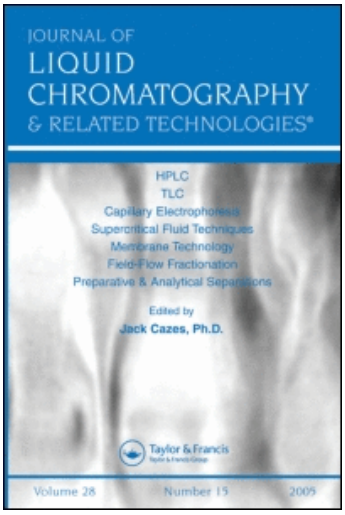

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Pairing Ion Effects in the Reversed Phase High Performance Liquid Chromatography of Peptides in the Presence of Alkylsulphonates

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PAIRING ION EFFECTS IN THE REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF PEPTIDES IN THE PRESENCE OF ALKYLSULPHONATES*

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ABSTRACT

The influence of pH and the concentration of the two lipophilic pairing ions, hexylsulphonate and camphor-10-sulphonate, on the retention of a group of small peptides to chemically bonded hydrocarbonaceous, microparticulate silicas has been further investigated. With low pH aqueous methanol mobile phases containing various concentrations of these surface active anions, the capacity factors of unprotected and C-protected peptides show similar dependencies on the concentration of the pairing ion. Column selectivity becomes essentially independent of pairing ion concentration above ca 25mM. At higher pH values, the influences of pairing ion interactions on peptide retention appear to diminish due to competing protic equilibria.

INTRODUCTION

Reversed phase high performance liquid chromatography (RP-HPLC) has gained wide popularity over the past decade.

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* High Performance Liquid Chromatography of Amino Acids, Peptides and Proteins, Part XXXIII. For Part XXXII see ref [1].

In part, the versatility of the technique resides in its ability to resolve ionic or ionisable solutes using aqueous mobile phases, based in many cases on hydro-organic solvent mixtures and combinations of compatible buffer reagents. Secondary chemical equilibria established between the polar solute molecules and these components present in the mobile phase can dramatically influence their retention behaviour on hydrocarbonaceous silicas. Manipulation of protonic equilibria is one way by which the retention of ionised compounds can be varied on these non-polar supports. Control over selectivity can also be achieved by means of suitable interactive lipophilic or hydrophilic ions added at an appropriate concentration to the aqueous mobile phase. In this circumstance, which is commonly referred to as 'ion-pair' reversed phase chromatography, the retention of ionised solutes can be augmented or attenuated depending on the polarity of the added buffer ion. With lipophilic ions, adsorption of the ion onto the hydrophobic surface of the column packing material can occur and this will result in the stationary phase effectively behaving as a dynamic solvent-generated ion-exchanger. With hydrophilic ions, adsorption to the hydrophobic surface of the column packing material is much less favoured and ion-pair formation may take place in the aqueous mobile phase followed by sorption of the solvated complex to the non-polar stationary phase surface. A variety of retention mechanisms have been proposed [2-6] to account for the influence of these competing secondary equilibria on the chromatographic behaviour of ionised solutes such as catecholamines and polar pharmaceuticals.

Similar concepts have been recently applied to the analysis and purification of peptides from synthetic and natural sources. Because of the flexibility which can be achieved over retention

and selectivity with interactive electrolyte systems, the use of RP-HPLC has consequently become very popular in peptide and protein chemistry. These areas of application have recently been reviewed [7-9]. The main objective of the present study was to further elucidate the influence of protonic and pairing-ion interactions on the retention of ionised peptides to chemically bonded hydrocarbonaceous stationary phases. We restricted this study to mobile phases containing low concentrations of alkylsulphonates in aquo-methanol mixtures over the range pH 2.5-7.0 and relatively simple peptides, some with their C-terminus protected.

EXPERIMENTAL

Apparatus:

All the data were collected under isocratic elution conditions using a Waters Associates (Milford, Mass., U.S.A.) HPLC system which included a M6000 A solvent delivery system, an U6K universal liquid chromatograph injector and a M450 variable wavelength UV monitor coupled to a Rikadenki dual channel recorder. The μBondapak C_{18} columns (10μm, 30cm x 4mm I.D.) were purchased from Anac (NZ) Ltd. Sample injections were made with Pressure-Lok liquid syringes, series B110, from Precision Sampling (Baton Rouge, La., U.S.A.).

Chemicals and Reagents:

All solvents were Analar grade and purified prior to use as described previously [10]. The amino acids and peptides used in this study were obtained from Sigma Chem. Co. (St Louis, Mo., U.S.A.) and Vega Biochemicals (Tucson, Ariz., U.S.A.). All amino acids except glycine were of the L-configuration. The alkylsulphonic acids were obtained from B.D.H. (Poole, Great Britain) or prepared from the corresponding alkylbromide and anhydrous sodium sulphite [11]. Orthophosphoric acid,

LINEAR SEQUENCES OF PEPTIDES USED IN THE PRESENT STUDY*

No.	Peptide	No.	Peptide
1.	G.	7.	V.L.
2.	G.G.	8.	F.L. amide
3.	A.G.	9.	A.K.
4.	G.F.	10.	G.L.Y.
5.	G.F. amide	11.	G.G.Y. amide
6.	R.F.	12.	R.F.A.

* The one letter code for the amino acids is as given by M.O. Dayhoff in Atlas of Protein Sequence and Structure (National Biomedical Research Foundation, Silver Spring, Md., U.S.A., 1972), G = gly, A = ala, F = phe, R = arg, V = val, L = leu, K = lys, and Y = tyr.

sodium hydroxide and sodium dihydrogen phosphate were obtained from May and Baker (Dagenham, Great Britain).

Methods:

All chromatograms were carried out at ambient temperature (ca 18^{o}). All peptides were made up in the eluent under study. Bulk solvents were degassed by sonication and the appropriate mobile phases prepared and equilibrated to operating conditions as reported previously [12]. All columns were equilibrated to new eluent conditions for at least 100 column volumes. Sample sizes were generally 10µg peptide material injected in 5- or 10-µl volumes. The capacity factors were calculated as reported previously [10] and the data analysed using a non-linear least squares fit programme developed for the ligand

adsorption model. Two modified subroutines "Miniz" and "Fun" were bound to a BMD 07R programme (Biomedical Computer Programs, University of California) to determine the curves of best fit from the non-linear least squares analysis on a Burroughs 6700 Computer. Copies of the modified subroutines of the programme appear in the appendix.

RESULTS AND DISCUSSION

At low pH, an unprotected peptide with only a single N-terminal amino group will exist in solution effectively as the zwitterionic species, HP_i, and as the positive ion, $H_2P_i^+$. The corresponding C-protected peptide will be present only in the protonated form, $H_2P_i^+$, under these conditions. The capacity factor for a C-protected peptide on a hydrocarbonaceous stationary phase with a mobile phase containing a surface active alkylsulphonate, e.g. sodium hexanesulphonate(Na^+Hex^-), can be given by

$$k' = \psi \cdot K_e \frac{[Na^+Hex^-]_s}{[Na^+]_m} \qquad \ldots.(1)$$

where ψ is the phase ratio, $[Na^+Hex^-]_s$ and $[Na^+]_m$ the concentrations of the bound sodium hexanesulphonate and sodium ions in the mobile phase respectively and K_e is the equilibrium binding constant for an ion-exchange event, i.e.

$$K_e = \frac{[H_2P_i^+Hex^-]_s[Na^+]_m}{[Na^+Hex^-]_s[H_2P_i^+]_m} \qquad \ldots.(2)$$

Similarly, the capacity factor for a diprotic unprotected peptide can be expressed in the form

$$k' = \psi\left[K_{e_1} + \frac{K_{e_2}K_{a_1}}{[H^+]}\right]\frac{[H^+]}{[H^+] + K_{a_1}} \cdot \frac{[Na^+Hex^-]_s}{[Na^+]_m} \qquad \ldots.(3)$$

where K_{e_1}, K_{e_2} are the equilibrium binding constants for the charged and zwitterion species respectively and K_{a_1} the dissociation constant for the ionisation event concerned. Alternative forms of equations 1 and 3 can be derived from the explicit hetaeric model of Horvath and coworkers [3,13], e.g. in the circumstance where the chromatographic process is represented by the limiting case of dynamic ion exchange, the capacity factor is given by

$$k' = \psi \frac{K_0[Hex^-]_s + K_1K_4[Hex^-]_m[Hex^-]_s}{(1 + K_1[Hex^-]_m)(1 + K_2[Hex^-]_m)} \quad \ldots.(4)$$

where $[Hex^-]_s$ and $[Hex^-]_m$ are the maximum surface concentrations of bound hexanesulphonate and the concentration of hexanesulphonate in the eluent, K_0 and K_1 are the equilibrium constants for the binding of the peptide and hexanesulphonate to the stationary phase surface, K_2 and K_4 are the equilibrium constants for ion-pair formation in the mobile phase and dynamic ion-exchange complex formation at the surface of the stationary phase respectively.

The effect of ionisation on the capacity factor for a C-protected monoprotic peptide under these conditions can be expressed as

$$k' = \frac{k_0 + k_1 \frac{[H^+]}{K_{a_1}}}{1 + \frac{[H^+]}{K_{a_1}}} \quad \ldots.(5)$$

where k_0 and k_1 are the limited capacity factors of the unionised and positively charged species and K_{a_1} is the protonic dissociation constant. In a similar fashion, the capacity factor of a diprotic peptide is given by

$$k' = \frac{k_o + k_{-1}\frac{K_{a_2}}{[H^+]} + k_1\frac{[H^+]}{K_{a_1}}}{\frac{K_{a_2}}{[H^+]} + \frac{[H^+]}{K_{a_1}} + 1} \quad \ldots.(6)$$

where k_o, k_{-1} and k_1 are the limiting capacity factors of the zwitterionic, anionic and cationic forms of the unprotected diprotic peptide and K_{a_1} and K_{a_2} are the first and second acid dissociation constants respectively.

Under constant low pH elution conditions, eqns 1-4 predict that the capacity factor for both unprotected and C-protected peptides will initially increase and then slowly decrease with increasing pairing ion concentrations, provided $K_1[Hex^-]_m$ and $K_2[Hex^-]_m$ are both of the order of unity at the highest pairing ion concentration. Thus a parabolic dependence of the capacity factor on pairing ion concentration is anticipated provided complex exchange processes [3] do not occur. An approximately parabolic dependence will also occur if the pairing ion forms, at a sufficiently high concentration, micelles into which the solutes can partition. In the case where one of the terms in the demoninator of eqn 4 vanishes, e.g. when binding of the pairing ion to the non-polar stationary phase is unfavoured and $K_1[X^-]_m$ is small, the plot of k' versus $[X^-]_m$ is expected to take the form of a rectangular hyperbola. Furthermore, as the pH is increased at constant pairing ion concentration, the capacity factors of unprotected peptides are anticipated to initially fall, reach minima values proximal to the pI values and then slowly increase at higher pH values. The capacity factors of C-protected peptides on the other hand should increase more rapidly at higher pH values.

Figure 1 shows the retention behaviour of the peptides listed in the Table as a function of the sulphonate concentra-

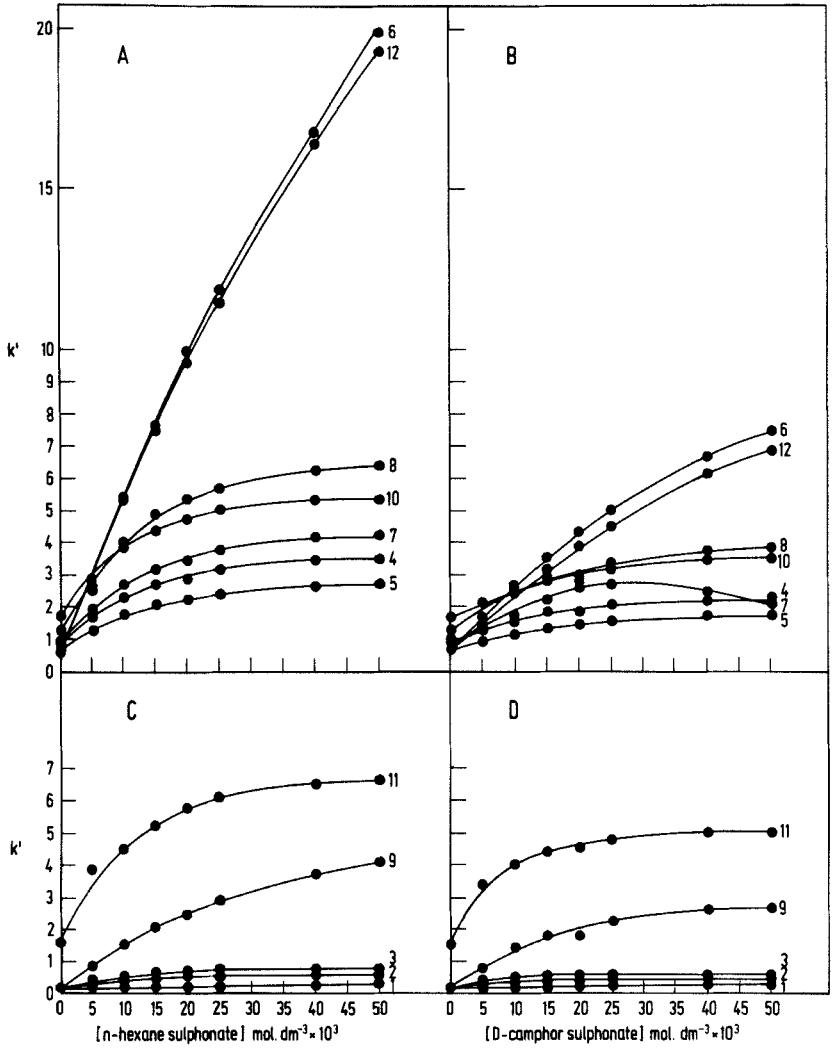

Figure 1. Dependence of the capacity factors of protonated peptides on the concentration of pairing ion in the mobile phase. Chromatographic conditions: column μBondapak C_{18}; flow rate, 2ml/min; temperature, 18°; mobile phases, (A) and (B) 25% methanol-water-50mM NaH_2PO_4-15mM H_3PO_4 with 10M NaOH to pH 3.0, and (C) and (D) 5% methanol-water-50mM NaH_2PO_4-15mM H_3PO_4 with 100mM NaOH to pH 3.0 containing various concentrations of the pairing ion reagents. Peptide key given in the Table.

tion for two alkylsulphonates in aqueous methanol eluents containing 50mM NaH_2PO_4, pH 3.0. These results obtained with hexanesulphonate and camphor-10-sulphonate are consistent with earlier observations [2,6-8,11,12,14] on the effect of anionic and cationic lipophilic reagents on peptide and amino acid retention to alkylsilicas. These previous studies have demonstrated the difficulty in making unambiguous interpretations of such chromatographic data in terms of either an exclusively ion-pair or a dynamic liquid-liquid ion-exchange mechanism because the formal dependence of retention on the lipophilic ion concentration in the mobile phase is the same in both cases. As noted above, the retention mechanism is dependent on a variety of experimental parameters and may change upon varying the mobile phase water content or, for that matter, differ slightly from one lipophilic counterion to another or from one peptide to another. Both of the alkylsulphonates used in the present study are, however, known to act as surface active anions as revealed by their adsorption isotherms which are of the L- or H-type and obey the relationship explicit to the Freundlich equation, i.e.

$$[X^-]_s = a\,[X^-]_m^b \qquad \ldots.(7)$$

According to the ligand adsorption model [2,3], the general form of the capacity factor dependence on $[X^-]$ can be expressed by the relationship

$$k' = (k_o + \beta[X^-])\cdot(1 + K_1[X^-])^{-1}\cdot(1 + K_2[X^-])^{-1} \qquad \ldots.(8)$$

where k_o is the capacity factor of the solute in the absence of a pairing ion and the meaning of β depends on the underlying physicochemical equilibria controlling retention, i.e. for dynamic liquid-liquid ion exchange $\beta = K_1K_4$ and for ion pair formation followed by distribution to the stationary phase $\beta = K_2K_3$ where K_3 is the ion pair equilibrium distribution

constant. Under chromatographic distribution conditions which involve only ion pairs, the dependence of the capacity factor for ionised solutes on pairing ion concentration can be expressed in terms of a modified form of equation 8, namely,

$$k' = (k_0' + K_2K_3[X^-])\cdot(1 + K_2[X^-])^{-1} \quad \ldots.(9)$$

Based on data obtained with 3-nitrobenzoic acid and sodium cromoglycinate as model solutes and alkylbenzyldimethylammonium chlorides as pairing ions, Riley et al. [15] concluded that ion-pairing in the mobile phase followed by distribution to the stationary phase was the dominant retention mechanism. Furthermore, this group demonstrated that the bulk phase liquid-liquid distribution coefficients for ion-pair association, K_D, are linearly related to the chromatographic ion-pair distribution constants, K_3, viz.

$$K_{3,i} = a_iK_{D,i} + b_i \quad \ldots.(10)$$

where a_i and b_i are the slope and intercept coefficients for solute i.

Analysis of the retention data for compounds (1) - (12) as a function of pairing ion concentration in terms of equations 7 and 8 was carried out on a Burroughs 6700 computer using a BMD computer programme modified to determine curves of best fit from the non-linear least squares analysis. In all cases better fit of the experimental data to the relationship inherent in the three parameter equations, i.e. eqn 8, was obtained. Although this circumstantial evidence may favour the involvement of dynamic ion exchange processes at the surface of the stationary phase as has been proposed in several studies [5,6,12-14], precise data on the magnitude of the association constants for protonated peptides and alkylsulphonates are required from suitable extrachromatographic experiments before such a postulated mechanism could be unequivocally substantiated.

With the peptides studied the trend was apparent with k′ (hexanesulphonate)>k′(camphor-10-sulphonate) although selectivity factors were similar. In Figure 2 are shown plots of $\Delta\alpha/\alpha$ versus pairing ion concentration. Above about 25mmol/l, the

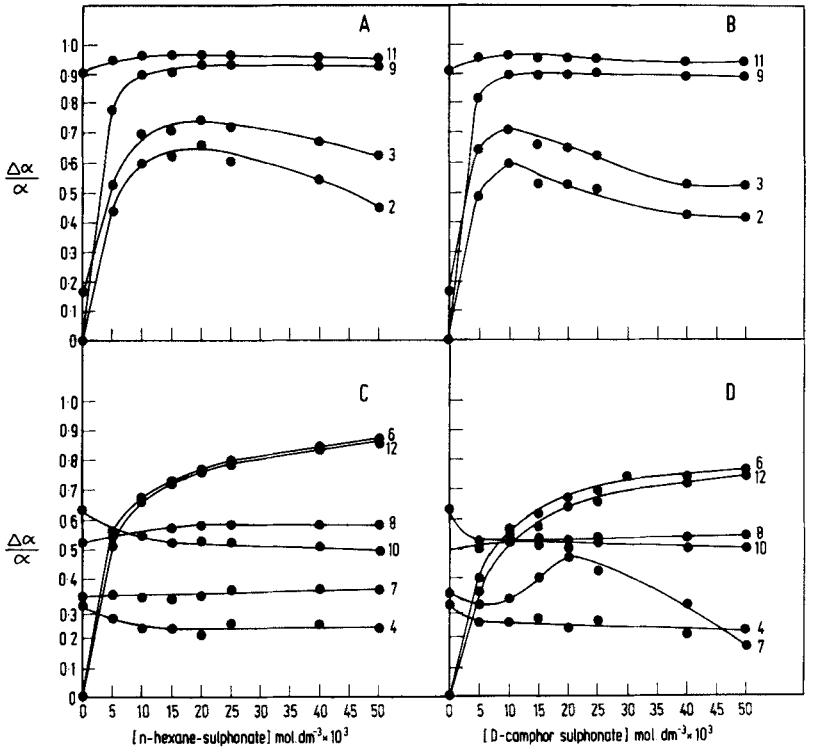

Figure 2. Plots of the dependence of selectivity parameters of protonated peptides on the concentration of pairing ion in the mobile phase. Chromatographic conditions: column, μ-Bondapak C_{18}; flow rate, 2ml/min; temperature, 18°; mobile phases, (A) and (B) 5% methanol-water-50mM NaH_2PO_4-15mM H_3PO_4 with 10M NaOH to pH 3.0 and (C) and (D) 25% methanol-water-50mM NaH_2PO_4-15mM H_3PO_4 with 100mM NaOH to pH 3.0 containing various concentrations of the pairing ion reagent. The α value of peptides (1) and (5) were taken as unity in the calculation of the $\Delta\alpha/\alpha$ values. Peptide key given in the Table.

selectivity coefficients become effectively independent of both the hexylsulphonate and the camphor-10-sulphonate concentration for these peptides, i.e. above this limiting value the capacity factors but not selectivities can be increased by appropriate increases in the pairing ion concentration. Similar observations have been made [12] for the retention behaviour of larger peptides on reversed phase silicas in the presence of lipophilic ions. The observed isocratic elution order for the peptides under the low pH, pairing ion conditions was consistent with the current concept that peptide selectivity in RP-HPLC to a large extent reflects differences in their interfacial hydrophobic contact areas. These contact area parameters can be related [2] to the relative hydrophobicities of the individual peptides. The side chain functionality, positional array and extent of ionisation all make significant contributions to the overall hydrophobicity of a peptide under these low pH and pairing ion conditions. As can be seen from Figures 1, 3 and 4 the capacity factors of the glycinyl peptides do not show a pronounced dependency on pairing ion concentration or on pH under the conditions examined, a result which suggests that the peptide chain may make only a small contribution to the sorption phenomena of peptides chromatographed under these conditions.

Shown in Figure 3 are typical plots of k' versus pH for several peptides chromatographed on a μBondapak C_{18} column with a phosphate based eluent. Two types of retention behaviour are evident from these plots. The first type, shown by the C-protected peptides, is characteristic of weak bases. As the pH increases, the charged ammonium moieties of the C-protected peptide deprotonate. With a reduction in the extent of ionisation, the k' values of these peptides increase. The observed dependency of k' on pH shown, for example, by peptide (8) is in good agreement with eqn 5, which predicts that plots

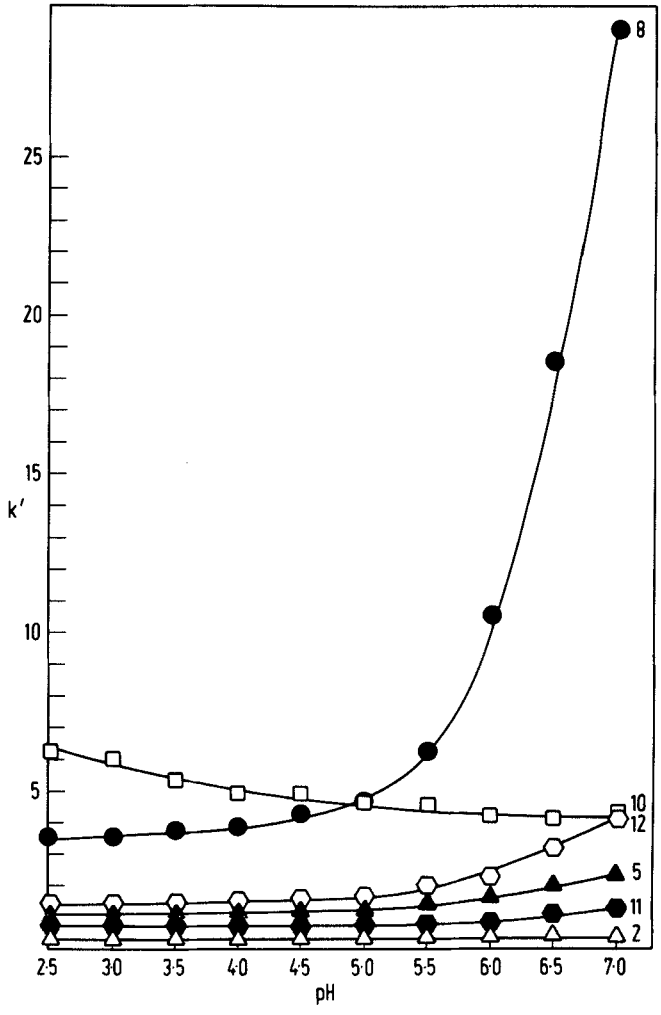

Figure 3. Capacity factors for several peptides on a μ-Bondapak C_{18} column as a function of pH. The eluent was 5% methanol-water-50mM NaH_2PO_4-15mM H_3PO_4 titrated with 10M NaOH over the range pH 2.5-7.0; flow rate 2ml/min; sample size, 5μg/5μl injections. Peptide key given in the Table.

of k´ versus pH for such weak monoprotic bases should be sigmoidal with the inflexion pH value corresponding to the pK_{a_1} value. Because of the instability of hydrocarbonaceous bonded silicas above ca pH 7.5 it was not possible to complete the pH titration although the trend is evident. Similar arguments can be applied to the treatment of k´ versus pH plots of N-protected peptides and other weak acids where the reverse situation exists, e.g. k´ increases with decreasing pH. With the small unprotected peptides, such as gly-leu-tyr and arg-phe-ala competing retention contributions due to the protonic equilibria of the amino and carboxyl groups will tend to be counterbalanced in the pH region proximal to the isoelectric point of the peptide and this will lead to maximal ionisation and minimum retention. At pH values higher than the pI, the retention behaviour of unprotected peptides will increasingly reflect the contribution made by the free amino group to the retention process. With polyprotic peptides, the effect of additional ionogenic centres on the dependence of k´ on pH can be accomodated in expanded expressions similar to eqn 6 with addition k'_n, K_{a_n} terms included for each additional ionogenic centre. In general k´ values will reflect the extent of ionisation of the solute, i.e. as the extent of ionisation increases, k´ will decrease.

Shown in Figure 4 are representative data on the influence of pH on the k´ values of unprotected and C-protected peptides at constant hexanesulphonate molarity of 10mM. At otherwise constant mobile phase composition, the pH strongly influences the retention of both classes of peptides under these conditions. As anticipated on the basis of eqns 2 and 3, the k´ values of all the peptides initially decreased when the pH was increased from pH 3.0. Comparable decreases in k´ values with increasing pH has also been observed with amino acids under similar

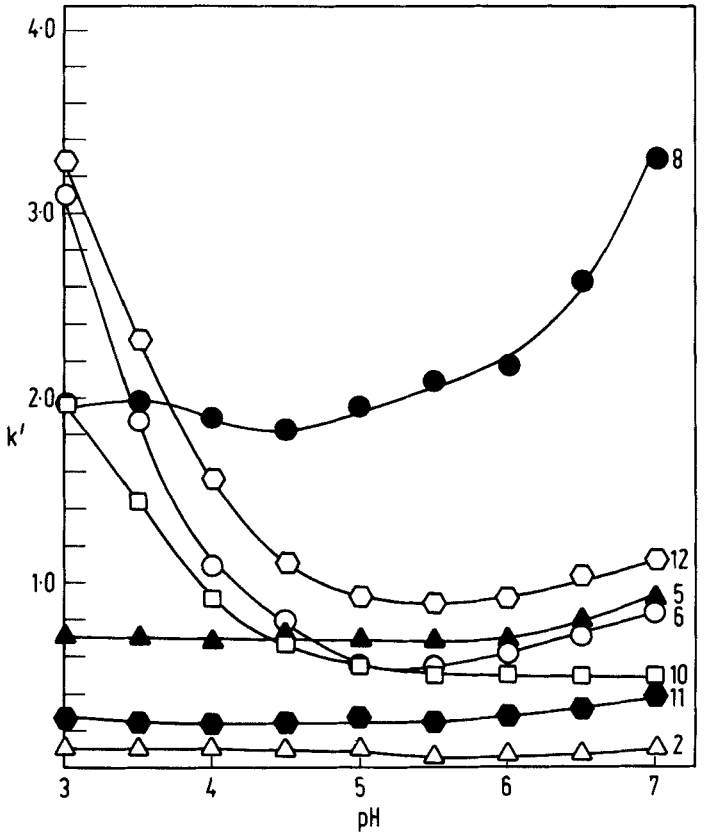

Figure 4. Capacity factors for several peptides on a μ-Bondapak C_{18} column as a function of pH in the presence of a low concentration of pairing ion reagent. The eluent was 20% methanol-water-50mM NaH_2PO_4-15mM H_3PO_4-10mM sodium hexane-sulphonate, titrated with 10M NaOH over the range pH 2.5-7.0; flow rate, 1ml/min; sample size 5μg/5μl injections. Peptide key given in the Table.

conditions with dodecylsulphonate system [14]. Above ca pH 5.0, pairing ion interactions appear to play less significant roles with the k′ values of the various peptides generally increasing with an elution order comparable to that observed with the corresponding phosphate based mobile phase with the

pairing ion deleted. As has been discussed elsewhere [8,12], the k′ versus pH plots determined in the presence of low concentrations of lipophilic pairing ions, can be used to assess the homogeneity of a particular peptide over a very wide range of ionisation conditions. Major selectivity changes can be easily achieved and related to the physicochemical basis of the retention mechanism under these conditions. Since several competing secondary chemical equilibria are simultaneously modulated in a controlled manner, excellent resolution can be achieved for very closely related peptides by the judicious choice of both the pH and pairing ion condition. Similar criteria can be proposed for the assessment of polypeptide and protein homogeneity and have been employed for the reversed phase HPLC analysis of the tryptic maps of proteins, including pituitary protein hormones [10,16,17].

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APPENDIX 1: SUBROUTINE MINIZ

```
0       $SET SEPARATE                                                                   00001000
1            SUBROUTINE MINIZ(X,A,F,NV,NC,NP1,TOL,EPS,EE,ND,MXIT,LLK,P0,P1,IW)  00001100
2       C       SUBROUTINE MINIZ FOR BMDX85                    APRIL  4, 1966   00002000
3            DIMENSION A(NP1,NP1),X(NV,NC),F(100),FP(100),D(100),P0(100),P1(10000003000
4           1),B(10)                                                                00004000
5            DIMENSION PP0(100)                                                     00005000
6       C                                                                           00006000
7       C                                                                           00007000
8            F(4)=X(2,1)                                                            00008000
9            WRITE(6,999) F(4)                                                      00008100
10       999 FORMAT(9HKVALUE = F5.2)                                                00008200
11      C                                                                           00008300
12           IF(IW.EQ.0)C=1.0                                                       00009000
13           NP=NP1-1                                                               00010000
14           MNAIL = 10                                                             00011000
15           NAIL = 0                                                               00012000
16           DO 557 I=1,NP                                                          00013000
17       557 PP0(I)=P(I)                                                            00014000
18      C                                                                           00015000
19           EPS1=5.*EPS+1.                                                         00016000
20           WRITE(6,23)(P0(I),I=1,NP)                                              00017000
21        23 FORMAT('1MINIMA',15X,1P9E12.4/(22X,1P9E12.4))                          00018000
22           WRITE(6,22)(P1(I),I=1,NP)                                              00019000
23        22 FORMAT('0MAXIMA',15X,1P9E12.4/(22X,1P9E12.4))                          00020000
24           WRITE(6,195)                                                           00021000
25       195 FORMAT('0ITERATION    ERROR     PARAMETERS'/14X,'MEAN'/14X,'SQUARE00022000
26          1')                                                                     00023000
27       194 LLL=5                                                                  00024000
28           LIT=0                                                                  00025000
29           HU=NC                                                                  00026000
30           H=0.                                                                   00027000
31           LLLL=5                                                                 00028000
32           EQ=7.2E65                                                              00029000
33           DO 556 I=1,NP                                                          00030000
34       556 P(I)=PP0(I)                                                            00031000
35           FNC=NC-NP                                                              00032000
36       29  DO 85 I=1,NP1                                                         00033000
37           DO 85 J=1,I                                                            00034000
38       85  A(I,J)=0.                                                             00035000
39           DO 2 L=1,NC                                                            00036000
40      C    CALL FUN(F(L),FP,X(1,L),P,LLK)                                         00037000
41           CALL FUN(Q,FP,P,X(1,L))                                                00038000
42           FP(NP1)=X(ND,L)-Q                                                      00039000
43           IF(IW.NE.0)C=X(IW,L)                                                   00040000
44           IF(C.LT.(1.E-20)) GO TO 2                                              00041000
45           DO 1 I=1,NP1                                                           00042000
46           FPIC=FP(I)*C                                                           00043000
```

```
      DO 1 J=1,I                                                        00044000
    1 A(I,J)=A(I,J)+FPIC*FP(J)                                          00045000
    2 CONTINUE                                                          00046000
      EE=A(NP1,NP1)/FNC                                                 00047000
    6 IF(EE.LE.E0) GO TO 871                                            00048000
      NAIL=NAIL+1                                                       00049000
      IF(NAIL.GT.MNAIL) GO TO 871                                       00050000
      DO 872 I=1,NP                                                     00051000
      P(I)=PP0(I)+D(I)/2.                                               00052000
872   D(I)=D(I)/2.                                                      00053000
      GO TO 29                                                          00054000
C                                                                       00055000
C                                                                       00056000
 871 WRITE(6,198)LIT,NAIL,EE,(P(I),I=1,NP)                              00057000
 198 FORMAT(I6,1X,I2,1X,1P10E12.4/(22X,1P9E12.4))                       00058000
     NAIL=0                                                             00059000
8191 LIT=LIT+1                                                          00060000
     CALL STEP(A,D,P,PO,P1,NP1,TOL)                                     00061000
     IF(LIT.GT.MXIT) GO TO 200                                          00062000
     IF(EE.EQ.0.) GO TO 470                                             00063000
     PC=1.                                                              00064000
     IF(E0.NE.7.2E65) PC=ABS((E0-EE)/EE)                                00065000
     E0=EE                                                              00066000
     IF(PC.LT.EPS) GO TO 100                                            00067000
     LLL=LLLL                                                           00068000
 121 DO 28 I=1,NP                                                       00069000
     PP0(I)=P(I)                                                        00070000
 28  P(I)=P(I)+D(I)                                                     00071000
     GO TO 29                                                           00072000
 200 WRITE (6,201)                                                      00073000
 201 FORMAT(30HOTHE PROCESS IS NOT CONVERGING)                          00074000
     GO TO 470                                                          00075000
 100 LLL=LLL-1                                                          00076000
     IF(LLL.NE.0) GO TO 121                                             00077000
 470 DO 778 I=1,NP                                                      00078000
 778 FP(I)=SQRT(ABS(A(I,I)*EE))                                         00079000
     WRITE (6,779) (FP(I),I=1,NP)                                       00080000
 779 FORMAT('0ASYMPTOTIC STANDARD DEVIATIONS OF THE PARAMETERS'//       00081000
    X(10X,1P10E12.4))                                                   00082000
     DO 841 I=1,NP                                                      00083000
     FP(I)=SQRT(ABS(A(I,I)))                                            00084000
     DO 841 J=1,I                                                       00085000
 841 A(J,I)=A(I,J)                                                      00086000
     WRITE(6,774)                                                       00087000
 774 FORMAT('0ASYMPTOTIC CORRELATION MATRIX OF THE PARAMETERS')         00088000
     L1=0                                                               00089000
 371 L0=L1+1                                                            00090000
     L1=MIN0(NP,L1+10)                                                  00091000
     WRITE(6,372)(L,L=L0,L1)                                            00092000
 372 FORMAT('0',10I12)                                                  00093000
     DO 842 I=1,NP                                                      00094000
     K=0                                                                00095000
```

APPENDIX 1 (Continued)

```
99              DO 373 J=L0,L1                                                  00096000
100             K=K+1                                                           00097000
101             IF(FP(I).EQ.0.0.OR.FP(J).EQ.0.0.OR.A(I,J).EQ.0.0)GO TO 843      00098000
102             B(K)=A(I,J)/(FP(I)*FP(J))                                       00099000
103             GO TO 373                                                       00100000
104          843 B(K)=0.0                                                       00101000
105          373 CONTINUE                                                       00102000
106          842 WRITE(6,374)I,(B(J),J=1,K)                                     00103000
107          374 FORMAT(I4,2X,10F12.5)                                          00104000
108              IF(L1.NE.NP)GO TO 371                                          00105000
109         1515 WRITE(6,553)                                                   00106000
110          553 FORMAT('1CASE        F              Y-F   STANDARD VARIABLES00107000
111             1'/30X,'DEVIATION'/30X,'OF ESTIMATE')                          00108000
112             DO 551 L=1,NC                                                  00109000
113             SDEST=0.0                                                      00110000
114             CALL FUN(Q,FP,P,X(1,L))                                        00111000
115             DO 24 I=1,NP                                                   00112000
116             DO 24 J=1,NP                                                   00113000
117           24 SDEST=SDEST+FP(I)*FP(J)*A(I,J)                                00114000
118             SDEST=SQRT(SDEST*EE)                                           00115000
119             TT=X(ND,L)-Q                                                   00116000
120          551 WRITE(6,552)L,Q,TT,SDEST,(X(I,L),I=1,NV)                      00117000
121          552 FORMAT(1X,I4,10F12.5/(41X,7F12.5))                            00118000
122             RETURN                                                         00119000

123             END                                                            00120000

** END OF LIST **
```

APPENDIX 2: SUBROUTINE FUN

```
0                 $SET SEPARATE                                                                    00120000
1                       SUBROUTINE FUN(F,D,P,X)                                                    00121000
2                 C         SUBROUTINE FUN FOR TEST PROBLEM OF BMDX85                              00122000
3                       DIMENSION X(1),D(3),P(4)                                                   00123000
4                       Q=P(4)                                                                     00123100
5                       F=(Q+P(1)*X(1))/(1+P(2)*X(1))                                              00124000
6                       D(1)=X(1)/(1+P(2)*X(1))                                                    00125000
7                       D(2)=(-X(1)*(Q+P(1)*X(1)))/ ((1+P(2)*X(1))**2)                             00126000
8                 C     D(3)=(-X(1)*(Q+P(1)*X(1)))/((1+P(2)*X(1)) * ((1+P(3)*X(1))**2))            00127000
9                       RETURN                                                                     00128000
10                      END                                                                        00129000

** END OF LIST **
```

```
0                 $SET SEPARATE
1                       SUBROUTINE FUN(F,D,P,X)                                                    00120000
2                 C         SUBROUTINE FUN FOR TEST PROBLEM OF BMDX85                              00121000
3                       DIMENSION X(1),D(3),P(4)                                                   00122000
4                       Q=P(4)                                                                     00123000
5                       F=(Q+P(1)*X(1))/((1+P(2)*X(1))*(1+P(3)*X(1)))                              00123100
6                       D(1)=X(1)/((1+P(2)*X(1))*(1+P(3)*X(1)))                                    00124000
7                       D(2)=(-X(1)*(Q+P(1)*X(1)))/((1+P(3)*X(1)) * ((1+P(2)*X(1))**2))            00125000
8                       D(3)=(-X(1)*(Q+P(1)*X(1)))/((1+P(2)*X(1)) * ((1+P(3)*X(1))**2))            00126000
9                       RETURN                                                                     00127000
10                      END                                                                        00128000
                                                                                                   00129000

** END OF LIST **
```