Journal of Chromatography, 586 (1991) 221–232
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 573

Multiparameter optimizations in micellar liquid chromatography using the iterative regression optimization strategy

Joost K. Strasters, Sang-Tae Kim☆ and Morteza G. Khaledi*

North Carolina State University, Department of Chemistry, P.O. Box 8204, Raleigh, NC 27695 (USA)

(First received January 3rd, 1991; revised manuscript received May 29th, 1991)

ABSTRACT

An extension of the iterative regression optimization strategy to multi-parameter optimizations is described and applied to the separation of ionic compounds (amino acids and peptides) by means of micellar liquid chromatography. The parameters examined are the concentration of surfactant, the concentration of 2-propanol and pH. Fairly regular (linear, weakly curved) retention behaviour of the compounds as a function of the parameters results in an efficient optimization using a relatively small number of initial experiments.

INTRODUCTION

The method development scheme in most forms of reversed-phase high-performance liquid chromatography (RP-HPLC) is relatively complex owing to a lack of theoretical relationships to predict retention behaviour under varying experimental conditions. This is especially true if the identity of one or more of the components in a mixture is unknown.

It is for this reason that a large number of approaches using more or less empirical relationships have been developed to obtain a satisfactory separation on the basis of a limited number of experiments, as indicated in a number of excellent reviews [1–3]. In addition, an overview of advances regarding computer applications in this area was published recently [4].

The necessity for an efficient experimental design becomes especially important when dealing with forms of liquid chromatography suitable for the simultaneous analysis of ionic and non-ionic compounds such as ion-pair liquid chromatography (IP-LC) and micellar liquid chromatography (MLC). Here the number of possible parameters can be large, *e.g.*, the type and concentration of surfactant or ion-pairing reagent, the type and concentration of organic modifier(s), pH, temperature and ionic strength. The method development strategy must provide the chromatographer with an answer as to which parameters are the most appropriate ones to use and how to set up initial experiments to search the selected parameter space in an efficient way. The problem of parameter selection in IP-LC and MLC has not been fully addressed as yet, although preliminary investigations in IP-LC have been described [5,6].

Here we are concerned with a multi-parameter experimental design which can be applied in RP-HPLC in general, although the largest gain with respect to finding a better separation can be expected in IP-LC and MLC owing to the large number of relevant parameters, as indicated above. The main difference between these two forms of chromatography is the amount of hydrophobic surfactant in the mobile phase: the concentration used in IP-LC is below the critical micelle concentration (CMC) and consequently only free surfactant

☆ Present address: Hyo-Mox 2-Dong 383-2, Dong-Gu Taegu, 701-032, Seoul, South Korea.

0021-9673/91/$03.50

ions are present in the solution. As the stationary phase is modified with surfactant, a variation in the surfactant concentration will strongly influence the characteristics of the stationary phase. Solutes form ion pairs either in the mobile phase or on the stationary phase and consequently a strong curvature will be observed in plots of ln k' (k' = capacity factor) as a function of the surfactant concentration in the mobile phase. Nonetheless, optimization of these separations is possible and has been described [7–9].

In MLC, on the other hand, the concentration of surfactant is above the CMC and the concentration of free surfactant does not vary nearly as much with the amount of surfactant as in IP-LC. Instead, a variation in the surfactant concentration is translated into an increase in the concentration of micelles in the solution. As a consequence, the characteristics of the modified stationary phase are much more stable, and generally a regular (*i.e.* linear or weakly curved) retention behaviour is observed as a function of both the surfactant and organic modifier concentration [10–15].

Previously we described the successful application of a two-parameter version of the iterative regression optimization strategy in MLC [15]. Owing to unique selectivity effects as a function of the surfactant or organic modifier concentration [13,14], a simultaneous variation of these parameters is required in order to exploit the full separation power of the method. Optimizing these two parameters often results in shorter chromatograms with superior resolution.

Here we are concerned with a further extension of the parameter space with a third variable, *i.e.* pH. As we are often dealing with weakly acidic or basic compounds [5,6], this parameter can play a major role in fine tuning the selectivity. However, one should be cautious owing to the inherent non-linearity in the resulting change in retention behaviour, as discussed below. In order to include an additional parameter in the optimization, an adjusted scheme of the iterative regression optimization strategy was applied which was used previously in IP-LC. Although two parameters usually suffice for samples of moderate complexity to obtain a satisfactory separation, inclusion of the third parameter will often further improve the quality of the obtainable optimum, with respect to both resolution and analysis time. In other words, by including additional, relevant parameters in the optimization, the required peak capacity for a given separation is further reduced [16].

THEORY

Optimization strategy

The iterative regression optimization strategy was originally described by Drouen *et al.* for both the one- and two-parameter cases [17,18]. Applications were mainly found in traditional RP-HPLC, although applications in IP-LC and MLC have also been reported [8,15]. Full details of the method can be found in the above references. In a previous paper [15], the two-parameter optimization in MLC was discussed in detail, and only the basic principles will be given here.

In the two-parameter case, the search for the "optimum" separation can be envisioned in a three-dimensional space: two axes are related to the parameter under investigation and the third dimension is used to express the quality of the separation by means of a criterion (*e.g.*, minimum resolution) observed in the chromatogram. Within the examined region of the parameter space, a chromatogram can be simulated at every combination of the two parameters and consequently a criterion value can be predicted at a given mobile phase composition. This results in a three-dimensional plot (like a mountainous landscape) called the response surface, where the highest (or lowest) peak will be related to the parameter values that produce the best chromatogram. The aim of interpretive optimization strategies is to produce an accurate representation of the response surface with a minimum number of experiments.

The iterative regression strategy assumes that in a first approximation, retention (ln k') is a linear function of the parameters within a selected portion of the parameter space. When represented in a three-dimensional space, with ln k' as the third dimension, this translates into a plane [15]. As three points are required to define a plane, the parameter space will be divided into triangular subspaces (Fig. 1a) [15]. The linear models derived on the basis of three experiments (chromatograms observed for the parameter values on the corners of the triangles) are used to predict the retention for each component for

other experimental conditions (parameter values). The computer is used to go through the parameter space with small steps and to use the predictions of ln k' at each point to reconstruct the chromatogram and consequently calculate the predicted quality of the separation. After calculating the criterion values over the parameter space (*i.e.*, the response surface), the mobile phase compositions for the optimal chromatogram can be predicted.

Subsequent measurements can be used to refine the response surface by a further subdivision of the parameter space into smaller triangles. Again it is assumed that the linear model holds within each subspace. Depending on the location of the additional measurements, a triangular subspace is subdivided either into three or two new triangles. In Fig. 1b, measurement 6 performed after the five initial experiments divides one of the original subspaces, triangle 3,4,5, into three new subspaces: 3,4,6; 3,5,6; and 4,5,6. Measurement 7, located in the triangle 4,5,6, creates three new triangles, etc. In Fig. 1c, measurement 6 is located on the side of the subspace 3,4,5 and consequently divides this subspace in triangles 3,5,6 and 4,5,6. Each additional measurement will further refine the subdivision and consequently the accuracy of the prediction within the affected sections of the parameter space.

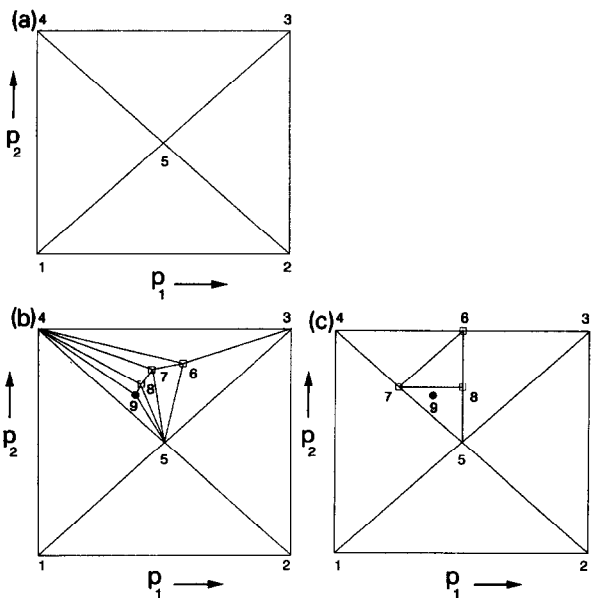

Fig. 1. Experimental design for a two-parameter optimization by means of the iterative regression strategy. The solid lines indicate the subspaces used to define the linear models and the numbers identify the location and order of the experiments. (a) The initial experiments; (b) a possible set of consecutive experiments in the case of a direct measurement of the predicted optimum in combination with a retention behaviour showing a strong curvature as a function of the parameters; (c) a possible set of consecutive experiments when the next measurement is chosen in the subspace containing the predicted optimum and is located as far from the other measurements as possible.

The selection of the location of the next measurement is governed by two, sometimes conflicting, considerations: on the one hand, one tries to measure the predicted optimum directly. When the measured and predicted chromatograms coincide, a confirmation of the assumed linearity is obtained in addition to a strong indication that the predicted global optimum actually is the true optimum (this proposition assumes that the observed linearity in the examined portion of the parameter space will also be maintained in the remainder of the parameter space). A disadvantage of this approach is illustrated in Fig. 1b: when strong deviations of the linear model are observed, the subsequent search of the parameter space will provide the location of a new optimum, and the process is repeated until the predicted and measured optima coincide. This will possibly result in an undesirably large number of experiments.

Possible solutions to this problem include the use of higher order models (and consequently a different experimental design with a larger number of initial experiments). Alternatively, one can adhere to the second consideration mentioned above, *i.e.*, try to obtain as much information as possible by means of an additional measurement. This can be relaized by locating the next measurement as far from the other measurements as possible, *i.e.*, on the long side of the triangle (Fig. 1c). In order to converge on the optimum, additonal measurements are always located in the subspace containing the predicted optimum. The procedure is stopped and the predicted optimum measured when the size of the resulting subspaces drops below a given size, dictated by the expected curvature of the retention behaviour as a function of the mobile phase composition. This approach will result in an inefficiently large number of experiments when the retention behaviour (ln k') is fairly linear.

Drouen *et al.* [17,18] proposed an intermediate

solution by shifting the composition of the next measurement in the direction of the point with the largest information content. One could also envision a design where the first additional experiment is performed according to the second consideration, while the locations of further measurements are based on the observed linearity of the retention behaviour. However, it is important to realize that the "best" approach is strongly dependent on the regularity of the observed retention, and will vary with the type of chromatography, the range of parameters examined and possibly also the nature of the solutes.

An inherent assumption in the application of the iterative regression strategy as described above is that deviations from linearity are limited to the extent that the quality of chromatograms in areas not examined and the area near the optimum is not much higher than predicted. This assumption seems to hold much better in MLC [15] than in IP-LC [8]. As indicated in these references, this can be checked by additional experiments.

Adjustments for three-parameter optimization

In order to perform the optimization for three or more parameters, a straightforward extension to a multi-parameter space must be performed [19]. For instance, in the case of a three-parameter optimization, the square in Fig. 1 is replaced by a cube and the triangles are replaced by tetrahedra, as indicated in Fig. 2, where the tetrahedron defined by measurements 2, 5, 8 and 9 has been emphasized. Each side of the cube is a two-parameter design identical with that presented in Fig. 1. Again, in order to obtain an unambiguous subdivision of the cube, the centre of the cube is included in the measurements, which results in a total of 24 tetrahedra. This requires a total of fifteen initial chromatograms. In analogy with the two-parameter optimization, a linear model relating the retention of a solute to the parameter values can be derived for each subspace. The models derived for all components in the mixture can then be used to predict and evaluate the chromatograms. However, as the parameter space is already three-dimensional, it would be difficult to envision a plot of the criterion value as a function of the three parameters, as this constitutes the fourth dimension (one possible way to visualize this is to think of a cube where the quality of the chromatogram in each

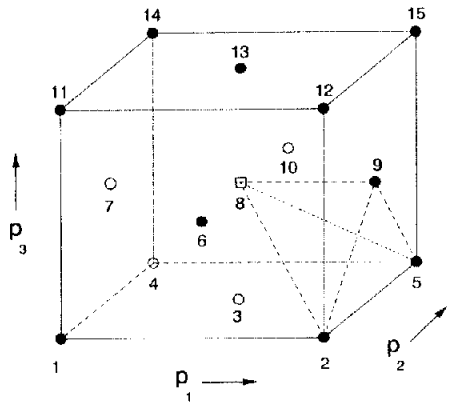

Fig. 2. Initial experiments in the case of a three-parameter iterative regression optimization. The solid dots represent points located on the "visible" outside of the cube, the open dots (3, 4, 7 and 10) are measurements on the "invisible" sides of the cube and the open square (8) is located in the centre of the cube. One of the subspaces, the tetrahedron (2, 5, 8, 9), is indicated by the dashed lines.

point is represented by a colour, ranging from dark blue for a bad separation to light red for good separations: the optimum would be apparent as an intensely coloured red cloud in the cube). The graphical representation is discussed further later.

It is apparent in the rigorous treatment described here that the number of initial experiments increases rapidly with each additional parameter, *i.e.*, two for one parameter, five for two parameters, fifteen for three parameters, etc. Therefore, it seems likely that this will limit the applied dimensionality (the number of parameters taken into consideration), rather than restrictions in calculation time [20]. This again emphasizes the need for efficient selection procedures to be applied before the actual optimization in order to keep the actual amount of experimental work to the minimum [5]. Alternative designs with fewer measurements can be envisioned, but these will require some form of regressiin; for instance, a pyramid described by the centre and four corners of the cube encloses a larger part of the parameter space (equivalent to four tetrahedra) and uses five measurements to derive a linear model with four parameters. As a consequence, deviations from linearity will strongly influence a larger section of the model and thus might require additional measurements in a later stage of the optimization. In addition, it will reduce the certainty in the statement

that the determined optimum in the examined section of the parameter space is the true optimum for that section.

The inclusion of additional experiments in a three-parameter optimization follows the same rules as the two parameter case (Fig. 3). A new measurement on the side of the triangle results in two new triangles (Fig. 1c), while a new measurement on the side of a tetrahedron divides the two sides of that tetrahedron and consequently results in two new tetrahedra (Fig. 3a). A new measurement inside a triangle divides that triangle into three new ones (Fig. 1b), while a new measurement on the side of a tetrahedron (*i.e.*, a triangle) again divides that triangle into three sections, thus creating three new tetrahedra (Fig. 3b). When the new measurement is located in the centre of the tetrahedron, four new tetrahedra will result (Fig. 3c). For each additional parameter, this scheme will be extended with one step, and will be extremely difficult to visualize.

In analogy with the two-parameter optimization, a new retention model is now derived for each compound in each newly created subspace and the chromatograms in these sections are predicted and evaluated.

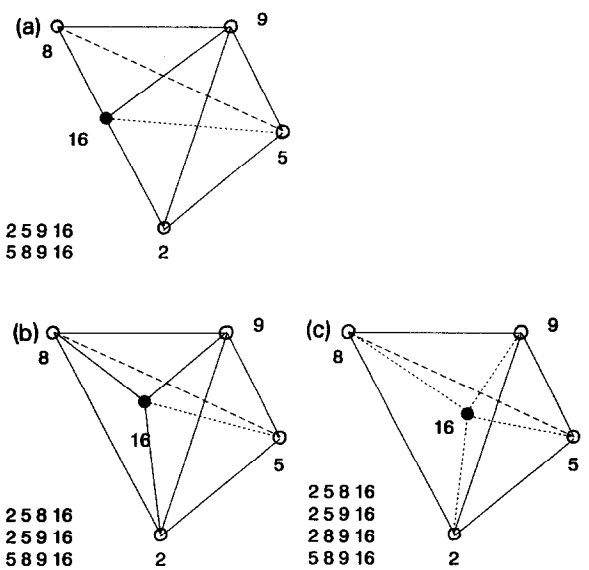

Fig. 3. Possible subdivisions of the tetrahedron shown in Fig. 2 by an additional measurement (16). The newly created tetrahedra are identified by the experiments listed on the lower left side of the figures. (a) Measurement 16 located on the line connecting experiments 2 and 8; (b) measurement 16 located on the plane defined by experiments 2, 8 and 9; (c) measurement 16 located in the centre of the original tetrahedron.

A separate problem is a simple graphical representation of the quality of the separation as a function of the various parameters. In the software version used for the example described under Experimental, this was done by presenting the user with two-dimensional slices at fixed values of the third parameter. In addition, it is also possible to extend this approach by calculating the slice at a given combination of two or more of the parameters. In this way it will be possible to obtain an impression of the quality of the separation for a more or less "isoeluotropic" set of conditions, although both parameters involved will influence the retention time. Further examples of the graphical representation are given under Results and Discussion.

EXPERIMENTAL

In order to illustrate the use and implications of a three-parameter optimization in MLC, the retention of a set of nine amino acids and peptides was examined as a function of pH, the concentration of surfactant and the concentration of organic modifier.

All experiments were performed on a 5-μm particle size LiChroCART C_{18} column (12.5 cm × 4 mm I.D.) (Merck, Darmstadt, Germany). The column was thermostated at 40°C and the flow-rate was 1 ml/min (dead volume 0.86 ml). A silica precolumn was employed to saturate the mobile phase with silicates and to protect the analytical column. The chromatographic equipment consisted of a pump (Model 2350; ISCO, Lincoln, NE, USA) and a V^4 absorbance detector set at 210 nm (ISCO).

The compositions of the mobile phases, the identities of the solutes (checked in the chromatograms by means of separate injection of the standard) and the observed retention times are listed in Table I. The solutes and the surfactant, sodium dodecyl sulphate (SDS), were obtained from Sigma (St. Louis, MO, USA). The surfactant solution was prepared by dissolving the required amount in doubly distilled, deionized water and filtering over a 0.45-μm nylon filter. The pH and ionic strength were adjusted by adding phosphate buffer such that the total buffer concentration of the final solution was 0.02 *M*. After adding the required amount of

TABLE I

CONCENTRATION OF THE SURFACTANT ([SDS]), THE PERCENTAGE OF 2-PROPANOL (PrOH) AND THE pH USED IN THE CHROMATOGRAPHIC EXPERIMENTS REGARDING THE MIXTURE OF NINE AMINO ACIDS AND PEPTIDES, TOGETHER WITH THE IDENTITIES AND RETENTION TIMES [t_r (min)] OF THE SOLUTES

Mobile phase		Composition				
[SDS] (*M*)		0.1	0.1	0.2	0.3	0.3
PrOH (%)		0.0	10.0	5.0	0.0	10.0
pH		2.5	2.5	2.5	2.5	2.5
Components		t_r (min)				
1 Arg	(R)	25.53	13.73	8.17	8.01	4.23
2 His	(H)	20.85	9.60	6.38	6.63	3.33
3 Leu	(L)	26.37	9.79	7.85	9.43	4.19
4 Tyr	(Y)	8.14	3.72	3.18	3.32	2.02
5 Ala–Tyr	(AY)	4.50	3.31	2.35	2.05	1.66
6 Gly–Phe–Leu	(GFL)	53.15	23.10	14.61	17.59	7.62
7 Aso–Phe	(DF)	11.69	7.39	4.89	4.44	2.97
8 Lys–Phe	(KF)	35.50	29.82	13.37	11.01	7.76
9 Leu–Trp	(LW)	32.02	13.65	9.06	11.13	4.78
Mobile phase		Composition				
[SDS] (*M*)		0.1	0.2	0.2	0.2	0.3
PrOH (%)		5.0	0.0	5.0	10.0	5.0
pH		3.0	3.0	3.0	3.0	3.0
Components		t_r (min)				
1 Arg	(R)	15.25	11.85	6.80	4.85	4.45
2 His	(H)	9.93	9.00	4.57	3.33	3.21
3 Leu	(L)	13.05	14.02	7.05	4.65	5.08
4 Tyr	(Y)	4.10	4.50	2.63	1.90	2.12
5 Ala–Tyr	(AY)	3.55	2.70	2.15	1.95	1.70
6 Gly–Phe–Leu	(GFL)	32.57	27.50	15.05	11.10	9.90
7 Asp–Phe	(DF)	8.46	6.38	4.37	3.62	3.16
8 Lys–Phe	(KF)	32.10	17.69	13.35	12.40	8.25
9 Leu–Trp	(LW)	18.75	17.59	9.05	6.73	6.23
Mobile phase		Composition				
[SDS] (*M*)		0.1	0.1	0.2	0.3	0.3
PrOH (%)		0.0	10.0	5.0	0.0	10.0
pH		3.5	3.5	3.5	3.5	3.5
Components		t_r (min)				
1 Arg	(R)	21.78	7.09	5.38	6.64	2.74
2 His	(H)	13.45	4.30	3.43	4.25	2.17
3 Leu	(L)	24.25	5.05	5.75	9.04	3.15
4 Tyr	(Y)	6.21	1.65	2.09	2.85	1.45
5 Ala–Tyr	(AY)	4.25	2.57	1.97	1.92	1.53
6 Gly–Phe–Leu	(GFL)	58.89	22.70	14.82	18.21	7.67
7 Asp–Phe	(DF)	10.27	4.18	3.70	3.92	2.28
8 Lys–Phe	(KF)	38.04	28.80	12.99	11.12	7.60
9 Leu–Trp	(LW)	35.50	12.75	8.85	11.47	4.65

organic modifier, 2-propanol (Fisher Scientific, Pittsburgh, PA, USA), the apparent pH was adjusted to the specified value.

The software to evaluate the separation at different mobile phase compositions was based on an extended version of the iterative regression optimization strategy [19] implemented by means of the Turbo-Pascal compiler version 5.5 (Borland, Scotts Valley, CA, USA). The program runs on a DeskPro 286 (COMPAQ Computer, Houston, TX, USA), equipped with a Model 80287 coprocessor, 640 kbyte of conventional and 1 Mbyte of expanded memory, and an Enhanced Graphics Adapter with colour monitor. The simulated chromatograms are based on a Gaussian peak shape, using the plate count (average 2500) and dead volume observed in the chromatographic experiments.

RESULTS AND DISCUSSION

Fig. 4 shows the parameter space selected for the separation of the mixture of nine peptides and amino acids listed in Table 1. Similarly to described procedure [15], the examined range of concentrations for surfactant and 2-propanol were determined on the basis of chromatographic insight and physical limitations: the minimum surfactant concentration is far above the CMC of SDS and was chosen such that a reasonable retention time for all components was obtained. Although in this instance information from previous experiments was used, a more objective selection on the basis of a micellar gradient will be a valid alternative to determine this concentration [21]. Likewise, the maximum micelle

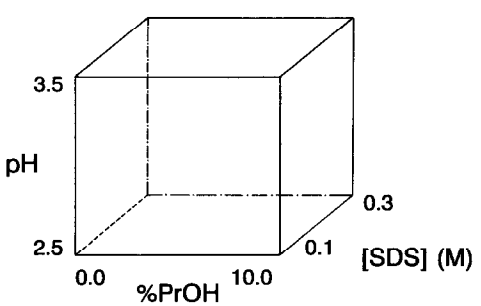

Fig. 4. Parameter space selected for the separation of the nine-component amino acid–peptide mixture. [SDS] indicates the concentration of surfactant (*M*) and %PrOH is the percentage of 2-propanol organic modifier.

concentration was selected such that the viscosity of the mobile phase was acceptable (*i.e.*, maximum pressure drop over the column within 2000 psi) and all capacity factors were higher than (approximately) 1.0. The upper limit of the propanol concentration was based on considerations regarding retention times, viscosity and micellar integrity: when the concentration of the organic modifier becomes too high, the characteristics of the micellar pseudophase change (by creating a micro-emulsion or complete disappearance of the micelles).

The range of pH values examined was intentionally selected to contain only a small portion of the full range between 2.5 and 7.0, *i.e.*, pH 2.5–3.5. This was done to prevent deviations from linearity in the retention behaviour: for larger ranges an S-shaped retention *vs.* pH curve will be observed when the component changes from its acidic to its basic form, which will require at least one additional measurement for an approximation with linear segments.

A number of the initial chromatograms are displayed in Fig. 5. The criterion used here to define the quality of the separation is the minimum resolution, *i.e.*, the resolution of the least separated pair of components in the chromatogram. The following remarks can be made.

None of the initial chromatograms shows sufficient separation of all components in the mixture (sufficient separation is usually defined as resolution between 1.0 and 1.5, where a resolution of 1.5 corresponds to "baseline" separation). This again illustrates the observations presented in previous papers [14,15] where a decrease in elution strength by decreasing the surfactant and/or organic modifier concentration does not automatically result in improved resolution due to the combined selectivity/elution strength observed in MLC.

The overall analysis time is not a function of pH, because the degree of dissociation of the latter components in the chromatograms (KF, GFL) hardly changes in the examined pH range. As a consequence, the pH can be used to fine tune the selectivity without influencing the elution strength of the mobile phase. However, the observation is strongly sample dependent.

The influence of the pH is dependent on the values of the other parameters, in other words, a simultaneous optimization of all variables is required in order to examine the full separation potential. This is

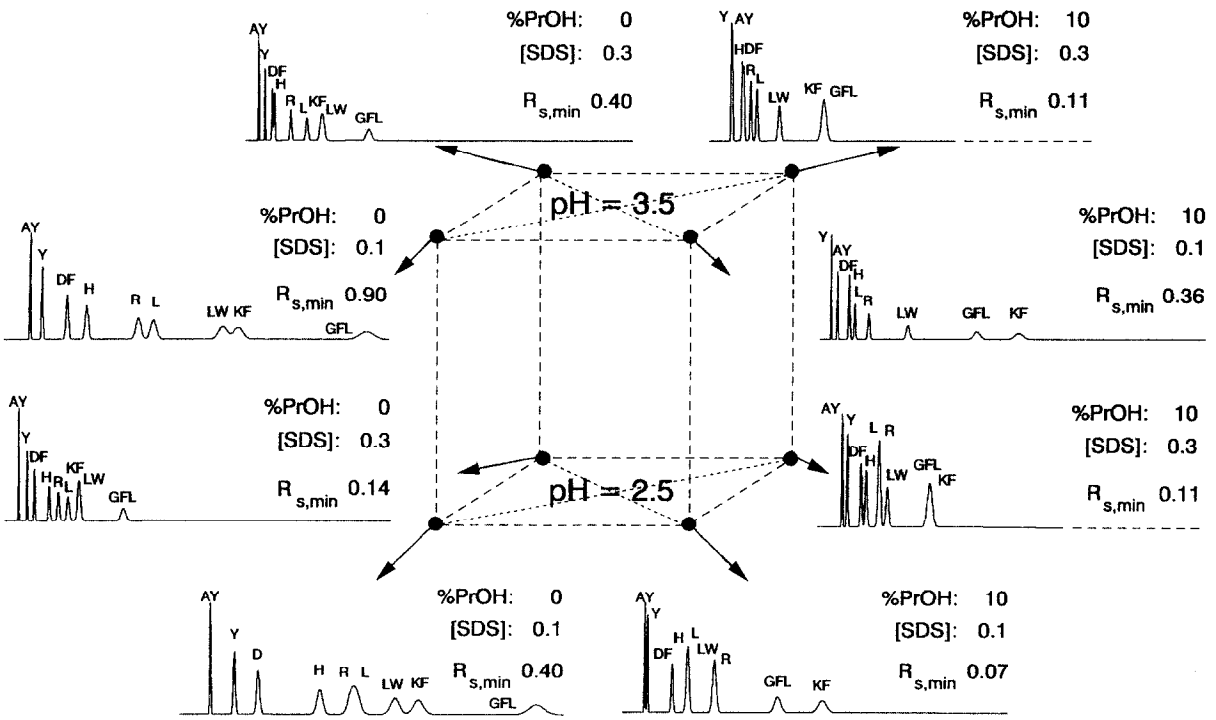

Fig. 5. Set of the initial experiments performed with the nine-component amino acid–peptide mixture, identified in Table 1, relative to their position in the parameter space. The minimum resolution observed in each chromatogram is indicated by $R_{s,min}$.

especially obvious when comparing the chromatograms on the left and the right sides of the cube: on the left side, the order of components AY and Y does not change as a function of pH. On the right side, on the other hand, the order of AY and Y does change on increasing the pH from 2.5 to 3.5. A possible explanation of this phenomenon is the micellar-induced pK_a shift [22], caused by a difference in the partitioning of the undissociated and dissociated species of a solute in the micellar pseudo-phase. As the dissociated form has the same charge as the micelles, the partitioning process will result in a surplus of this form in the aqueous phase with respect to the thermodynamic equilibrium, and consequently a reduction in the amount of dissociation. Hence the apparent pK_a of a solute will increase or, in other words, the S-shaped retention behaviour of a solute will be shifted to the right. Here this shift is more apparent for AY than for Y, such that the decrease in retention for AY as a function of pH is less pronounced at high concentrations of surfactant whereas the decrease in the retention of Y is influenced less by the surfactant concentration.

The above observations are further emphasized in Fig. 6, where the results of the first step of two two-parameter optimizations and the three-parameter optimization are compared. The lower iso-response surface (containing lines which connect points with equal criterion values) refers to pH 2.5. Apparently, a good separation is predicted at 4% propanol, 0.10 *M* SDS. The corresponding chromatogram is displayed on the right. The resolution is more than adequate ($R_{s,min}$ = 1.6) but the analysis time is relatively long (*ca.* 40 min). The same applies to the optimum predicted at pH 3.5, where similar resolution and analysis time are observed at 1% propanol, 0.14 *M* SDS. However, when the full parameter space is taken into consideration, an even better separation is obtained. This is represented by the iso-response surface at the pH where the optimum criterion value for the full parameter space is observed, *i.e.*, pH 3.1. Not only is the resolution

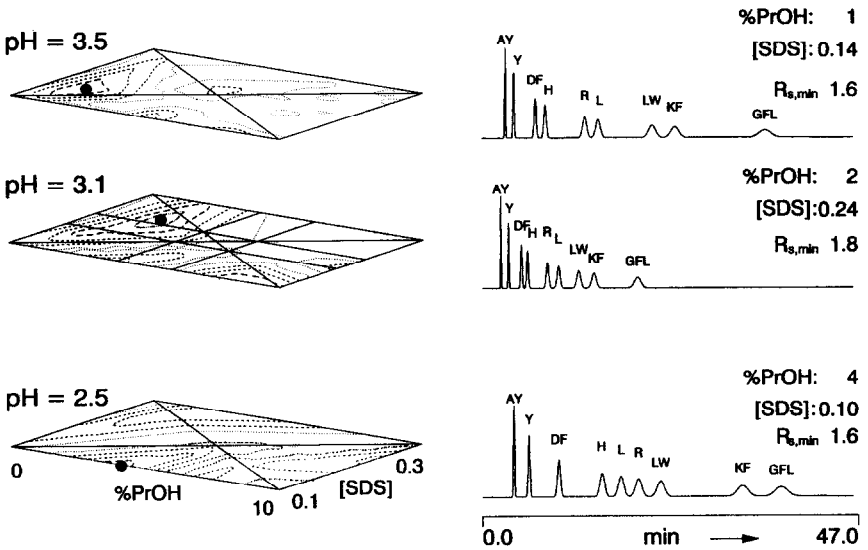

Fig. 6. Three intersections in the original parameter space at the specified pH values. In each intersection, isoresponse lines show the behaviour of the criterion, *i.e.*, the minimum resolution. In addition, the predicted optimum at each pH, location indicated by the solid dot, is displayed on the right. The optimum at pH 3.1 is also the predicted global optimum of the three-parameter optimization.

improved from 1.6 to 1.8 (in fact an unnecessary improvement, as 1.5 is already a sufficient separation for components with approximately equal responses and concentrations) but, more important, the analysis time can be drastically reduced from 38 to 20 min. Hence two parameters are sufficient to obtain a sufficient separation for this mixture but three parameters enable us to achieve a better chromatogram with respect to both separation and analysis time. Further discussion of this topic is presented later.

The full three-parameter optimization predicts an optimum mobile phase composition of 2% propanol, 0.24 *M* SDS and pH 3.1. However, when this chromatogram was actually measured, a slight difference was observed between the predicted and measured chromatograms, resulting in a new location of the predicted global optimum. This is illustrated in Fig. 7: another slice of the full parameter space is shown for a constant value of the surfactant concentration (0.24 *M* SDS). The intersection between this plne and the various tetrahedra is indicated by the solid lines. Chromatogram 16 (2% propanol. 0.24 *M* SDS, pH 3.1) results in the formation of four new tetrahedra within tetrahedron (4, 7, 10, 14) as indicated in the middle left of the square. The measured chromatogram is indicated by the asterisk, and is surrounded by the intersections of tetrahedra (4, 7, 10, 16), (4, 10, 14, 16), (4, 7, 14, 16) and (7, 10, 14, 16) with the plane ([SDS] = 0.24 *M*).

The change in the location of the global optimum and consequently the necessity for an additional

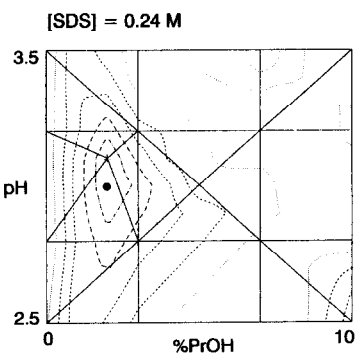

Fig. 7. Intersection of the full parameter space at a surfactant concentration [SDS] = 0.24 *M* after sixteen experiments. The iso-response lines (dashed lines) connect points with identical criterion values. The solid lines show intersections with the various tetrahedra defined by previous experiments. The asterisk indicates the location of experiment 16, and the solid dot defines the location of the predicted optimum, shown in Fig. 8.

measurement are caused by a deviation from the assumed linear retention behaviour (ln k') as a function of the parameters. In this instance the deviation was most noticeable in the direction of varying pH, as was to be expected given the nature of the parameter. The location of the next measurement is indicated by the dot, and is positioned at 2% propanol, 0.24 *M* SDS and pH 3.0. In addition, the shape of the response surface, described by the dashed lines, indicates a fairly stable optimum (gradual change in the criterion value as a function of the parameters). Of course, this statement only holds here for the two parameters examined in this figure (pH and propanol concentration), but examination of the plane perpendicular to that presented in Fig. 7 at 2% propanol shows a similar behaviour of the response (not shown).

The predicted and observed chromatograms at this mobile phase composition are presented in Fig. 8. Since the predicted chromatogram is the result of a simulation, the impurities (origin unknown) in the beginning of the measured chromatogram in Fig. 8b are absent. In addition, the resolution in the measured chromatogram is slightly less than that in the predicted chromatogram, mostly because of some peak asymmetry, not expressed in the plate count and consequently not taken into consideration in the calculations. Refinement of the criterion by including asymmetry in the resolution calculation [23] will improve the results. Nonetheless, the two chromatograms are very similar, indicating that the linear model applied was sufficient to describe most

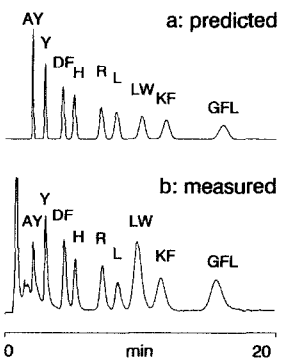

Fig. 8. Predicted and measured chromatograms of the nine-component amino acid–peptide mixture defined in Table 1 at [SDS] = 0.24 *M*, 2% propanol and pH 3.0.

of the observed retention behaviour. This is indicative of a fairly regular retention behaviour as a function of the examined parameters, which is similar to the conclusion obtained for the two-parameter case [15].

As mentioned before, the use of minimun resolution is not the best criterion if one wants to optimize both separation and analysis time. This is especially true if sufficient separation (R_s = 1.5) is achieved for a number of parameter settings: further improvement of the resolution is not sensible, while improvement in analysis time is not expressed by the criterion (if strongly varying concentrations or detector responses of the components are observed, an adjusted definition of resolution might be applied [24] and the above statement still holds true). In order to circumvent these problems, several solutions have been proposed, *e.g.*, the use of the so-called "multi-criterion decision making" [24]. Alternatively, a much better defined response surface with respect to the goal of the chromatographer is obtained if a so-called threshold criterion is applied [2]: the parameter space is searched for the minimum analysis time, taking into consideration only chromatograms with sufficient separation. The results of using a variation of this criterion (*i.e.*, the inverse of the retention time of the last peak, unless the resolution drops below a critical value in which case the criterion is set to zero) for the peptide–amino acid mixture are presented in Fig. 9. The intersections show that the global optimum is much better defined by this approach, and indeed results in a better chromatogram with respect to analysis time (*ca.* 14 min) compared with those presented in Fig. 6, with sufficient resolution of all components.

CONCLUSIONS

The iterative regression optimization strategy is easily extended to multi-parameter optimizations. However, an inhibitively large number of initial experiments limits the application to three parameters.

The regular retention behaviour of solutes in MLC as a function of the concentration of organic modifier, concentration of surfactant and pH permits the use of the simple linear model, as long as the examined range of pH values is limited. This regularity also allows a direct measurement of the predicted

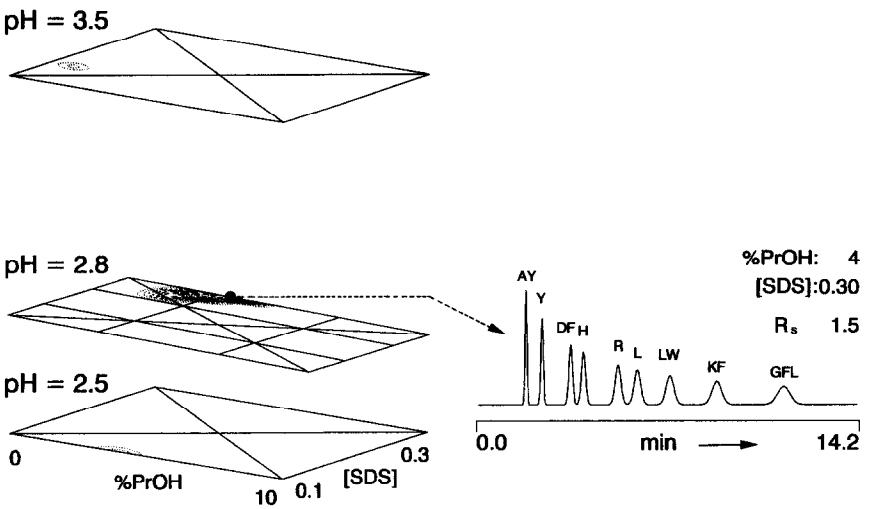

Fig. 9. Three intersections in the original parameter space at the specified pH values. In each intersection, iso-response lines show the behaviour of the criterion, the inverse retention time of the last component unless the minimum resolution is lower than 1.5, in which event the criterion is set to zero. In addition, the predicted global optimum, location indicated by the solid dot, is displayed on the right.

optimum mobile phase composition, resulting in a fast optimization procedure. However, the iterative part of the procedure remains essential to correct for slight deviations in the observed retention behaviour.

Separate optimization of, *e.g.*, pH apart from the other parameters is inefficient and can result in sub-optimum separations owing to the dependence of the response on the values of surfactant and organic modifier concentration. This is inherent in the chromatographic technique, where for instance a change in surfactant concentration influences the overall dissociation of the components.

The next step in the development of a comprehensive optimization strategy for this type of chromatography is an intelligent and efficient selection of the relevant parameters and especially the examined range in the parameter space. In this way the advantages of a fast and easy method development protocol in MLC can overcome some of the disadvantages of this type of chromatography, such as the limited efficiency.

ACKNOWLEDGEMENT

This work was supported by a grant from the National Institutes of Health (FIRST Award, GM38738).

REFERENCES

1 J. C. Berridge, *Techniques for Automated Method Optimization of HPLC Separations*, Wiley, Chichester, 1985.
2 P. J. Schoenmakers, *Optimization of Chromatographic Selectivity —a Guide to Method Development*, Elsevier, Amsterdam, 1986.
3 L. R. Snyder, J. L. Glajch and J. J. Kirkland, *Practical HPLC Method Development*, Wiley, New York, 1988.
4 J. L. Glajch and L. R. Snyder (Editors), *Computer-Assisted Method Development for High-Performance Liquid Chromatography*, Elsevier, Amsterdam, 1990; *J. Chromatogr.*, 485 (1989).
5 G. K.-C. Low, A. Bartha, H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, 478 (1989) 21.
6 A. Bartha, G. Vigh and J. Stahlberg, *J. Chromatogr.*, 485 (1989) 403.
7 A. P. Goldberg, E. Nowakowska, P. E. Antle and L. R. Snyder, *J. Chromatogr.*, 316 (1984) 241.
8 H. A. H. Billiet, J. Vuik, J. K. Strasters and L. de Galan, *J. Chromatogr.*, 384 (1987) 153.
9 P. M. J. Coenegracht, N. V. Tuyen, H. J. Metting and P. J. M. Coenegracht-Lamers, *J. Chromatogr.*, 389 (1987) 351.
10 D. W. Armstrong and F. Nome, *Anal. Chem.*, 53 (1981) 1662.
11 M. Arunyanart and L. J. Cline Love, *Anal. Chem.*, 56 (1984) 1557.
12 J. G. Dorsey, M. T. DeEchegaray and J. S. Landy, *Anal. Chem.*, 55 (1983) 924.
13 M. G. Khaledi, *Anal. Chem.*, 60 (1988) 876.
14 M. G. Khaledi, J. K. Strasters, A. H. Rodgers and E. D. Breyer, *Anal. Chem.*, 62 (1990) 130.
15 J. K. Strasters, E. D. Breyer, A. H. Rodgers and M. G. Khaledi, *J. Chromatogr.*, 511 (1990) 17.

16 D. P. Herman, H. A. H. Billiet and L. de Galan, *Anal. Chem.*, 58 (1986) 2999.
17 A. C. J. H. Drouen, H. A. H. Billiet, P. J. Schoenmakers and L. de Galan, *Chromatographia*, 16 (1982) 48.
18 A. C. J. H. Drouen, *Ph.D. Thesis*, Delft University of Technology, Delft, 1985.
19 A. van Renesse, J. K. Strasters, H. A. H. Billiet and L. de Galan, presented at the *13th Symposium on Column Liquid Chromatography, Stockholm, June 25–30, 1989*, (poster) M/TU-P-164.
20 H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, 485 (1989) 27.
21 L. S. Madamba-Tan, J. K. Strasters and M. G. Khaledi, *Anal. Chem.*, submitted for publication.
22 M. G. Khaledi and A. H. Rodgers, *Anal. Chim. Acta*, 239 (1990) 121.
23 P. J. Schoenmakers, J. K. Strasters and A. Bartha, *J. Chromatogr.*, 458 (1988) 355.
24 P. M. J. Coenegracht, A. K. Smilde, H. J. Metting and D. A. Doornbos, *J. Chromatogr.*, 485 (1989) 195.