Journal of Chromatography, 203 (1981) 65–84
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,128

SURFACE SILANOLS IN SILICA-BONDED HYDROCARBONACEOUS STATIONARY PHASES

II. IRREGULAR RETENTION BEHAVIOR AND EFFECT OF SILANOL MASKING

KLAAS E. BIJ, CSABA HORVÁTH*, WAYNE R. MELANDER and AVI NAHUM
Chemical Engineering Group, Department of Engineering and Applied Science, Yale University, New Haven, CT 06520 (U.S.A.)

SUMMARY

Dibenzo-crown ethers and certain peptides with unprotected amino groups do not exhibit the regular linear dependence of the logarithmic retention on the composition of binary hydro–organic eluents on silica-bonded C_8 and C_{18} hydrocarbonaceous stationary phases but the plots have minima. Similar results are found when the stationary phase is "naked" silica having no ligates. The results are interpreted in terms of a dual retention mechanism that postulates solute retention as a result of both solvophobic (hydrophobic) and silanophilic interactions between the eluite and stationary phase even in the case of "naked" silica. Masking of the surface silanols by increasing the water concentration of the eluent or by the addition of a suitable amine, which can also be a buffer component, is shown to attenuate silanophilic interactions so that regular retention behavior is observed with both crown ethers and peptides. Beyond its traditional use, this approach may make possible the measurement of chromatographic data without interference by silanophilic effect and, thus, facilitate the development of a retention index system. On the other hand, silanol groups at the surface of bonded phases may be essential to obtain adequate selectivity as illustrated by reversed-phase chromatography of a peptide mixture. The similarity between the retention data obtained with aqueous eluents on "naked" silica gel and on C_8 and C_{18} alkyl-silicas implies that the relative polarity of the mobile and stationary phase is the chief distinguishing mark for classification of various chromatographic systems. Furthermore, it also suggests that the dual retention mechanism may be a rather common phenomenon which needs to be taken into account for interpretation of retention data and designing chromatographic separations.

INTRODUCTION

The predominance of silica-bound hydrocarbonaceous stationary phases in high-performance liquid chromatography (HPLC) has evoked great interest in explicating the mechanism of retention in reversed-phase chromatography (RPC).

0021-9673/81/0000–0000/$02.50

Assuming reversible binding of the eluite molecules to the alkyl ligates at the surface of the stationary phase we adapted the rather comprehensive solvophobic theory[1] to quantify the major factors involved in the retention process which is treated essentially as a solvent effect[2–4]. Although the properties of the stationary phase surface are idealized in this treatment, the results have greatly facilitated our understanding of the physico-chemical phenomena and the prediction of retention and selectivity, particularly when water-rich eluents and neutral or anionic eluites are employed. Recent study[5] has demonstrated that despite the variations observed among commercial long-chain alkyl-silica bonded phases in practice, their intrinsic retention behavior under the conditions stated above is essentially identical, *i.e.*, it manifests itself in homoenergetic retention in agreement with the predictions of the solvophobic theory.

However, as noted by numerous chromatographers, retention in RPC does not always exhibit the "regular" behavior expected on the basis of the solvophobic (hydrophobic) effect. Some anomalous phenomena have been explained as being due to secondary chemical equilibria[6,7] in the eluent. Perhaps most irregularities can be attributed to eluite interaction with silanol groups at the surface of the stationary phase. The impact of such silanophilic interactions on retention is particularly pronounced when the eluent is rich in organic solvent and the eluite molecule contains amino or other similar functions.

Alternatively specific binding of one or more components of the eluent by the stationary phase may also give rise to changes in the surface properties with concomitant alteration of retention behavior. This phenomenon in RPC has been hardly explored and its importance cannot yet be assessed except in obvious cases, *e.g.* when the stationary phase surface is converted into a dynamically-coated ion exchanger by an ion-pairing hetaeron present in the eluent[8].

Recently the effect of silanol groups in RPC has been analyzed by using a dual retention mechanism model[9] that predicts the minimum observed in plots of logarithmic retention factor against eluent composition for the chromatography of a crown ether on octadecyl-silicas with methanol–water mixtures as the eluent. The goal of the present work is to shed further light on the nature of silanophilic interactions believed to be responsible for irregular behavior including non-linear retention behavior.

Particular attention is paid to the use of silanol-masking agents in the eluent that allow us to obliterate silanophilic retention, thus facilitating regular retention behavior. The latter is essential for the development of a hydrophobic index system[10] of sufficiently broad scope for characterizing the properties of eluites, solvent mixtures and columns and for optimizing chromatographic separations.

Of course, heterogeneity of the stationary phase surface is one of the most prominent and time-honored problems of chromatography. Solutions ranged from impregnation of charcoal with long-chain fatty acids[11] through poisoning active sites with KCN or H_2S[12] to the use of octanol as a "saturator" to deactivate the stationary phase[13], for instance. In HPLC the use of amino compounds has been recommended[14,15] to mediate untoward effect of residual silanols in the stationary phase. Considering the wealth of data on tailing reducers and other meritorious ingredients in chromatographic systems the present work does not claim novelty for the concepts presented here. Yet, it is a step in our continuing effort to gain further insight in the chromatographic process by attempting a quantitative treatment of data obtained with presently available precision measuring devices.

Theory of silanol masking

As discussed elsewhere[9] the dual retention model postulates that the retention factor k of an eluite that is bound in two different ways to the stationary phase can be expressed as the sum of the retention factors for the two processes

$$k = k_1 + k_2 \tag{1}$$

where

$$k_1 = \varphi_1 K_1 \tag{2a}$$

and

$$k_2 = \varphi_2 K_2 \tag{2b}$$

where K and φ are the thermodynamic equilibrium constants and the phase ratios of the available binding sites, respectively, and the subscripts 1 and 2 are used to distinguish the above quantities as being solvophobic and silanophilic, respectively.

A masking agent such as a long-chain aliphatic amine added to the eluent is expected to bind to a silanol (and to one or more hydrocarbonaceous ligates), a phenomenon for which the term "silanophilic interaction" is used. It is assumed that upon binding of the masking agent the surface concentration of the accessible silanols [SiOH] and the corresponding phase ratio φ_2 are reduced, whereas φ_1 remains unchanged.

The reversible binding of the masking agent A to the stationary phase is characterized by the corresponding equilibrium constant

$$K_A = \frac{[\text{SiOH}\cdot\text{A}]}{[\text{SiOH}]\,[\text{A}]} \tag{3}$$

where [A] is the concentration in the mobile phase, [SiOH] is the concentration of the surface silanols and [SiOH·A] is the surface concentration of the complex. Masking reduces the surface concentration of the available silanols and the actual phase ratio for silanols becomes

$$\varphi_2 = \frac{\varphi_{2,\max.}}{(1 + K_A[\text{A}])} \tag{4}$$

where $\varphi_{2,\max.}$ is the phase ratio of silanols in the absence of masking agent. In view of eqn. 2b the silanophilic retention factor increment will also decrease with increasing concentration of the masking agent. The value of the overall retention factor is given by

$$k = k_1 + \frac{k_2}{1 + K_A[\text{A}]} \tag{5}$$

where k_2 is the retention factor increment for silanophilic binding in the absence of a masking agent.

Since k_1 and k_2 are properties of the column and the solvents used in the eluent, they are expected to be invariant with changing concentration of the masking agent. Eqn. 5 shows that with increasing concentration of the masking agent the retention factor decreases to its solvophobic limit.

Measurement of the binding constant for the masking agent

The potency of the masking agent in attenuating silanophilic retention is conveniently quantified by K_A, which can be used to rank various masking agents according to their efficacy. Two methods are proposed here for the evaluation of K_A from chromatographic measurements.

The retention factor k obtained at a certain concentration [A] of the amine in the mobile phase can be subtracted from the retention factor, k_0, obtained in the absence of hetaeron to yield

$$k_0 - k = k_2 K_A[A]/(1 + K_A[A]) \tag{6}$$

and eqn. 6 can be rearranged to

$$\frac{[A]}{k_0 - k} = \frac{1}{k_2 K_A} + \frac{[A]}{k_2} \tag{7}$$

According to eqn. 7, plots of $[A]/(k_0-k)$ against [A] yield straight lines with the reciprocal of the silanophilic retention factor as the slope. The stability constant of the silanol complex, K_A, is given by the slope to intercept ratio. Various other linear transformations of eqn. 6 may be made for the evaluation of the stability constant[16] and the silanophilic retention factor.

The graphical methods for determination of the stability constant require that retention data be obtained at several concentrations of a given masking agent. Obtaining these data may represent an unjustifiable expenditure of effort when the objective is to compare rapidly the masking efficacy of several agents. In such cases a simplified method such as that described below can be used for evaluation of K_A.

Let us define the variable Δ_I from the retention factor in the absence of masking k_0 and the retention factor k_I obtained at concentration $[A]_I$ of masking agent according to

$$\Delta_I = (k_0 - k_I)/[A]_I \tag{8a}$$

In a similar fashion the variable Δ_{II} can be defined as

$$\Delta_{II} = (k_0 - k_{II})/[A]_{II} \tag{8b}$$

where k_{II} is the retention factor of an eluite when the concentration of the masking agent is $[A]_{II}$. Combination and rearrangement of eqns. 5, 8a and 8b yield the following expression for the equilibrium constant for silanol masking

$$K_A = \frac{\Delta_I - \Delta_{II}}{[A]_{II}\Delta_{II} - [A]_I\Delta_I} \tag{9}$$

The results obtained by using eqn. 9 are, of course, more sensitive to experimental error than those obtained by using eqn. 7 with data measured at a relatively large number of concentrations and therefore caution should be used in their interpretation.

EXPERIMENTAL

Materials

Dibenzo-18-crown-6 (DB18C6) was obtained from Eastman (Rochester, NY, U.S.A.) and dibenzo-24-crown-8 (DB24C8) was from Strem Chemicals (Newburyport, MA, U.S.A.). All quaternary amines were Eastman products, and 2-ethylaminoethanol and diisopropylamine were from Aldrich (Milwaukee, WI, U.S.A.). N,N-Dimethyldodecylamine, *n*-butylamine and other chemicals were purchased from Chem Service (West Chester, PA, U.S.A.). The peptides used, N-*tert.*-butyloxycarbonyl-L-valyl-L-methionyl-L-alanylglycyl-L-valyl-L-isoleucylglycine ethyl ester, the hydrochloride of L-valyl-L-methionyl-L-alanylglycyl-L-valyl-L-isoleucylglycine ethyl ester, N-*tert.*-butyloxycarbonyl-L-leucyl-L-leucyl-L-isoleucyl-(O-benzyl)-L-seryl-(O-benzyl)-L-tyrosylglycine ethyl ester, the trifluoroacetate salt of L-leucyl-L-isoleucyl-(O-benzyl)-L-seryl-(O-benzyl)-L-tyrosylglycine ethyl ester, were a gift of R. E. Galardy (presently at the Department of Biochemistry, University of Kentucky, Lexington, KY, U.S.A.). Acetonitrile, methanol, tetrahydrofuran, ethyl acetate, methylene chloride, and *n*-hexane were "distilled in glass" from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Distilled water was prepared with a Barnstead distilling unit.

Instruments and columns

The chromatographic system consisted of a Model FR-30 pump (Knauer, Berlin, G.F.R.) with a Rheodyne (Berkeley, CA, U.S.A.) Model 7010 sampling valve having a 20-μl sample loop and a Kratos-Schoeffel (Westwood, NJ, U.S.A.) Model 770 variable-wavelength UV detector. In experiments with crown ethers we used a Kratos-Schoeffel Model FS-970 fluorometric detector with excitation wavelength and emission filter cut-offs set at 218 and 300 nm, respectively. Chromatograms were obtained with a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 56 dual-pen recorder. The column temperature was controlled by recirculating water through an insulated stainless-steel jacket from Model K-2/R thermostatted water bath (Messgeraete-Werk, Lauda, G.F.R.). Whatman (Clifton, NJ, U.S.A.) supplied 10-μm Partisil silica, Partisil ODS, ODS-2 and ODS-3 columns, and 10-μm LiChrosorb RP-18 and 7-μm LiChrosorb RP-8 columns were obtained from Knauer. 5-μm Supelcosil LC-8 and LC-18 columns were from Supelco (Bellefonte, PA, U.S.A.). The dimensions of the above columns were 250 × 4.6 mm I.D. or 150 × 4.6 mm I.D. The 5-μm Hypersil ODS column was from Shandon Southern Products (Runcorn, Great Britain). The 10-μm LiChrosorb RP-8 (Merck, Darmstadt, G.F.R.) columns (40 × 4.6 min I.D.) were prepared in our laboratory by slurry packing of the particles with isopropanol. In addition to alkyl-silica columns, columns packed with cross-linked porous polystyrene particles were also employed. Amberlite XAD-2 was obtained from Rohm and Haas (Philadelphia, PA, U.S.A.), ground and classified by sedimentation in our laboratory[17]. Styragel having a particle size of 35–75 μm was obtained from Waters Assoc. (Milford, MA, U.S.A.). Both particulate preparations were slurry packed using methanol into columns having dimensions of 150 × 4.6 mm I.D.

Procedures

In experiments involving crown ethers the reversed-phase columns were conditioned by washing sequentially with methanol, tetrahydrofuran, ethyl acetate, methylene chloride and *n*-hexane, as described previously[9].

Peptides were usually dissolved in 2,2,2-trifluoroethanol at a concentration of *ca.* 0.5 mg/ml and detection of the peptides was achieved by monitoring the absorbance of the column effluent at 220 nm.

The retention time of fructose was used as the retention time of an unretarded component. The retention time of an eluite was evaluated at the peak maximum. The retention factors k were calculated in the usual way[4,5].

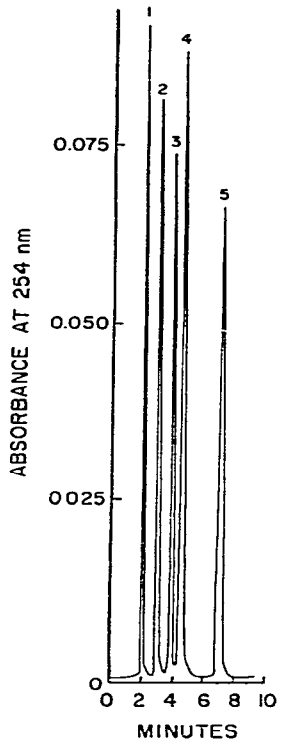
Spacefilling molecular structures of dibenzo-18-crown-6, dibenzo-24-crown-8, N,N-dimethyldodecylamine and triethylamine were constructed using the PROPHET system.

RESULTS AND DISCUSSION

Silanophilic interactions with crown ethers

Plots of the logarithmic retention factor of DB18C6 obtained on either Partisil ODS or Partisil ODS-2 against the composition of methanol–water eluent were found to pass through a minimum[9]. On the other hand, such plots are usually linear over the entire composition range of binary eluents in RPC of relatively small and less polar eluites, although similar irregular retention behavior has been observed with other heterocyclic molecules[18–21]. Conformational changes have also been found to give rise to irregular retention behavior[6]. The results obtained with DB18C6, however, were interpreted as the consequence of retention by both silanophilic and solvophobic mechanisms with the former dominant at low water concentrations and the latter significant at high water concentrations in the eluent[9].

In order to test further the hypothesis that silanol groups at the surface are responsible for the retention of crown ethers on silica-bonded hydrocarbonaceous stationary phases in contact with water-lean eluents, DB18C6 and DB24C8 were chromatographed on Hypersil ODS, LiChrosorb RP-8 and LiChrosorb RP-18 columns by using methanol–water mixtures rich in methanol. A typical chromatogram, Fig. 1, illustrates the separation of a mixture containing the two crown ethers. Plots of the logarithmic retention factors obtained with the above columns for each crown ether *versus* eluent composition are shown in Fig. 2. In the case of DB18C6 each plot passes through a minimum when the aqueous eluent contains 20–30% methanol in agreement with earlier results[9] obtained by using Partisil ODS and Partisil ODS-2 columns. Plots for DB24C8, however, do not pass through a minimum although they manifest curvature, indicating greater retention at low water concentrations than expected from retention observed at high water concentration upon assuming regular behavior, *i.e.*, linear relationship between logarithmic retention factor and solvent composition. As irregular retention behavior has been observed on five different alkyl-silicas, we may conclude that it is a general property of silica-bonded hydrocarbonaceous stationary phases rather than an idiosyncracy of a particular type of column material. The results depicted in Fig. 3 were obtained in binary hydro–organic solvent with methanol, acetonitrile and tetrahydrofuran as the



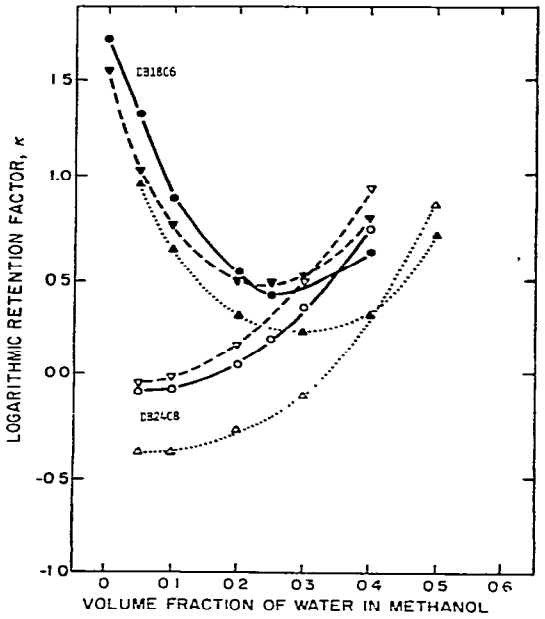


Fig. 1. Typical chromatogram illustrating the separation of a mixture containing crown ethers by reversed-phase chromatography. Column, 10-μm Partisil ODS-3 (250 × 4.6 mm); eluent, 0.1 M $NaClO_4$ in methanol–water (7:3); temperature, 25°C; flow-rate, 1.5 ml/min. Sample components: 1 = resorcinol; 2 = DB18C6; 3 = nitrobenzene; 4 = DB24C8; 5 = toluene.

Fig. 2. Graph illustrating the dependence of the logarithmic retention factor for crown ethers on the volume fraction of water in the methanol–water mixture used as the eluent. Solid and open symbols represent DB18C6 and DB24C8, respectively. The stationary phases employed are Hypersil ODS (○, ●), LiChrosorb RP-8 (△, ▲), and LiChrosorb RP-18 (▽, ▼).

organic solvent component. It is seen that for DB18C6 all κ *vs.* ψ plots* exhibit a minimum whereas with acetonitrile–water mixtures a minimum is obtained also for DB24C8. These results support the earlier finding[9] that the minimum does not arise from specific solvation effects and that the observed retention behavior is unlikely to be the result of secondary chemical equilibria with organic components of the mobile phase. A common feature of the results is increasing retention with decreasing water concentration. This is consistent with an interpretation that the retention is due to silanophilic interactions at low water concentrations[9] whereas water "masks" silanolic sites at higher water concentrations so that retention is due to solvophobic (hydrophobic) interactions under these conditions.

The cause of the different retention behavior of the two crown ethers is not clear. Silanophilic interactions are likely to involve hydrogen bonding between the silanols and the oxygens of the crown ring. Examination of spacefilling models of the

* According to earlier convention[9] the volume fraction of water in the binary hydro–organic eluent and the logarithm of the retention factor, k, are denoted by ψ and κ, respectively.

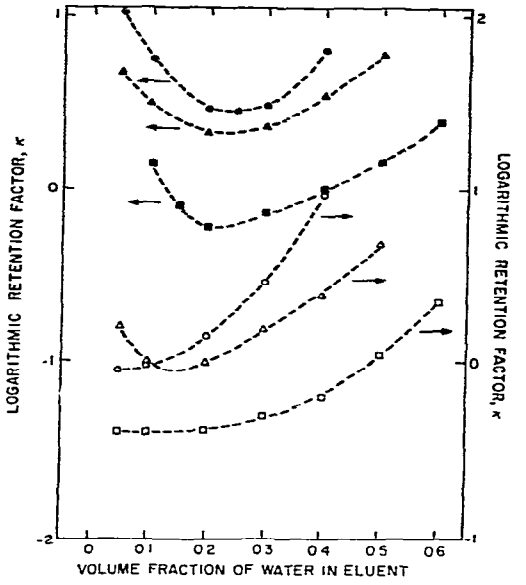


Fig. 3. Graph illustrating plots of the logarithmic retention factor of crown ethers on LiChrosorb RP-18 as a function of the composition of various hydro–organic eluents. Solid and open figures correspond to DB18C6 and DB24C8, respectively. The eluents are as follows: methanol–water (○, ●); acetonitrile–water (△, ▲); and tetrahydrofuran–water (□, ■).

two crown ethers revealed that the ring of DB24C8 is much larger than that of DB18C6 and therefore can accommodate larger guest ions, as stated by others[23], but gave no insight into silanophilic interactions of crown ethers, insofar as neither ring seemed to accommodate a silanol uniquely well. Furthermore, the crown usually binds cations rather than neutral species or anions such as the ionized silanol. To obtain additional information the two molecules were constructed graphically by using the PROPHET system, so that the ring energy was minimized according to the Wipke method[24] and the results are shown in Fig. 4. It is seen that the two crown

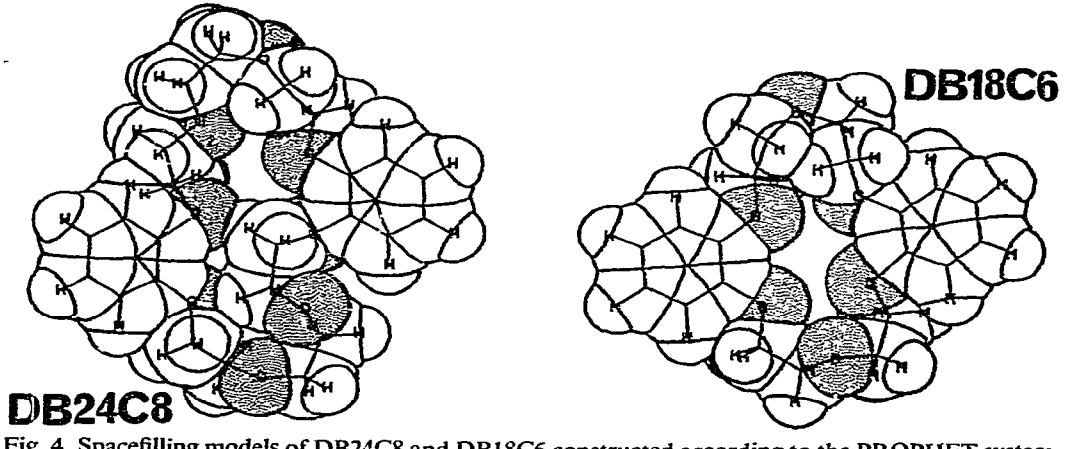


Fig. 4. Spacefilling models of DB24C8 and DB18C6 constructed according to the PROPHET system. For emphasis, the oxygens are shaded.

ethers have significantly different three-dimensional oxygen density. As silanols are expected to form hydrogen-bonds with ring oxygens of crown ethers, the proximity of the oxygen atoms in the ring should result in enhanced binding. Such a geometric effect may be responsible for the greater silanophilic retention of DB18C6 compared to that of DB24C8.

Evidence for significant participation of silanols in retention is found in retention data for the crown ethers obtained under non-aqueous reversed-phase conditions. Neat methanol, acetonitrile and their mixtures were used as eluents. Because acetonitrile is the stronger eluent in RPC[25,26], decrease in retention factor with increase in acetonitrile concentration is expected and, as shown in Fig. 5, this behavior is indeed observed. However, as the concentration of acetonitrile exceeds 60% (v/v) the retention factors of the crown ethers increase. Whereas this behavior is totally unexpected on the basis of the solvophobic theory[1,3], it can be readily interpreted by the dual retention model[9]. If silanols bind methanol in preference to acetonitrile, it is quite clear that the retention cannot occur by silanophilic or solvophobic interactions alone because each mechanism would predict a monotonic decrease in retention factor with decrease in concentration of one of the solvent components.

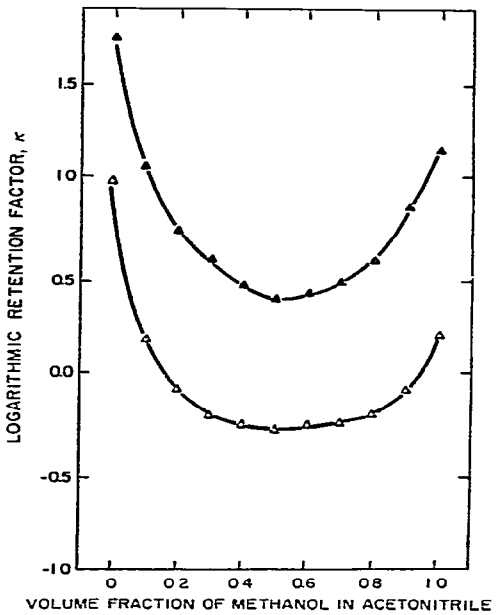


Fig. 5. Graph illustrating the dependence of the logarithmic retention factor of crown ethers on Partisil ODS as a function of the composition of the methanol–acetonitrile mixtures used as the non-aqueous eluent. Data for DB18C6 and DB24C8 are shown by the symbols ▲ and △, respectively.

Amines are generally believed to interact strongly with silanols[14,15] and aliphatic amines have been used to reduce tailing in reversed-phase chromatography. If an amine, which competes well with an eluite for silanols, is included in the mobile phase at a sufficiently high concentration, retention of eluite will occur only by solvophobic

mechanism and linear κ *vs.* ψ plots will be obtained. Indeed, as shown in Fig. 6, reversed-phase chromatography of DB18C6 and DB24C78 with methanol–water mixtures containing 10 m*M* hexadecyltrimethylammonium bromide yields quasi-linear plots in contrast to the parabolic plots obtained for the crown ethers with otherwise identical mobile phases without the amine. This observation also supports the postulate that the irregular retention behavior which manifests itself in non-linear κ *vs.* ψ plots is due to the effect of surface silanols provided no specific solvent effects or conformation changes[6] are involved.

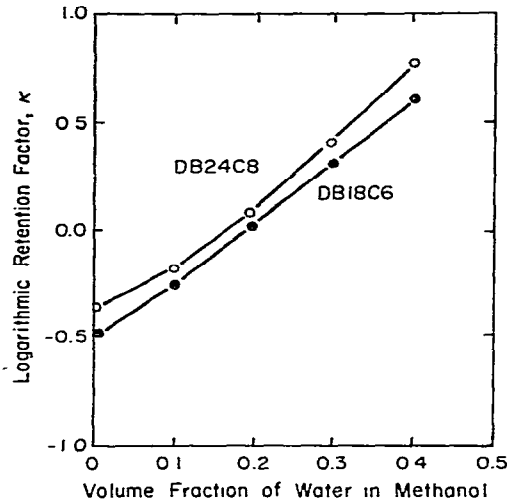
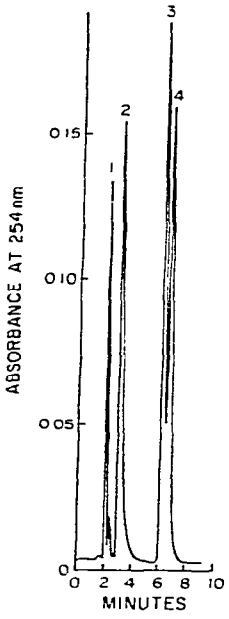


Fig. 6. Plots of the logarithmic retention factors of DB18C6 and DB24C8, obtained on Partisil ODS-2, against the eluent composition. The water–methanol mixture used as the eluent contained 10 m*M* trimethylhexadecylammonium bromide.

Reversed-phase chromatography on naked silica

The name reversed-phase chromatography is usually associated with the use of a non-polar stationary phase and a polar mobile phase[27]. These two terms, however, defy precise definition and it may be preferable to denote reversed-phase systems as consisting of a stationary phase less polar than the mobile phase. Within this nomenclature the use of silica gel columns with a neat aqueous or water-rich hydro–organic eluent would also fall into the category of reversed-phase chromatography. In our study on crown ethers and peptides, we have indeed found that under the above conditions RPC could be carried out by using naked silica, *i.e.*, unaltered silica gel, as the stationary phase. Fig. 7 shows a chromatogram obtained with naked silica with gradient elution from plain water at increasing methanol concentration and it is seen that sample components eluted in order of decreasing polarity, *cf.* Fig. 1.

Further examination of the retention behavior of large eluite molecules with polar functions which interact with silanols also evidences that a chromatographic system consisting of naked silica and a hydro–organic eluent may exhibit properties attributed to RPC systems. Fig. 8 illustrates that the retention factors of the two crown ethers, DB18C6 and DB24C8, on naked silica have a dependence on the composition of the methanol–water eluent similar to that found when the stationary phase



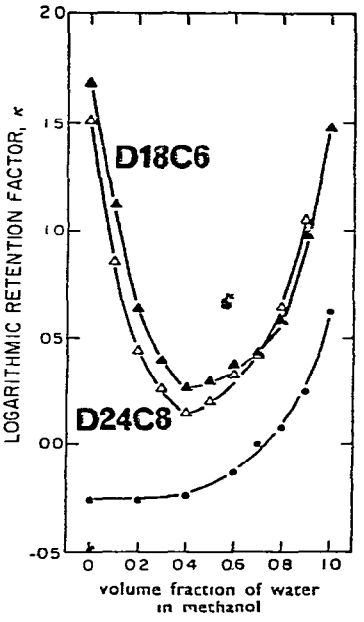


Fig. 7. Reversed-phase chromatogram showing the separation of a mixture containing crown ethers on naked silica. Peaks: 1 = nitrobenzene; 2 = resorcinol; 3 = DB18C6; 4 = DB24C8. Column: 10-μm Partisil silica, 250 × 4.6 mm I.D. Mobile phase: from 0 to 2 min, 10% methanol in water; from 2 to 10 min, a gradient from 10% to 25% methanol in water. Flow-rate, 1.5 ml/min; temperature, 25°C; detection, UV absorbance at 254 nm.

Fig. 8. Graphs illustrating the relationship of the retention factor of crown ethers on naked silica gel and the composition of the hydro-organic eluent. The eluites are DB18C6 (▲) and DB24C8 (△). The retention of propylbenzene (●) having no polar groups is significant only at high water concentrations, *i.e.* in the reversed-phase chromatographic mode.

is alkyl-silica, such as octyl- or octadecyl-silica (Fig. 3). Analogous retention behavior is observed on naked silica with an otherwise non-polar heptapeptide which has a free amino group at the N-terminus as illustrated by the κ *vs.* ψ plot depicted in Fig. 9. When the terminal amino group is blocked, however, the logarithmic retention factor becomes a linear function of the eluent composition as also shown in Fig. 9. Comparison of these results with those obtained with octadecyl-silica (Figs. 2 and 3) for instance, as well as the chromatographic behavior of peptides discussed below, leads to the conclusion that when the polarity of the eluent is sufficiently high, silica gel proper can act as a "non-polar" stationary phase. Thus, it can be used for the separation of hydrophobic peptides not only in the "normal"[28] but also in the "reversed-phase" mode.

In the hermeneutics of the traditional chromatographic classification this behavior of silica may be considered anomalous although it simply demonstrates that the polarity of the eluent relative to that of the stationary phase determines whether the retention behavior is "normal" or "reversed". It is known that the siloxane groups at the silica surface are essentially hydrophobic[29,30]. Thus, the data can be interpreted in the light of the dual retention mechanism[9] which occurs due to the presence of "hydrophilic" silanol and "hydrophobic" siloxane groups at the stationary phase

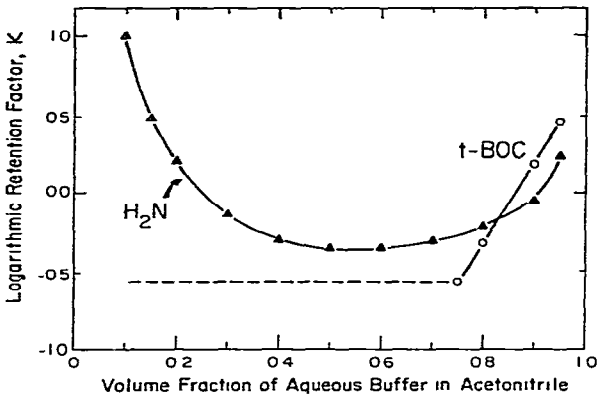


Fig. 9. Retention of protected and unprotected heptapeptides on naked silica as a function of the eluent composition. ○-○, Boc-Val-Met-Ala-Gly-Val-Ile-Gly-OEt; ▲-▲, NH_2-Val-Met-Ala-Gly-Val-Ile-Gly-OEt. Column, 10-μm Partisil, 250 × 4.6 mm I.D.; eluent, acetonitrile–aqueous 50 m*M* sodium phosphate buffer, pH 2.25; flow-rate, 1.5 ml/min; detection, UV at 220 nm; temperature, 25°C. For volume fractions of aqueous buffer of 0.1 to 0.75 the κ values of the Boc-heptapeptide were smaller than the value indicated by the dashed line.

surface. An analysis similar to that given for the irregular retention of crown ethers on octadecyl-silica can be employed to interpret the minima of κ *vs.* ψ plots observed with data obtained by using columns packed with naked silica. In any case the Janus-faced behavior of various silica gels —and for that matter perhaps of all other stationary phases— should serve as a caveat in mechanistic interpretation of chromatographic data.

Silanophilic interactions with peptides

It has been frequently observed in RPC of nitrogenous compounds that plots of the logarithmic retention factor *versus* the composition of binary hydro-organic eluents are not linear[19–22,31,32] and even show minima[19–22]. In addition, peaks of such eluites are often broad and asymmetric, and those phenomena have been attributed to interaction with silanols[15]. With these facts in mind, we have investigated the retention behavior of some hydrophobic peptides with the N-terminal amino group free or blocked with a *tert.*-butyloxycarbonyl (Boc) group and the C-terminus in the ethyl ester form. The peptides used are fragments of glycophorin A[33] and for convenience, we shall use the notation Boc-G_7 and NH_2-G_7 for heptapeptide fragments (G_7) having blocked and free N-terminus, respectively. The amino acid sequence of G_7 is Val-Met-Ala-Gly-Val-Ile-Gly.

The results obtained with NH_2-G_7 and Boc-G_7 in RPC on Supelcosil LC-8 by using aqueous methanol or acetonitrile that contained 10 or 50 m*M* phosphate buffer, pH 2.25, are depicted in Fig. 10. It is seen that κ *vs.* ψ plots for NH_2-G_7 pass through a minimum at relatively low water concentrations. In contradistinction Boc-G_7 exhibits regular retention as seen from the linear κ *vs.* ψ plot. Comparison of data suggests that the minimum observed for the unprotected peptide arises from a dual retention mechanism involving silanophilic interactions with the free amino group in addition to the solvophobic effect usually responsible for retention in RPC. Indeed, the interaction may be electrostatic as shown by the approximately five-fold

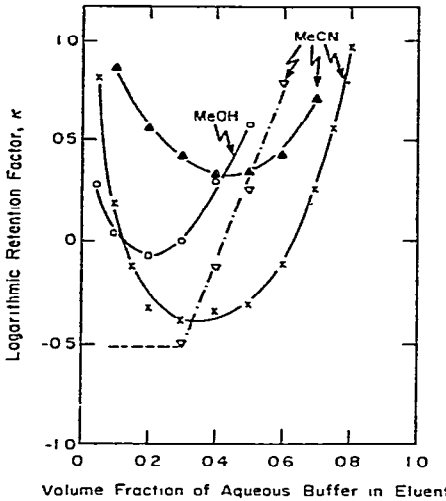


Fig. 10. Retention of NH_2-Val-Met-Ala-Gly-Val-Ile-Gly-OEt and Boc-Val-Met-Ala-Gly-Val-Ile-Gly-OEt on Supelcosil LC-8 as a function of eluent composition. For volume fractions of aqueous buffer of 0.1 to 0.3 the κ value of the Boc-derivative (▽) was less than the value indicated by the dashed line. Column, Supelcosil LC-8, 150 × 4.6 mm I.D.; eluents, acetonitrile–aqueous 10 m*M* sodium phosphate, pH 2.25 (▲); acetonitrile–aqueous 50 m*M* sodium phosphate, pH 2.25 (× and ▽); and methanol–aqueous 50 m*M* sodium phosphate, pH 2.25 (○); flow-rate, 1.5 ml/min; temperature, 25°C; detection, UV at 220 nm.

increase in the retention factor upon decreasing the phosphate concentration from 50 to 10 m*M*.

In order to investigate further the interaction between the stationary phase and amines, the retention of benzyltriethylammonium bromide was measured on LiChrosorb RP-8 with methanol–0.1 *M* aqueous sodium phosphate, pH 7.0 (10:90) as the mobile phase at different sample loads. As shown in Fig. 11, the retention factor

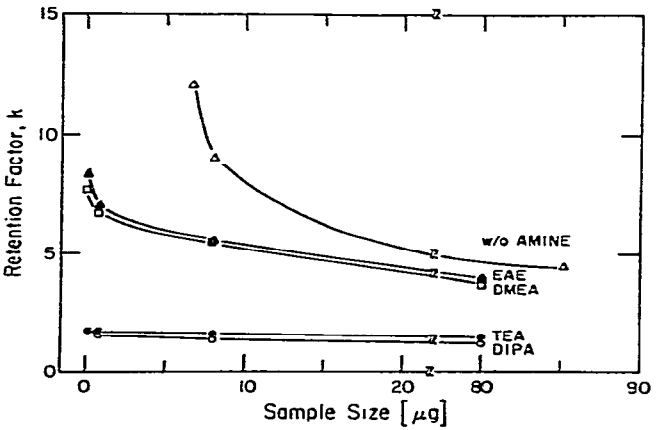


Fig. 11. Effect of different amines on the retention of benzyltriethylammonium bromide. Column, 10-μm LiChrosorb RP-8, 40 × 4.6 mm; eluent, methanol–aqueous 0.1 *M* sodium phosphate, pH 7.0 (10:90) without amine or containing 2.0 m*M* amine; flow-rate, 2.0 ml/min; temperature, 25°C. EAE = 2-ethylaminoethanol; DMEA = dimethylethanolamine; TEA = triethylamine; DIPA = diisopropylamine.

decreases dramatically as the amount of sample injected increases in the range under investigation. However, upon adding N-ethylaminoethanol or dimethylethanolamine to the eluent, the effect is reduced and when triethylamine or diisopropylamine is used, retention is practically independent of the sample load in the range investigated. The results suggest that there is a competition between the amines and the eluite for surface silanols and triethyl- or diisopropylamine block the stronger binding sites so that they are no longer available to the eluite. Ethanolamines are also expected to bind to the silanols via the amine group. Yet, the masking effect of these substances, which contain hydroxyl function, appears to be much lower than that of those without polar groups besides the amine function. Triethylammonium buffers are particularly popular in chromatography[14,34–37] and other amine buffers have also been found to be useful in RPC[17].

The effect of sample size on the retention was also investigated with benzyltriethylammonium bromide and 4-methoxy-1-methylpyridinium iodide at both pH 2.12 and pH 8.80 and the results are presented in Fig. 12. Whereas at pH 2.12 sample size effects on the retention are rather small, at pH 8.80 the sample load dramatically affects retention. This behavior can be readily explained by the ionization of silanols at alkaline pH that results in a large increase in silanophilic interactions with positively charged eluites.

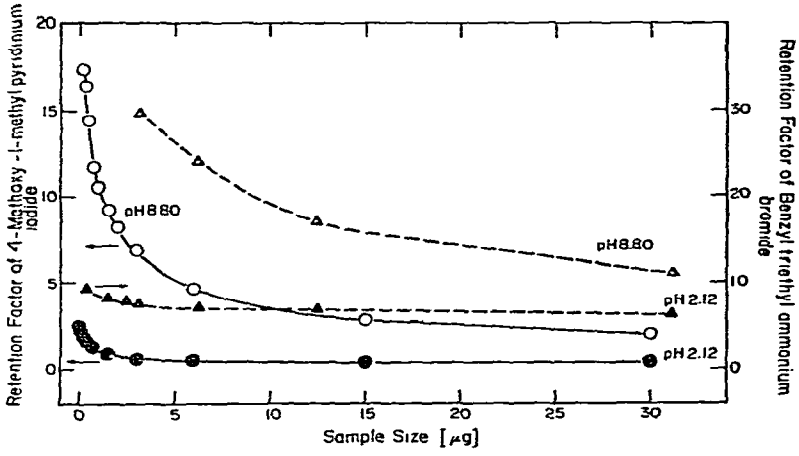


Fig. 12. Effect of sample size and pH on the retention of 4-methoxy-1-methylpyridinium iodide (○ and ●) and benzyltriethylammonium bromide (△ and ▲). Column: LiChrosorb RP-8, 40 × 4.6 mm I.D.; eluents, methanol–aqueous 0.1 *M* sodium phosphate, pH 2.12 (10:90), and methanol–aqueous 0.1 *M* Tris, pH 8.80 (10:90); for both eluents the ionic strength was brought to 1.125 by the addition of sodium sulfate; flow-rate, 2 ml/min; temperature, 25°C; detection, UV at 254 nm.

Masking of silanols

Silanophilic interactions are conveniently reduced or eliminated by incorporation of an amine, which binds sufficiently strongly to surface silanols, in the eluent. The spacefilling molecular structures in Fig. 13 illustrate the large size difference between the two types of amine that are potentially useful silanol-masking agents in RPC. Relatively small molecules such as triethylamine are sufficiently soluble and can also serve as buffer components. In order to reduce excess band spreading and

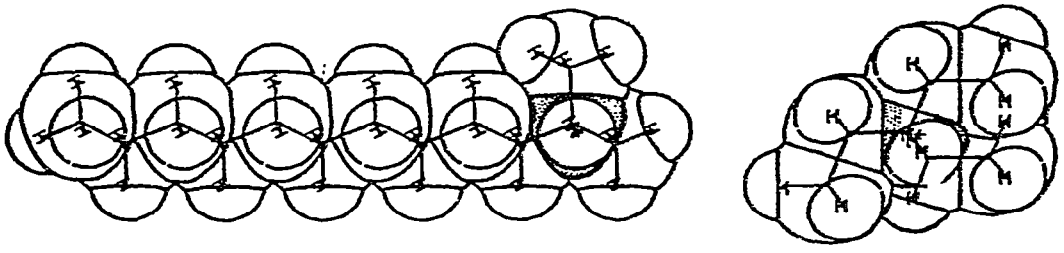


Fig. 13. Spacefilling structure of N,N-dimethyldodecylammonium and triethylammonium ions.

other untoward manifestations associated with silanophilic interactions, sometimes high concentrations of these substances are required to be effective. On the other hand, amines having a bulky alkyl moiety such as N,N-dimethyldodecylamine are usually effective at concentrations as low as 1 m*M* because the silanol–amine complex is stabilized by hydrophobic interactions between the hydrocarbonaceous ligates of the stationary phase and the alkyl chain of the amine.

The silanol-masking effect of decyltrimethyl-, dodecyltrimethyl- and hexadecyltrimethylammonium ions was investigated by measuring the retention of NH_2-G_7 on both octyl- and octadecyl-silica. The eluent was 90% acetonitrile and 10% 10 m*M* phosphate in water, pH 2.2, and contained either 1 or 5 m*M* of the quaternary ammonium compound. We used eqn. 9 to estimate silanophilic binding constants of the amines, K_A, for both stationary phases and the results are listed in Table I. It is seen that the most potent silanol scavenger of those tested is hexadecyltrimethylammonium bromide, and that efficacy increases with carbon number. Thus, the results conform to the observation that the efficacy of the amines in reducing silanophilic interactions increases with the length of the alkyl chain. The reciprocal of the binding constant is equal to the concentration of the masking agent at which saturation of the silanols is half-maximal. On the other hand, a recommended amine concentration, C_A^*, for use in RPC can be calculated as that amine concentration which reduces the silanol effect by 90%. The values of C_A^* for the three amines are also included in Table I. These results should be considered tentative because the method used for determination of the stability constant is highly sensitive to experimental error.

TABLE I

FORMATION CONSTANTS, K_A, FOR COMPLEXES FORMED BETWEEN SURFACE SILANOLS AND QUATERNARY AMINES ADDED TO THE ELUENT AS WELL AS THE RECOMMENDED AMINE CONCENTRATIONS, C_A^*, IN THE ELUENT

Both octyl-silica (C_8) and octadecyl-silica (C_{18}) were used and the eluent contained 90% (v/v) of acetonitrile and 10% (v/v) of 10 m*M* phosphate in water, pH 2.2. The columns were Supelcosil LC-8 and Supelcosil LC-18, respectively. The parameters were calculated from the data by using eqn. 9. The recommended amine concentration, C_A^*, is taken as $10\ K_A^{-1}$.

Alkylammonium compound	$K_A \times 10^{-3}\ [M^{-1}]$		$C_A^*\ [mM]$	
	C_8	C_{18}	C_8	C_{18}
Decyltrimethyl	2.3	7.4	4.4	1.3
Dodecyltrimethyl	7.1	16.0	1.4	0.6
Hexadecyltrimethyl	16.0	23.0	0.6	0.4

However, they are expected to serve as a useful guide in the choice of amine concentration. Table I contains data obtained with both octyl- and octadecyl-silicas. It is seen that the latter stationary phase binds the silanol-masking agents more strongly than does octyl-silica; therefore, silanophilic interactions with such monomeric type stationary phases can be obliterated at a lower concentration of the amine when C_{18} alkyl ligate is used instead of C_8 ligate.

Regular retention behavior due to silanol masking

Silanol groups are frequently regarded as undesirable in RPC[34]. Indeed, as shown in a previous study[5] silanophilic interactions may be responsible for the selectivity variation observed with different columns. Moreover, any retention index system in RPC[10] requires predictable monotonic retention behavior. As shown here, when both solvophobic and silanophilic interactions are significant, irregular behavior occurs and therefore the construction of such an index for the prediction of retention or the tentative identification of sample components becomes exceedingly difficult. A rather simple way to circumvent the effect of silanophilic interactions, however, is to incorporate an amine in the eluent to scavenge the silanols. The data presented here suggest that several amines could be used as effective masking agents at sufficiently low concentration to avoid ion-pairing effects. Amines containing dodecyl or hexadecyl functions and having modest solubility in the eluent appear to be appropriate for use in RPC.

Amines can be effective, not only in reducing the dependence of retention on sample size as well as in improving column efficiency and sample recovery, but also in normalizing the retention behavior of nitrogen-bearing eluites. This is clearly seen

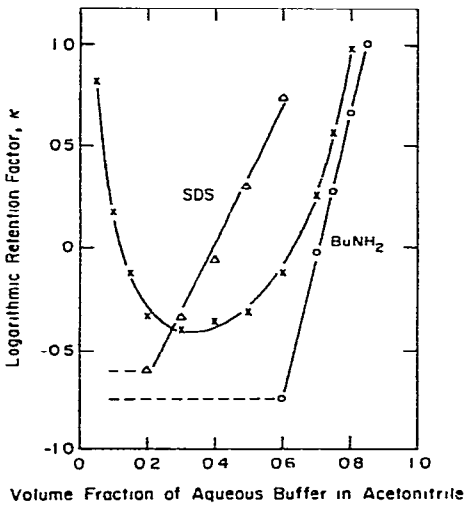


Fig. 14. Influence of sodium dodecyl sulfate (SDS) and *n*-butylamine ($BuNH_2$) on the retention of NH_2-Val-Met-Ala-Gly-Val-Ile-Gly-OEt. Column, Supelcosil LC-8, 150 × 4.6 mm I.D.; eluents, acetonitrile–50 m*M* sodium phosphate in water, pH 2.25, containing no additive (×), 20 m*M* SDS (△) or 10 m*M* $BuNH_2$ (○); flow-rate, 1.5 ml/min; temperature, 25°C; detection, UV at 220 nm. For volume fractions of aqueous buffer of 0.1–0.2 for SDS and of 0.1–0.6 for $BuNH_2$ the κ values were smaller than those indicated by the dashed lines.

in Fig. 14, which shows retention factor data for NH_2-G_7 obtained on Supelcosil LC-8 with mobile phases composed of acetonitrile and 50 m*M* phosphate buffer, in water, pH 2.25, in various proportions with or without 10 m*M* *n*-butylamine as silanol scavenger. In the absence of butylamine the retention factor is a parabolic function of the solvent composition but when butylamine is present the κ *vs.* ψ plot is linear. The observed regular retention behavior suggests that at this concentration of butylamine silanophilic interactions are eliminated and the retention occurs essentially by solvophobic mechanism. However, regular retention behavior is observed also when sodium dodecylsulfate (SDS) is added to the eluent instead of the amine. In this case, the dodecylsulfate hetaeron may form an ion-pair with NH_2-G_7 and thereby preclude silanophilic interactions. Alternatively the κ *vs.* ψ plot may be linear due to solvophobic interactions that govern retention in ion-pair chromatography[3,8,38]. In any case the result shows that silanophilic retention can be eliminated not only by blocking surface silanols but also by masking that function of the eluite molecule which interacts with the silanols.

Silanophilic interactions and selectivity

Notwithstanding the general opinion that silanols at the stationary phase surface are undesirable in RPC, silanophilic interactions may be quite useful in obtaining adequate selectivity for a given separation in chromatographic practice. Fig. 15 shows chromatograms of a peptide mixture containing Boc-G_7, NH_2-G_7, Boc-Leu-Leu-Ile-Ser(Bzl)-Tyr(Bzl)-Gly-OEt, and NH_2-Leu-Ile-Ser(Bzl)-Tyr(Bzl)-Gly-OEt. In Fig. 15A an acceptable separation of the peptides with good resolution is observed by using acetonitrile–aqueous 50 m*M* sodium phosphate, pH 2.25 (90:10) as the mobile phase. When decyltrimethylammonium chloride is added to the mobile phase, however, the resolution deteriorates as shown in Fig. 15B. The loss of resolution occurs because the retention of the Boc-peptides remains unchanged but the

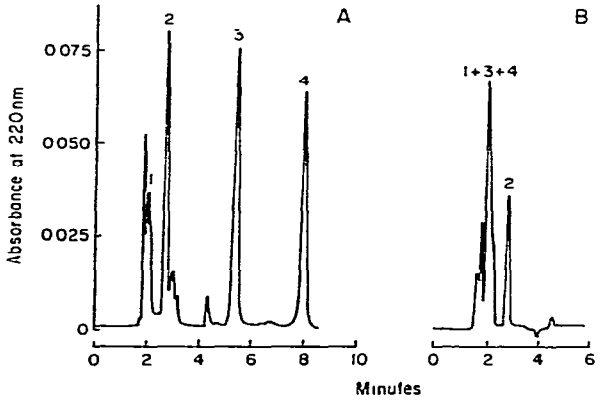


Fig. 15. Effect of silanol-masking on peptide separation. Peptides: 1 = Boc-Val-Met-Ala-Gly-Val-Ile-Gly-OEt; 2 = Boc-Leu-Leu-Ile-Ser(Bzl)-Tyr(Bzl)-Gly-OEt; 3 = NH_2-Val-Met-Ala-Gly-Val-Ile-Gly-OEt; 4 = NH_2-Leu-Ile-Ser(Bzl)-Tyr(Bzl)-Gly-OEt. Column, Supelcosil LC-8, 250 × 4.6 mm I.D.; eluent, acetonitrile–50 m*M* sodium phosphate in water, pH 2.25, (90:10) without (A) or with (B) 5 m*M* dodecyltrimethylammonium chloride; flow-rate: 1.5 ml/min; temperature, 25°C; detection, UV absorbance at 220 nm.

peptides with free amino group elute much more rapidly and are therefore co-chromatographed with one of the Boc-peptides in the presence of the silanol-masking agent.

Silanophilic interactions can be used to advantage in optimization of separations, and may be particularly availing in preparative-scale chromatography. A mixture of interest contained two Boc-peptides and NH_2-G_7. As seen in Fig. 16, the retention factor of NH_2-G_7 is nearly the same when 60% and 80% acetonitrile–10 m*M* aqueous phosphate buffer, pH 2.25, are the mobile phases. However, the retention of both Boc-peptides is reduced at higher acetonitrile concentrations so that the retention of the amine and neutral species are commensurate. This effect is a consequence of the minimum in retention factor–eluent composition curve for the amino peptide (Fig. 14) and the regular elution behavior of the protected peptide. Whereas any given retention factor for the peptide with free amino group can be obtained at two eluent-compositions, for the blocked peptide each retention factor value corresponds to only one composition. Therefore a sufficiently hydrophobic peptide and its N-blocked derivative can be conveniently separated in RPC on silica-bonded hydrocarbonaceous stationary phases with a hydro–organic eluent without silanol-masking agent. Our experience has indicated that blocked hydrophobic peptides such as these used here have retention comparable to their unblocked congeners in water-lean hydro–organic mobile phases and that the retention of the amino-peptide is much greater than that predicted by extrapolation of data obtained in water-rich eluent.

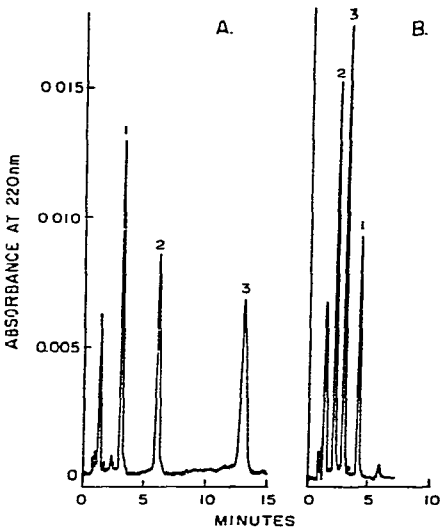


Fig. 16. Effect of solvent composition on peptide separation. Peptides: 1 = NH_2-Val-Met-Ala-Gly-Val-Ile-Gly-OEt; 2 = Boc-Ile-Ser(Bzl)-Tyr(Bzl)-Gly-OEt; 3 = Boc-Leu-Leu-Ile-Ser(Bzl)-Tyr(Bzl)-Gly-OEt. Column, Supelcosil LC-8, 150 × 4.6 mm I.D.; eluents: A, acetonitrile–10 m*M* sodium phosphate in water, pH 2.25 (60:40); B, acetonitrile–10 m*M* sodium phosphate in water, pH 2.25 (80:20); flow-rate, 1.5 ml/min; temperature, 25°C; detection: UV absorbance at 220 nm.

CONCLUSIONS

(1) When retention factors measured on silica-bound hydrocarbonaceous stationary phases with binary hydro–organic eluents are plotted against the eluent composition, minima are observed not only with crown ethers but also with amino peptides.

(2) A dual retention mechanism involving both solvophobic and silanophilic interactions is believed to be responsible for such irregular retention behavior.

(3) With the above substances RPC occurs when columns packed with naked silica gel and aqueous eluents are used.

(4) Consequently, the term RPC may be applied to any chromatographic system that entails a mobile phase more polar than the stationary phase and is not necessarily restricted to the use of so-called non-polar stationary phases.

(5) Irregular retention behavior observed in RPC with naked silica is also attributed to the dual retention mechanism.

(6) With hydrocarbonaceous bonded phases regular retention behavior, *i.e.*, linear dependence of the logarithmic retention factor on the composition of the binary hydro–organic eluent, is obtained upon masking the silanol groups at the stationary phase surface with a suitable amino compound in the eluent.

(7) Blocking the cationic functions of the eluite molecule has the same effect.

(8) Despite the sometimes favorable effect of silanophilic interactions on chromatographic selectivity, we recommend that in RPC with silica-bonded hydrocarbonaceous stationary phases silanol-masking agents be used in the eluent in order to obtain reproducible results and the regular retention behavior needed for a hydrophobic index system based on chromatographic data.

(9) Methods are presented for the evaluation of the efficacy of silanol-blocking agents.

ACKNOWLEDGEMENTS

The generous supply of peptides and valuable advice by R. E. Galardy as well as the assistance of Jackeline Spong in performing some of the experiments are gratefully acknowledged. This work was supported by the respective grant Nos. CA21948, GM20993 and GM25702 (to Richard Galardy) from the National Cancer Institute and National Institute for General Medical Sciences, U.S. Public Health Services, Department of Health and Human Services.

REFERENCES

1 Cs. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129.
2 Cs. Horváth and W. R. Melander, *Amer. Lab.*, 10, No. 10 (1978) 17.
3 W. R. Melander and Cs. Horváth, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography —Advances and Perspectives*, Vol. 2, Academic Press, New York, 1980, pp. 113–319.
4 Cs. Horváth, W. R. Melander and I. Molnár, *Anal. Chem.*, 49 (1977) 142.
5 W. R. Melander, J. Stoveken and Cs. Horváth, *J. Chromatogr.*, 199 (1980) 35.
6 W. R. Melander, A. Nahum and Cs. Horváth, *J. Chromatogr.*, 185 (1979) 129.
7 B. L. Karger, J. N. LePage and N. Tanaka, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography —Advances and Perspectives*, Vol. 1, Academic Press, New York, 1980, pp. 113–206.

8 Cs. Horváth, W. R. Melander, I. Molnár and P. Molnár, *Anal. Chem.*, 49 (1977) 2295.
9 A. Nahum and Cs. Horváth, *J. Chromatogr.*, 203 (1981) 53.
10 W. R. Melander, B.-K. Chen and Cs. Horváth, *J. Chromatogr.*, 185 (1979) 99.
11 D. E. Weiss, *Nature (London)*, 162 (1948) 372.
12 A. Tiselius, *Kolloid-Z.*, 105 (1943) 101.
13 J. Porath and C. H. Li, *Biochim. Biophys. Acta*, 13 (1954) 268.
14 A. Wehrli, J. C. Hildenbrand, H. P. Keller, R. Stampfli and R. W. Frei, *J. Chromatogr.*, 149 (1978) 199.
15 A. Sokolowski and K.-G. Wahlund, *J. Chromatogr.*, 189 (1980) 299.
16 Cs. Horváth, W. Melander and A. Nahum, *J. Chromatogr.*, 186 (1979) 371.
17 W. R. Melander, J. Stoveken and Cs. Horváth, *J. Chromatogr.*, 185 (1979) 111.
18 D. J. Pietrzyk and C. H. Chu, *Anal. Chem.*, 49 (1977) 860.
19 P. J. Twitchett and A. C. Moffat, *J. Chromatogr.*, 111 (1975) 149.
20 N. E. Hoffman and J. C. Liao, *Anal. Lett.*, A11 (1978) 287.
21 S. Eksborg, H. Ehrsson and U. Lönroth, *J. Chromatogr.*, 185 (1979) 583.
22 S. Eksborg, *J. Chromatogr.*, 149 (1978) 225.
23 I. M. Kolthoff, *Anal. Chem.*, 51 (1979) 1R.
24 W. P. Rindone and T. Kush (Editors), *PROPHET, Molecules: A User's Guide to the Molecule Facilities of the PROPHET System*, Bolt Beranek and Newman Inc., Cambridge, MA, April 1980.
25 L. R. Snyder, J. W. Dolan and J. R. Gant, *J. Chromatogr.*, 165 (1979) 3.
26 H. Colin, N. Ward and G. Guiochon, *J. Chromatogr.*, 149 (1978) 169.
27 G. A. Howard and A. J. P. Martin, *Biochem. J.*, 46 (1950) 532.
28 F. Naider, R. Sipzner, A. S. Steinfeld and J. M. Becker, *J. Chromatogr.*, 176 (1979) 264.
29 K. K. Unger, *Porous Silica*, Elsevier, Amsterdam, 1979.
30 R. K. Iler, *Chemistry of Silica*, Wiley, New York, 1979.
31 S. H. Unger, J. R. Cook and J. S. Hollenberg, *J. Pharm. Sci.*, 67 (1978) 1364.
32 J. K. Baker, *Anal. Chem.*, 51 (1979) 1693.
33 M. Tomita and V. T. Marchesi, *Proc. Nat. Acad. Sci. U.S.*, 72 (1976) 2964.
34 J. Porath, *Nature (London)*, 175 (1955) 478.
35 I. C. Caldwell, *J. Chromatogr.*, 44 (1969) 331.
36 M. Dizdaroglu and M. G. Simic, *J. Chromatogr.*, 195 (1980) 119.
37 W. S. Hancock, C. A. Bishop, R. L. Prestidge, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 153 (1978) 391.
38 W. R. Melander and Cs. Horváth, *J. Chromatogr.*, 201 (1980) 211.