Journal of Chromatography, 511 (1990) 17–33
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 402

Simultaneous optimization of variables influencing selectivity and elution strength in micellar liquid chromatography

Effect of organic modifier and micelle concentration

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(First received October 9th, 1989; revised manuscript received February 20th, 1990)

ABSTRACT

Previously, the simultaneous enhancement of separation selectivity with elution strength was reported in micellar liquid chromatography (MLC) using the hybrid eluents of water–organic solvent–micelles. The practical implication of this phenomenon is that better separations can be achieved in shorter analysis times by using the hybrid eluents. Since both micelle concentration and volume fraction of organic modifier influence selectivity and solvent strength, only an investigation of the effects of a simultaneous variation of these parameters will disclose the full separation capability of the method, *i.e.* the commonly used sequential solvent optimization approach of adjusting the solvent strength first and then improving selectivity in reversed-phase liquid chromatography is inefficient for the case of MLC with the hybrid eluents. This is illustrated in this paper with two examples: the optimization of the selectivity in the separation of a mixture of phenols and the optimization of a resolution-based criterion determined for the separation of a number of amino acids and small peptides.

The large number of variables involved in the separation process in MLC necessitates a structured approach in the development of practical applications of this technique. A regular change in retention behavior is observed with the variation of the surfactant concentration and the concentration of organic modifier, which enables a successful prediction of retention times. Consequently interpretive optimization strategies such as the iterative regression method are applicable.

INTRODUCTION

During recent years, the number of applications of reversed-phase high-

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0021-9673/90/$03.50

performance liquid chromatography (RP-HPLC), has been greatly extended by the use of secondary chemical equilibria in the mobile phase[1,2]. The use of these equilibria is mostly aimed at an enhancement of selectivity, and examples include ion-suppression, ion-pairing, complexation and use of micelles. One of the consequences of the use of additional equilibria is an extension of the number of parameters influencing the retention and selectivity observed in the chromatographic process. The chromatographer will only be able to exploit the full potential of the implemented separation mechanisms, if a systematic approach is available to describe or examine the effect of all important parameters involved. The results of such an investigation can then be used to predict the optimal conditions for a given separation problem.

A rigorous analysis of the chemical principles of the effect of secondary equilibria on the separation has been used to derive the setpoint for the optimal separation of one pair of components[2,3]. However, in practical applications it is necessary to consider the interactions of the parameter related to the secondary equilibrium with other parameters: for instance a change in organic modifier concentration will influence the dissociation constants of the solutes. Furthermore, different regions of the parameter space will often be associated with different critical peak-pairs, necessitating an analysis involving all components.

In a previous paper[4], we reported the simultaneous enhancement of separation selectivity and solvent strength in micellar liquid chromatography (MLC) using hybrid eluents of water–micelles–organic solvents. This selectivity enhancement occurs systematically, *i.e.* peak separation increases monotonically with volume fraction of organic solvent added to micellar eluents. This phenomenon in MLC was attributed to the existence of competing equilibria and/or the compartmentalization of solutes and organic modifier by micelles which have a great influence on the solvent strength parameter, S [*i.e.* slope of log capacity factor (k') *vs.* volume fraction of organic modifier (φ_{org})]. This phenomenon was observed for different groups of ionic and nonionic compounds with a variety of functional groups and for both anionic and cationic micellar eluents. The generality of the simultaneous selectivity enhancement with elution strength in hybrid systems indicates that the possible cause(s) is (are) not the same as the typical solvent selectivity effect in hydro–organic mobile phases, as the latter is only observed for a limited group of compounds which selectively interact with a given solvent. In fact, it has been shown recently that although maximum selectivity can be observed at intermediate solvent strength values in certain ternary or quaternary hydro–organic mixtures and for selected compounds, the general trend is higher selectivity at lower eluent strength[5].

There are two general approaches for optimization of solvent strength and selectivity in RP-HPLC with conventional hydro–organic eluents. Snyder and co-workers[6–8] have shown that adequate resolution can be achieved by varying the concentration of the organic modifier in the mobile phase. This method is only suitable for simple samples that contain components of different molecular size or dissimilar chemical functionality[6]. However, in cases where this method is applicable, good separations can be achieved with a few initial experiments[6–8]. The change in band spacing as a result of an increase in organic modifier concentration in hydro–organic RP-HPLC is usually not possible for samples that contain solutes with equal (or close) S values or when there is a strong correlation between S values and the corresponding retention[6–8]. As a consequence, most approaches in RP-HPLC with respect to

a systematic improvement of the isocratic separation of more complex mixtures are based on the following steps[6–11]: first the required elution strength is determined, preferably by means of a gradient. Then the selectivity of the mobile phase is varied, *e.g.* by changing the type of organic modifier, keeping the overall elution strength constant. Recently, the selectivity effect of the weak solvent (*i.e.* water) in ternary/quaternary hydro–organic eluents, was discussed[5]. However, in order to describe this effect for a given sample, a number of additional experiments will be required for the optimization of the ternary/quaternary mobile phases. As described below, the most important implication of the simultaneous increase of selectivity with solvent strength in MLC is that sequential optimization of solvent strength and selectivity is not effective and a more structured approach is required due to the complex nature of the retention process.

Optimization of the selectivity has received much attention in the literature and excellent reviews are available[8–10]. One differentiates between two types of procedures: (a) non-interpretive strategies which make no assumptions on the individual retention behavior of the components and solely evaluate the quality of the observed chromatograms, thus avoiding the need for peak-tracking; (b) interpretive optimization strategies which model the retention behavior of the individual components, thus requiring fewer experiments to derive an acceptable separation. However, specific information on the retention of all components is essential. Although most of these optimization strategies have been developed for standard RP-HPLC, a number of applications in ion-pairing chromatography have been described[12–16]. In most of these cases the addition of anionic or cationic surfactant is aimed at selectively decreasing the elution strength of the mobile phase for the ionic compounds, while keeping the retention of the uncharged solutes constant, as indicated in a recent review of optimization strategies in ion-pairing chromatography by Low *et al.*[17]

However, when the concentration of surfactant is further increased (*i.e.* when working above the critical micelle concentration, CMC), the retention mechanisms involved change dramatically as compared to ion-pairing chromatography. The presence of the micellar pseudo-phase enables additional partitioning of solutes between the stationary and the mobile phase, either because of electrostatic interaction with the charged outer-layer of the micelles and/or because of hydrophobic interactions with the lipophilic interior, thus influencing both the retention of neutral and ionized species. It is because of this dualistic influence on the retention of different solutes that strong changes in selectivity are observed in response to the variation of different parameters[4,18–20]. Obviously, one of the major parameters influencing retention is the concentration of the micellar pseudo-phase, equal to the total concentration of surfactant minus the CMC. Models described in the literature[21,22] indicate a linear relation between the inverse of the capacity factor, k', and the micelle concentration $[M]$:

$$k' = \frac{P_{SW} \cdot \Phi}{v \cdot (P_{MW} - 1) \cdot [M] + 1} \tag{1}$$

P_{MW} and P_{SW} represent distribution coefficients describing the partitioning of the solute between aqueous and micellar phase and aqueous and stationary phase respectively, v is the molar volume of the micellar pseudo-phase and Φ is the

chromatographic phase ratio. A number of parameters can be varied to influence the elution characteristics: in addition to the concentration, the charge of the headgroup and the length of the hydrophobic chain are factors related to the surfactant. Traditionally, only low concentrations of organic modifier are used (*e.g.* 3% propanol) to influence the column efficiency[23–26]. Only recently, the influence of the type and concentration of the organic modifier on the selectivity was described[4,20]. Because of the role of the electrostatic interactions, the pH is an important parameter to direct the retention of protonated species[27], for instance in the case of amino acids and peptides[28]. The ionic strength of the mobile phase will influence the elution as well, again depending on the combination of hydrophobic and electrostatic interactions[27]. Apart from the effect of the temperature on efficiency[23], the influence of this variable on the separation has not yet been examined systematically.

It is the aim of this paper to demonstrate that a sequential approach, *i.e.* an initial determination of the elution strengh followed by the optimization of the selectivity, is not feasible in the case of MLC and that the complex retention behavior necessitates a combined examination of the influence of the independent parameters involved. In this first investigation, we concentrate on two parameters which cause a large change in retention and selectivity and which are easy to vary: the concentration of the surfactant [either myristyltrimethylammonium bromide (CTAB) or sodium dodecyl sulfate (SDS)] and the concentration of the organic modifier, 2-propanol.

EXPERIMENTAL

In order to examine the changes in selectivity caused by a change in concentration of both organic modifier and micellar pseudo-phase, the retention times of two mixtures, a set of fifteen phenols and a set of thirteen amino acids and small peptides, were collected using reversed-phase columns.

The initial experiments regarding the phenol mixture were performed on a 5-μm particle LiChroCart C_{18} column (Merck, Darmstadt, F.R.G.), 12.5 cm × 4 mm I.D. The column was thermostated at 40°C and the flow was 1 ml/min. The column dead volume (0.67 ml) was measured by making multiple injections of water samples. A silica precolumn was employed to saturate the mobile phase with silicates and to protect the analytical column. The chromatographic equipment consisted of a dual-pump system (Model 2350, ISCO, Lincoln, NE, U.S.A.) and a V^4 absorbance detector set at 254 nm (ISCO), controlled by Chemresearch Chromatographic Data Management/System Controller software (ISCO) running on a PC-88 Turbo Personal Computer (IDS, Paramount, CA, U.S.A.). The analysis of the mixture was performed on the same instrument, using two identical LiChroCart columns as described above, and operating with a flow of 1.5 ml/min. In this case the dead volume was 1.40 ml.

The compositions of the mobile phase, the identities of the solutes and the observed capacity factors are listed in Table I. The solutes and the surfactant, CTAB, were obtained from Aldrich (Milwaukee, WI, U.S.A.). The surfactant solution was prepared by dissolving the required amount in doubly distilled deionized water and filtering through a 0.45-μm nylon filter. The ionic strength was adjusted by adding phosphate buffer (pH 7) such that the total buffer concentration of the final solution was 0.05 *M*. After adding the required amount of organic modifier, 2-propanol (Fisher Scientific, Pittsburgh, PA, U.S.A.), the pH was adjusted to 7.

TABLE I

THE CONCENTRATION OF THE SURFACTANT, [CTAB], AND THE PERCENTAGE OF 2-PROPANOL, USED IN THE CHROMATOGRAPHIC EXPERIMENTS REGARDING THE MIXTURE OF FIFTEEN PHENOLS

In addition the identities and capacity factors k' of the solutes are listed.

Mobile phase						
[CTAB], *M*		0.04	0.04	0.08	0.12	0.12
% 2-Propanol		0.0	10.0	5.0	0.0	10.0
Components				k'		
1	4-Benzamidephenol	4.7	1.9	2.3	2.2	1.2
2	4-Hydroxybenzyl alcohol	7.2	2.5	3.1	3.9	1.6
3	4-Hydroxyphenemethyl alcohol	11.4	3.4	4.3	5.4	2.0
4	4-Hydroxybenzylcyanide	20.1	5.9	7.0	8.3	3.4
5	4-Hydroxyacetophenone	29.0	9.5	9.9	10.9	4.4
6	Phenol	29.9	11.6	11.2	12.7	6.0
7	4-Hydroxybenzaldehyde	31.4	11.8	11.2	10.8	4.9
8	4-Fluorophenol	37.8	15.1	13.4	14.5	6.8
9	4-Methylphenol	43.6	18.4	15.3	16.4	8.1
10	4-Hydroxypropiophenone	50.7	19.3	16.5	17.3	7.5
11	4-Nitrophenol	63.8	29.7	22.0	17.5	10.2
12	4-Isopropylphenol	73.4	32.9	23.8	27.1	12.9
13	4-Hydroxybenzophenone	75.5	35.5	24.2	22.2	11.3
14	4-Hydroxydiphenylmethane	77.4	37.8	24.8	26.0	13.5
15	4-*tert.*-Butylphenol	86.8	38.2	27.7	32.6	14.9

All experiments regarding the amino acid–peptide mixture were performed on the same chromatographic system, using two identical columns in series with a total dead volume of 1.40 ml and with the detector set at 210 nm. The compositions of the mobile phase, the identities of the solutes and the capacity factors are listed in Table II. The solutes and the surfactant, SDS, were obtained from Sigma (St. Louis, MO, U.S.A.). The flow-rate was varied between 0.7 and 1.5 ml/min (dictated by the pressure limit of the system). The final analysis of the mixture was run at 1 ml/min. The preparation of the mobile phase was identical to the one described above with the following exceptions: the molarity of phosphate ions in the final solution was 0.02 *M*, and the pH was adjusted to 2.5.

The software to evaluate the separation at different mobile phase compositions was based on an extended version of the iterative regression optimization strategy[26,27] implemented by means of the Turbo-Pascal compiler version 4.0 (Borland, Scotts Valley, CA, U.S.A.). The program runs on a DeskPro 286 (COMPAQ Computer Corporation, Houston, TX, U.S.A.), equipped with a 80287 coprocessor, 640 kByte of conventional and 1 MByte of expanded memory, and an Enhanced Graphics Adapter with color monitor. The simulated chromatograms are based on a Gaussian peak-shape, using the plate-count and dead-volume observed in the chromatographic experiments.

TABLE II

THE CONCENTRATION OF THE SURFACTANT, [SDS], AND THE PERCENTAGE OF 2-PROPANOL, USED IN THE CHROMATOGRAPHIC EXPERIMENTS REGARDING THE MIXTURE OF THIRTEEN AMINO ACIDS–PEPTIDES

In addition the identities and capacity factors k' of the solutes are listed.

Mobile phase							
[SDS], *M*			0.1	0.1	0.25	0.4	0.4
% 2-Propanol			0.0	15.0	7.5	0.0	15.0
Components					k'		
1	Ala–Tyr	(AY)	5.5	3.2	1.7	1.4	0.9
2	Tyr	(Y)	11.3	3.0	2.4	2.9	1.1
3	Asp–Phe	(DF)	16.5	7.9	4.2	3.9	1.9
4	Leu–Tyr	(LY)	17.0	6.0	3.6	4.3	1.5
5	Gly–Leu–Tyr	(GLY)	18.3	6.9	3.7	4.3	1.5
6	Met	(M)	25.1	4.4	4.1	6.6	1.8
7	Trp	(W)	30.1	9.9	6.0	7.1	2.5
8	Arg	(R)	38.0	14.8	6.8	7.4	2.6
9	Leu–Trp	(LW)	49.3	15.1	8.3	11.3	3.2
10	Lys–Phe	(KF)	52.1	44.7	13.2	10.5	5.4
11	Gly–Phe–Leu	(GFL)	81.3	26.6	14.1	17.7	5.1
12	Phe–Phe	(FF)	81.5	21.8	12.6	18.2	4.6
13	Arg–Phe	(RF)	85.3	50.8	16.7	17.5	6.3

RESULTS AND DISCUSSION

In order to predict the properties of a chromatogram, such as the retention times and selectivities of the solutes that will be observed at a given mobile phase composition, we used the iterative regression optimization strategy originally described by Drouen *et al.*[29] and extended by Van Renesse *et al.*[30]. This is an interpretive optimization method which was also applied in ion-pairing chromatography[12,14]. First, the retention of all individual compounds in a mixture is modeled as a linear function of the variable(s) using a minimum number of initial experiments. For instance, in the case of one variable situation, φ_{org}, a linear model of ln k' *vs.* φ_{org} is derived on the basis of two initial values of φ_{org}. The parameter space for a given optimization is encompassed by the initial parameter values. Then, the retention of the solutes at mobile phase compositions other than those used in the actual measurements within the parameter space will be predicted through interpolation of the assumed linear model of ln k' *vs.* parameter(s). Based on the predicted retention values of all the compounds in the mixture, the quality of separation at all mobile phase compositions within the parameter space is calculated and an optimum is predicted. If the observed quality of the separation at the predicted optimum composition is not satisfactory, more experiments will have to be performed (*i.e.* through an iterative process) in order to locate the global optimum in the parameter space. The success or failure of finding the optimum parameter values with a minimum number of initial experiments would depend on the correctness of the linearity assumption of the model. In the case of

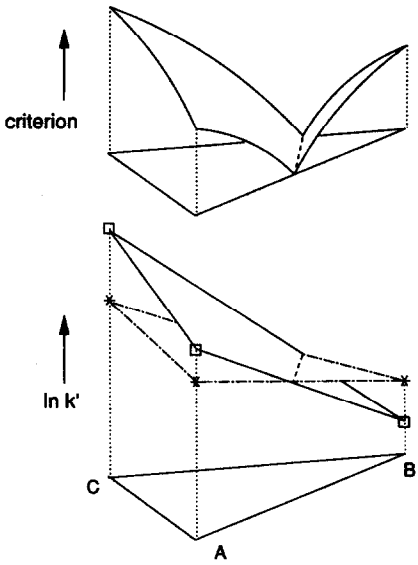

Fig. 1. An example of the retention-planes of a two component mixture, predicting the retention ln k' in the parameter space defined by mobile phase compositions A, B and C (lower part). The quality of the predicted chromatograms is expressed by means of a criterion, resulting in the response surface displayed in the top figure.

MLC, excellent agreement between the predicted and observed optimum chromatograms was observed with a minimum number of experiments as shown below. This is attributed to the regular change in retention behavior as a function of the variables. In this case we are dealing with two parameters, the concentration of surfactant and of 2-propanol respectively, and consequently the linear model will consist of a plane fitted through the retention data observed in three initial chromatograms. The three chromatograms are selected such that they are enclosing the parameter space currently under investigation. The model is then used to predict the retention of the component at a point located within this parameter space. This process is repeated for all components in a mixture, and the quality of the resulting estimated chromatograms is expressed by means of a suitable criterion. Fig. 1 shows an example with two components.

On the basis of a theoretical derivation using the solubility parameter concept, it was found that the relation between ln k' and the concentration of organic modifier in RP-HPLC is in fact quadratic[31] and the assumed linearity will only hold over a limited range of compositions. To compensate for deviations from linearity in the selected parameter space, further refinement of the model using additional measurements is usually required in an actual optimization in conventional RP-HPLC with hydro–organic eluents. In the case of MLC only a small range of organic modifier concentrations is examined and we can expect that deviations from linearity will be limited. The actual relation between the capacity factor and micelle concentration is given in eqn. 1. However, due to the fact that all examined micelle concentrations are of the same order of magnitude, only small deviations from linearity will be observed in relations between ln k' and $[M]$.

In order to derive an unambiguous definition of the examined parameter space[14], the retention is determined at five mobile phase compositions as indicated in Tables I and II: four measurements at the corners of the selected two-dimensional parameter-space and one measurement in the center. The square parameter space consists of four triangle subspaces. A separate linear model is determined for each of the four subspaces defined by three of the five measurements, *i.e.* two corner points and the central point. The extreme values of the parameters are dictated by the practical limitations of the chromatographic system: the lower surfactant concentration must be well above the CMC (*ca.* 8 m*M* for SDS and 0.9 m*M* for CTAB at ambient temperatures and without organic modifier) and must be strong enough to cause elution of all components. The upper surfactant concentration is determined by a combination of the solubility of the surfactant, the viscosity of the resulting mobile phase and degradation of the efficiency at higher concentrations. The organic modifier concentration is limited to a maximum of *ca.* 15% propanol to ensure the integrity of the micelles.

First we are concerned with a general description of the observed selectivity over the full parameter space, and the initial five compositions will suffice. However, in order to derive practical separations additional measurements in the areas of interest must be checked against the applied model and if necessary the model must be refined. The following discussion will first focus on the phenol mixture, followed by a description of the results regarding the amino acid–peptide mixture.

Elution strength

As expected, a decrease in retention time was observed when either the surfactant concentration (and hence the micelle concentration) or the concentration of organic modifier is increased. This is illustrated in Fig. 2 for 4-*tert.*-butylphenol: the mobile phase compositions where the same retention is expected on the basis of the linear interpolation are connected, resulting in "iso-retention" (dashed) lines. It is obvious that when both micelle and modifier concentration are increased, the respective effects will be combined and an even shorter retention is observed. In this way the retention of 4-*tert.*-butylphenol decreases from a capacity factor of 87.8 in the

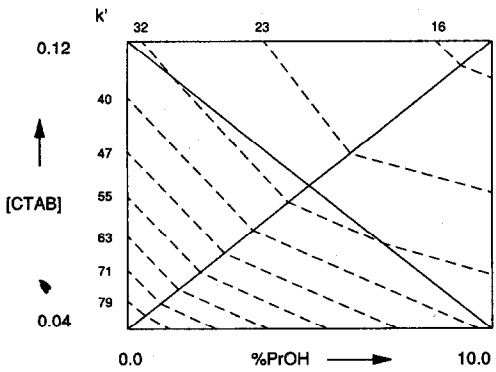

Fig. 2. The capacity factor of 4-*tert.*-butylphenol, *k'*, as a function of the surfactant concentration [CTAB] (*M*) and the percentage of 2-propanol, %PrOH. The dashed lines connect points defined by equal capacity factors. The solid lines connect the measured compositions thus defining the retention planes.

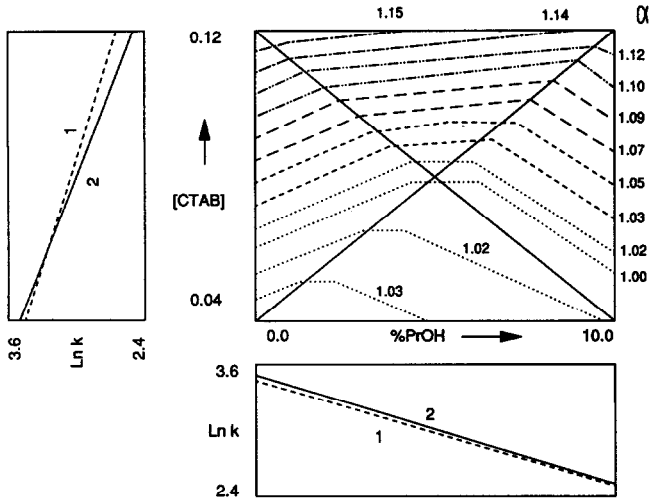

Fig. 3. Retention, ln k', of phenol (1) and 4-hydroxybenzaldehyde (2) as a function of the 2-propanol concentration (%PrOH) at 0.04 *M* CTAB (lower frame) and as a function of the surfactant concentration [CTAB] (*M*) at 0% 2-propanol (upper left frame). The selectivity α of the two components as a function of both surfactant and 2-propanol concentration: the dashed lines connect points with identical selectivity (upper right frame).

lower left corner (0.04 *M* CTAB, 0% 2-propanol) to 14.8 in the upper right corner (0.12 *M* CTAB, 10% 2-propanol).

Selectivity

Although the above trend is observed for all components, it must be stressed that the amount of reduction will not be the same, thus resulting in the desired change in selectivity. This is further illustrated by examining the changes in selectivity, α, which is defined as the ratio of the capacity factors of two components where numerator and denominator are selected such that the resulting value is larger than 1. Fig. 3 shows the effect of varying surfactant and propanol concentration on the selectivity of phenol and 4-hydroxybenzaldehyde: when the propanol concentration is increased a general decrease in selectivity is observed, due to a similar change in retention (lower frame of Fig. 2). However, by increasing the surfactant concentration an increase in selectivity is observed due to the fact that 4-hydroxybenzaldehyde shows a stronger drop in retention than phenol. Although the selectivity goes through a minimum as the two components coelute somewhere between 0.04 and 0.12 *M* CTAB, the final separation in 0.12 *M* CTAB is clearly superior to the ones observed at other mobile phase compositions.

The reverse is observed when the retention behavior of 4-hydroxy-propiophenone and 4-nitrophenol is examined (Fig. 4): a reduction of the selectivity occurs when the surfactant concentration is increased, since the two components are coeluting in 0.12 *M* CTAB. When the 2-propanol concentration is increased from 0 to 10% the selectivity increases, since the reduction in retention of 4-hydroxy-propiophenone is stronger than the change in retention of 4-nitrophenol.

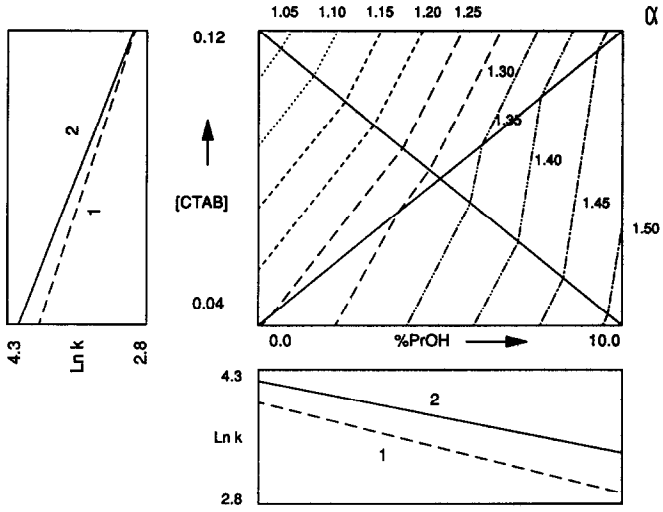

Fig. 4. Retention, ln k', of 4-hydroxypropiophenone (1) and 4-nitrophenol (2) as a function of the 2-propanol concentration at 0.04 *M* CTAB (lower frame) and as a function of the surfactant concentration [CTAB] (*M*) at 0% 2-propanol (upper left frame). The selectivity α of the two components as a function of both surfactant and 2-propanol concentration: the dashed lines connect points with identical selectivity (upper righ frame).

Simultaneous evaluation of selectivity and elution strength

Apparently in MLC different components react in different ways to changes in concentration of surfactant and/or organic modifier. In this way the chromatographer is supplied with additional tools to refine a separation and optimize the selectivity for a given multi-component mixture. However, the described advantage can only be exploited to the fullest when both parameters are simultaneously taken into account, *i.e.* by only using the surfactant or the organic modifier concentration, or varying one after optimizing the other, a better separation can be easily missed. This is generally true for other HPLC methods where co-optimization of the variables is necessary, *e.g.* [ion pair] and pH in ion-pair LC as demonstrated by Kong *et al.*[32].

It is possible that in MLC the optimal selectivity is observed when mobile phase compositions are used with a higher elution strength, depending on the components involved. Although this will complicate the design of an efficient optimization strategy, the gain in control over the separation can be considerable and will outweigh this disadvantage. As an example, the chromatograms of the mixture of fifteen phenols at the five mobile phase compositions are displayed in Fig. 5 (the chromatograms were simulated on the basis of the retention data in Table I). The best overall separation (in terms of selectivity) is observed in chromatogram E, which also happens to be the chromatogram with the minimum overall retention time. The other chromatograms show a severe coelution of a larger number of components. This is further emphasized in Fig. 6, showing the minimum selectivity which will be observed in chromatograms of the fifteen-component mixture recorded at different mobile phase compositions. The maximum values of this minimum selectivity are observed in the upper right corner, *i.e.* in the region with high elution strength.

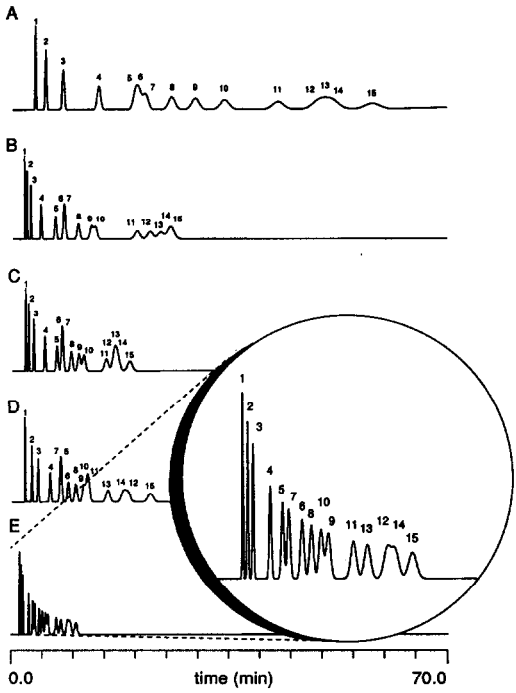

Fig. 5. Five simulated chromatograms using retention data of the fifteen components from Table I and assuming 2500 plates and equal areas for all components. Mobile phase compositions: (A) 0.04 *M* CTAB, 0% 2-propanol; (B) 0.04 *M* CTAB, 10% 2-propanol; (C) 0.08 *M* CTAB, 5% 2-propanol; (D) 0.12 *M* CTAB, 0% 2-propanol; (E) 0.12 *M* CTAB, 10% 2-propanol. The identification numbers of the solutes refer to Table I.

Optimization of selectivity

As can be observed in Fig. 5, showing the simulated chromatograms for the phenol mixture, the best selectivity for this mixture is observed at high micelle and propanol concentrations. However, the resolution of components 12 and 14 is still

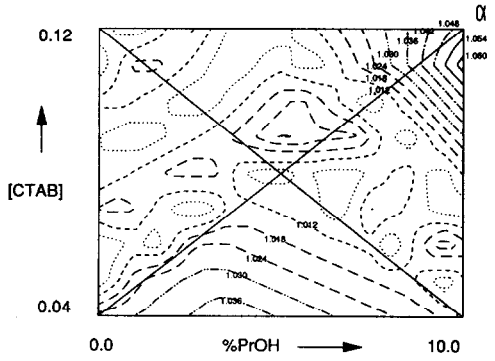

Fig. 6. A contourplot indicating the minimum selectivity that will be observed in chromatograms of the fifteen-component mixture described in Table I, when recorded at different mobile phase compositions. The lines connect points with equal minimum selectivity.

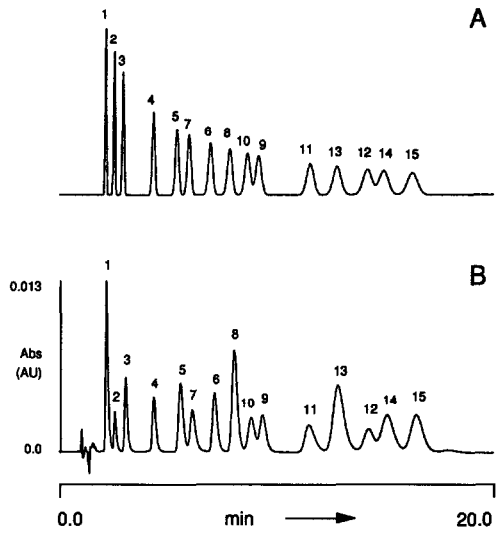

Fig. 7. Chromatograms of the fifteen-component mixture recorded at 0.11 *M* CTAB and 10% 2-propanol. (A) The chromatogram predicted on the basis of the linear retention model used in the optimization, assuming 4500 plates and equal areas for all components. (B) The measured chromatogram using two 12.5-cm columns and a flow of 1.5 ml/min. (for further details see Experimental section). The numbering refers to table I.

insufficient. In order to better resolve these two peaks, we examined the minimum α plot (for the fifteen-component mixture) over the full parameter space (Fig. 6). Apparently, a maximum value for the minimum selectivity in the chromatograms will be observed at 0.11 *M* CTAB and 10% 2-propanol. In order to test the accuracy of the predicted optimum, a chromatographic experiment with the fifteen-component mixture was performed at the above mobile phase composition. To increase the resolution, a two-column system was used, resulting in an average plate-count of 4500. The increase in retention time was partially compensated for by operating at 1.5 ml/min.

Both the predicted and observed chromatograms are shown in Fig. 7. The change in mobile phase composition has indeed improved the selectivity of components 12 and 14 without deteriorating the selectivity of the other separations (observed minimum selectivity 1.066). Furthermore, the observed retention data (Fig. 7B) correspond well with the predicted capacity factors (Fig. 7A), indicating that the assumed linear model is reasonable. Additionally, it will be unlikely that a better selectivity will be observed anywhere within the examined parameter space. In order to derive a truly optimal separation other parameters, such as pH, must be involved in the optimization procedure (in fact the five initial experiments originated from a parallel investigation into the application of MLC in quantitative structure–activity relationship studies of phenols, which requires experimental circumstances different from those more suited for a separation problem).

The amino acid–peptide mixture

In the foregoing discussion we concentrated on the observed selectivity in the

various chromatograms to stress the lack of correlation between elution strength and selectivity in MLC. However, the use of selectivity as an optimization criterion has a number of disadvantages, one of which is the fact that the observed separation (resolution) is determined by a combination of selectiviy, retention and efficiency. However, other optimization criteria which express the goal of the chromatographer in a better way can also be used without changing the overall optimization strategy[11]. This is illustrated by optimizing the separation of thirteen amino acids and small peptides using iterative regression design and a resolution based criterion (Table II). In this case the normalized resolution product r (ref. 29), was chosen as the optimization criterion which aims at an even distribution of all peaks over the available separation space:

$$r = \prod_{i=1}^{n-1} R_{s_{i+1,i}} \Big/ \left[\sum_{i=1}^{n-1} R_{s_{i+1,i}} / (n-1) \right]^{n-1} \tag{2}$$

where $R_{s_{i+1,i}}$ represents the resolution between peaks $i+1$ and i out of a total of n components. Since the product of all observed (expected) resolutions is used, coelution will effectively cause the criterion to drop to zero. On the other hand, extremely long chromatograms with a number of unnecessarily large resolution values will also be represented by low criterion values. This criterion has its own disadvantages: due to the normalization, a very short chromatogram with all

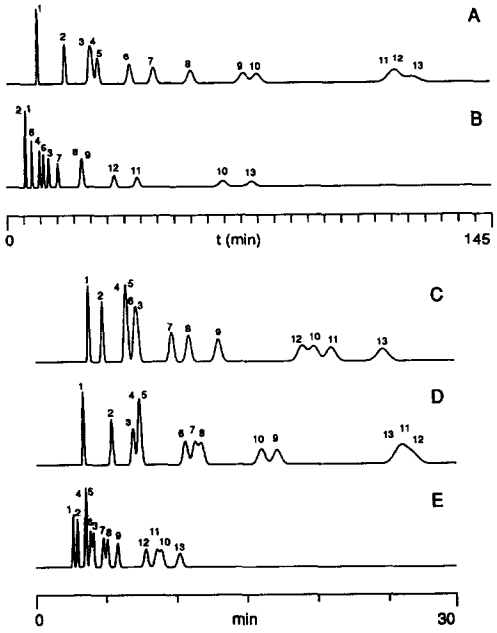

Fig. 8. Five simulated chromatograms using retention data of the thirteen components from Table II and assuming 4000 plates and equal areas for all components. Mobile phase compositions: (A) 0.1 *M* SDS, 0% 2-propanol; (B) 0.1 *M* SDS, 15% 2-propanol; (C) 0.25 *M* SDS, 7.5% 2-propanol; (D) 0.4 *M* SDS, 0% 2-propanol; (E) 0.4 *M* SDS, 15% 2-propanol. The identification numbers of the solutes refer to Table II.

components evenly distributed will still be heavily favored, even if the maximum observed resolution is small. If this is the case, only an increase of the plate-count will provide the desired separation (*not* a decrease of the solvent strength due to the effect on selectivity as described above). Alternatively, a response surface related to a different criterion can be examined.

The initial chromatograms are displayed in Fig. 8, and observations similar to those made for the phenol mixture are apparent: either increase in micelle concentration or increase in organic modifier concentration will decrease the overall retention, but will influence different solutes in different ways. This is best seen by looking at components 6, 11 and 12, showing a much stronger change in retention with varying conditions than the surrounding components, causing numerous cases of coelution and peak-crossings in intermediate mobile phase compositions. A satisfactory separation can only be found through a systematic search of the parameter space, as displayed in Fig. 9A. In this case (Fig. 9A) the predicted optimum criterion value is located at an intermediate elution strength at 0.16 *M* SDS and 12% 2-propanol. The predicted chromatogram at this composition is shown in Fig. 10A, showing an almost full separation of all components. Although the reduction in analysis time (not included in the criterion and consequently not considered in a systematic way in this optimization) is not as dramatic as in the previous example (Fig. 7), the chromatogram still shows a threefold reduction as compared to the chromatogram at 0.1 *M* SDS. What is more important however, is the fact that the actual chromatogram recorded at the specified mobile phase composition (Fig. 10B) is almost identical to the predicted one (Fig. 10A), with the exception of the last two components which show a slightly lower retention than predicted (resulting in a slightly lower criterion value of 0.170 instead of 0.175). This means that the response surface, predicted on the basis of the first five experiments, will hardly change when the new data point is included in the modeling, resulting in the same location for the optimum. A stable response surface is indicative of a reliable optimum, and no further experiments are necessary to further refine the location of the optimal mobile phase composition. Consequently, the regular retention behavior observed in these MLC

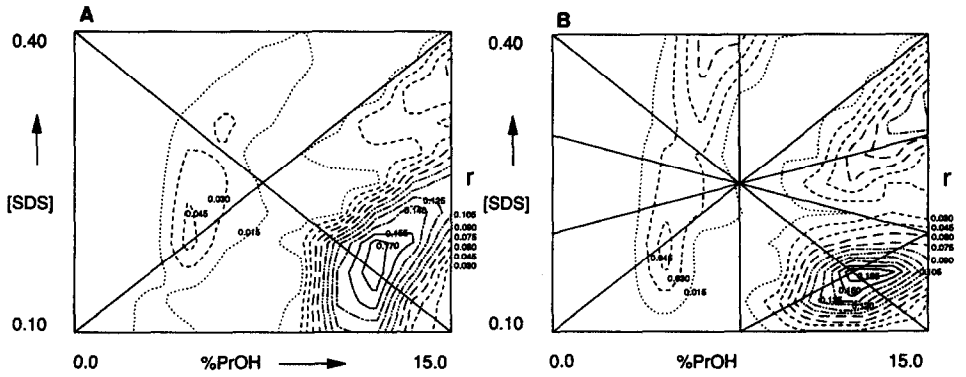

Fig. 9. A contourplot indicating the normalized resolution product *r* that will be observed in chromatograms of the thirteen-component mixture described in Table II, when recorded at different mobile phase compositions. The lines connect points with equal criterion values. (A) The response surface on the basis of five measurements. (B) The response surface on the basis of twelve experiments.

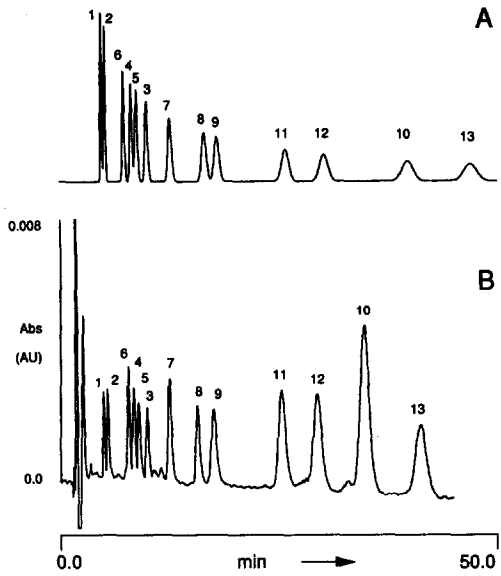

Fig. 10. Chromatograms of the thirteen-component mixture at 0.16 *M* SDS and 12% 2-propanol. (A) The chromatogram predicted on the basis of the linear retention model used in the optimization, assuming 4000 plates and equal areas for all components. (B) The measured chromatogram (further details in experimental section). The numbering refers to Table II.

examples, which is responsible for the stability of the response surface, enables an optimization on the basis of a relatively small number of experiments. As a final proof, Fig. 9B displays the response surface observed when a larger number of chromatograms is measured and included in the retention modeling, one of them the actual optimum displayed in Fig. 10B. The additional data-points are used to define a finer grid of triangles over the parameter-space, resulting in a more accurate response surface. Although a number of secondary, local, maxima are slightly more prominent, the important characteristics of the response surface such as location, criterion value and stability of the global optimum remain aproximately the same.

CONCLUSION

The two examples described in the previous section illustrate the following points:

Due to the complex retention mechanisms involved, a separate optimization of elution strength and selectivity in MLC is inefficient. In fact, it is observed that an increase in elution strength can coincide with an enhancement of the selectivity. Since the concentration of surfactant and organic modifier both influence the above factors, it is essential that a variation of these two parameters is examined simultaneously.

Although retention and selectivity vary strongly with varying concentrations of surfactant and/or organic modifier, the observed changes are very regular and are well described by a simple linear model with ln k' as dependent variable. As a consequence, it is possible to predict the retention behavior of the solutes on the basis of a limited

number of experiments (in the above examples five), even though these experiments are relatively far apart in the parameter space. In this way it is easy to estimate the likelihood of finding the desired separation, defined by the applied criterion, within the selected parameter space, thus reducing the required effort with respect to actual experiments.

The major drawback of practical separations applying MLC still is the low chromatographic efficiency caused by the resistance to mass transfer in the processes involving the micelles and the with surfactant modified stationary phase. This is especially important when the increased micelle concentrations cause a decrease in plate count, resulting in a varying efficiency over the parameter space. In this respect it is worthwhile to examine the inclusion of the expected efficiency and peak shape in the expression of the chromatographic quality in order to reduce the problem as much as possible, for instance by applying the adjusted resolution equations described by Schoenmakers *et al.*[33]. In addition to improvements in the chromatographic efficiency, inclusion of additional parameters such as pH and temperature will further increase possibility of harnessing the separation power supplied by secondary equilibria.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support by the National Institutes of Health (GM 38738). Acknowledgement is also made to the donors of the Petroleum Research Funds administered by the American Chemical Society.

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