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Effect of mobile phase additives on peptide retention in reversed-phase chromatography with pellicular and totally porous sorbents

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Abstract

The effect of two mobile phase additives, trifluoroacetic acid and phosphoric acid, on the energetics of peptide retention in reversed-phase chromatography was investigated using Hy-Tach C_{18} micropellicular and Vydac C_4 and C_{18} totally porous stationary phases. The effect of the relatively low phase ratio of columns packed with micropellicular sorbents was also examined. The logarithmic retention factors, of two model peptides, Ac-RGGGGLGLGK-amide and Ac-RGAGGLGLGK-amide, were evaluated with different columns and additives in a practical range of eluent strength. The dependence of the logarithmic retention factor on the concentration of acetonitrile in the mobile phase was linear in all cases. The higher sensitivity of the retention to the organic modifier concentration in the case of the Hy-Tach C_{18} column is attributed to the relatively low phase ratio of this column. Pairwise plots of the logarithmic retention factors were linear. The plots of data obtained with the two additives has unit slopes and thus reveal *homoenergetic* retention behavior. On the other hand data obtained on two different columns manifest *homeoenergetic* retention, the slopes of plots are different from unity. The analysis has yielded consistent results and validated the assumption that the retention free energy can be divided into two components arising from mobile phase and stationary phase contributions. The approach also allowed an estimation of the relative phase ratios of the columns and the Vydac C_{18} column was found to have an 3 and 8 times higher phase ratio than the Vydac C_4 and the Hy-Tach C_{18} column, respectively.

Keywords: Mobile phase composition; Stationary phases, HPLC; Reversed-phase chromatography; Thermodynamic parameters; Peptides

1. Introduction

A traditional approach to enhance the speed and efficiency of separation in HPLC employs pellicular column packing that consists of fluid impervious microspheres with a retentive layer of the stationary phase proper [1–8]. Due to the pellicular configuration, intraparticular mass transfer resistances are confined to the very thin outer shell of the particles and therefore drastically reduced. A paradigm of this approach is represented by Hy-Tach C_{18} columns that are packed with 2-μm fused-silica beads coated with a thin porous octadecylsilica layer and has been employed for rapid separation of peptides and proteins at high resolution [8]. Such columns, which have superior thermal stability, are frequently operated at elevated temperatures in order to reduce the mobile phase viscosity with a concomitant enhancement of the rate of diffusion.

In general, stationary phases of the pellicular

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configuration have adsorption capacities lower than the conventional mesoporous sorbents, particularly in the chromatography of small molecules. On the other hand, the loading capacity of traditional columns were found to be only 3 times higher in the chromatography of large molecules such as proteins than that of equally sized columns packed with the micropellicular stationary phases [6]. Such a slight difference in the column loading capacity is more than compensated by the high speed and efficiency obtained with pellicular packings in analytical work.

We recall that according to a conventional definition the phase ratio is the volume of the octadecyl functions accessible to the eluite divided by the volume of the mobile phase in the column [9]. The phase ratio of columns packed with pellicular stationary phases is lower than that of those packed with conventional sorbents. One of the goals of this study is to examine the effect of the low phase ratio in reversed-phase chromatography of peptides. The other aims at the role of the mobile phase additives trifluoroacetic acid (TFA) and phosphoric acid (PA) that are most commonly used in eluents containing acetonitrile (ACN) for the reversed-phase chromatography of peptides. TFA is known to enhance the solubility of most peptides and to reduce their electrostatic interaction with the residual silanol groups at the chromatographic surface [10–14]. TFA has low UV transparency and high volatility, the latter is of advantage in preparative separations. Phosphoric acid is also a popular additive in the reversed-phase chromatography of peptides and proteins and its advantage over TFA is that it facilitates the elution of peptides at lower ACN concentrations. Despite the wide employment of the two additives in the HPLC of peptides over the last twenty years, the mechanistic understanding of their effect is rather poor.

In the present study the retention behavior of two closely related decapeptides [15–18] is investigated using Hy-Tach C_{18} micropellicular as well as Vydac C_4 and C_{18} totally porous stationary phases with water–ACN mobile phases containing either TFA or PA. Both thermodynamic and extra-thermodynamic analyses of the data were used to examine the properties of the stationary phases employed, the significance of the phase ratio differences on the retention free energy and to shed light on the mechanistic aspects associated with the use of the two mobile phase additives.

2. Experimental

2.1. Materials

HPLC-grade acetonitrile (ACN), reagent-grade sodium hydroxide, phosphoric acid (PA) and trifluoroacetic acid (TFA) were from J.T. Baker (Phillipsburgh, NJ, USA). Synthetic decapeptides S_2 and S_3 [15] having the sequence Ac–RGGGGLGLGK-amide and Ac–RGAGGLGLGK-amide were obtained from Pierce (Rockford, IL, USA).

2.2. Preparation of eluents

ACN and additive required for the concentration stated were mixed in a beaker with 90% (v/v) of the total water volume estimated and the pH of the solution was adjusted under stirring at room temperature. Thereafter the solution was transferred to a volumetric flask and its final volume was adjusted with water. The apparent pH was measured by using a Model pHM82 pHmeter with a No. GK 473901 LL2 glass electrode from Radiometer America (Cleveland, OH, USA) and it was taken as the pH of the solution. A 0.5 *M* aqueous sodium hydroxide solution was used for pH adjustment of eluents containing PA, whereas solution containing TFA were used as such. Deionized water was obtained by a NanoPure unit (Barnstead, Boston, MA, USA), filtered through a 0.5-μm membrane filter from Millipore (Bedford, MA, USA) and degassed by sparging with helium before use.

2.3. HPLC unit and columns

Most experiments were performed using a Hewlett-Packard (Palo Alto, CA, USA) Model HP 1090 liquid chromatograph, equipped with a Model DR5 ternary solvent delivery system, cooled autosampler, thermostated column compartment, and diode-array detector. The column effluent passed through the heat exchanger (Part No. 79880-67304, Hewlett-

Packard) in the diode-array detector before entering the flow-cell.

The chromatographic system was controlled and data evaluation was performed by a Series 79994A ChemStation computer and the chromatograms were recorded on a ColorPro graphic plotter, both from Hewlett-Packard. Other experiments were carried out with an HPLC unit assembled from a Series 400 pump, a Model LC 95 variable-wavelength detector, both from Perkin Elmer (Norwalk, CT, USA), and a Rheodyne (Cotati, CA, USA) Model 7125 injection valve. Before entering the column the eluent passed through a heat exchanger coil and the injector valve, which were placed together with the column in a Model DL-8 constant temperature bath (Haake-Bucher, Saddlebrook, NJ, USA). The dead volume of fittings and tubing was kept to the minimum and the flow-cell of the detector was kept under a pressure of 1000 p.s.i. (6.89 MPa). Chromatograms were obtained with a Shimadzu (Columbia, MD, USA) Model C-R3A Chromatopac integrator. Vydac columns (150×4.6 mm I.D.) containing 5-μm butylsilica or 5-μm octadecylsilica were purchased from The Separations Group (Hesperia, CA, USA). Hy-Tach columns (30×4.6 mm I.D. and 105×4.6 mm I.D.) packed with spherical 2-μm micropellicular octadecyl silica were obtained from Glycotech (Hamden, CT, USA).

2.4. Chromatographic measurements

Water–ACN mixtures containing 2% to 10% (v/v) of the organic modifier and 0.1% TFA or 20 m*M* PA as the additive were used for isocratic elution at 25°C.

3. Theoretical

In interactive chromatography the retention factor k', phase ratio ϕ and the thermodynamic equilibrium constant K for the reversible binding of eluite to the stationary phase are related as

$$k' = \phi K \tag{1}$$

The logarithmic retention factor κ is proportional to the Gibbs free energy change associated with retention ΔG° according to the following relationship,

$$\kappa = \Phi - \frac{\Delta G^\circ}{2.3RT} \tag{2}$$

where R, T and Φ, are the gas constant, absolute temperature and the logarithm of the phase ratio, respectively. Thus, analysis of the logarithmic retention factors offers a mean to gain insight into the physico-chemical phenomena underlying the retention process.

In order to study the energetics of retention in reversed-phase chromatography, κ–κ plots were introduced as diagnostic tools [19]. According to this approach the retention energetics of a set of eluites on two different columns A and B, can be compared by plotting the corresponding κ values according to the relationship

$$\kappa_A = \kappa_B + (\Phi_A - \Phi_B) + \left(\frac{\Delta G^\circ_B}{2.3RT} - \frac{\Delta G^\circ_A}{2.3RT}\right) \tag{3}$$

When the Gibbs free energies for the binding of a particular eluite to the stationary phase at a given mobile phase composition are identical on both columns, the third term on the right hand side of Eq. 3 becomes zero and we obtain that

$$\kappa_A = \kappa_B + (\Phi_A - \Phi_B) \tag{4}$$

Thus, a linear κ–κ plot is obtained according to Eq. 4 when the pairwise retention free energies of the eluite are the same with both stationary phases. According to Eq. 4 the slope of the corresponding κ–κ plots is unity and the intercept is the logarithmic quotient of the two phase ratios. In this case the retention process on the two columns is conveniently termed *homoenergetic* [19], and in reversed-phase chromatography such behavior was found with certain alkyl-silica stationary phases and low-molecular-mass eluites in the absence of specific interactions with the stationary phase. As suggested by Eq. 4, when the retention behavior is *homoenergetic*, the differences observed in the magnitude of retention with a given eluite on various columns by using the same eluent are essentially due to differences in the phase ratios of the columns.

If the corresponding retention free energies on the two columns are not identical but proportional to each other, then

$$\Delta G^{\circ}_{A} = \alpha \Delta G^{\circ}_{B} \tag{5}$$

where α is the proportionality constant. Combining Eqs. 3 and 5 we get

$$\kappa_{A} = \alpha \kappa_{B} + (\Phi_{A} - \alpha \Phi_{B}) \tag{6}$$

Eq. 6 shows that linear $\kappa - \kappa$ plots are obtained also when the ratio of retention free energies in the two columns is constant but in this case the slope will be given by the value of α. This retention behavior has been called *homeoenergetic* [19] because of the similar physico-chemical basis of the retention in the two chromatographic systems.

3.1. Retention on the same column with different additives in the eluent.

Let us assume that the retention free energy consists of two parts arising from contributions by the stationary phase $\Delta G^{\circ}_{S}$and mobile phase ΔG°_{M}, so that

$$\Delta G^{\circ} = \Delta G^{\circ}_{S} + \Delta G^{\circ}_{M} \tag{7}$$

Let us further assume that the mobile phase additive affects only the value of ΔG°_{M} but not that of ΔG°_{S} and Φ. Then we can express the logarithmic retention factors κ_{T} and κ_{P} of a given eluite, which is chromatographed on the same column with the respective additive TFA and PA present in the mobile phase, as

$$\kappa_{T} = \Phi - \frac{\Delta G^{\circ}_{S}}{2.3RT} + \frac{\Delta G^{\circ}_{M,T}}{2.3RT} \tag{8}$$

and

$$\kappa_{P} = \Phi - \frac{\Delta G^{\circ}_{S}}{2.3RT} + \frac{\Delta G^{\circ}_{M,P}}{2.3RT} \tag{9}$$

where ΔG°_{S} is the stationary phase contribution and $\Delta G^{\circ}_{M,T}$ and $\Delta G^{\circ}_{M,P}$ are the appropriate mobile phase contributions to the retention free energy. Subtraction and rearrangement of Eqs. 8 and 9 yield

$$\kappa_{T} = \kappa_{P} + \frac{\Delta G^{\circ}_{M,T}}{2.3RT} + \frac{\Delta G^{\circ}_{M,P}}{2.3RT} \tag{10}$$

Eq. 10 predicts that, when the above assumptions hold, linear $\kappa - \kappa$ plots of the retention data measured on the same column with eluents containing different additives will be linear with unit slope that is characteristic for *homoenergetic* retention behavior.

3.2. Retention on different columns with the same mobile phase additive.

When two different columns are employed with the same eluent the contributions of the stationary phase effects to the Gibbs free energy may not be identical. Yet, the two chromatographic systems still could exhibit *homeoenergetic* behavior when the corresponding stationary phase contributions to the retention free energy are proportional, that is

$$\Delta G^{\circ}_{S,V} = \alpha' \Delta G^{\circ}_{S,H} \tag{11}$$

where α' is a constant and the subscripts V and H refer to the two columns, which are Vydac and Hy-Tach columns in the present investigation. Eq. 11 can be combined with Eqs. 8 and 9 to yield

$$\kappa_{V} = \Phi_{V} - \alpha' \frac{\Delta G^{\circ}_{S,H}}{2.3RT} + \frac{\Delta G^{\circ}_{M,V}}{2.3RT} \tag{12}$$

and

$$\kappa_{H} = \Phi_{H} - \frac{\Delta G^{\circ}_{S,H}}{2.3RT} + \frac{\Delta G^{\circ}_{M,H}}{2.3RT} \tag{13}$$

Upon rearranging Eqs. 12 and 13 we obtain the relationship between the corresponding κ_{V} and κ_{H} values as

$$\kappa_{V} = \alpha' \kappa_{H} + (\Phi_{V} - \alpha' \Phi_{H}) + \left(\frac{\Delta G^{\circ}_{M,V}}{2.3RT} - \alpha' \frac{\Delta G^{\circ}_{M,H}}{2.3RT} \right) \tag{14}$$

Eq. 14 predicts that when the stationary phase contributions to the retention free energy do not depend on the particular additive in the mobile phase

employed, linear $\kappa-\kappa$ plots with slope α' are obtained. As a result the retention on the column pair exhibits *homeoenergetic* behavior.

4. Results and discussion

4.1. Dependence of Φ on ACN concentration

The retention factors of the S_2 and S_3 peptides were measured as a function of the ACN concentration in the mobile phase containing either TFA or PA as the additive. The three columns used in this study were packed with micropellicular C_{18}, totally porous C_4 and C_{18} stationary phases and the plots of κ against the ACN concentration are depicted in Fig. 1. In each case, the ACN concentration was selected so as to obtain accurately measurable retention factors. The plots are linear with correlation coefficients greater than 0.998, so that the data reflect well behaving reversed-phase chromatographic systems.

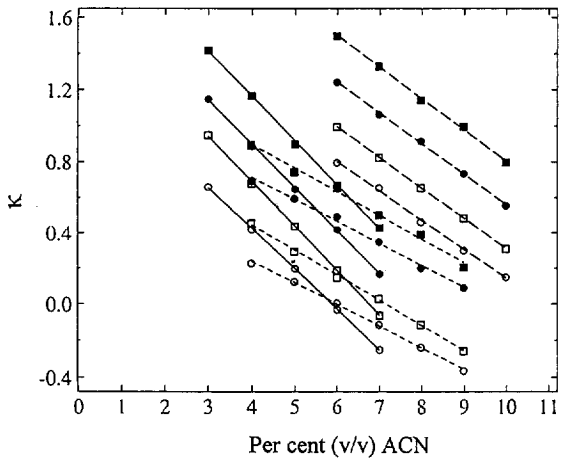

Fig. 1. Effect of the stationary phase and the mobile phase additive on the retention of the S_2 and S_3 peptides. The logarithmic retention factors measured on Hy-Tach C_{18} (solid lines), Vydac C_4 (long dashed lines) and Vydac C_{18} (short dashed lines) columns are plotted against the acetonitrile concentration in the eluent. Data points for the peptides S_2 and S_3 are shown by circles and squares, whereas the additives TFA and PA are indicated by solid and open symbols.

As seen in Fig. 1, the intercepts of lines corresponding to TFA are always greater than those corresponding to PA and this reflects that the eluent strength with TFA as the additive is lower than with PA. As expected from its relatively low phase ratio the peptides were eluted on the Hy-Tach micropellicular column at ACN concentrations lower than those required for the elution of the two peptides with the same retention factor on the totally porous C_4 or C_{18} stationary phases. It is noted that the slopes of the plots for both peptides are significantly steeper with data obtained on the micropellicular octadecylsilica column than on the other two columns. This observation suggests that the slope of these plots depends on the phase ratio of the column in a way that the lower phase ratio results in a higher slope. In our case the slopes of 0.24 for the micropellicular C_{18} sorbent compares to 0.17 and 0.14 for the C_4 and C_{18} totally porous stationary phases, respectively.

4.2. Analysis of $\kappa-\kappa$ plots

One column and two mobile phase additives

The logarithmic retention factors of the S_2 and S_3 peptides were measured on Hy-Tach C_{18} as well as Vydac C_4 and C_{18} columns with PA or TFA in the eluent under otherwise identical conditions. In Fig. 2 the data obtained with one additive was plotted against that with the other. As seen the resulting $\kappa_T-\kappa_P$ plot of the combined data is linear with unit slope and a correlation coefficient of 0.999 or better. According to Eq. 10, linear $\kappa_T-\kappa_P$ plots with unit slopes are manifestations of *homoenergetic* retention behavior with the two mobile phase additives on a given column. The intercept is the difference between the Gibbs free energy contributions arising from mobile phase effects and was found to be 0.44. The results in Fig. 2 are in good agreement with the prediction of Eq. 10 and show that the additives affect only the free energy contribution of the mobile phase but not that of the stationary phase.

When Eq. 10 holds for the chromatographic system under investigation, the ratio of the retention factors obtained with the two additives by using a given column and eluite should be constant. Indeed, examination of the pertinent retention data obtained

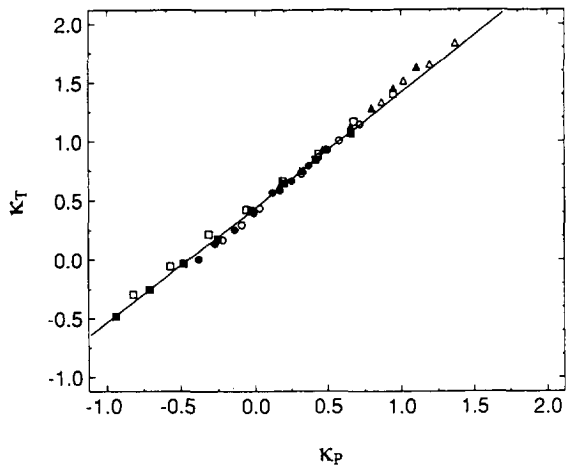

Fig. 2. $\kappa_T - \kappa_P$ plots of retention data obtained with peptides S_2 and S_3 on three different columns by using TFA or PA as the mobile phase additive. Data with peptides S_2 and S_3 are shown by solid and open symbols, respectively. Data referring to the micropellicular Hy-Tach C_{18}, the totally porous C_4 and C_{18} columns are indicated by squares, circles and triangles, respectively.

with TFA and PA shows that the ratio of the respective k' values is 2.75 for each of the three columns. This quantifies the empirical observation that in the case of reversed-phase chromatography of peptides and proteins the eluent strength is lower with TFA than with PA as the mobile phase additive [19] under otherwise identical conditions. Furthermore, it suggests that the two additives affect the properties of the mobile phase in the same way independently of the stationary phase.

Two columns and one mobile phase additive

The logarithmic retention factors of the two peptides obtained with the three columns using TFA and PA in the eluent were plotted pairwise and the results are depicted in Fig. 3. As seen the $\kappa-\kappa$ plots are linear for all three column pairs: Vydac C_4 vs. Hy-Tach C_{18}, Vydac C_{18} vs. Hy-Tach C_{18} and Vydac C_4 vs. Vydac C_{18}. In each case the plots of data obtained with TFA or PA have essentially identical slopes. In view of Eq. 14 this represents a *homeoenergetic* behavior and shows that the free energy contributions attributed to the stationary phase do not depend on the nature of the additive. The differences in the intercepts of the lines representing data obtained with a given column-pair and different additives reflect the dependence of the free energy contribution by the mobile phase containing the additive.

As similar behavior is observed with each column-pair, only the results obtained with the column-pair Vydac C_4 vs. Hy-Tach C_{18} are discussed. In this case the two $\kappa-\kappa$ plots representing the use of TFA and PA in the eluent have the same slope of 0.55 and the respective intercepts of 0.247 and 0.054. The slopes and intercepts obtained from the above analysis can be conveniently used to validate this analytical method and thus verify the assumptions described in the theoretical section.

Since in our case, the phase ratios are fixed and α' is a constant, the intercepts i of the $\kappa-\kappa$ plots of data obtained on the two columns Vydac C_4 and Hy-Tach C_{18} with TFA and PA can be expressed as follows

$$i_T = (\Phi_V - \alpha'\Phi_H) + \left(\frac{\Delta G^\circ_{M,V,T}}{2.3RT} - \alpha'\frac{\Delta G^\circ_{M,H,T}}{2.3RT}\right) \quad (15)$$

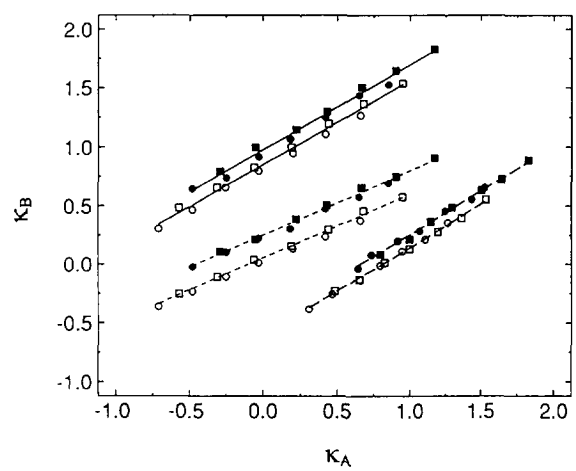

Fig. 3. $\kappa_A - \kappa_B$ plots of retention data obtained with peptides S_2 and S_3 on three column pairs by using two mobile phase additives in the case of each column pair: Vydac C_4 *vs* Hy-Tach C_{18} (solid lines), Vydac C_{18} vs. Hy-Tach C_{18} (long dashed lines), Vydac C_4 vs. Vydac C_{18} (short dashed lines). Data with peptides S_2 and S_3 are represented by circles and squares whereas the open and solid symbols refer to the use of the additives PA and TFA, respectively.

and

$$i_P = (\Phi_V - \alpha'\Phi_H) + \left(\frac{\Delta G^\circ_{M,V,P}}{2.3RT} - \alpha'\frac{\Delta G^\circ_{M,H,P}}{2.3RT}\right) \quad (16)$$

Subtraction and rearrangement of Eqs. 15 and 16 yield an expression for the difference of the intercepts in terms of Gibbs free energy contributions as

$$\Delta i = i_T - i_P = \left(\frac{\Delta G^\circ_{M,V,T} - \Delta G^\circ_{M,V,P}}{2.3RT}\right) - \alpha'\left(\frac{\Delta G^\circ_{M,H,T} - \Delta G^\circ_{M,H,P}}{2.3RT}\right) \quad (17)$$

The Gibbs free energy differences in Eq. 17 are available from $\kappa_T - \kappa_P$ plots of data obtained on single columns with the two mobile phase additives and were found both equal to 0.44 as described previously. The value of α' was obtained as 0.55 from the slope of the $\kappa_V - \kappa_H$ plots of data measured on the two columns according to Eq. 14. With these data Δi is calculated by Eq. 17 as 0.198 and this value is in very good agreement with Δi obtained as the difference of the intercepts of the $\kappa_T - \kappa_P$ plots obtained with data measured on the two columns with TFA and PA that is 0.247−0.054=0.193.

The analysis of retention data obtained with the two different additives on Vydac C_{18} and Hy-Tach C_{18} columns also revealed *homeoenergetic* retention behavior as seen by the corresponding κ_V vs. κ_H plots in Fig. 3. Both plots are linear with identical slopes of 0.74, whereas the intercepts obtained with TFA and PA are 0.960 and 0.850, respectively. Thus the difference of the intercepts is 0.96 − 0.85=0.11. This conforms very well to the Δi value calculated as 0.114 by Eq. 17 with the α' value obtained from the $\kappa_T - \kappa_P$ plot on Vydac C_{18} and Hy-Tach C_{18} columns, see Fig. 2.

The observed and predicted Δi values are summarized in Table 1. The consistency of the results lends further support to the validity of the assumptions and the analytical methods used in our approach. According to our analysis of the $\kappa - \kappa$ plots, the stationary phase contributions to the Gibbs free energy are not effected by the mobile phase additives and the ratios of the mobile phase contributions to the Gibbs free energy are about the same for the different reversed-phase columns. It follows that the two additives have the same effect on retention energies with both columns in the experimental range of ACN concentration in the mobile phase. Consequently, the variations observed in the retention of peptides with different additives in the eluent are likely to arise from the interaction of the additives with the peptides. This is not unexpected since the eluents containing either additives were strongly acidic and thus the ionization of the silanol groups at the chromatographic surface were suppressed. It is believed therefore, that the mechanism of retention was dominated by solvophobic interactions that can be interpreted within the hermeneutics of the solvophobic theory [19].

Besides lowering the pH and thus suppressing ionization of the residual silanols at the chromatographic surface, both TFA and PA are believed to interact with the amino functions of the peptides via ion-pair formation [20]. Furthermore, both additives exhibit strong hydrogen-bonding properties [21]. Phosphoric acid acts both as donor and the acceptor [22] and in most cases, oxygen atoms of the phosphate moiety can engage in more than one hydrogen bond due to the tetrahedral symmetry [23]. Extensive

Table 1
The slopes, intercepts, as well as the observed and predicted differences of the intercepts Δi of the $\kappa_A - \kappa_B$ plots of retention data obtained on three column pairs with trifluoroacetic acid and phosphoric acid in the aqueous ACN eluents

Column Pairs		Slope	Intercept		Intercept difference	
A	B		TFA	PA	Observed[a]	Predicted[b]
Vydac C_4	Hy-Tach C_{18}	0.55	0.247	0.054	0.193	0.198
Vydac C_{18}	Hy-Tach C_{18}	0.74	0.960	0.850	0.110	0.114
Vydac C_4	Vydac C_{18}	0.75	−0.470	−0.579	0.109	0.110

[a] Directly from the plots in Fig. 3.
[b] By Eq. 17 with intercept in Fig. 2 and the corresponding slope in Fig. 3.

hydrogen bonding of the phosphate group to peptide molecules may enhance the virtual polarity of the eluite molecules. TFA acts as a proton acceptor via its fluoro atoms and the carbonyl group [21] and is less polar than PA. Interaction with TFA is expected to reduce the virtual polarity of the peptides in acidic media. Consequently the retention of peptides is greater when the eluent contains TFA rather than PA as the additive under otherwise identical conditions. In turn, with TFA as the mobile phase additive the acetonitrile concentration has to be higher than with PA to elute a peptide at the same k' value. This can also be seen from the results in Fig. 1 and Table 2.

Columns packed with micropellicular stationary phases have lower phase ratios than those packed with totally porous stationary phases having the same bonded moiety. A simple estimate for the ratio of the phase ratios of the Vydac C_{18} and the Hy-Tach C_{18} column can be obtained as follows. Let us assume *homoenergetic* $\kappa-\kappa$ plots despite the fact that the slope of such plots for this system is about 0.75. Then the intercept which is found to be 0.91 can be taken as the quotient $\Phi_{\text{Vydac C18}}/\Phi_{\text{Hy-Tach C18}}$. Antilog of this value gives 8.2 as the ratio of the phase ratios for the Vydac C_{18} and Hy-Tach C_{18} columns. Thus, according to this approximation the totally porous Vydac C_{18} column has about eight times higher phase ratio than the micropellicular Hy-Tach C_{18} column for the eluites investigated.

It is noted that the volume of mobile phase in the Hy-Tach column packed with micropellicular stationary phase is about the half of that in the Vydac C_{18} column having the same dimensions due to the intraparticle porosity of the latter stationary phase. From this we may infer in view of the above estimate for the phase ratio in the totally porous column the amount of the stationary phase volume is about 16 times greater than in the column packed with the pellicular sorbent. If the loading capacity of the column was proportional to the amount of stationary phase, the loading capacity of the Vydac column were 16 times greater than that of the Hy-Tach column. For small molecules this is in agreement with experimental observations. However, for large molecules such as proteins the chromatographic surface of totally porous stationary phase is diminished because of the inaccessibility of the relatively small pores. Consequently, the difference between effective phase ratios as well as the loading capacities of columns packed with pellicular and with porous sorbents for macromolecules is expected to be smaller [6].

Due to their higher phase ratio, columns packed with totally porous sorbents require stronger eluents, i.e., higher organic modifier concentration than columns packed with pellicular sorbents having the same functionality, in order to elute a sample component with the same retention factor. This is shown in Table 2 that lists the concentrations of acetonitrile required in the eluent for the elution of the two peptides, S_2 and S_3, with a retention factor of unity on the three columns having different phase ratios. It should be noted that according to Eq. 1, the equilibrium constant must be greater to obtain a given value of the retention factor when the phase ratio is smaller under otherwise identical conditions.

The above estimation can be extended to a comparison between the phase ratios of Vydac C_{18} and Vydac C_4 columns. The intercept of the corresponding $\kappa-\kappa$ plots yields 2.8 for this phase ratio quotient, i.e., the C_{18} column has a phase ratio about three times higher than the C_4 column. This appears to be somewhat low considering that the octadecyl moieties occupy, on the same support and at a

Table 2
Acetonitrile concentration in the mobile phase needed to elute peptides and S_3 with $k'=1$ on the three columns described in Fig. 1

Column	Percent (v/v) acetonitrile					
Additive	Hy-Tach C_{18}		Vydac C_4		Vydac C_{18}	
	PA	TFA	PA	TFA	PA	TFA
S_2 peptide	5.8	7.8	6.9	10.1	11.0	13.4
S_3 peptide	6.7	8.8	8.2	11.2	11.9	14.0

commensurable surface coverage, a significantly greater volume than the butyl functions, and that the mobile phase volume in the C_{18} column is expected to be smaller than in the C_4 column due to the greater volume of the bonded stationary phase layer. However. the accessibility of the octadecyl functions to molecules as large as decapeptides is likely to be limited and large difference in the chain length of the alkyl functions may also affect the effective surface area and thus the retention behavior of the two stationary phases. Therefore, the estimate of a three times higher phase ratio for the C_{18} column over that of the C_4 column appears to be reasonable.

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