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Effect of the characteristics of the phase system on the retention of proteins in hydrophobic interaction chromatography

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Abstract

In contrast to reversed-phase chromatography (RPC), where the stationary phases available show only minor differences as regards the retention and selectivity of separation, in hydrophobic interaction chromatography (HIC) the various stationary phases may show different effects depending on the type of support and ligand applied. In this work, the data obtained on four commercially available HIC columns with five standard proteins using salts of different types were investigated. It was found that the slope–intercept plot derived from the ln k *vs.* salt molality relationship can furnish a useful characterization of the hydrophobic interaction chromatography of proteins. A new hydrophobicity index introduced in RPC can also be applied as an overall parameter to characterize hydrophobic interaction. By using the above parameters the effects of both the column type and salt can be evaluated. Characterization of the HIC columns can be of considerable assistance in the selection of the appropriate column for a given separation.

1. Introduction

In the last decade, high-performance hydrophobic interaction chromatography (HIC) has developed into a powerful and popular technique for the analytical and preparative separation of proteins and other biopolymers [1–16]. Although the molecular interaction involved, London-type dispersion, is similar to that operating in reversed-phase liquid chromatography (RPLC), there are substantial differences as regards the separation of biologically active materials. Table 1 shows the main characteristics of the two techniques.

In RPLC packings the functional groups are long alkyl chains, very densely distributed, producing strongly hydrophobic surfaces. As a result, organic solvents must be used to desorb the proteins. As a consequence of the strong interaction and the organic solvent used, most of the proteins are subjected to unfolding and could loose some or all of their biological activity.

In contrast, in HIC packings there are short alkyl or cyclic functional groups, much more sparsely distributed, producing moderately hydrophobic surfaces and resulting in mild hydrophobic interaction. As a result, elution can be accomplished by buffers without addition of organic solvents. Proteins are retained at high initial salt concentrations (1–3 M) and eluted

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Table 1
Comparison of RPLC and HIC

Parameter	RPLC	HIC
Interaction	Dispersion	Dispersion
Stationary phase:		
Ligand type	Long alkyl chains (C_8–C_{18})	Short alkyl chains (C_2–C_4), phenyl, CH_x
Ligand density	High	Low
Hydrophobicity	Strong	Moderate
Mobile phase:		
Type	Aqueous–organic (CH_3OH, CH_3CN)	Solution of different salts
Operation	Gradient	Reverse gradient
Protein:		
Structure on the stationary phase	Unfolded	Native (folded, three-dimensional)
Dominant feature (retention)	Overall hydrophobicity (primary sequence)	Surface hydrophobicity
Loss of biological activity	Considerable	Small

selectively by decreasing the salt concentration using reverse gradients under conditions that promote the preservation of the biological activity of most proteins [3,4].

In recent years, several stationary phases have been developed for HIC on various matrices with different functional groups and with widely different retention characteristics [1,2,5,10–12]. In addition, various mobile phase parameters (type of salt, initial salt concentration, gradient time, flow-rate, temperature, pH, addition of organic modifiers) are applicable for the modulation of retention and selectivity [3–9,13–16].

In our laboratory, measurements have been carried out under gradient conditions on four commercially available HIC columns with different types of salts [17–19]. By comparison and evaluation of the data obtained, it was demonstrated that the retention and selectivity of protein separations depend on both the type of stationary phase and the salt used [17,19,20]. In contrast to RPLC, where the stationary phases show only minor differences as regards the absolute and relative retentions of various solutes [21–25], in HIC the stationary phases may show different effects depending on the type of support and ligand applied [4–12].

In this study, we investigated the characteristics that can be used to describe the hydrophobic interaction and retention of proteins on HIC columns of different types and using different types of salts in the mobile phase.

2. Experimental

The experimental approach used in this study was taken from the papers of Szepesy and Rippel [17,19], where exact descriptions of the materials and the chromatographic conditions are given. The data obtained under gradient conditions were evaluated according to the linear solvent strength (LSS) model of gradient elution with a program written in C and run on the computer used as a part of the chromatographic system. (For the theory of the LSS model and for the details of the evaluation procedure see ref. 19 and references cited therein.)

2.1. *Columns [17,19]*

TSK-Phenyl 5-PW (PHE) (Beckman, San Ramon, CA, USA), Synchropak-Propyl (PRO) (Synchrom, Linden, IN, USA), Spherogel CAA-HIC (CAA) (Beckman) and Alkyl-Superose HR 10/10 (ALK) (Pharmacia–LKB, Uppsala, Sweden) columns were used.

2.2. *Proteins [17]*

Cytochrome *c* (CYT), ribonuclease A (RNA), ovalbumin (OVA), lysozyme (LYS), α-chymotrypsinogen A (CHY) were obtained from Sigma (St. Louis, MO, USA).

3. Results and discussion

Users often find the behaviour of proteins on HIC media to be unpredictable. Generally it is difficult to identify the characteristics of a protein, *e.g.*, its hydrophobicity, that will predict its interaction with the HIC packing. The significance of hydrophobicity in stabilizing protein structure has been recognized and is amply reviewed in the literature. However, a quantitative description and understanding of hydrophobic interaction and its exact role, *e.g.*, in the folding of proteins, is still being investigated.

In the literature over 80 more or less different set of parameters have been reported to describe the hydrophobicity of amino acids [26]. These parameters were used to predict the overall hydrophobicity of the proteins or to characterize their surface regions, on the basis of the primary amino acid sequence. In several studies it was found that very few of these descriptors derived for hydrophobicity correlate well. In addition, most of the widely used set of hydrophobicity parameters show very poor correlation with the retention behaviour of the proteins [26,27]. According to the most recent theory suggested, the hydrophobicity is not constant and is closely related to the hydration of amino acids in a changing environment [28,29]. For this reason, we tried to find some parameters derived directly from the chromatographic measurements with standard proteins to characterize interactions in HIC.

Several models have been suggested to describe the retention of proteins under HIC conditions. The theoretical backgrounds of these models are different, *e.g.*, preferential interaction [30,31], displacement model of chromatography [32] or a combination of the hydrophobic and electrostatic interactions existing in an HIC phase system [33], and none of them has gained general acceptance. It was found that at the higher salt concentrations regularly used in HIC the retention of solutes can be readily described with a two-parameter linear function:

$$\ln k = \ln k_w + S m_s \quad (1)$$

where m_s is the molality of the salt used, $\ln k_w$ is the intercept, *i.e.*, the hypothetical retention in pure water, and S is the slope of the function. We have found that our data correlate well with Eq. 1 [19], and we therefore studied the influence of the operating parameters on the retention of proteins according to this relationship.

This type of equation is very similar to that used in RPLC to describe the retention behaviour of the solutes and characterize their hydrophobicity [24,25,35]. The S index for a particulate solute was observed to be nearly constant on different C_{18} packing materials [34]. On the other hand, $\ln k_w$ was suggested as a descriptor of hydrophobicity for various solutes [24,25].

In HIC, both parameters of Eq. 1 have also been suggested to describe hydrophobic interaction. Fausnaugh and Regnier [6] presented $\ln k_w$ as the "strength of hydrophobic interaction". Horváth and co-workers [7,33] introduced S as a "hydrophobic interaction parameter". However, none of these parameters has been found adequate to describe the hydrophobic interaction of various proteins under different operating conditions. The retention characteristic of a protein depends on both the intercept and slope. For this reason, these parameters should be combined, *e.g.*, by plotting the slope as a function of the intercept, which can be given as

$$S = p + q \ln k_w \quad (2)$$

where p and q are constants for a given stationary phase in a given salt solution. Gehas and Wetlaufer [35] investigated this relationship for dansylamino acids. They found that a good correlation existed between the slope and intercept values, which suggests that both are dependent variables. Whereas the slope depends on the contact surface area of the molecule, the intercept seems to characterize the strength of

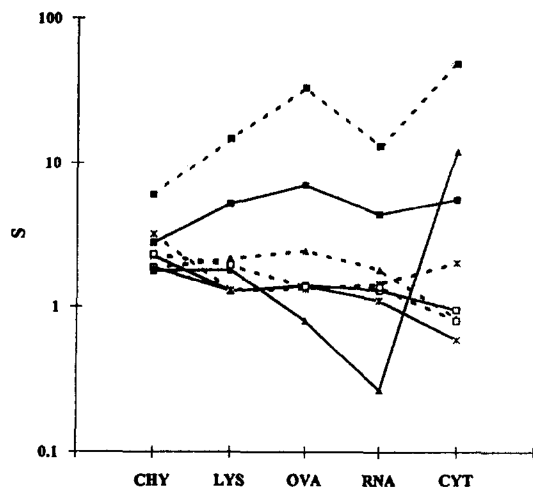

Fig. 1. Slopes of the ln k *vs.* salt molality relationship obtained in ammonium sulphate (solid lines) and in sodium citrate (dashed lines) for the columns and proteins investigated. Column: ■ = ALK; ▲ = CAA; * = PHE; □ = PRO.

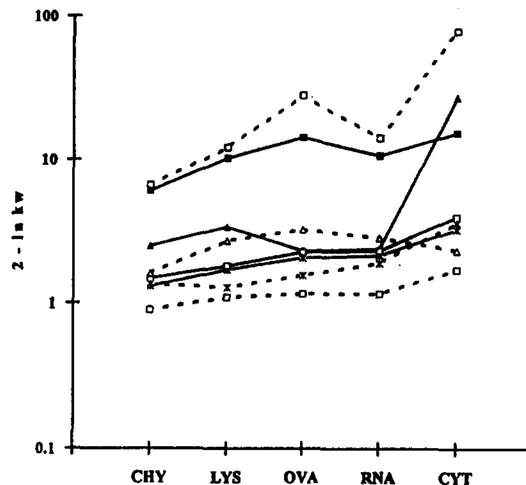

Fig. 2. Intercepts of the ln k *vs.* salt molality relationship obtained in ammonium sulphate (solid lines) and in sodium citrate (dashed lines) for the columns and proteins investigated. Column: ■ = ALK; ▲ = CAA; * = PHE; □ = PRO.

the interaction between the solute and stationary phase [6,7,33,35].

In Fig. 1, the slopes of the ln k *vs.* salt molality relationships obtained in ammonium sulphate (AS) and sodium citrate (SC) for the columns and proteins investigated are shown. With both salts the S values are very close on the PHE and PRO columns, reflecting the similar characteristics of these columns [17]. The S values obtained on the CAA column are different and the largest deviations are observed on the ALK column [19].

Fig. 2 shows the intercept values in AS and in SC for the columns and proteins investigated. In order to present all the data on the same figure, the scale has been transformed as indicated, *i.e.*, inverse scaling is used. The courses of these plots are very similar to those presented in Fig. 1. The PHE column produces similar values for all proteins, the PRO and CAA columns slightly different values and the deviations on the ALK column are the largest.

The effect of the salt type on the parameters can be evaluated by plotting the values obtained in the different salt solutions against each other. Fig. 3a shows a comparison of the slope values and Fig. 3b that of the intercept values for the two salts investigated. The data obtained on the PHE column are on or around the diagonals, indicating a minor effect of the salt change. On the PRO column the impact is more pronounced on the intercepts and lower on the slopes. The data obtained on the CAA and ALK columns show considerable changes in the characteristics when using SC instead of AS. On all columns the extent of effect is highly dependent on the type of protein, *i.e.*, it is not the same for the different proteins.

Fig. 4 shows the slope–intercept relationships obtained on the various columns. (Note that the lines on the figure do not cover the values relating to the proteins; they only demonstrate the effect of salt change. The markers are not real values but are used only for designation.) On the PHE column the regression lines almost coincide, *i.e.*, the salts affect the retentions similarly. On the PRO column the positions of the lines are very different. The slopes (q in Eq. 2) are positive on both of these columns. On the CAA and ALK columns the change of salt results in distinctly different courses of the data. On these columns the slopes in Eq. 2 are negative and the spread of the points indicates an opposite tendency of modifications. The extent of the changes on the CAA column is similar to those on the PRO column but in the opposite direction. The largest changes were

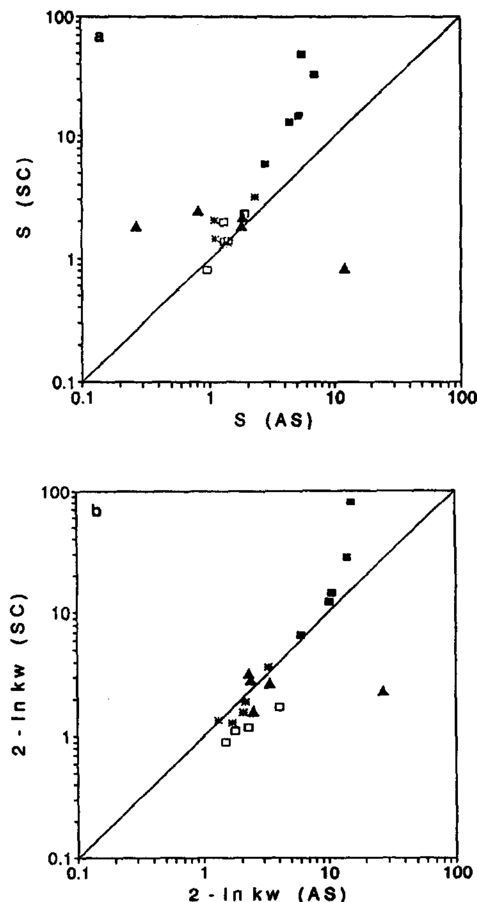

Fig. 3. Comparison of (a) the slope and (b) the intercept values obtained in ammonium sulphate (AS) and those obtained in sodium citrate (SC) on the columns investigated. Column: ■ = ALK; ▲ = CAA; * = PHE; □ = PRO.

obtained on the ALK column (cf. Figs. 1 and 2). This column was also tested with sodium acetate [19] and the results are also presented.

The intercepts of the regression lines (p in Eq. 2) also show different courses. On the PHE and PRO columns the intercept decreased, whereas on the CAA and ALK columns it increased on replacing AS with SC. This indicates that the characteristics of the phase system are not modified in the same way on changing the type of salt in the eluent.

Most recently Valkó and Slégel [36] suggested a new hydrophobicity index (φ_0) of solutes under RPLC conditions which can be calculated as the

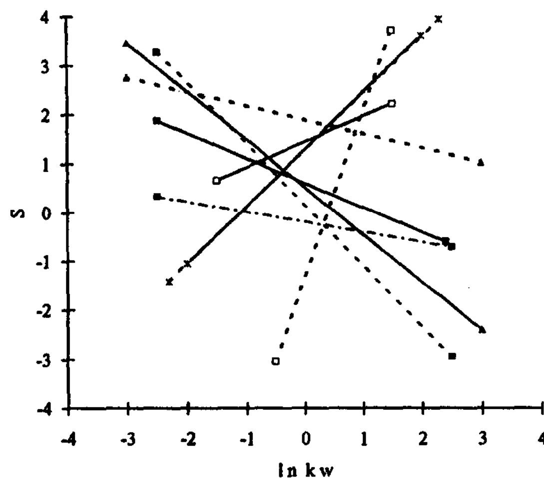

Fig. 4. Slope–intercept relationships obtained in ammonium sulphate (solid lines), in sodium citrate (dashed lines) and in sodium acetate (dot-dashed lines) on the columns investigated. Column: ■ = ALK; ▲ = CAA; * = PHE; □ = PRO. The lines do not cover the region of the values obtained experimentally; they only represent the fit of Eq. 2. For more details, see text.

ratio of the intercept and the slope values obtained from the log k $vs.$ φ (volume fraction of the organic modifier) relationship. Under HIC conditions this parameter can be designated by m_0:

$$m_0 = -\ln k_w / S \tag{3}$$

As m_0 is the salt molality, where $\ln k = 0$ ($cf.$ Eq. 1), this means that when using this eluent the molar concentrations of the related compound are identical in the stationary and mobile phases. This parameter can also be used as a descriptor similar to $\ln k_w$. With $\ln k_w$ the mobile phase composition is fixed ($m = 0$) and the extent of retention is used for the characterization. With m_0 the reference condition is a fixed retention ($\ln k = 0$) and the salt concentration of the mobile phase in which it is obtained is taken into consideration.

In Fig. 5, the m_0 values determined in AS and in SC are shown for all the columns and proteins investigated. In some instances the values obtained are fictitious as m_0, $i.e.$, the characteristic concentration of the salt, is lower than zero. This means that in these instances the reference

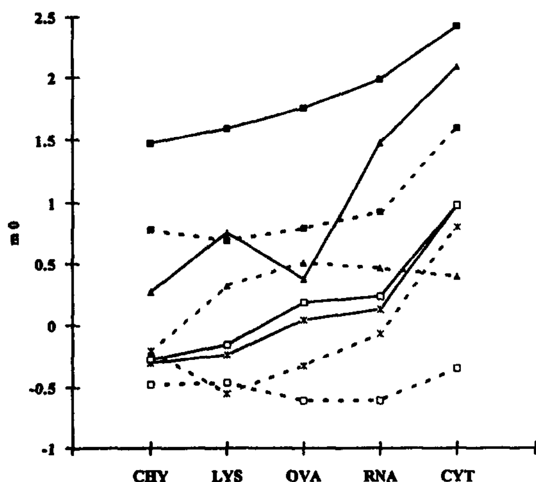

Fig. 5. m_0 values determined in ammonium sulphate (solid lines) and in sodium citrate (dashed lines) for the columns and proteins investigated. Column: ■ = ALK; ▲ = CAA; * = PHE; □ = PRO.

condition cannot be maintained under real conditions, *i.e.*, the ln k_w values of the related components are higher than zero. However, the value are applicable for comparison.

The plots obtained are very similar to those presenting the slope and intercept values (Figs. 1 and 2), but as m_0 involves both of these values it

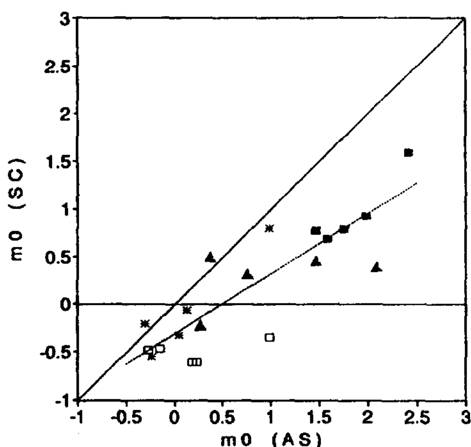

Fig. 6. Comparison of the m_0 values determined in ammonium sulphate (AS) and in sodium citrate (SC) on the columns investigated. Column: ■ = ALK; ▲ = CAA; * = PHE; □ = PRO. Dashed line = fit. For details, see text.

can be regarded as an overall parameter reflecting the "retentive strength" of the related phase system. The positions of the markers for the individual columns indicate the relative strength of the hydrophobic interaction between the stationary phase and the proteins investigated because the larger is m_0 the weaker is the interaction.

As regards the strength of the stationary phases, in AS the PHE and PRO columns are the strongest and they are very similar. The CAA column is weaker and the ALK column has the lowest strength. In SC the strength of the interaction is increased (the m_0 values are lower), *i.e.*, the retention on the same column is always higher in SC than in AS. The order of the columns seems to remain the same but the differences between the columns are smaller.

In order to evaluate the effect of salt on the columns the m_0 values obtained with the different salts can be plotted against each other. Fig. 6 shows the m_0 values obtained with AS and SC for the four columns. The spread of the points indicates that some correlation exists within the two sets. A straight line could have been fitted to the data with a good correlation. The values relating to the different columns are located in different regions along this line. These regions for the PHE and PRO columns are partly overlapped, which is an indication of the similarity of these stationary phases, but the effects of the salt change are different. The slope of the regression line equals the rate of the molal surface tension increments of the two salts. As regards the behaviour of the proteins on the columns, the larger is the distance of the points from the diagonal, the greater is the effect of the salt change.

4. Conclusions

The slope–intercept relationship derived from the ln k *vs.* salt molality plot can furnish a useful characterization of the hydrophobic interaction chromatography of proteins. The new hydrophobicity index (φ_0) introduced in RPLC can

also be applied in HIC, providing an overall parameter to describe hydrophobic interaction. By using this parameter the effects of both the column type and salt can be evaluated.

On the PHE and PRO columns, which furnish data with positive slopes of the slope *vs.* intercept plot, the change of salt has a minor effect on the retention of proteins although the relative retention, *i.e.*, the selectivity, may change considerably. These phase systems can be designated as stationary phase controlled.

In contrast, on the CAA and ALK columns, the data produce negative slopes of the slope–intercept plot. The change of salt type has a large effect on the retention of proteins and on the selectivity of separation. These phase systems can be termed mobile phase or salt controlled.

In contrast to RPLC, where the stationary phases show only minor differences as regards the retention and selectivity of separation for various solutes, in HIC the stationary phases may show different effects depending on the type of support and ligand applied. For this reason it is of practical importance to characterize every HIC column in order to predict the performance to be expected in a given separation. If sufficient separation of a sample cannot be achieved in a stationary phase-controlled system, it is expedient to try a column of the salt-controlled type.

As regards the prediction of retention of the individual proteins under different HIC conditions, especially a theoretical framework remains to be developed that can adequately describe the conformation and surface properties of proteins under various operating conditions. For the time being the selection of the phase system in HIC is carried out by trial and error. For this process the above characterization of the stationary phases can provide considerable assistance.

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