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# High-performance liquid chromatography of amino acids, peptides and proteins

## CX[a]. Principal component analysis of four sets of group retention coefficients derived from reversed-phase high-performance liquid chromatography of peptides

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ABSTRACT

Principal component analysis procedures have been used to further characterise peptide retention behaviour in reversed-phase (RP) high-performance liquid chromatography. In particular, the analysis was performed with four new scales of group retention coefficients (GRCs) in conjunction with fourteen physicochemical descriptors of the side chain functionalities of the twenty naturally occurring amino acids. The results demonstrate a negative correlation between GRCs derived from peptide retention data with RP-C18 or RP-C8 stationary phases and aqueous trifluoroacetic acid–acetonitrile solvents and amino acid parameters which describe electronic characteristics. Conversely, there was a positive correlation between GRCs derived from peptide retention data with a RP-C18 stationary phase and aqueous trifluoroacetic acid–acetonitrile–2-propanol mixtures and amino acid parameters related to steric and volume characteristics. These results are interpreted in terms of the solvophobic theory. The relevance of quantitative structure–retention relationships to the mechanistic basis of peptide and protein interactions with chromatographic surfaces is discussed.

## INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) has in recent times become the most widely used technique for the purification of peptides and polypeptides. In addition, the use of RP-HPLC has enabled various physicochemical parameters associated with peptide and protein surface interactions and folding hierarchies to be evaluated rapidly and quantitatively [1].

Despite the ever increasing usage of RP-HPLC for the separation and analysis of peptide and proteins fully developed mechanistic models are not yet available to describe the interactive processes that occur between the non-polar stationary phase,

[a] For part CIX, see ref. 23.

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the mobile phase and the peptide or protein solute. The development of such physical models would provide the basis for significant improvement in the practical application of optimisation protocols. In addition their development would enable elucidation of the key parameters that control the physicochemical basis of peptide and protein binding to hydrocarbonaceous surfaces.

Solvophobic theory, as developed by Sinanoglu and co-workers [2–6] and adapted by Horváth and co-workers [7,8] for isocratic reversed-phase chromatography, endeavours to provide a mechanistic and thermodynamic basis for the interpretation of solute, solvent and stationary phase interactions in reversed-phase chromatographic systems. While providing insight into the overall free energy, $\Delta G^0$, considerations that control the RP-HPLC retention process, the practical implementation of solvophobic theory can present difficulties. For example, full evaluation of solvophobic parameters requires *inter alia* knowledge of the values of the microthermodynamic surface tension and dielectric constant of the mobile phase. For multicomponent eluents such values are not generally available from literature sources. The lack of practical and accessible mechanistic models has forced most investigators to rely upon non-mechanistic, empirical models to describe peptide or protein retention behaviour with non-porous and porous, chemically modified *n*-alkylsilicas. The most fully developed and available of these models is the Linear Solvent Strength (LSS) gradient model as originally developed by Snyder [9,10]. This model provides a quantitative basis for the evaluation of peptide and protein retention behaviour under ideal reversed-phase conditions and allows a rational selection of chromatographic parameters in order to achieve a set of optimal chromatographic conditions. While the LSS retention model provides a useful basis for the optimisation of peptide or protein separation it does not allow the assessment of the fundamental physicochemical and structural parameters of the biosolute which underlie the retention process.

One approach that can be employed to elucidate the biosolute parameters which control the interaction of peptides and proteins with the reversed-phase chromatographic environment is to examine chromatographically derived amino acid retention coefficients. We have previously developed four new sets of chromatographically derived retention coefficients [11] which allow a more detailed analysis of reversed-phase retention processes involving peptide or protein interactions than previously reported hydrophobicity coefficients. Central to the development of these new group retention coefficients (GRCs) were a number of important considerations, including: (1) each of the four sets of retention coefficients were derived from peptides eluted from different chromatographic systems with well defined aquo–organic mobile phase conditions and *n*-alkylsilica stationary phases, (2) they were derived from the reversed-phase retention data of more than 1300 peptides which in effect produce coefficients that represent the RP-HPLC retention characteristics of each amino acid in a wide range of peptide environments, (3) the retention data used to calculate the coefficients are measures of the dynamic interaction of the peptide with the alkyl chains of the stationary phase, and (4) the large number of peptide examples were required to provide a high level of statistical significance.

The various chromatographic systems included in the development of these new GRCs included three different chemically bonded *n*-alkysilica reversed-phase stationary phases, namely, RP-C18, RP-C8 and RP-C4, and two different organic solvents in

the aquo–organic mobile phase, acetonitrile (ACN) and 2-propanol–ACN. The range of experimental conditions used to derive the GRCs thus allows the influence of these different mobile and stationary phases on the GRCs to be assessed. The derivation and implications of these coefficients have been described in detail previously [11].

This study explores the fundamental physicochemical parameters that underlie the retention process with peptides in each of the four chromatographic systems. Each of the four sets of GRCs were examined with principal component analysis in conjunction with a range of fundamental parameters that describe the various characteristics of the amino acid side chains. This detailed analysis allows the retention coefficients to be resolved down to fundamental parameters that can be related to overall properties of the amino acid side chains in a peptidic environment and a specific chromatographic condition.

## MATERIALS AND METHODS

The GRCs used in this study were derived from the retention data of over 1300 peptides, as described previously [11]. In this study, four sets of GRCs are considered. Each of the four sets of GRCs has been generated from peptides eluted from different chromatographic conditions (as shown in Table I).

These four sets of GRCs were subjected to principal component analysis in conjunction with the parameters described by Fauchere *et al.* [12]. The principal component analysis was performed with use of the SPSS$^x$ program upon the Monash University Vax computer.

## RESULTS AND DISCUSSION

### *Theoretical considerations*

According to the solvophobic theory, the physicochemical basis of the retention processes in RP-HPLC phase is largely a result of incremental changes in the microscopic surface tension. More specifically, the binding process can be visualised in terms of the preferential interaction of the solute with the non-polar surface as a result of its expulsion from the polar mobile phase of higher surface tension. A number of factors such as size and composition of the peptide solute and the precise composition of the mobile phase will therefore impinge upon the binding process.

TABLE I

THE FOUR DIFFERENT CHROMATOGRAPHIC CONDITIONS FROM WHICH PEPTIDES WERE ELUTED TO DERIVE THE GROUP RETENTION COEFFICIENTS AND THEIR ABBREVIATED NAMES

| Abbreviated name | Chromatographic conditions |
|---|---|
| C18 | RP-C18 stationary phase; aqueous trifluoroacetic acid (TFA)–ACN mobile phase |
| C8 | RP-C8 stationary phase; aqueous TFA–ACN mobile phase |
| C4 | RP-C4 stationary phase; aqueous TFA–ACN mobile phase |
| TPA | RP-C18 stationary phase; aqueous TFA–ACN–2-propanol mobile phase |

The retention of a solute in a reversed-phase chromatographic system can be described by the isocratic retention factor, $k'$, such that under near equilibrium conditions:

$$\ln k' = -\frac{1}{RT}\Delta G^0 + \ln\left(\frac{RT}{PV}\right) + \Phi \tag{1}$$

where $\Delta G^0$ is the overall difference in free energy of the solute between the mobile phase and the stationary phase, $V$ is the mean molar volume of the solvent, $P$ is the operating pressure, $T$ the temperature, $R$ the molar gas constant and $\Phi$ is a constant related to the density of the accessible ligands. The $\Delta G^0$ term can be further represented as:

$$\Delta G^0 = \Delta G^0_{\text{cav}} + \Delta G^0_{\text{es}} + \Delta G^0_{\text{vdw}} + \Delta G^0_{\text{assoc}} + \Delta G^0_{\text{red}} \tag{2}$$

where $\Delta G^0_{\text{cav}}$ is the difference between the mobile phase and stationary phase in free energy associated with cavity formation, $\Delta G^0_{\text{es}}$ is the free energy change derived from any electrostatic interactions, $\Delta G^0_{\text{vdw}}$ is the free energy change caused by Van der Waals interactions, $\Delta G^0_{\text{assoc}}$ is the free energy change from ligate–eluite association and $\Delta G^0_{\text{red}}$ is essentially a correction term for non-ideal behaviour that accounts for any other change in the difference in the free energy of the solute moving between the mobile phase and stationary phase.

According to solvophobic theory, the capacity factor of a solute eluted with a particular column and eluent composition can be related to physical and chemical properties of the solute and the mobile phase according to the following expression:

$$\ln k' = A' + B'\left(\frac{1-\lambda}{2\lambda}\right)\frac{\mu_s^2}{v_s}\frac{1}{1-(\alpha_s/v_s)} + C'\Delta A \tag{3}$$

where the constants $A'$, $B'$ and $C'$ are given by

$$A' = \Phi - \frac{\Delta G_{\text{vdw.assoc}}}{RT} + \frac{\Delta G_{\text{vdw.s}}}{RT} + \frac{4.836N^{1/3}(\kappa^e - 1)V^{2/3}\gamma}{RT} + \ln\frac{RT}{PV} \tag{4}$$

$$B' = ND/RT \tag{5}$$

where $N$ is Avagadro's number, and

$$C' = N\gamma/RT \tag{6}$$

The parameter $\lambda$ is defined as:

$$v_{\text{SL}} = \lambda v_s \tag{7}$$

where $v_{\text{SL}}$ and $v_s$ are the molecular volumes of the solute–ligand complex and the unbound solute, respectively. The term $D$ is the dielectric constant of the medium,

while $\mu_s$ and $\alpha_s$ refer to the static dipole moment and the polarisability of the solute, respectively.

Experimentally it has been shown that the solvophobic forces which have the greatest influence on $k'$ are those which are dependent on surface tension, $\gamma$ [7]. Eqn. 3 can therefore be simplified to

$$\ln k' = A'' + B''\gamma \tag{8}$$

where $A''$ is the sum of all the terms present in eqn. 3 which do not contain the surface parameter and

$$B'' = \frac{N\Delta A_h + 4.836N^{1/3}(\kappa^e - 1)V^{2/3}}{RT} \tag{9}$$

The parameter $\kappa^e$ is defined as the ratio of the energy required for the formation of a cavity with a surface area equal to solute areas and the energy required to extend the planar surface of the liquid by the same area. The dependency of $k'$ on the surface tension, $\gamma$ and hydrophobic contact area, $\Delta A_h$, as described by the solvophobic theory has been experimentally validated for low-molecular-weight solutes [7] and small peptides [13]. In the case of small peptides, each constituent amino acid will contribute to the overall retention according to intrinsic physical properties such as hydrophobicity, charge and/or polarity, size and shape. The relative magnitude of the contribution of each residue within a peptide which is intrinsic to the experimental retention data of a peptide solute has been derived in our previous studies from multiple linear regression analysis of a retention data base of over 1300 peptide retention times. These chromatographically derived retention coefficients were therefore subjected to factor analysis to identify the underlying parameters which control the interactive properties of each amino acid residue within a peptidic environment. A summary of the various chromatographic conditions and the abbreviations used to describe these conditions are listed in Table I.

*Statistical analysis of the group retention coefficients*

Principal component analysis is a statistical technique used to identify a relatively small number of factors that can be used to represent relationships among sets of many interrelated variables. The aim of the present study was to derive insight into the molecular and atomic forces behind peptide retention processes in RP-HPLC. Each of the four sets of GRCs were therefore subjected to principal component analysis in conjunction with a range of independently determined amino acid parameters.

Fourteen physicochemical descriptors of the side chains of the 20 naturally occurring amino acids were used and are listed in Table II. It has been shown previously that these parameters describe either steric, volume or electronic characteristics of the amino acid side chains [12]. The first of these steric parameters, $\Xi$, listed in Table II describes the steric influence of the side chain in terms of complexity, branching and symmetry, while $v$ and $v_{reg}$ are a function of the minimal Van der Waals radius. The next five parameters characterise the volume rather than the shape of the side chain. These include values for the length of the amino acid side chain, $L$, the

TABLE II

PARAMETERS DESCRIBED BY FAUCHERE *ET AL.* [12] THAT WERE USED IN THE PRINCIPAL COMPONENT ANALYSIS

| Parameter | Description | Factor |
|---|---|---|
| $\Xi$ | Measure of steric influence, including three attributes: complexity, branching and symmetry | Steric |
| $v$ | A function of the minimum Van der Waals radius | Steric |
| $v_{reg}$ | Smoothed steric parameter | Steric |
| $L$ | Length of the amino acid side chain | Volume |
| $B1$ | Minimum width of the amino acid chain | Volume |
| $B5$ | Maximum width of the amino acid chain | Volume |
| $\alpha$ | Polarisability of the amino acid side chain | Volume |
| $V_v$ | Normalised Van der Waals volume | Volume |
| $\sigma$ | Inductive field effects | Electronic |
| $n_H$ | Number of hydrogen bond donors | Electronic |
| $n_N$ | Number of full non-bonding orbitals on oxygen and nitrogen | Electronic |
| $i_A$ | Number of positive charges | Electronic |
| $i_B$ | Number of negative charges | Electronic |
| $pK_a$ | Dissociation of hydrogens from side chains | Electronic |

minimum and maximum width of the side chain, $B1$ and $B5$, respectively, the polarisability, $\alpha$, which is related to molar refractivity and is a function of the molecular volume, and $V_v$ which is the normalised Van der Waals volume. The remaining six parameters represent descriptors of the electronic properties of the side chains. These include $\sigma$, which describes inductive field effects, $n_H$, the number of OH and NH bonds, $n_N$, the number of full non-bonding orbitals on O and N atoms, $i_A$ and $i_B$, the number of positive and negative charges, respectively and the $pK_a$ of each amino acid side chain. As noted by Fauchere *et al.* [12] these parameters probably do not describe all the possible components of amino acid side chains, and redundancy between individual parameters cannot be ruled out. However, these categories are sufficient to give a reasonable indication of the major parameters that control the chromatographic retention process and to explain the differences between the four sets of retention coefficients.

The initial statistics of the principal component analysis indicated that five factors described approximately 85% of the total variance of the fourteen parameters plus the four sets of retention coefficients. The pattern of factor loading is displayed in Fig. 1. Factor loadings in the rotated factor matrix of less then 0.35 were set to zero. The first factor (factor 1) which includes the TPA set of retention coefficients, also contains parameters that are related to steric and volume parameters of the amino acid side chains. Factors 2 and 4 do not contain any of the retention coefficient sets. However, these factors validate the analysis in that factor 2 represents four of five volume related parameters while factor 4 includes four of the six electronic-related parameters. Factor 3, contains both the C18 and C8 sets of retention coefficients. This factor has a predominantly electronic character including the number of full non-bonding orbitals on oxygen, $n_H$, and the number of positive charges, $i_A$. The results of linear correlation analysis of the four GRC scales are shown in Table III. The C18 and C8 sets of GRCs have a high correlation coefficient and were expected to be

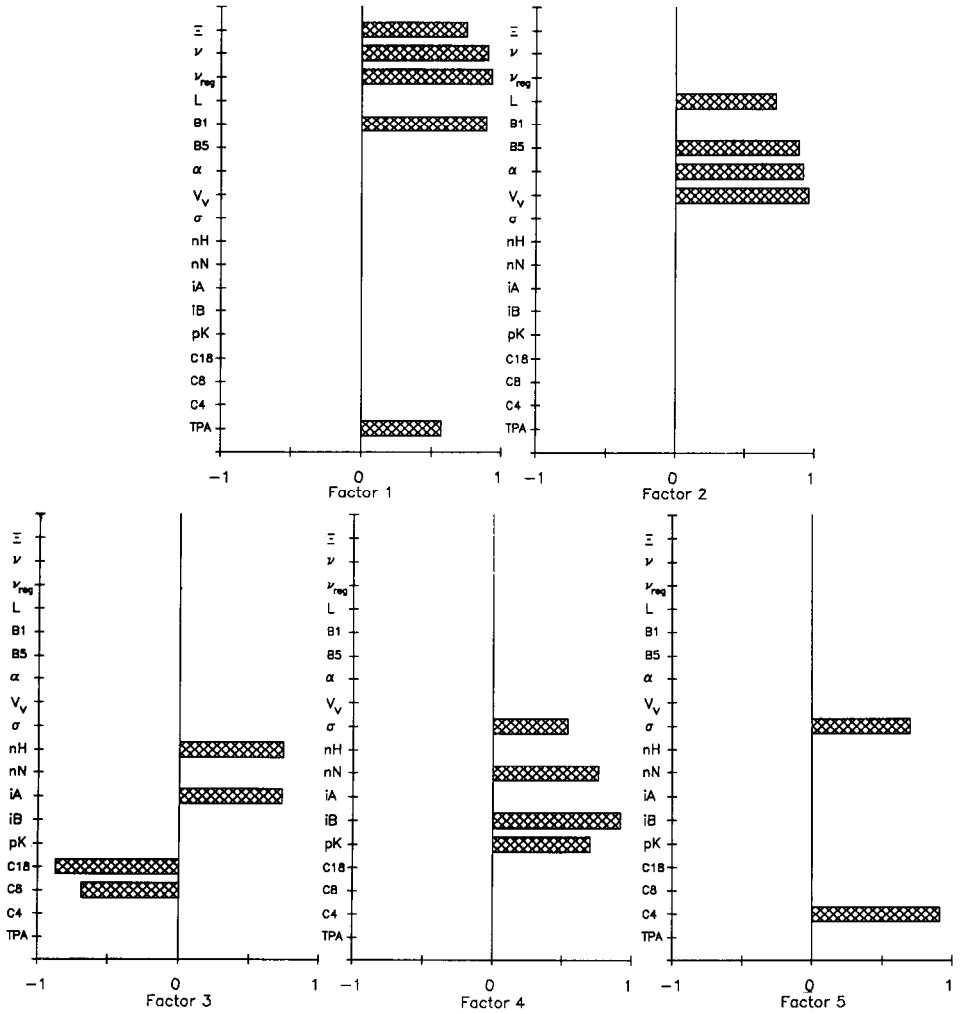


Fig. 1. Pattern of factor loading for each of the five factors.

part of the same factor. The fifth factor, (factor 5) contains two parameters, the C4 set of retention coefficients and $\sigma$, the parameter that describes the inductive field effects of the amino acid side chains. Table IV lists the percentage of the total variance accounted for by each of the 5 factors. Five factors were chosen because they accounted for all four of the group retention coefficient scales and further, each of these five factors provided a significant contribution of the total variance of the analysis [14], Table IV.

*The effect of the organic modifier in the mobile phase on the group retention coefficients*

The role of the organic modifier in the retention process can be analysed through comparison of the correlation results for the C18 and TPA GRCs sets. The results of

TABLE III

CORRELATION COEFFICIENTS FROM LINEAR REGRESSION ANALYSIS OF THE FOUR GROUP RETENTION COEFFICIENT SCALES

| | C18 | C8 | C4 |
|---|---|---|---|
| C8 | 0.80 | | |
| C4 | −0.05 | −0.05 | |
| TPA | 0.44 | 0.30 | 0.15 |

linear regression analysis are shown in Table III. The data indicated that while the C18 and C8 coefficient sets had a high degree of linearity, both the C4 and TPA showed no linearity with any of the other coefficient sets. The only difference between the C18 and TPA retention coefficient sets (Table I), is that the peptides used to generate these two sets of retention coefficients were eluted with a different mobile phase. The C18 retention coefficients were calculated from peptides eluted with a gradient of TFA–ACN–water with a RP-C18 stationary phase, while the TPA coefficients were generated from peptides eluted with a gradient of TFA–2-propanol–ACN–water and with the same RP-C18 stationary phase. Even though the TPA coefficients were not generated from peptides eluted in 2-propanol but a mixture of 2-propanol–ACN any differences in the retention process as reflected in the GRCs must be due to the presence of 2-propanol. It is therefore the effect of 2-propanol in the mobile phase that is controlling the differing RP-HPLC retention processes.

Table V lists the dielectric constant and solvent polarity of 2-propanol, ACN and water for comparison. Since 2-propanol has a lower dielectric constant and solvent polarity than ACN, charge interactions therefore appear to play a limited role in the solute–solvent component of the retention process in the TPA chromatographic environment. Furthermore, it is apparent from the principal component analysis that the parameters which are related to the TPA retention coefficient set are steric or volume measures. Thus, these facets of the size of the amino acid side chains control the region of the peptidic solute that can interact with the alkylsilica stationary phase in RP-HPLC systems when 2-propanol is used in part as a co-organic modifier in the mobile phase.

TABLE IV

THE PERCENTAGE OF TOTAL VARIANCE ACCOUNTED FOR BY EACH OF THE FIVE FACTORS DERIVED FROM PRINCIPAL COMPONENT ANALYSIS

| Factor | Percentage of total variance |
|---|---|
| 1 | 31.9 |
| 2 | 21.9 |
| 3 | 14.1 |
| 4 | 10.2 |
| 5 | 6.7 |
| Total | 84.8 |

TABLE V

DIELECTRIC CONSTANT AND SOLVENT POLARITY OF 2-PROPANOL, ACN AND WATER

| Solvent | Dielectric constant | Solvent polarity |
|---|---|---|
| 2-Propanol | 20.3 | 4.0 |
| ACN | 37.5 | 5.8 |
| Water | 80.0 | 10.2 |

The C18 and C8 coefficients sets were generated from peptides eluted with a mobile phase gradient of TFA–ACN–water and, stationary phases of 18 and 8 carbon alkyl chains, respectively. Fig. 1 indicates that the coefficients of the two GRCs sets correspond in an opposing way to the electronic parameters, that is, the C18 and C8 sets have a negative correlation with the electronic parameters. In other words, the analysis indicates that as the electronic characteristics of an amino acid side chain increase, then the smaller will be its retention coefficient.

In terms of the solvophobic theory, the parameters which will be directly affected by changes in the organic modifier include, $D$, the dielectric constant and $\gamma$, the surface tension. It is evident from the data listed in Table V that 2-propanol–ACN–water mixtures will have a lower dielectric constant and polarity than ACN–water mixtures. This will in turn influence the ability of the peptide solutes to interact with the solvent molecules which will then directly impinge upon the resultant retention coefficients for each amino acid residue. For small solutes such as amines or individual amino acids, the dominant force behind solute–surface interactions can be related to the surface tension of the mobile phase and the hydrophobic surface area of the solute [7]. In addition, the parameters related to the dipole moment, $\mu_s$, the polarisability, $\alpha_s$, and the molecular volume, $v_s$ of each constituent amino acid residue within a particular peptide sequence will be closely dependent on the properties of the mobile phase and the neighbouring amino acids within the peptide. As a consequence, there is a fine balance between the relative contribution of all the possible factors involved in the retention process. The results of principal component analysis reveal that for peptides eluted with TFA–water–ACN mixtures, there is a negative correlation between the amino acid GRCs and the electronic properties of each residue. This can be contrasted with the positive correlation between the GRCs and the steric and volume properties of amino acids with peptides eluted with TFA–water–ACN–2-propanol mixtures. Thus in relative terms, the residues with high dipolar characteristics contribute less to retention as they can interact more easily with the relatively polar solvent molecules. In contrast, in the presence of 2-propanol, the larger residues interact more strongly with the stationary phase rather than the mobile phase. This behaviour can be understood in terms of the solvophobic theory if one considers the solvent cavity created about the solute. Because 2-propanol has a low dielectric constant, $D$, charge or dipole interactions between the solute and solvent will be minimised. Therefore, the solvent forming the wall of the cavity will become more ordered than the bulk solvent, thus reducing the entropy, $\Delta S^0$, of the system. The change in free energy of any system is related to the change in entropy as follows:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \tag{10}$$

where $\Delta H^0$ is the change in enthalpy. Any decrease in entropy is energetically unfavourable. Thus, it is energetically unfavourable in terms of entropy for a cavity to be formed around larger amino acid side chains. As a consequence a favourable decrease in $\Delta G^0$ will occur upon binding to the stationary phase sorbent. Our findings are in accord with the results predicted by the solvophobic theory since eqns. 3–6 show that there should be a linear relationship between the solvent dielectric constant and log $k'$. Moreover, similar conclusions have been reached by Horváth *et al.* [7], and, Fausnaugh *et al.* [15] from studies of proteins eluted from reversed-phase and hydrophobic interaction chromatographic sorbents.

*The effect of alkyl chain length on the group retention coefficients*

Further examination of the results of the principal component analysis indicated that the C18 and C8 retention coefficients are negatively correlated to two electronic parameters in factor 3, while the C4 coefficients are positively correlated with the electronic parameter $\sigma$, inductive field affect, factor 5, Fig. 1. These three coefficient sets were generated from peptides eluted with the same mobile phase, a gradient of TFA–ACN–water. It is therefore the differing length of the *n*-alkyl chain (*i.e.*, C4 *versus* C18 or C8) of the stationary phase ligands that is controlling the retention processes observed between C18 and C8 retention coefficients, and the C4 coefficients. Since the C18 and C8 coefficients show a strong linear relationship with each other the solute retention processes are very likely to be similar.

The similarity of the C18 and C8 retention coefficients and their differences with the C4 coefficients have been previously observed with retention behaviour of peptides [11]. For example, the retention behaviour of a family of paracelsin peptide analogues were examined under isocratic conditions on a series of reversed-phase sorbents which varied in alkyl chain length from C1 to C18. Plots of $k'$ *versus* chain length showed a retention maxima between $n = 2$ and 4, which was followed by a significant decrease for $n \leqslant 5$ carbon atoms [16]. In addition, nuclear magnetic resonance studies have documented differences in the dynamic structure of the C18 (or C8) and C4 *n*-alkyl ligands [17]. From measurement of the spin lattice relaxation times of reversed-phase ligands with a range of alkyl chain lengths it was found that the RP-C4 alkyl ligands had a far more rigid conformation than the 18- and 8-carbon alkyl ligands. The extreme rigidity of the RP-C4 alkyl ligands could mean that this stationary phase provides an essentially planar surface for the interaction of the solute with the stationary phase. However, since the RP-C4 retention coefficients only account for 6.7% of the total variance of the principal component analysis and because these coefficients were correlated with only one other parameter, it is difficult from these data to draw more specific conclusions on the factors which are important in the interactions of peptide solutes with C4 stationary phases.

## CONCLUSIONS

A large number of chromatographically derived amino acid hydrophobicity coefficients have been published in recent years (for compendia see refs. 11 and 18). The main use for these scales has been associated with the prediction of the retention position of peptides of known amino acid composition or alternatively for the prediction of internal and external areas within a protein structure through the

analysis of hydropathy profiles [11,18,19]. However, there has been no detailed analysis of the precise physicochemical basis of the retention coefficients in terms of the underlying forces which control the peptide–stationary phase interaction. It is evident from the present study that the relative interactive potential of peptides is intimately dependent on the specific nature of both the stationary phase ligand and the mobile phase composition. While this result is anticipated on the basis of the large body of literature on the empirical separation of peptides by RP-HPLC and solvophobic theory, the present study characterises more completely the complex nature of peptide–surface interactions in more quantitative terms. Furthermore, it has been assumed previously that a single coefficient value will represent the binding contribution capacity of an individual amino acid in all possible sequence arrangements. This assumption is clearly untenable in view of the results of the present study as well as examples [18,20] which demonstrate chromatographic resolution between peptides of identical amino acid composition but different sequences. These experimental observations simply reflect that the interaction of peptides with chromatographic surfaces is orientation specific. The results of the present study will thus underpin further investigations to unravel the physicochemical factors which determine the ability of amino acid residues within a particular peptide sequence to participate in the binding process. Such studies on the atomic and molecular forces which determine the contact region(s) of peptides and proteins in the adsorptive modes of HPLC [21,22], including RP-HPLC are currently underway in these laboratories.

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