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Systematic method development in hydrophobic interaction chromatography
I. Characterization of the phase system and modelling retention

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Abstract

In order to rationalize the selection of the phase system (the stationary phase and the mobile phase) applicable for the separation of some proteins, gradient measurements were carried out on three columns of different types using three salts as eluent constituents. The slope and intercept of the ln k–salt concentration relationship for proteins varied with the different phase systems. The hydrophobicity index (c^*) showed how the alteration of the type of phase system affects the selectivity of separation. Two-dimensional mapping along the parameters of the slope–intercept relationship revealed the extent of the difference among the phase systems. In addition, the effect of solutions of binary and ternary salt mixtures on the retention of proteins was investigated. Measurements were carried out according to a mixture design and the retention time was modelled with the predictive method of Jandera and by fitting a polynomial to the data according to the Sentinel method. The accuracy of the former method were dependent on the type of phase system. The fit of second-order polynomial gave very good results but a first-order model was also acceptable.

1. Introduction

The separation of bio-macromolecules is currently of major interest. Reversed-phase chromatography (RPC) is now the most commonly used technique for HPLC separations. Numerous reports [1–4] have shown that RPC has very high resolving power for proteins and peptides. However, the strong interaction between the highly hydrophobic stationary phase and the protein, on the one hand, and the use of organic modifiers as eluent constituents, on the other, can be very detrimental to the native structure of the proteins. As a consequence of these interactions, most of the proteins are subjected to unfolding and denaturation, and can lose some or all of their biological activity.

Hydrophobic interaction chromatography (HIC) has developed into a real alternative to RPC for the separation and purification of proteins. In HIC the proteins are retained by a weakly hydrophobic stationary phase at high salt concentration and are eluted by a decreasing salt gradient [5–7]. The main advantage of HIC over RPC for separating proteins is that because of the milder interaction between the proteins and

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the phase system, most biomolecules retain their activity and suffer very little denaturation.

Retention and selectivity in HIC can be modulated by means of several operating parameters such as the type and characteristics of the stationary phase, type of salt, parameters of the gradient (initial and final salt concentration, gradient time), flow-rate, pH, temperature and addition of organic modifiers [7].

The retention behaviour of proteins in HIC can be described in general by the thermodynamic model of Horváth and co-workers [8–10]. The solvophobic theory relates the retention of proteins to the molar surface tension increment of the salt [11–13]. The validity of the model requires that there be no specific binding of the salt to the protein molecule and no change in the protein structure under different operating conditions. Although it has been found that the molar surface tension argument is an oversimplification of the process and cannot be generally applied because of the unpredictable specific salt-binding effects [14–16], the solvophobic theory provides a good theoretical basis for understanding the basic processes of HIC, and the molal surface tension increment can be used to give a first guess of the effectiveness of a salt.

In contrast to RPC, 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. It has been also demonstrated that the various salts affect differently the retention of proteins on the different stationary phases [17,18]. In addition, it has been found that the salts have different effects on the retention of the hydrophilic and hydrophobic proteins [18,19]. For these reasons, the selection of an appropriate stationary phase is of great importance in the design and optimization of HIC separations. Notwithstanding, few studies on the characterization and influence of the stationary phase have been published [20–22]. The above results also imply that the effect of a salt cannot be predicted in advance but should always be determined experimentally in the given phase system for all the proteins to be investigated.

The selective variations of retention of the individual proteins due to salt exchange in the eluents [16,18,19] indicate that by using binary or ternary salt gradients of different kinds and compositions, fine tuning of the selectivity of separations can be accomplished. However, the effect of the salt mixtures in the eluent has only been studied recently by El Rassi et al. [23]. One of our aims was to explore the potential of the application of salt compositions.

Within the past few years, a new generation of products for computer-assisted HPLC method development has emerged. The results have been summarized in several books and in many publications [24–29]. Because RPC is the most frequently used technique of HPLC, most of the softwares was released for optimization of separations of this type under isocratic or gradient conditions. Some methods developed for small molecules have been successfully applied also for peptide and protein samples [30–34]. Nevertheless, no publication has appeared on the application of computer-assisted method development for HIC separations.

Despite the results of intensive investigations revealing the effect of many factors that influence the retention of proteins under HIC conditions, the design and optimization of HIC separations are not trivial. At present, the selection of the phase system and the operating conditions is established on the basis of subjective preferences and local experience, and the optimization, if at all, is carried out mainly by trial and error. The other objective of our study was the application of some methods, originally developed and generally applied for RPC separations, in the design and optimization of HIC separations.

In this paper we discuss the possibilities of modelling the retention of proteins on different columns using single and multiple salt solutions as eluent constituents; in the next part the rational selection of the phase system and the systematic optimization of HIC separations will be discussed.

2. Experimental

2.1. Apparatus

A Merck–Hitachi (Merck, Darmstadt, Germany) LiChrograph, consisting of an L-6200 programmable pump with accessories for low-pressure eluent mixing, a Rheodyne Model 7125 injector with a 10-μl loop and an L-4250 UV–Vis programmable detector operated at 280 nm, was used. System control and data acquisition and evaluation were performed with a D-6000 HPLC Manager running on an IBM PC AT compatible computer.

2.2. Columns

The main characteristics of the columns used are listed in Table 1.

2.3. Materials

Analytical-reagent grade ammonium sulphate, sodium acetate and sodium sulphate were purchased from Reanal (Budapest, Hungary). Distilled water was prepared by double distillation and ion-exchange.

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

2.4. Procedures

For the scanning exercises, measurements were carried out at three different gradient times (15, 30 and 45 min) using linear inverse gradients from 2 to 0 *M* ammonium sulphate and sodium sulphate and from 4 to 0 *M* sodium acetate in 0.05 *M* sodium phosphate buffer adjusted to pH 7 with 0.1 *M* NaOH. The flow-rate was 0.5 ml/min for sodium acetate and 1 ml/min for the other two salts. For the binary (2:1 and 1:2) and ternary (1:1:1) salt systems, the above solutions were mixed volumetrically in advance, and the measurements were carried out with 30-min gradients generated by the above buffer at a flow-rate of 1 ml/min.

Samples were prepared by dissolving about 2 mg of each protein in 1 ml of water. All the measurements were repeated at least twice and the average data are used throughout this paper.

3. Results and discussion

A systematic method development process must consist of some or all of the following steps [24,27]: initial exercises, retention optimization,

Table 1
Characteristics of the columns used

Material	Source	Ligand type	Support	Dimensions (mm × mm I.D.)	Particle size (μm)	Pore size (Å)
Synchropak Propyl	Synchrom (Linden, IN, USA)	Propyl	Silica	250 × 4.1	6.5	300
Hema-BIO 1000 Phenyl	Tessek (Prague, Czech Republic)	Phenyl	HEMA[a]	80 × 8.0	10	1000
POROS PH	PerSeptive Biosystems (Cambridge, MA, USA)	Phenyl	PSDVB[b]	100 × 4.6	10	8000/1000 (bimodal)

[a] Hydroxyethyl methacrylate.
[b] Styrene–divinylbenzene copolymer.

selectivity optimization, kinetic optimization, i.e., the optimization of the efficiency of the separation, and validation. All of these steps can be further divided. The initial exercises involve, among others, the precise formulation of the goal of separation, the selection of the phase system that is appropriate for the separation and the definition of the parameter space within which the optimum is presumed to be located. The last steps means the identification of the parameters affecting the retention of components to be separated and the definition of their range relevant to a practicable optimization of the separation. A method or algorithm must be also selected that provides an efficient and rapid way to find the optimum.

For RPC separations, most of the above steps can be considered as "standardized". There are some sort of stationary phases (octadecyl, octyl and, to a lesser extent, phenyl or cyano types) which are used very frequently, and the organic modifiers are almost always methanol, acetonitrile or in some instances tetrahydrofuran, used alone or in mixtures. In contrast, in HIC neither the type of stationary or mobile phase nor the range of the eluent strength can be selected in this way. Recently, a wide variety of stationary phases suitable for HIC separations have become available [7,10,22] and, theoretically, all the salts having "salting-out" properties can be considered as modifiers [8–10]. This, on the one hand, provides great flexibility for the selection of the phase system but, on the other, this means "too much freedom", because at present there is no method either for rational selection or even for reliable characterization of the phase system.

It has been found [17–19] that the combination of different salt solutions and columns of different types leads to unique phase systems, i.e., by varying the type of salts in the eluents and/or the type of the stationary phase, not only the retention of the components changes but also the selectivity of the separations.

In a recent study [35], we investigated some RPC methods suggested for the characterization of stationary phases. The results obtained for columns used in RPC and in HIC showed that the overall strength of the stationary phases can be evaluated fairly well but the results regarding polarity and/or hydrophobicity are misleading, especially for the HIC columns. It was also found that not only the type of ligand but also the type of support may have a significant effect on the retention.

Three widely differing stationary phases were selected for this study. The strength of HEMA fell in the range of that of the RPC columns, PRO was the weakest column in the RPC mode [35] but one of the strongest in HIC mode [18], and on POROS no retention could have been obtained under RPC conditions but excellent separations were reported under HIC conditions. However, these stationary phases differ not only in the overall strength but also in the type of ligand and the type and geometry of the support. Here we postulate that the more different the characteristics of the columns are, the greater the effect on retention and selectivity would be.

As regards salts, the above rule of thumb was applied again. Ammonium sulphate is the most commonly used salt in HIC [5,6]. In another study [19], we found that compared with ammonium sulphate, sodium acetate exerted selective effects on the proteins, i.e., it increased the retention of the hydrophobic proteins but decreased that of the hydrophilic proteins.

On the basis of our earlier results [7,17–19], sodium citrate should have been the third candidate but it was left out because some solubility problems occurred during the preparation of the multiple salt solutions. Instead, sodium sulphate was selected, because a special effect (deviation from the predictions of the solvophobic theory) was found also for this salt [5].

This kind of experimental design (using three different columns and three different modifiers) can be considered as an adaptation of that used in RPC when, in addition to an alkyl-type stationary phase, phenyl and cyano types are also tested for tuning the selectivity of separations [36–38]. This approach is especially recommended for samples with which more common methods of adjusting selectivity (modifier type and/or strength, pH, temperature, etc.) are impossible, problematic or inconvenient.

The HIC separations are always performed

under gradient conditions. The theory and practice of gradient elution has been well developed, and it has been discussed and summarized in many books and other publications [30–33]. It was found [19,39] that the so-called linear solvent strength (LSS) model of gradient elution is a good approximation under HIC conditions. When using only one salt in the eluent, it makes possible, on one hand, the estimation of the parameters of the retention profiles of the proteins and, on the other, the systematic and efficient optimization of HIC separations.

In RPC, there are two ways of modelling the retention of the components in multi-solvent eluents. The first is the Sentinel method, which was introduced originally for the optimization of selectivity under isocratic conditions and was later extended to the optimization of gradient separations [40–42]. First, "iso-eluotropic" binary eluents are designed that provide more or less the same retention for the components. Next, each of these gradients represents one "mixture variable" in a mixture design technique approximating the retention of components in multi-solvent systems by a second-order polynomial. In the other method [43–46], the parameters of the retention profiles of components in the binary systems are estimated, and then the retention of components in ternary and quaternary eluents can be calculated from these parameters in an iterative way.

In HIC, the logarithmic retention factor of components ($\ln k$) can be well approximated as

$$\ln k = \ln k_w + Sc \quad (1)$$

where c is the concentration of the salt used in the eluent, and $\ln k_w$ (the extrapolated $\ln k$ value to pure water) and S are constants characteristic of the phase system applied. If a linear gradient of salt is used, the net retention time of the components can be given as

$$t'_g = \ln (Smt_m k_0 + 1)/Sm \quad (2)$$

where m is the slope of the gradient, t_m is the hold-up time and k_0 is the retention of component at the initial eluent composition (c_0). From data for two gradient runs, the parameters of Eq. 1 can be estimated. If more than two gradient data are available, the validity of Eq. 1 can also be checked. The method has been described in details elsewhere [19].

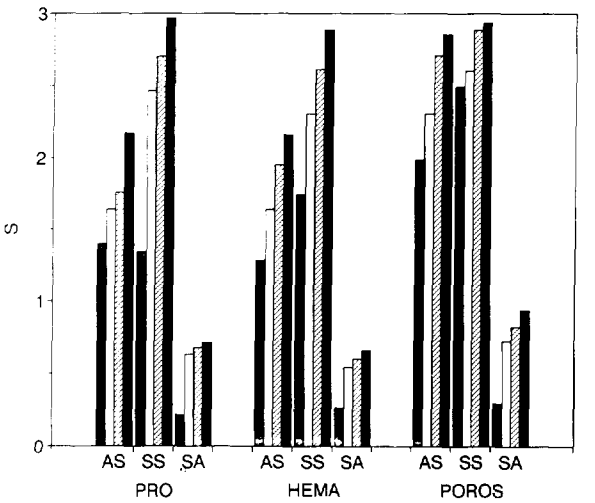


Fig. 1. Slope of retention profiles (S in Eq. 1) obtained in the different phase systems. The bars represent RNA (grey), OVA (white), LYS (hatched) and CHY (black).

The slope (S) and intercept ($\ln k_w$) values obtained with the different systems are shown in Figs. 1 and 2, respectively. (In most instances the data for CYT could not be evaluated because of

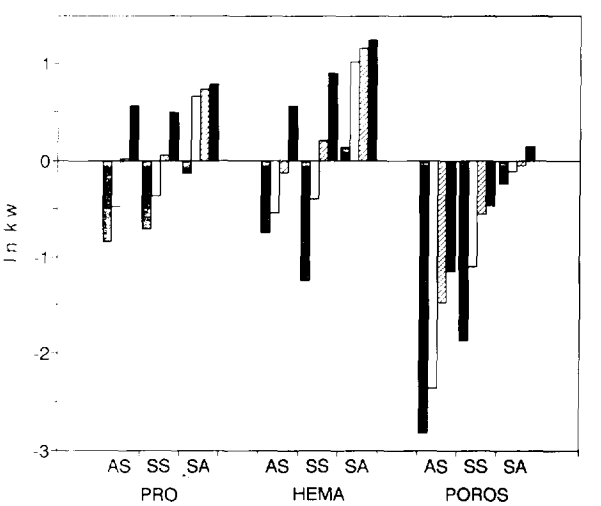


Fig. 2. Intercept of retention profiles ($\ln k_w$ in Eq. 1) obtained in the different phase systems. The bars represent RNA (grey), OVA (white), LYS (hatched) and CHY (black).

pre-elution or because of the serious overlap with the system peak, so this component was omitted from the evaluation, but not from the later optimization.) It is immediately seen that the differences between the columns are not as large as would have been expected from characterization in the RPC mode [35]. This is a direct confirmation of our earlier presumption that the tested methods provide reliable characteristics for the RPC columns but misleading results for the HIC columns.

The S parameters (Fig. 1) are similar on PRO and HEMA and are higher on POROS, but the order of columns cannot be established unambiguously on the basis of this parameter. The rates of alterations generated by the salt exchange in the eluent are also very similar. The use of sodium sulphate (SS) instead of ammonium sulphate (AS) increases slightly and replacing ammonium sulphate with sodium acetate (SA) decreases significantly the values of this characteristic, i.e., the salt type had a much larger effect on S than the type of stationary phase.

Larger differences can be observed in the values of $\ln k_w$ (Fig. 2). In general, this characteristic is the lowest in ammonium sulphate (AS) and the highest in sodium acetate (SA), but it is clear that here the type of stationary phase is the dominant factor, i.e., a change in the type of stationary phase exerts a larger effect on $\ln k_w$ than that of the salt type.

As the retentions of proteins as reflected by the above parameters are affected by the type of stationary phase and by the type of salt, a complete characterization of the phase systems must take into account both of them. Recently, a new hydrophobicity index of solutes under RPC conditions was suggested [47] which we adapted to HIC conditions [18]. It can be calculated as

$$c^* = -\ln k_w / S \qquad (3)$$

where c^* is the salt molality at which $\ln k = 0$ (cf., Eq. 1), i.e., the molar concentrations of the related compounds are identical in the stationary and mobile phases. (It must be noted that earlier we designated this parameter m_0, but this is a confusing term because in the literature m is used for the slope of the gradient and the subscript zero relates to the initial conditions of the gradient.)

The calculated values for the proteins in the different phase systems are shown in Fig. 3. The course of the data seems to be some sort of mirror image of the $\ln k_w$ values but as c^* involves both $\ln k_w$ and S, it can be considered as a measure of the overall strength of the phase system. The lower is c^* the larger is the "retentive strength" of the phase system. Related to ammonium sulphate (AS), sodium acetate increases c^* on all the stationary phases, but the extent of the changes varies from column to column. The effect of sodium sulphate seems to be specific to the stationary phase. The range of c^* values decreases on PRO and increases on HEMA, but the average values are more or less the same. On POROS the range and the absolute values also decreased. It is also seen that the order of the columns relating to strength cannot be established; it depends greatly on the type of salts. The order obtained in sodium acetate (SA) is consistent, but in sodium sulphate there are considerable differences between the stationary phases.

As regards the individual proteins, the changes in the difference between the c^* values corre-

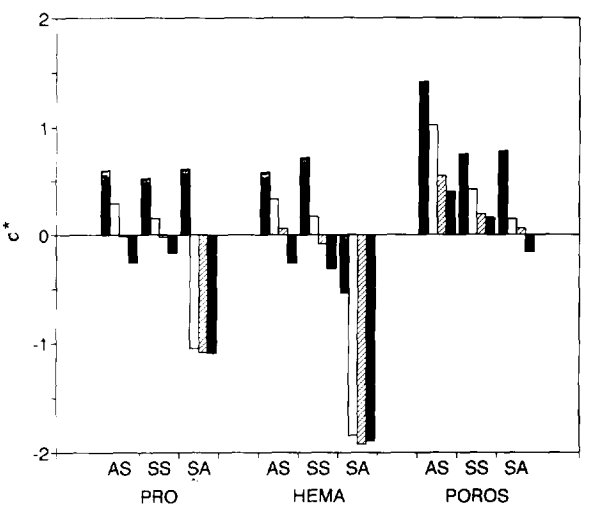


Fig. 3. Hydrophobicity index (c^*) calculated for the proteins. The bars represent RNA (grey), OVA (white), LYS (hatched) and CHY (black).

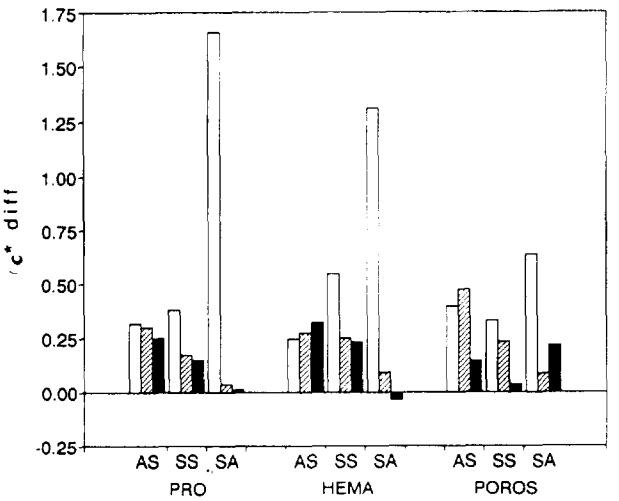


Fig. 4. Difference in c^* for pairs of proteins. The bars represent RNA–OVA (white), OVA–LYS (hatched) and LYS–CHY (black).

spond to the changes in the selectivity of the phase systems. The calculated differences are shown in Fig. 4. It is seen that neither the extent nor the order of these values are the same in the different systems. This means that changes in the type of the stationary phase and/or the salt type always modifies the selectivity of the phase system.

Under RPC conditions, S and $\ln k_w$ are not independent but show a good correlation [35]. Earlier we found a similar relationship between these two parameters also under HIC conditions [21], which can be formulated as

$$S = q + p \ln k_w \quad (4)$$

where q and p are constants. The characterization of a series of stationary phases that were different with respect to the type and surface concentration of the ligands and also the material and geometry of the support showed that these parameters are not constant but are specific to the phase system [35]. When mapping the columns with regard to these parameters obtained under RPC conditions, the RPC and HIC columns formed two distinct clusters, indicating the different strengths of the stationary phases.

On applying Eq. 4 to the data obtained in this study, good correlations were obtained ($r > 0.981$). The two-dimensional mapping of the phase system investigated is shown in Fig. 5. The values obtained in ammonium sulphate (AS) and in sodium sulphate (SS) on all the columns fall in or near the region of that of the RPC columns obtained for low-molecular-mass (LMM) components under RPC conditions (cf. Fig. 9 in Ref. [35]). This means that the HIC columns exhibit more or less the same strength for proteins under HIC conditions as the RPC columns for the LMM components in the RPC mode.

The smaller effect of changing from ammonium sulphate (AS) to sodium sulphate (SS) is clearly seen. The points relating to HEMA almost coincide and those for PRO are very close to each other. The difference on POROS is greater. Using sodium acetate (SA) instead of ammonium sulphate (AS) decreases all the q values and spreads the points over a wider range of p. As at present the meaning of these parameters under HIC conditions is not clear, further evaluation of changing the type of phase system cannot be accomplished, but the similarities and differences are clearly seen.

Having evaluated the gradient data obtained in single-salt systems, all the parameters are

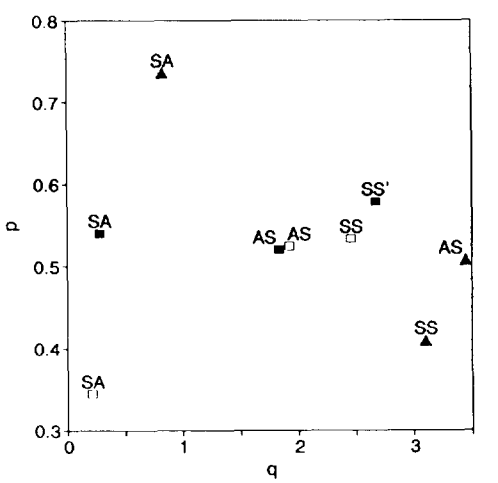


Fig. 5. Two-dimensional mapping of the phase systems using the parameters of Eq. 4 obtained in ammonium sulphate (AS), sodium sulphate (SS) and sodium acetate (SA). ■ = PRO; □ = HEMA; ▲ = POROS.

present for checking the applicability of the equation proposed by Jandera [43–46] for calculating retention in multi-solvent gradients under RPC conditions. The net retention time of a component can be given as

$$t'_g = \ln (t_m S_g k_{0g} + 1)/S_g \quad (5)$$

where

$$S_g = \sum S_i m_i$$

$$k_{0g} = \exp\left(\sum S_i c_{0i} + \sum c_{gi} \ln k_{wi} \Big/ \sum c_{gi}\right)$$

$$c_{gi} = c_{0i} - m_i t'_g/2$$

where t_m is the hold-up time of the system and i refers to the ith modifier (here salt). This iterative or recursive equation was applied for calculating the retention times of proteins in binary and ternary salt systems listed under Experimental. In addition, measurements were also carried out using these eluents.

The retention times obtained on the columns are shown in Fig. 6 and are listed in the third columns in Tables 2–4. Note that the figures correspond to the (unfolded) edges of the triangular mixture design. For a better overview, the apex corresponding to ammonium sulphate (AS) is put at both ends. MIX refers to the 1:1:1 solution. It is clearly seen that the gradient time of components changes according to a smooth function as the eluent composition changes from the solution of one pure salt to another. In most binary solutions this function seems to be a straight line.

The possibility of modelling retention was investigated in three ways. First, according to Eq. 5 the retention times were calculated from the parameters (S, ln k_w) obtained from the evaluation of the gradient data. The results are summarized in the fourth columns (calcd.) of Tables 2–4. Next, a first-order polynomial of the form

$$t_g = a_0 + a_1 c_{01} + a_2 c_{02} + a_3 c_{01} c_{02} \quad (6)$$

was fitted to the data, where c_{01} and c_{02} are the initial concentrations of ammonium sulphate (AS) and sodium sulphate (SS) and a_0–a_3 are

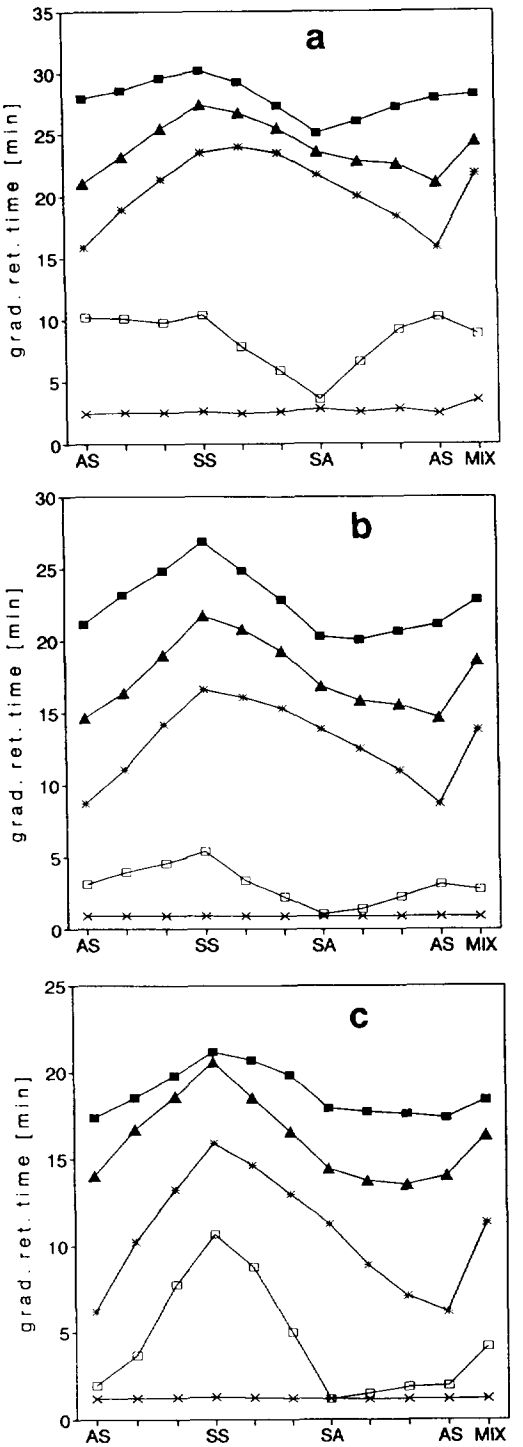


Fig. 6. Retention times obtained experimentally on (a) PRO, (b) HEMA and (c) POROS. ■ = CHY; ▲ = LYS; * = OVA; □ = RNA; × = CYT.

Table 2
Gradient retention times obtained experimentally (measd.) on HEMA and calculated from Eq. 5 (calcd.), from Eq. 6 (fit1) and from Eq. 7 (fit2)

Compound	Salt	Measd.	Calcd.	Fit1	Fit2
RNA	AS	3.09	3.29	3.08	3.15
		3.95	4.91	3.78	3.87
		4.55	4.55	4.48	4.60
	SS	5.38	5.19	5.18	5.33
		3.38	4.76	3.70	3.49
		2.21	2.90	2.22	2.07
	SA	1.04	0.91	0.93	1.06
		1.40	2.04	1.52	1.46
		2.25	3.04	2.30	2.16
	AS	3.09	3.29	3.08	3.15
	MIX	2.77	3.87	3.00	2.82
OVA	AS	8.69	8.14	8.81	8.61
		11.00	10.95	11.45	11.30
		14.13	13.57	14.10	13.96
	SS	16.57	15.88	16.74	16.59
		16.04	16.96	15.93	16.17
		15.25	15.92	15.12	15.24
	SA	13.87	12.99	14.30	13.82
		12.50	12.80	12.47	12.66
		10.97	11.40	10.64	10.93
	AS	8.69	8.14	8.81	8.61
	MIX	13.81	14.36	13.28	13.56
LYS	AS	14.62	14.42	14.35	14.64
		16.37	17.20	16.87	16.64
		18.95	19.58	19.39	18.97
	SS	21.70	21.59	21.91	21.63
		20.77	21.59	20.36	20.96
		19.24	19.78	18.80	19.34
	SA	16.83	15.85	17.25	16.77
		15.81	16.78	16.28	16.06
		15.50	16.48	15.32	15.35
	AS	14.62	14.42	14.35	14.64
	MIX	18.59	19.27	17.84	17.99
CHY	AS	21.08	20.94	20.93	21.06
		23.09	23.18	22.95	23.07
		24.77	25.01	24.96	24.97
	SS	26.82	26.51	26.97	26.74
		24.82	25.43	24.72	24.87
		22.83	22.96	22.47	22.67
	SA	20.25	19.30	20.22	20.14
		20.01	20.27	20.46	20.25
		20.59	21.20	20.70	20.55
	AS	21.08	20.94	20.93	21.06
	MIX	22.83	23.56	22.71	22.77

Table 3
Gradient retention times obtained experimentally (measd.) on PRO and calculated from Eq. 5 (calcd.), from Eq. 6 (fit1) and from Eq. 7 (fit2)

Compound	Salt	Measd.	Calcd.	Fit1	Fit2
RNA	AS	10.24	10.11	10.66	10.28
		10.08	10.20	10.43	10.02
		9.75	10.29	10.20	10.01
	SS	10.39	10.39	9.97	10.24
		7.78	8.51	8.00	8.01
		5.85	5.88	6.04	5.78
	SA	3.60	3.43	4.07	3.55
		6.60	5.92	6.27	6.87
		9.19	8.51	8.46	9.12
	AS	10.24	10.11	10.66	10.28
	MIX	8.84	8.51	8.23	8.44
OVA	AS	15.88	15.84	16.16	15.87
		18.89	18.98	18.84	18.94
		21.35	21.52	21.51	21.46
	SS	23.52	23.52	24.18	23.44
		23.97	24.37	23.57	24.11
		23.47	23.63	22.95	23.50
	SA	21.69	21.23	22.34	21.61
		20.00	20.59	20.28	20.22
		18.35	19.01	18.22	18.31
	AS	15.88	15.84	16.16	15.87
	MIX	21.81	22.04	20.89	21.48
LYS	AS	21.07	20.97	21.22	21.09
		23.20	23.63	23.37	23.25
		25.48	25.60	25.52	25.37
	SS	27.41	27.08	26.67	27.44
		26.72	27.18	26.43	26.74
		25.47	26.01	25.18	25.39
	SA	23.61	23.19	23.94	23.40
		22.79	23.25	23.03	23.14
		22.57	22.66	22.12	22.37
	AS	21.07	20.97	21.22	21.09
	MIX	24.45	25.29	24.28	24.58
CHY	AS	27.95	27.90	27.90	28.01
		28.55	28.88	28.78	28.72
		29.57	29.62	29.66	29.48
	SS	30.24	30.19	30.55	30.29
		29.23	29.55	28.82	29.17
		27.29	27.87	27.09	27.43
	SA	25.05	24.47	25.36	25.08
		26.05	26.24	26.21	26.05
		27.17	27.37	27.05	27.03
	AS	27.95	27.90	27.90	28.01
	MIX	28.25	28.62	27.93	28.08

Table 4
Gradient retention times obtained experimentally (measd.) on POROS and calculated from Eq. 5 (calcd.), from Eq. 6 (fit1) and from Eq. 7 (fit2)

Compound	Salt	Measd.	Calcd.	Fit1	Fit2
RNA	AS	1.96	1.80	1.32	1.49
		3.70	4.33	4.46	4.95
		7.75	7.37	7.60	8.20
	SS	10.68	10.57	10.74	11.25
		8.77	9.08	7.60	8.00
		5.00	5.06	4.46	4.74
	SA	1.16	1.12	1.32	1.49
		1.51	2.58	1.32	1.49
		1.86	3.20	1.32	1.49
	AS	1.96	1.80	1.32	1.49
	MIX	4.18	5.93	4.46	4.85
OVA	AS	6.19	6.36	6.16	6.19
		10.25	9.44	9.59	10.16
		13.25	12.62	13.01	13.39
	SS	15.91	15.74	16.44	15.88
		14.65	15.54	14.60	14.60
		12.95	13.77	12.76	13.05
	SA	11.25	10.70	10.92	11.23
		8.89	10.92	9.34	8.85
		7.07	9.71	7.75	7.16
	AS	6.19	6.36	6.16	6.19
	MIX	11.35	12.64	11.18	11.25
LYS	AS	14.05	14.47	13.94	14.06
		16.68	16.54	16.24	16.58
		18.59	18.54	18.53	18.75
	SS	20.61	20.48	20.83	20.58
		18.53	19.38	18.61	18.55
		16.57	17.09	16.38	16.52
	SA	14.43	13.54	14.16	14.49
		13.76	15.27	14.09	13.74
		13.52	15.71	14.02	13.60
	AS	14.05	14.47	13.94	14.06
	MIX	16.37	17.57	16.31	16.25
CHY	AS	17.37	17.24	17.15	17.35
		18.51	18.58	18.55	18.38
		19.81	19.91	19.95	19.67
	SS	21.17	21.22	21.35	21.22
		20.67	20.92	20.30	20.66
		19.83	19.66	19.25	19.56
	SA	17.91	17.48	18.20	17.91
		17.71	18.56	17.85	17.73
		17.59	18.59	17.50	17.54
	AS	17.37	17.24	17.15	17.35
	MIX	18.41	19.77	18.90	18.97

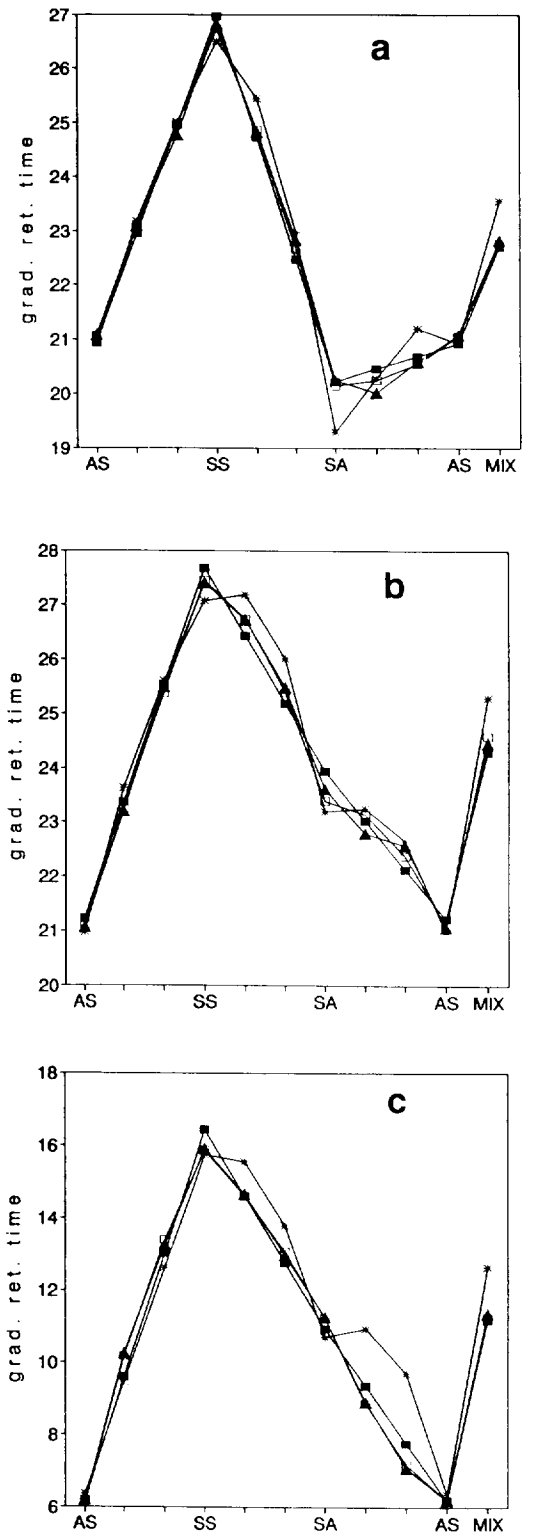
constants. The results (fit1) are summarized in the fifth columns of Tables 2–4. Finally, a second-order polynomial of the form

$$t_g = b_0 + b_1 c_{01} + b_2 c_{02} + b_3 c_{01} c_{02} + b_4 c_{01}^2 + b_5 c_{02}^2 \quad (7)$$

was also fitted, where c_{01} and c_{02} are the same as above and b_0–b_5 are constants. The results (fit2) are summarized in the last columns of Tables 2–4. For demonstration, some selected results are shown in Fig. 7.

As regards retention times predicted by Eq. 5, the fit is always very good in the ammonium sulphate–sodium sulphate (AS–SS) system, acceptable in the sodium sulphate–sodium acetate (SS–SA) mixtures but weak in the sodium acetate–ammonium sulphate (SA–AS) solutions. The model always predicts concave surfaces in the phase systems investigated which sometimes results in local maxima (cf., SS–SA mixtures in Fig. 7b) not indicated by the measurements, or in opposite curvature (concave instead of convex as in SA–AS mixtures in Fig. 7a). As the error of measurements was fairly high (±1 min), sometimes the values are erratic (cf., SA–AS mixtures in Fig. 7b), and hence it cannot be established how significant is the curvature indicated by these values. With this technique the smallest errors were obtained in the AS–SS mixtures on PRO and the largest in the SA–AS system on POROS. For the latter case the difference between the measured and the calculated retention times exceeds 2 min in some solutions. It seems that the closer are two systems on the p–q plot (Fig. 4), the better is the prediction.

In single-salt solutions, Eq. 5 reduces to Eq. 2, hence the data are applicable for checking the accuracy of the parameters of Eq. 1 obtained from the evaluation of the gradient data. It is seen that the largest error occurs in sodium acetate (SA) where, in most instances, Eq. 5 underestimates the retention times. This inaccuracy can be attributed to the error of measurements being the highest in this solution. It is also possible that the LSS conditions do not hold true in this instance, i.e., Eq. 2 is only an approxi-



mation of the retention time. However, further investigation is needed to reveal the full reason for this systematic error.

In order to fit the underlying model of the mixture design, one of the single-salt solutions must be regarded as a diluter and the other two as modifiers. For the former sodium acetate (SA) was selected, because the retention time was always the lowest in this system under the conditions applied.

The fit of the first-order polynomial (Eq. 6) is much better ($r > 0.957$) than that of the results provided by the predictive model (Eq. 5); however, the statistical evaluation of the parameters (a_0–a_3) showed that the fourth term on the right-hand side (the so-called cross-effect term) is almost always negligible. This means that simple interpolation gives retention times with good accuracy in multiple salt solutions under gradient conditions. This is supported by the fact that the retention time in MIX is always very close to the average of that measured in the single-salt solutions.

The second-order polynomial (Eq. 7) gave the best estimate of retention time ($r > 0.988$); however, the full polynomial was always redundant, i.e., some terms of the model were insignificant in all instances.

The above results could indicate that the second-order polynomial should always be used for modelling, but this kind of approach has some shortcomings. First, it is applicable only for the prediction of retention in "solvent strength gradients", i.e., when the amount of modifiers decreases during the gradient but their ratio remains constant. However, it is only one of the possible designs. The predictive model can estimate retention in gradients where only or also the ratio of the modifiers changes [43–46], hence, we consider that the advantages and

Fig. 7. Gradient retention times obtained experimentally (▲) and calculated from Eq. 5 (*), Eq. 6 (■) and Eq. 7 (□) for (a) CHY on HEMA (the values correspond to the respective columns in Table 3), (b) LYS on PRO. (the values correspond to the respective columns in Table 2) and (c) OVA on POROS (the values correspond to the respective columns in Table 2).

limitations of this model should be further investigated.

Another deficiency of the mixture design technique is that it tells nothing about the effect of the third salt. A complete investigation must take into account all the combinations of "diluters" and "modifiers", i.e., which selection of the salt solutions entered in the model of Eq. 7 (or Eq. 6) gives the most adequate description of the effect of salt compositions.

4. Conclusions

A systematic method development must include the selection of the phase system (the stationary phase and mobile phase) which is appropriate for the separation. At present this step is carried out by trial and error in HIC. The rationalization of the choice can be accomplished by characterization of the phase system. For this purpose, gradient measurements were carried out at three different gradient times on three columns of different kinds using three salts as eluent constituents.

First, the slope and intercept values of the ln k–salt concentration relationship of proteins were calculated according to the LSS model of gradient elution. Although alterations of the selectivity of the phase systems such as changing the type of salt and/or that of the stationary phases was indicated, a clear characterization could not have been made with these values. Next, the hydrophobicity index (c^*) characteristic of the protein and of the phase system used was calculated. These parameters and even their differences showed how the variation of the type of phase system affected the selectivity of separation. Finally, two-dimensional mapping with regard to the parameters (p, q) of the slope–intercept relationships revealed the similarities and differences among the systems.

Another step in the optimization process is the selection of the model describing the retention in the phase system chosen. The effect of salt compositions on retention was investigated using solutions of binary and ternary salt mixtures. For describing the retention in these systems, three models were applied and compared.

The accuracy of the predictive model developed by Jandera was dependent on the type of phase system. The distance on the p–q plot seemed to be an indication of the applicability.

According to the Sentinel method, a second-order polynomial was fitted to the retention times and very good correlations were obtained. On the other hand, the effect of all the salts used could not be evaluated. The application of a first-order model also gave acceptable results, which makes the description the retention of proteins in binary and ternary salt solutions under gradient conditions very easy.

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