero. When the sorption process involves competing polar and non polar equilibrium interactions, the intercept term, d, may

diverge from zero. Curvilinear relationships between k'_{app} (actual) and k'_{app} (predicted) will also result in these circumstances. Using data obtained with 72 different solutes (Table 1),chromatographed on the same μBondapak C_{18} column under identical elution conditions, the assumptions explicit to the solvophobic model have been examined using two methods of numerical analysis, namely multiple regression via an iterative forcing procedure and by a mathematical routine for solving linear equations. Both these methods of numerical analysis can be expanded to include memory parameters which accomodate amino acid residue triplet orders and hence recognise sequence features. Since these expanded subroutines would require for the common protein amino acids at least 9261 data points, we have limited the present analysis to non-isomeric peptides.

Forcing Approach.

The statistical framework of multiple regression analysis with forcing, i.e. the introduction of a few arbitarily chosen data points such that the intercept term, d, has a negligible value and the predicted and actual values are equal, has previously been applied [12,13,22] for the evaluation of sets of equations of the type of eqn. 7 to obtain the desired set of hydrophobic group contribution coefficients for polar solutes. Meek has used [6] this approach to derive retention coefficient values for peptides chromatographed on a BioRad ODS support. In the present study, the starting values for the group retention contribution, χ_n, for the 26 amino acids and end groups were obtained by plotting the k' (apparent) values of oligomeric peptides versus the number of residues, n. The slope of these plots equals the amino acid group retention contribution per residue, the intercept at n=0 represents the end group contribution. In other cases, starting values of the group retention contribution were computed from data reported

TABLE I.

Linear Sequences of Peptides used in the Present Study*

1. LW
2. LWMRF
3. LWMR
4. LWM
5. RF
6. RFA
7. MRF
8. MRFA
9. AY
10. PY
11. LY
12. VY
13. YYY
14. AK
15. YTPKA
16. KG
17. AG
18. GV
19. GF
20. VL
21. FA
22. GLY
23. VV
24. DRVYIHPFHL
25. DRVYIHPF
26. GIVEQCCASVCSLYQLENYCN
27. FVNQHLCGSHLVEALYLVCGERGFFYTPKA
28. FVQWLMNT
29. GLA
30. HSQGTFTSDYSKYLDSRRAQDFVQYLMNT
31. FPTIPLSR
32. LFDNAMLR
33. AHR
34. LHQLAFDTYEEFEEAYIPK
35. EQK
36. YSFLQDPETSLCFSESIPTPSNRNYGLLYCFR
37. EETQK
38. SNLQLLR
39. ISLLLIQSWLEPVEFLR
40. SVFANSLVYGASNSDVYDLLK
41. DLEEGIETLMGR
42. LEDGSPR
43. TGQIFK
44. QTYSK
45. ETYSK
46. FDTNSHNDDALLK
47. DMDKVETFLR
48. IVQCRSVEGSCGF
49. LHQLAFDTYEEFDPETSLCFSESIPTPSNRNYGLLYCFR
50. DRVYIHP
51. FF
52. FFF
53. FFFF
54. FFFFF
55. YGGFLTSEKSQTPLVTLFKNAIIKNAHKKGQ
56. YGGFMTSEKSQTPLVTLFKNAIIKNAHKKGQ
57. YGGFMTSEKSQTPLVTL

* The one letter code for the amino acids is as given by Dayhoff in Atlas of Protein Sequence and Structure (National Biomedical Research Foundation, Silver Spring, Md., U.S.A., 1972), see also Table II. Also included in the compilation of χ_n values were additional chromatographic data for 15 amino acids and homologues.

by Meek [6,7]. With the set of peptides listed in Table I, the predicted k′ (apparent) values were then calculated by summing the group retention contribution for each amino acid and end groups. After calculating the correlation coefficient between the predicted and observed k′ (apparent) values, 0.2 (or any other value which can be specified in the imput of programme 1) was added to the χ-value of an amino acid or end group. The predicted k′ (apparent) values of all peptides were then recalculated and new correlation coefficients between these and observed k′ (apparent) values were again calculated. If the new correlation coefficients improved, i.e. greater than the correlation coefficient before modification, then 0.2 (or another appropriate value) was added to the χ-value of that amino acid or end group, otherwise the orginal χ-value was kept. After all 26 amino acids and end groups were tested to see whether modification of the original χ-value was needed, 0.2 (or another appropriate value) was sequentially subtracted from each amino acid χ-value in turn and correlations were again calculated after each substraction to decide whether the change was needed. These two cycles were repeated for the times specified in the input of programme 1, which in general, involved at least 20 repeat entries. At the end of the cycling procedure the slopes of the plots of k'_{app} (observed) versus k'_{app} (predicted) were calculated, the χ-values normalised [6] and the above cycles repeated until optimal correlation was obtained. Fig. 1, shows the plot of k'_{app} (observed) versus k'_{app} (predicted) for the peptide data analysed in this way.

Multiple Linear Equation Approach.

In this approach the group retention contribution of each amino acid is treated as an unknown x_i. Peptides with observed retention times and known amino acid compositions are listed as a series of equations by the computing programme 2 in the form

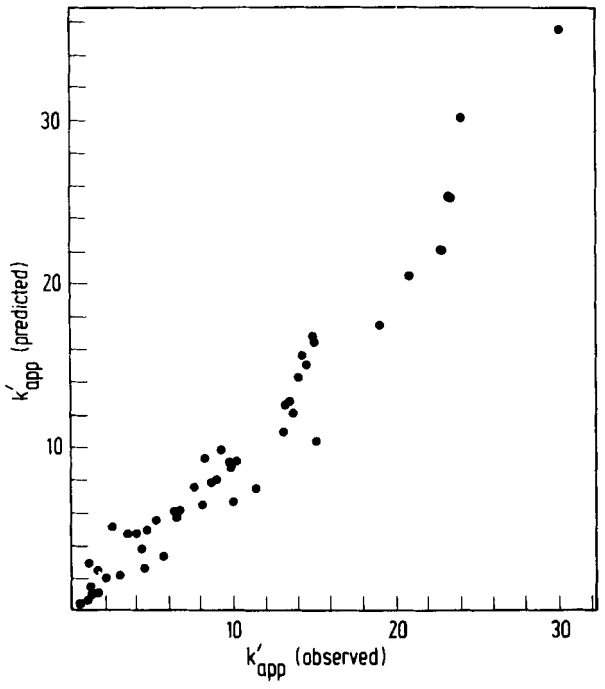

Figure 1. Correlation of observed k´ (apparent) versus k´ (apparent) predicted from the summation of the group retention contributions for amino acids and end groups. Values for the group retention contributions were computed via a forcing approach using chromatographic data for peptides listed in Table 1.

$$a_{11}x_1 + a_{12}x_2 + \ldots + a_{1n}x_n = b_1$$

$$a_{21}x_1 + a_{22}x_2 + \ldots + a_{2n}x_n = b_2$$

$$\vdots$$

$$a_{m1}x_1 + a_{m2}x_2 + \ldots + a_{mn}x_n = b_n$$

Computing programme 3 rewrites these equations in a more usual mathematical form: AX=b where

$$A = \begin{bmatrix} a_{11} & a_{12} & \cdots & a_{1n} \\ & \vdots & & \\ a_{m1} & a_{m2} & \cdots & a_{mn} \end{bmatrix}$$

$$X = \begin{bmatrix} x_1 \\ \vdots \\ x_n \end{bmatrix}$$

$$b = \begin{bmatrix} b_1 \\ \vdots \\ b_n \end{bmatrix}$$

Here we have more equations than the number of unknowns, therefore a unique solution will not usually exist. Because the observed retention times are subject to experimental errors, instead of choosing a particular number, n, from this set of equations, the method of least squares was applied to find a solution. This method uses all the information in the complete set of equations. The solution is obtained by solving $(A^TA)x = (A^Tb)$, which is a set of linear equation with n equations and n unknowns [26]. These equations can then be solved by standard procedures, e.g. by the Gaussian elimination procedure. Fig. 2 shows the plot of k'_{app} (observed) versus k'_{app} (predicted) using the calculated χ_n values for the amino acids and end groups obtained by this procedure.

Comparison of Predicted Versus Observed Retention Behaviour of Peptides.

Several studies have concluded [2,4,5] that the peptide chain proper makes only a very small contribution to the retention process for peptides on reversed phases eluted under

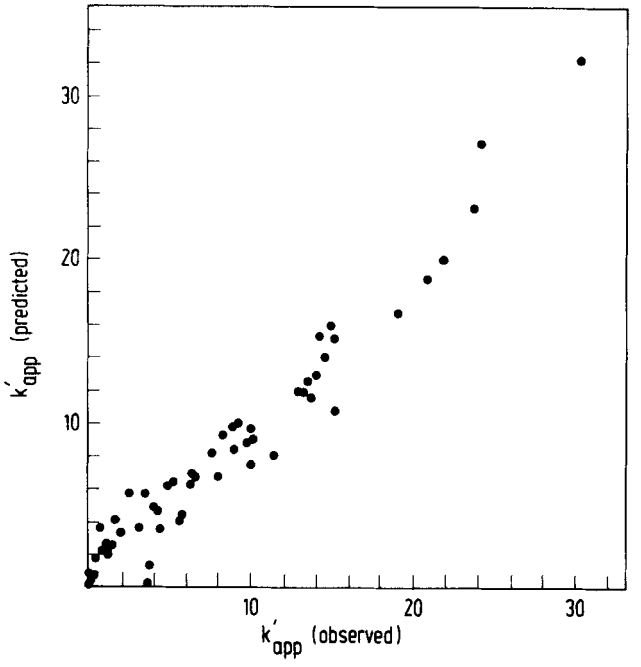

Figure 2. Correlation of observed k´ (apparent) versus k´ (apparent) predicted from the summation of the group retention contributions for amino acids and end groups. Values for the group retention contributions were computed via the multiple linear equation approach using chromatographic data for peptides listed in Table 1.

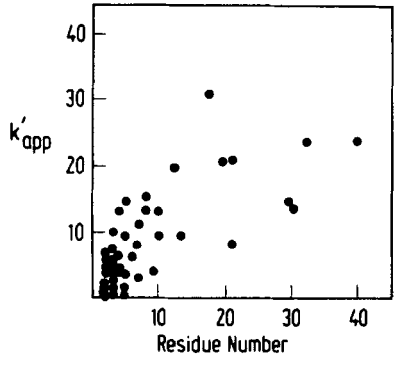

Figure 3. Plot of observed k´ (apparent) versus number of amino acid residues.

low pH conditions with aquo-organic solvent eluents. Furthermore, it has been concluded [2,5,28] that peptide size, per se, has little effect on retention which depends rather on the effective hydrophobic contact area. The trends evident from the data shown in Figs. 1-3 provide further support for these conclusions and the corollary that retention behaviour of a particular peptide follows the extent of its ionisation. For example, as the pH of the mobile phase was increased over the range pH 2.5-7.0 the retention of peptides rich in acidic amino acid residues, e.g. peptides 37, 41, tended to decrease whilst peptides rich in basic amino acid residues, e.g. peptides 6, 30, tended to have increased k´ (apparent) values. Because peptides containing basic amino acid residue experience diminished retention with phosphate mobile phases of low pH, it is likely that the phosphate ion acts via hydrophilic pairing ion effects augmented by the usual dependency of capacity factor on ionic strength.

As is evident from Fig. 1 and 2, a high degree of correlation is obtained between k'_{app} (observed) and k'_{app} (predicted) using the values of χ_n (Table II) derived by both methods of numerical analysis. It is apparent from these and associated studies reported by Meek and coworkers [6,7] that amino acid side chain hydrophobicities are the major solute factor in determining peptide retention to alkylsilicas with low pH eluents containing hydrophilic buffer ions. However, as can be seen from Figs. 1 and 2, and as manifested by the correlation coefficients of 0.973 and 0.972 obtained by the forcing and the multiple linear equation approaches, the fit of the data points to a straight line falls short of the expectations based solely on the solvophobic model. Several of the assumptions made in these calculations may be responsible for the observed divergence from the predicted linear relationships. For instance, no correlation terms have been included in the

TABLE II.

Group Retention Contributions for Amino Acids Residues

Entry	Code	Name	Frequency	χ_n(forcing)	χ_n(solving)
1.	A	Ala	23	-0.296	-0.121
2.	B	nor Val	-	-	-
3.	C	Cys	11	-1.500	-1.252
4.	D	Asp	20	2.171	1.918
5.	E	Gln	28	0.454	0.442
6.	F	Phe	47	2.782	2.522
7.	G	Gly	27	-0.770	-0.816
8.	H	His	13	-3.276	-2.673
9.	I	Ile	17	6.196	5.800
10.	J	nor Leu	-	-	-
11.	K	Lys	24	-0.405	-0.532
12.	L	Leu	53	3.424	3.160
13.	M	Met	9	3.562	3.566
14.	N	Asn	19	-1.687	-1.757
15.	O	hydroxy Pro	-	-	-
16.	P	Pro	19	-0.306	-0.396
17.	Q	Glu	22	-0.651	-0.764
18.	R	Arg	21	-1.184	-1.370
19.	S	Ser	36	0.405	0.664
20.	T	Thr	28	-0.987	-0.790
21.	U	pGlu	-	-	-
22.	V	Val	25	1.253	1.079
23.	W	Trp	2	-0.099	-0.278
24.	X	homo Ser	-	-	-
25.	Y	Tyr	34	1.145	0.896
26.	Z	hydroxy Lys	-	-	-
27.	@ [	NH_2, COOH end group	57	0.829	1.537

calculations to accomodate differences in specific electrostatic and hydrogen bonding interactions which are known to arise during the distribution of ionised peptides between polar mobile phases and hydrocarbonaceous silicas. The heterogeneity of the stationary phase surface of alkyl-bonded silicas has been examined [24,25] in several detailed investigations on peptide selectivity. Even with well 'capped' alkylsilicas of high carbon coverage, unprotected peptides show dual retention behaviour typified by the concave binodal dependence of $\ell n\ k'$ on the volume fraction of water in the mobile phase. This dual retention behaviour has been attributed to composite solvophobic-silophilic interactions. In addition, specific solvation effects dependent on the nature and concentration of the organic solvent modifier can lead[5,9] to individual selectivity divergencies for some peptides. From the scattering of the data points, it is unlikely that these electrostatic, hydrogen bonding or solvation components in the sorption process remain constant for different peptides. A further assumption, as yet little discussed in the literature on peptide separation by gradient elution RP-HPLC, relates to the nature of the gradient shape. For linear solvent strength gradient elution, it is assumed that the plots of $\ell n\ k'$ for each solute S_i, S_j ... versus separation time, t, after the start of the gradient will be linear and will have the same slope. As is evident from gradient elution studies of tryptic digests of proteins [2,29,30] linearity of $\ell n\ k'$ versus t over a wide range of t cannot always be anticipated with peptides.

The results reported here, nevertheless, give ample evidence that the hydrophobic group retention contribution of the amino acid residues in small peptides have an essentially additive effect on peptide retention to octadecylsilicas. With larger peptides, where secondary and tertiary structural features are likely to be important, greater deviations from

an additive effect can be anticipated. With such solutes, the choice of mobile phase composition will have a profound effect on resolution and sample recovery. For example, it is a common experience with larger or more hydrophobic peptides and polypeptides that efficient chromatography can only be achieved on reversed phase silicas over a very narrow range of organic solvent percentages. Because of the participation of competing retention processes, desorption of the intercalated domains of polypeptides and proteins from the solvated surface of the stationary phase will only be possible with mobile phases of sufficient elutropic strength to overcome the sum of the hydrophobic interactions without augmenting polar solute-stationary phase interactions. Gradient elution conditions can generally be chosen to satisfy this requirement even with macroglobulins which show extreme dependencies of $\ell n\ k'$ on ψ. In an associated study, we have applied these RP-HPLC procedures, and attendant methods of selectivity analysis, to the separation of proteins, including thyroglobulins and protein hormones.

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