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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

XLVI*. SELECTIVITY EFFECTS OF PEPTIDIC POSITIONAL ISOMERS AND OLIGOMERS SEPARATED BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The reversed-phase chromatographic retention behaviour of selected peptide homologues on an octadecylsilica stationary phase has been investigated in isocratic elution experiments using low pH binary mobile phases composed of water and methanol or water and acetonitrile. Good correlation for linear relationships between the logarithmic capacity factor, ln *k'*, and the surface tension, γ, of the eluent were obtained from the experimental data over the organic solvent content range up to ψ_s 0.6. In accord with predictions based on solvophobic considerations, linearity was also observed in the plots of In *k' versus* residue number, n_{res}, of the peptide oligomers over a wide range of ψ_s values. The retention behaviour of peptide positional isomers has also been examined with aqueous acetonitrile mobile phases and the selectivity differences which arise between isomer pairs discussed in terms of specific solvation processes.

INTRODUCTION

At the present time, reversed-phase high-performance liquid chromatography (RP-HPLC) is by far the most important liquid chromatographic technique available for the separation of protected and unprotected peptides'. It is now recognised largely from empirical studies that peptide retention and selectivity on microparticulate chemically bonded alkylsilicas may be manipulated by a variety of mobile phase parameters including the chemical nature and concentration of the added pairing or buffer ions, the pH and the composition and concentration of the organic solvent modifier. Although very polar small peptides can often be eluted from alkylsilicas with neat aqueous eluents of low to intermediate pH values in the absence of hydrophobic surface active pairing ions, in general, binary or tertiary aquo–organic solvent combinations are required if practical chromatographic separations are to be achieved

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As the volume fraction of the organic solvent modifiers increases up to *ca.* ψ_s 0.5. the retention of a particular peptide on an alkylsilica column usually decreases monotonously. In the reversed-phase mode, as the organic solvent content of a **mobile** phase increases, the elution strength of the eluent also increases as a consequence of changes in several of the physical properties of the mobile phase. In particular, variations in the mobile phase water content are associated with changes in the bulk surface tension, γ, the static dielectric constant, ε, and the viscosity, η. Variations in either of the physical parameters γ or ε are anticipated on the basis of solution theory to have important sequelae as far as the retention behaviour of peptides on alkylsilicas whilst changes in η can directly influence separation efficiencies. According to the solvophobic theory as elaborated by Horváth *et al.*[2] the change in the capacity factor, k', for simple organic compounds with change in composition of a binary mobile phase is due primarily to the change in the surface tension and the microscopic cavity factor, κ^e, a parameter which expresses the ratio of the energy changes involved in the creation of a cavity in the eluent with the molecular dimensions of the solute in eluent and in expanding the planar surface of the eluent by the same molecular surface area. In previous studies, we have demonstrated[3,4] that solvophobic theory can also provide a useful theoretical framework to evaluate peptide retention behaviour under regular reversed-phase chromatographic conditions. In the present study, we have investigated further the dependency of the k' values of small peptides on the eluent composition and bulk surface tension. The experimental data verify that over ranges of solvent composition where reversed-phase selectivities prevail (i.e., where peptides are eluted from alkylsilicas in the order of increasing relative hydrophobicities), changes in retention with isocratic changes in solvent composition essentially follow a linear dependency of the logarithmic capacity factor on γ. In addition, the influence of solvent composition on the retention behaviour of several dipeptide positional isomers has been examined,

MATERIALS AND METHODS

The peptides used in this study were purchased from Sigma (St. Louis, MO, U.S.A.) and purified by established RP-HPLC procedures. All amino acids except glycine were of the L configuration. The one letter code for the amino acids is used as given by Dayhoff[5]. Orthophosphoric acid, sodium dihydrogen phosphate, sodium sulphate and sodium hydroxide were obtained from May & Baker (Dagenham, Great Britain). Acetonitrile and methanol were all HPLC grade solvents from Waters Assoc. (Milford, MA, U.S.A.) or Burdick & Jackson Labs. (Muskagon, MI, U.S.A.). Water was double distilled and deionised using a Milli-Q system (Millipore. Bedford, MA, U.S.A.).

The chromatographic data were collected with a Waters Assoc. HPLC system, which consisted of two M6000A solvent-delivery systems, a M660 solvent programmer and a U6K universal injector. The detector was a Waters M450 variable-wavelength UV monitor coupled to a Data module and an Omniscribe dual-channel recorder. All chromatograms were carried out at 20°C. For isocratic elution experiments, all peptides were dissolved in the mobile phase at 1 mg/ml, whilst for gradient elution experiments the initial eluent was employed. Bulk solvents were degas'sed separately under sonication and appropriate mobile phases were prepared and col-

umns equilibrated to operating conditions as described previously[3]. The μBondapak C_{18} columns were obtained from Waters Assoc. The surface tension values for the methanol- and acetonitrile-water isocratic combinations were obtained by established procedures[6,7]. Sample injections were made with Pressure Lok liquid syringes (0–25 μl) from Precision Sampling (Baton Rouge, LA, U.S.A.) or Model 50A syringes from SGE (Melbourne, Australia).

Sample sizes varied between 2 and 5 μg of peptide material. The ionic strength and pH of the phosphate buffers were chosen on the basis of our earlier studies[8,9]. The pH measurements were performed with a glass combination electrode on a Radiometer PHM64 research pH meter. The capacity factors were calculated in the usual way with $NaNO_3$ to calibrate the column, Data were analysed by linear regression routines on a Hewlett-Packard Model 97 programmable calculator. The solvent strength parameters and the capacity factors were calculated as described previously'.

RESULTS AND DISCUSSION

Peptide retention on alkylsilicas is very responsive to changes in eluent composition, particularly variations in the water content. In recent experimental studies with peptides, polypeptides and proteins separated on chemically bonded reversed-phase silicas, concave bimodal dependencies have been observed[8–13] between the logarithmic capacity factors, ln k', and the volume fraction of the organic solvent modifier, ψ_s. This retention behaviour suggests that peptides can independently interact in two different ways with the surface of the alkylsilica stationary phase. One of these processes has been discussed[1,14] in terms of solvophobic phenomena with the retention component envisaged as arising as a consequence of the hydrophobic expulsion of the peptidic solute from a polar mobile phase with concomitant interaction between a hydrophobic domain (or domains) on the surface of the solute and the hydrocarbonaceous n-alkyl ligand. The second process is believed to be due to polar solute-stationary phase interactions and may arise from the interplay of silanophilic effects and hydrogen bonding events due to extracted solvent multi-layers at the surface of the stationary phase. In view of the influence which secondary chemical equilibria are known to have on chromatographic distribution equilibrium, changes in the ionisation state and extent of solvation of the peptidic solute associated with changes in the water content of the mobile phase would also be anticipated to significantly affect the magnitude of the stationary phase interaction for a particular peptide. The simplest form of this dependence of the capacity factor of a peptide, i, on eluent composition for isocratic systems can be expressed as

$$k'_i = k'_{i,w} e^{f(-s\psi_s)} + [k'_{i,0} Bf(\psi_s)]^{1} \quad (1)$$

where $k'_{i,w}$ is the capacity factor of the peptide, P_i, in a neat aqueous eluent; s is the solvent strength parameter for the peptide, P_i, which will depend on the molecular characteristics of the peptide and organic solvent; $k'_{i,0}$ is the capacity factor of the peptide, i, with the limiting less polar solvent and B is a chromatographic system constant. In the more generalised situation, the solvophobic and the silanophilic terms in eqn. 1 should both be weighted by the mole fraction of peptide binding in each mode. Elution conditions can be chosen in RP-HPLC separations of peptides

where selectivity is governed by either solvophobic or polar interactions. Most commonly conditions for regular reversed-phase elution involve mobile phases of a high water content, low pH and low ionic strength, in the absence of hydrophobic surface-active ions. Under these mobile phase conditions, the retention of peptides on reversed-phase silicas decreases as the organic solvent content in the mobile phase increases. Structurally different peptides show regular reversed-phase retention behaviour in so far that their elution order follows the order of increasing relative molecular hydrophobicities[1]. This behaviour is in agreement with a reduced form of eqn. 1 in which the polar adsorption term becomes insignificant. In this reduced form, eqn. 1 can be simplified further to the familiar linearized expression proposed by Snyder and co-workers[15] for the relationship between k' and ψ_s for small neutral and anionic solutes chromatographed on reversed phases, namely:

$$\ln k' = \ln k'_{i,w} - s\psi_s \quad (2)$$

With eluents encompassing the solvent modifier range of practical importance in either isocratic or gradient-elution reversed-phase separations of peptides on alkylsilicas, i.e., $0 < \psi_s < 0.5$, the dependency between $\ln k'$ and ψ_s can be evaluated in terms of this linear relationship although more detailed analysis of the experimental data invariably reveals curvilinear plots[3,9]. In the following theoretical treatment, it is assumed that with mobile phases encompassing a limited organic solvent range such as $0.1 < \psi_s < 0.4$, peptide retention to alkylsilicas is governed by solvophobic effects and that both the electrostatic and hydrogen bonding effects and changes in the entropy of mixing of the mobile phase components remains essentially constant with increasing organic solvent content. Under these conditions and at constant ionic strength, the relationship between the capacity factor, the surface tension, γ, and the relative surface area, ΔA_i, of the peptide molecule, P_i, in contact with the ligand *n*-alkyl chains, can be expressed[2] as

$$\ln k'_i = C + \gamma \cdot \frac{N\Delta A_i + 4.836N^{1/3}(\kappa_i^e - 1)\ V_m^{2/3}}{R\ T} \quad (3)$$

where C is a constant, and N, V_m, R and *Tare* Avogadro's number, the average molar volume of the mobile phase, the gas constant and the absolute temperature, respectively.

The cavity factor term, κ_i^e, is related to the surface area of peptide P_i and in fact can be defined as the ratio of energy required to create a cavity for a solvent molecule to the energy required to extend the planar surface of the mobile phase by the surface area of the peptide, P_i. In addition, for water-organic solvent combinations the term C in eqn. 3 is largely related to the Van der Waals component of the free energy of interaction of the peptide, P_i, with the mobile phase. Both the κ^e and the C term will thus vary with different organic solvent modifiers and change with the molecular size of the solute. Such relationships account, in part for the very steep dependencies of $\ln k'$ on γ and the very large extrapolated $k'_{i.w}$ values observed with larger polypeptides and proteins[8,9]. Previously we have shown" that with low-pH mobile phases, similar to those employed in the present study peptide retention was not significantly influenced by small changes in ionic strength. However, at relatively high ionic

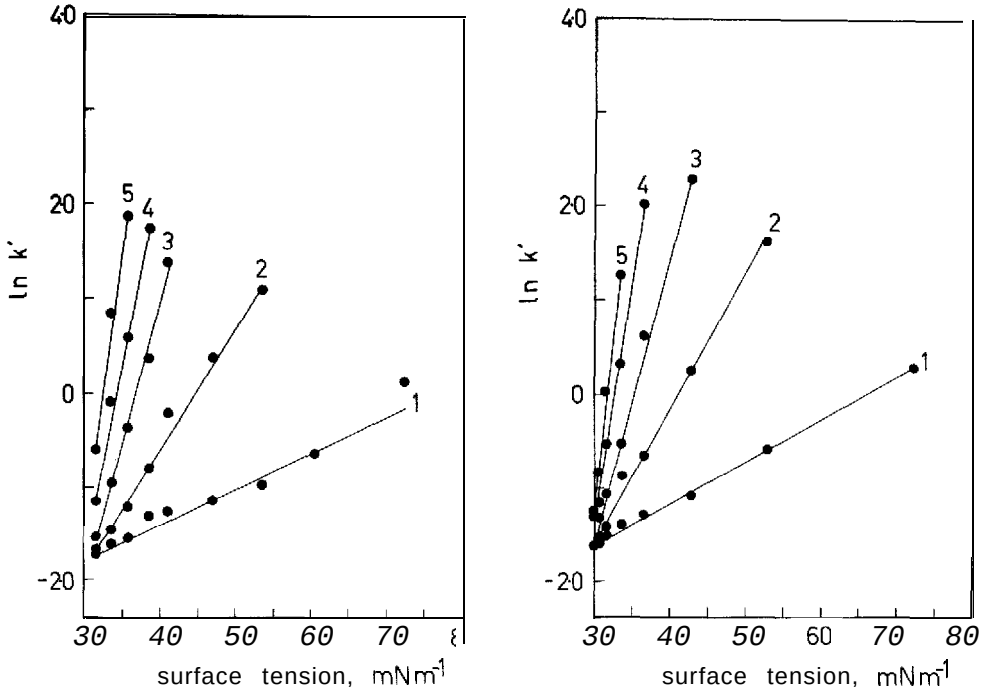

Fig. 1. Plot of the logarithmic capacity factor *versus* surface tension for several phenylalanine oligomers. Chromatographic conditions: column, μBondapak C_{18}; flow-rate, 2 ml/min; mobile phase, methanol–water-20 mM orthophosphoric acid, isocratic elution. with the methanol content adjusted between measurements. Peptides: 1 = F; 2 = FF; 3 = FFF; 4 = FFFF; 5 = FFFFF.

Fig. 2. Plot of the logarithmic capacity factor versus surface tension for several phenylalanine oligomers. The chromatographic conditions are the same as in the legend of Fig. 1 except that acetonitrile replaced methanol.

strengths, retention is expected to respond to variations in ionic strength since the surface tension of the eluent increases with the concentration of inorganic salts and both the cavity factor and the electrostatic terms are also affected. When retention is governed by solvophobic interactions, plots of ln k' *versus* γ are anticipated from eqn. 3 to be linear, with chromatographic selectivity, $\alpha_{i,j}$, for two peptides, i and j, separated under the same isocratic conditions directly related to $\Delta\Delta A_{i,j}$ and κ^e. Since pure water has the largest γ value of the common solvents, as the volume fraction of the organic solvent modifier increases, both ln k' and γ will decrease. When the $\Delta\Delta A_{i,j}$ term remains constant, as may occur, for example with a homologous series of peptides at the same level of ionisation and conformational state, plots of In k' *versus* residue number, n, should also be linear. The present study provides experimental verification of these retention relationships for methanol- and acetonitrile-based eluents (over the range $0.0 < \psi_s < 0.5$) with several homologous peptides and tyrosinyl dipeptide isomers. These model peptides were chosen because greater deviation in retention behaviour due to solvent-mediated changes in ionisation or buffer ion interactions can be expected with small peptides compared to larger polypeptides where very steep dependencies between ln k' snd ψ_s often exist[8,9,13].

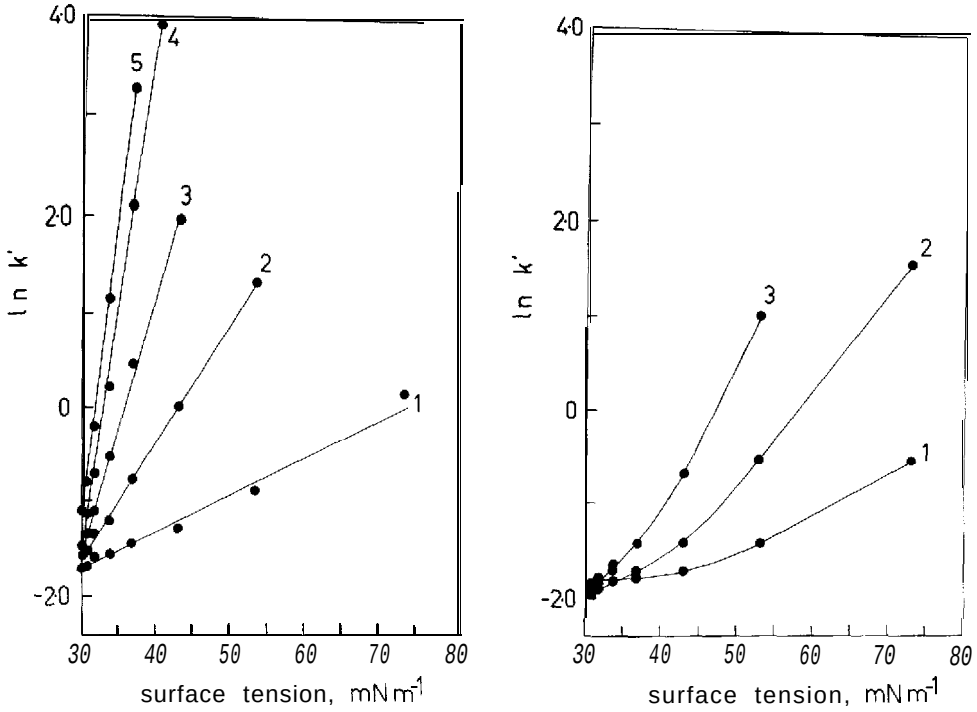

Fig. 3. Plot of the logarithmic capacity factor *versus* surface tension for several phenylalanine oligomers. The chromatographic conditions were the same as in the legend to Fig. 2 except that the primary mobile phase was water-4 m*M* sodium sulphate–15 m*M* orthophosphoric acid.

Fig. 4. Plot of the logarithmic capacity factor *versus* surface tension for several tyrosine homologues. The chromatographic conditions were the same as given in the legend to Fig. 3. Peptides: 1 = Y; 2 = YY; 3 = YYY.

Figs. 1 3 and Fig. 4 respectively show plots of In k' *versus* γ for several phenylalanine and tyrosine homologues eluted under low pH isocratic conditions from μBondapak C_{18} columns using aqueous methanol or acetonitrile mobile phases. As is evident from these figures, In k' exhibits an essentially linear dependency on γ over the range *ca.* 30–75 mN m^{-1}. Interestingly, the slopes of the plots of ln k' *versus* γ obtained with the phenylalanine homologues for the two acetonitrile systems (Table 1) did not greatly diverge despite the differences in added sulphate and phosphate buffer ion concentration. This suggests that the influence of polar hydrophilic ions such as sulphate and phosphate on peptide retention are essentially constant over the concentration range of acetonitrile examined. However, calculation of ln k' at $\psi_s = 0$, i.e., ln k'_w, according to this linear dependency does indicate subtle variation in the estimated In k'_w values due to difference in ionic strength of the neat aqueous eluent in each case, the predicted trend being in accord with available experimental data. These observations may be useful contrasted with the cooperative effects noted[13,16] in the RP-HPLC of proteins where conformational unfolding processes can also be induced by these ions.

Differences in regular reversed-phase retention of amino acids or peptides on a

TABLE I

CORRELATION OF COMPARATIVE RETENTION BEHAVIOUR FOR SMALL PEPTIDES WITH ELUENT SURFACE TENSION

The logarithmic capacity factor in a neat aqueous eluent, $\ln k'_w$, with $\psi_s = 0$ was calculated at 73 mN m^{-1} according to a modified form of eqn. 3, namely $\ln k' = a\gamma + C$. The slope and correlation coefficient, r^2, of this linear dependency was determined by linear regression.The one letter code for the amino acids is F = phenylalanine, Y = tyrosine. Elution systems: 1, methanol-water-20 m*M* orthophosphoric acid; 2, acetonitrile-water-20 m*M* orthophosphoric acid; 3, acetonitrile-water-4 m*M* sodium sulphate-15 m*M* orthophosphoric acid. In each case, the percentage of the organic solvent modifier was varied over the range 0–56%.

Peptide	*Elution system 1*			*Elution system 2*			*Elution system 3*		
	ln k'w	*Slope*	r^2	*ln k'w*	*Slope*	r^2	*ln k'w*	*Slope*	r^2
F	-0.04	0.04	0.967	0.29	0.04	0.994	0.08	0.04	0.985
FF	3.68	0.13	0.988	4.47	0.14	0.994	3.86	0.13	0.999
FFF	10.72	0.30	0.991	11.52	0.30	0.996	10.35	0.28	0.997
FFFF	15.31	0.40	0.998	20.14	0.50	0.996	17.21	0.43	0.983
FFFFF	23.03	0.57	0.982	28.09	0.68	0.997	27.02	0.68	0.999
Y							-0.55	0.04	0.999
YY							1.57	0.11	0.999
YYY							4.35	0.17	0.999

defined alkylsilica with different isocratic elution systems where $\psi_s < 0.5$ can be interrelated',” through the expression

$$\ln k'_{i,m} = z \ln k'_{i,n} + e \qquad (4)$$

where $k'_{i,m}$ and $k'_{i,n}$ are the capacity factors in elution systems m and n respectively and z is the relative eluotropic strength parameter. Table 11 lists the relative differences in the elution strength ($z_{relative}$) of isocratic mobile phases of different methanol or aceto-

TABLE II

RELATIONSHIP BETWEEN RELATIVE ELUOTROPIC STRENGTH AND SOLVENT PERCENTAGE DETERMINED WITH THE PHENYLALANINE PEPTIDES

The value of the relative eluotropic strength, z, was arbitrarily taken as unity for water-acetonitrile at 8 % and water-methanol at 16 % respectively.

Percentage solvent	$z_{relative}$	
	Organic solvent	
	Methanol	*Acetonitrile*
8	–	1.0
16	1.0	1.08
24	1.37	1.57
32	1.41	2.82
40	1.60	4.23
48	1.82	9.17
56	2.31	15.71

nitrile content derived from data obtained with the phenylalanine peptide series. As the acetonitrile content is increased from 16 to 56 %, there is a calculated change in relative eluotropic strength between the two isocratic mobile phases of *ca.* 14.5. The corresponding value for methanol over the same percentage range was *ca.* 2.3. The magnitude of these values is compatible with the incremental changes in retention observed[3] in the plots of In k' *versus* ψ_s for these same peptides with low-pH mobile phases containing either acetonitrile or methanol over the range $0 < \psi_s < 0.6$.

It is apparent from the above data that plots of ln k' *versus* γ (or alternatively ln k' *versus* ψ_s) can be used to evaluate relative differences in peptide retention as well as differences in the eluotropic strength parameters of various mobile phases now commonly employed in the RP-HPLC separation of peptides. At the present time, one of the major impediments to the quantitative translation of retention behaviour for different peptides from one set of chromatographic conditions to another has been the lack of reliable system coefficients such as those which can potentially be derived from In k' *versus* γ determinations, contact angle measurements or Hildebrand solubility parameter indices. Barford *et al.*[18] have addressed this issue in a recent study on several proteins whilst Van Oss *et al.*[19] have also considered a similar solubility parameter *versus* solvent composition approach to evaluate hydrophobic effects associated with "salting out" phenomena. Clearly, detailed evaluation of retention behaviour of peptides in terms of surface tension relationships and eluotropic strength-value dependencies would be most useful for the selection and optimisation of mobile-phase parameters for isocratic and gradient elution RP-HPLC of peptides. For example, for a gradient elution system where $d\psi_s/dt$ is constant and the ψ_s value of the terminating mobile phase is < 0.6, the relationship between the apparent capacity factor, k'_{app}, and γ can be approximated[3,9] to a linear function. As can be seen from Fig. 5, the plot of k'_{app} *versus* ψ_s for the phenylalanine series is in good agreement with this linear approximation. It is, however, worth noting from Fig. 5 that under conditions where $d\psi_s/dt$ is constant, which in the present case was 0.83 % per min, resolution may not necessarily be optimal for peptide components emerging later. How-

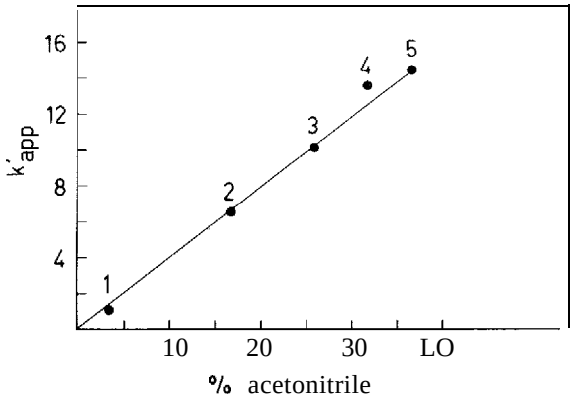

Fig. 5. Plot of k'_{app} *versus* volume fraction of the organic solvent modifier for several phenylalanine oligomers. Chromatographic conditions: column, μBondapak C_{18}; flow-rate, 1 ml/min; mobile phase, linear gradient of water-50 m*M* sodium dihydrogen phosphate-15 m*M* orthophosphoric acid (A) to 50 % acetonitrile in A in 60 min. Peptides: 1 = F; 2 = FF; 3 = FFF; 4 = FFFF: 5 = FFFFF.

ever, this can be achieved with linear solvent strength (LSS) gradients where the gradient slope is adjusted to accommodate differences in peptide *s* values[9].

It has been commonly argued that the retention behaviour of small- to medium-sized peptides reflects little or no secondary or tertiary structure under reversed-phase chromatographic conditions. Several predictive approaches for peptide retention have been developed[4,20], based on the assumption that retention can essentially be related to amino acid composition, with secondary or tertiary conformational effects playing an insignificant role. In these treatments, peptide retention has been equated with the summated hydrophobic influence of all the amino acid residues with linear plots for ln *k' versus* residue number, n_{res} an anticipated corrollary for oligomers. As is evident from Fig. 6a and b, In ***k'*** does exhibit an essentially linear dependency on n_{res} for the phenylalanine series over a wide range of ψ_s values. Similar observations have been briefly reported' [7,21,22] with other amino acid homologues. However, the data shown in Figs. 6 and 7 also indicate that divergences from the expected linear behaviour may arise at inappropriate isocratic ψ_s values where a particular peptide becomes unretained or where polar interactions with the stationary phase become more pronounced. Further, at higher concentrations of organic solvent in the mobile phase decreases in the relative selectivities are evident. This loss of selectivity at intermediate to high organic solvent concentrations has also been observed' with polypeptides. Since similar divergencies have been found[23,24] with homologous series of aliphatic alcohols and barbiturates, this anomalous retention be-

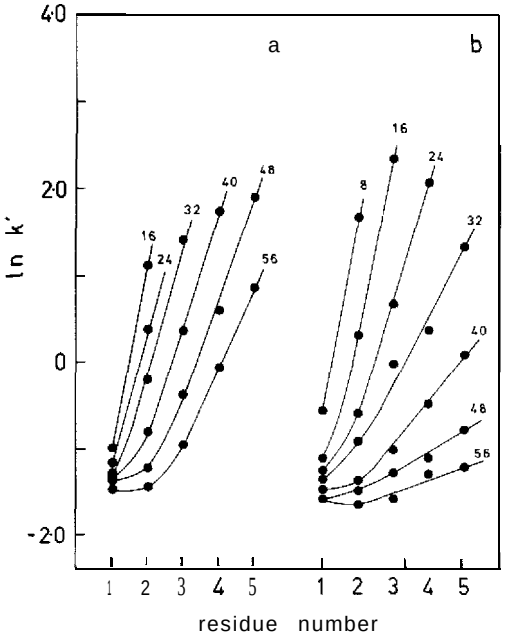

Fig. 6. a, Plot of the logarithmic capacity factor *versus* residue number, n_s, for several phenylalanine oligomers at different methanol compositions. Chromatographic conditions were the same as given in the legend to Fig. 1. b, Plot of the logarithmic capacity factor versus residue number, n_{res}, for several phenylalanine oligomers at different acetonitrile compositions. Chromatographic conditions were the same as given in the legend to Fig. 2.

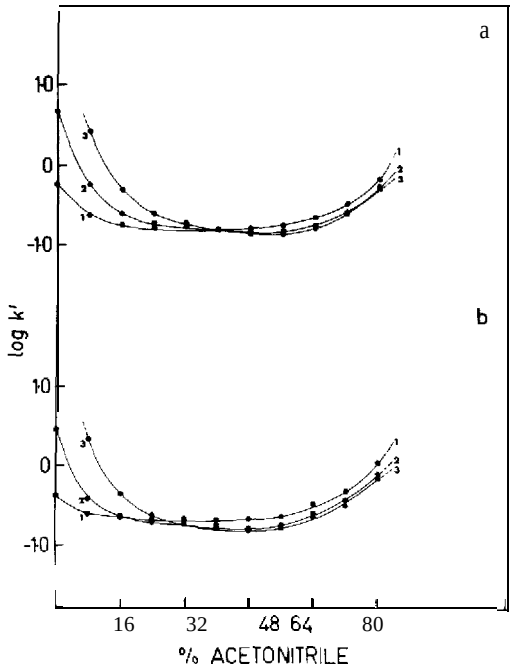

Fig. 7. Plots of the logarithmic capacity factors of Y (1), YY (2) and YYY (3) against the volume fraction of the organic solvent in water-acetonitrile isocratic mobile phases. The chromatographic conditions were: column, μBondapak C_{18}; flow-rate, 2 ml/min, mobile phases water- 4 mM sodium sulphate-15 mM orthophosphoric acid (a) and water-4 mM sulphuric acid-15 mM orthophosphoric acid-15 mM triethylamine (b), the acetonitrile content being adjusted over the ψ_s range 0.0–0.8.

haviour cannot be considered solely as a solute-specific phenomenon but rather an expression of the dual retention mechanism exhibited by silica-based reversed phases. Interestingly, the slope of the plots $\ln k'$ *versus* n_{res} shown in Fig. 6a and b were consistently steeper with methanol systems compared to acetonitrile systems at the same volume fraction, a result in accord with known differences in the eluotropic values of these two solvents.

Figs. 7 and 8 show the relationship between $\ln k'$ and ψ_s for several tyrosine homologues and various tyrosinyl positional isomer dipeptide pairs respectively. Compared to larger polypeptides eluted under the same mobile phase conditions[8,9,12] the slope curvature parameters and the k'_w values, of these small peptides are considerably smaller (Table 111). For these experiments, the peptides were chromatographed on μBondapak C_{18} columns with either aqueous 4 mM sodium sulphate–15 mM orthophosphoric acid, or with aqueous 4 mM sulphuric acid–15 mM orthophosphoric acid-l 5 mM triethylamine, 'the acetonitrile content being adjusted over the ψ_s range 0.0–0.8. In all cases, the plots of $\ln k'$ versus ψ_s pass through minima, characteristic selectivity reversals from a reversed-chase separation mode to a polar phase separation mode being clearly evident. With this selection of positional isomer dipeptides both YV and YA are eluted before their counterparts VY and AY with water-rich eluents with an elution order reversal evident at high acetonitrile compositions. However, the opposite reten-

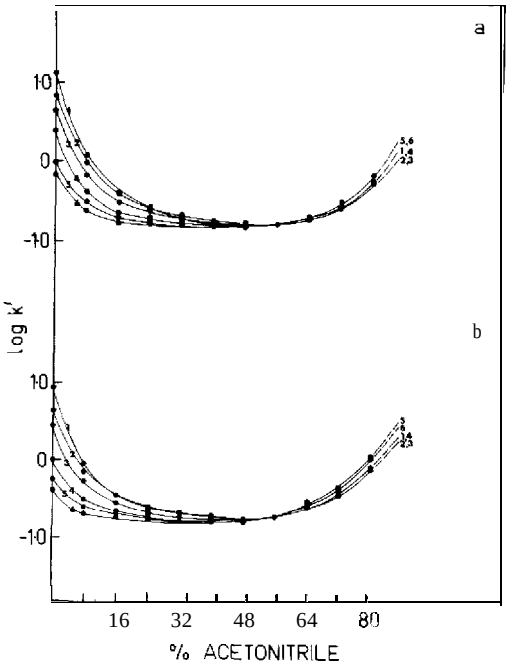

Fig. 8. Plots of the logarithmic capacity factors of several dipeptide positional isomers *versus* the volume fraction of **the** organic solvent in water-acetonitrile isocratic mobile phases. The chromatographic conditions are given in the legend to Fig. 7. Peptides: 1 = VY; 2 = YL; 3 = LY; 4 = YV; 5 = AY; 6 = YA.

tion behaviour was 'found for the pair YL and LY. YL being eluted less rapidly than LY at lower solvent content. A possible explanation for the retention behaviour seen with these isomeric pairs of dipeptides when water-rich eluents are used may be found in the preferential aqueous solvation of the protonated amino group attached to the more hydrophobic residue. Under the two mobile-phase conditions used, both the N-terminal amino group and the phenolic hydroxyl group of a tyrosinyl dipeptide will be fully protonated. The relative hydrophobicities of the respective component amino acids of the selected dipeptides follow the order A < V < Y < L[4,20,21]. Thus. if we consider. for example, the peptide pair LY and YL we anticipate that the H^+L moiety is more highly solvated than the H'Y moiety, and as a consequence, H^+LYOH will be eluted before H^+YLOH. A similar rationale can be applied to the other peptide pairs. These retention behaviours and the attendant selectivity reversals at higher solvent concentrations are reminiscent of the changes in elution order observed[25–28] previously with these and similar dipeptides in studies on the influence of pH on k' values. For example, at high pH the peptide Gly-Leu is eluted from a reversed-phase column before the peptide Leu-Gly, but the reverse is found at low pH. With aquo-organic eluents, the extent of ionisation of a particular peptide will vary according to the solvent composition due to a non-linear dependency of protonic equilibria on the dielectric constant of the mobile phase and the participation of specific solvation effects. Solvation processes have a direct bearing on ionisation constants of amphoteric compounds, with the derived p*K* values of peptides responsive to the particular organic solvent used. As has been discussed else-

TABLE III

RELATIVE RETENTION PARAMETERS FOR SMALL PEPTIDES

Calculated according to eqn. 2 by **regression** analysis of the retention data, regression coefficients were $r^2 > 0.95$. n.e. indicates peptide is not eluted by a neat aqueous eluent from μBondapak C_{18} columns.

Peptide	*In k'_w (observed)*	*Elution system*	
		In k'_w (calculated)	*s* value
F	0.15	0.05	
FF	3.0	2.01	5.8
FFF	n.e.	5.07	8.3
FFFF	n.e.	7.60	10.5
FFFFF	n.e.	9.90	12.2
YL*	1.90	1.73	7.8
LY*	1.49	1.31	7.3
YA*	-0.38	-0.49	3.8
AY*	-0.05	-0.15	4.3
YV*	0.88	0.67	6.4
VY*	2.55	2.34	9.5

* Determined over the range $0 < \psi_s < 0.16$.

where[1,14], significant retention and selectivity changes can occur in the RP-HPLC of peptides and other ionogenic solutes when the mobile phase pH corresponds to a value near to a p*K* value. Specific solvation of the stationary phase via selective multilayer extraction of the organic solvent modifier will give rise to variations in the apparent dielectric constant of the stationary phase, and this in turn will influence the retention behaviour of ionised peptide solutes. The influence of specific solvation is also highly relevant to the estimation of peptide retention on alkylsilicas since with adequate quantitation solvation effects could be included into existing forcing or quadratic expansion routines[4,22] used to compute peptide retention with indices derived from topological relationships. Clearly at this stage, further study is needed to evaluate the role of specific solvation in peptide and protein separation by RP-HPLC before such indices can find wide acceptance and reliable usage.

In summary, the experiments described in this paper validate the predicted dependency, based on solvophobic considerations, of peptide retention on the bulk surface tension of binary mobile phases in RP-HPLC. As a consequence, it should also prove feasible to estimate peptide retention with alkylsilicas when multi-component eluents are used, provided the surface tension *versus* composition relationships are known. In addition, our study has further indicated the importance of solvation phenomena in the control of peptide selectivity in RP-HPLC separations.

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