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PRACTICAL OPTIMIZATION OF THE SEPARATION OF A LIMITED SUB-SET OF COMPONENTS BY USING ISOELUOTROPIC TERNARY ELUENT MIXTURES IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A fully integrated procedure is presented for the separation optimization of all or a limited subset of components by means of isoeluotropic ternary solvent mixtures along with the iterative regression method in reversed-phase high-performance liquid chromatography. The optimization search area is defined for both full and limited optimization by utilizing a statistical approach in combination with an extended gradient isocratic scanning procedure. Starting eluent compositions are selected with respect to the complexity of the sample mixture, and the merits of the procedure are evaluated by performing the full and limited (3-out-of-9) optimization of a nine-component aromatic solute mixture. Three resolution-based optimization criteria are used and selected sequentially to achieve satisfactory resolution for the peaks of interest in the shortest possible analysis time. In order to guide the analyst through the selection of the criteria and to assist in the evaluation of the optimum found, an "expert" algorithm has been integrated in the optimization program. The use of the algorithm in the optimization of the analysis time is also demonstrated (while resolution is maintained at the required level) by applying a suitable criterion and allowing column length variation.

INTRODUCTION

The development and application of computerized optimization procedures in high-performance liquid chromatography (HPLC) is a steadily growing area of research. Most of the currently proposed selectivity optimization schemes revolve around the separation of all components in the sample mixtures with some minimum resolution. However, the analyst is not always interested in the quantification of all the components, but rather in a limited subset of key components. The merits of performing such a "limited" optimization, rather than the separation of all solutes in a complex mixture ("full" optimization), have been evaluated by Herman *et al.*[1] using

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computer-simulated examples. It has been shown that the peak capacity (and therefore the analysis time) required to separate a solute mixture at a given probability level can be substantially reduced for moderately complex mixtures when the number of solutes of interest is less than half of the total number of solutes. Generally, the eluent optimization of limited sample subsets will (i) save analysis time (the peak capacity requirement for a successful separation is lower) or (ii) allow mixtures containing a larger number of components to be analyzed. In the same paper, a procedure was formulated for selecting appropriate starting eluent compositions for solvent optimization in reversed-phase (RP) HPLC on a statistical basis[1]. The approach allows also the analyst to define starting conditions for the optimization of a limited subset of key components (NI) in a sample containing more solutes (M $\geq$ NI).

The application of predictive optimization procedures such as the iterative regression method[2] to limited optimization procedures requires (besides the recognition of the solutes in the successive chromatograms) optimization criteria which reflect only the separation of the peaks of interest. In a recent paper[3], different resolution-based optimization criteria were adapted for limited optimization. To avoid the ambiguity of multi-purpose criteria, a sequential approach was formulated for three resolution-based criteria. As a primary goal, we aim to find satisfactory resolution for the peaks of interest. The secondary goal can be to achieve the shortest possible analysis time, while resolution is maintained at the required level.

In this paper the utilization of the rational selection of the starting eluent compositions on a statistical basis and the sequential use of optimization criteria are evaluated, respectively, by performing both the full and limited optimization of the same sample by using ternary solvent mixtures in RP-HPLC. Another possible approach is the use of the Multi-Criteria Decision Making (MCDM) procedure suggested by Smilde et al.[4] which also can perform resolution and time optimization simultaneously. We favour the sequential approach for several reasons. The MCDM plots do not provide information on the ruggedness of the resolution optimum. Also the sequential approach lends itself more readily to automation than the MCDM procedure because it shows directly how the chromatography can be adapted to gain analysis time. To guide the chromatographer through the selection of criteria and to help in the evaluation of the optimum found, an "expert" algorithm has been developed and integrated into the binary and ternary eluent optimization programs. The application of this integrated procedure in the optimization of the (satisfactory) resolution of the sample components and the minimum analysis time, by allowing column length variation, is also demonstrated.

EXPERIMENTAL

HPLC-grade organic solvents were obtained from Rathburn (Walkerburn, U.K.). Distilled, deionized water was prepared by means of a Milli-Q water purification system (Millipore, Molsheim, France). Solutes for the sample mixtures were from Fluka (Buchs, Switzerland) and E. Merck (Darmstadt, F.R.G.).

The stationary phase was 5-μm ODS-Hypersil (Shandon Southern Products, U.K.) slurry-packed into 4.6 mm I.D. HPLC columns of length 7.5, 12.5 or 20 cm. The chromatographic system consisted of a Model 1090 chromatograph, equipped with an auto-injector and a Model 1040A linear photodiode-array UV–VIS spectrometer (Hewlett-Packard, Waldbronn, F.R.G.). All measurements were made at 25°C.

The optimization programs for limited optimization were developed in PRO/ BASIC on a Waters 840 Data Management System, equipped with 512K memory, dual diskette drive (2 × 400K), integral 10M Winchester disk drive, extended bit map graphics with colour monitor and a Letterprinter LA-100 (all from Digital Equipment Corporation, Maynard, MA, U.S.A.).

RESULTS AND DISCUSSION

Determination of the optimization search area

Recently, an efficient procedure has been described by Herman *et al.*[5] for predicting solute retention in the three common binary solvent mixtures used for ternary eluent optimization in RP-HPLC. The method combines the results from a single water-to-methanol gradient scan and one or two additional isocratic measurements. The statistical approach[1] and the above procedure have been integrated into a common procedure, which allows selection of the most suitable eluent compositions on a statistical basis for either full or limited optimization at constant or variable eluotropic strength. The operation of this integrated method for the experimental selection of the initial eluent compositions for a full optimization example has been discussed by De Galan *et al.*[6] in detail. Here, the application of this start-up procedure will be demonstrated for both full and limited optimization of ternary eluent mixtures of "fixed"eluotropic strength (isoeluotropic) for the separation of a sample containing nine aromatic solutes.

First, a water-to-methanol gradient scan is carried out in 15 min (Fig. 1). The first solute peak is eluted at 1.21 min, the last one at 13.96 min (see Fig. 1 for other experimental details). The polarity range of this sample (defined according to ref. 1) is found to be 5 from the gradient experiment. Once the polarity range and the number of the solutes are known (the latter can also be estimated as described in ref. 6), the peak capacity needed to solve the separation problem at a preselected probability level can be determined from the probability curves described in ref. 1.

We target the separation of (i) all nine components and (ii) three solutes out of nine at the probability level 0.75. In ref. 1 an empirical relationship was derived to relate a NI-out-of-M limited optimization problem to the equivalent m'-out-of-m' full
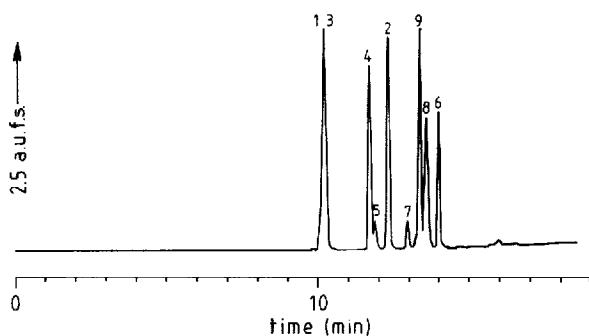

Fig. 1. Gradient elution chromatogram of an aromatic solute mixture. For solutes see Table III. Linear gradient from water to methanol in 15 min; void time, 1.41 min; flow-rate, 1 ml/min; column, 20 cm × 4.6 mm I.D. packed with 5-μm ODS-Hypersil; detection, 210 nm.

TABLE I

DETERMINATION OF THE INITIAL ISOCRATIC BINARY ELUENT COMPOSITIONS FOR THE FULL OPTIMIZATION OF A NINE-COMPONENT SAMPLE MIXTURE

The polarity range of the sample is 5, the preselected level of probability of separation success is 0.75 and the necessary peak capacity is 26.

Organic solvent		Predicted retention*		Measured retention*		Probability
Type	Concentration					
Methanol	57.1	1.71	10.16	1.66	6.00	0.341
	48.5	2.81	11.50	2.40	14.26	0.797
	49.9	2.26	12.39	2.08	11.2	0.738
Acetonitrile	40	2	11	1.41	6.53	0.552
	35.6	1.72	10.20	1.90	13.9	0.858
	37.5	1.67	9.99	1.67	10.03	0.754
THF	33.4	2	11	2.15	9.65	0.623
	30.8	2.46	13.26	2.47	13.9	0.775

* Capacity factors of first and last peaks.

optimization problem, in terms of the total number of components. The 3-out-of-9 separation for ternary optimization was calculated to be equivalent to a full optimization of seven components.

Next, the corresponding plots of probability vs. peak capacity (cf., Fig. 2 in ref. 1) are selected according to the polarity range index and the (equivalent) number of the components. The required peak capacities for 0.75 probability are found to be 26 (nine components) and 18 (seven components), respectively. According to the above approach, in order to maintain the same probability of separation success with ternary solvent mixtures, the initial binary eluents should have the same (predetermined) peak capacities. Once the probability level (peak capacity of the chromatogram) is closely identical in each of the three binary mixtures *and* the solute retention is within acceptable limits, the ternary eluent optimization procedure can be initiated.

The results on the determination of the starting eluent compositions are summarized in Tables I and II. In the first line of Table I (9-out-of-9 separation) the

TABLE II

DETERMINATION OF THE INITIAL ISOCRATIC BINARY ELUENT COMPOSITIONS FOR THE LIMITED OPTIMIZATION OF THREE SOLUTES OF INTEREST FROM A NINE-COMPONENT SAMPLE MIXTURE

The polarity range of the sample is 5, the preselected level of probability of separation success is 0.75 and the necessary peak capacity is 18.

Organic solvent		Predicted retention*		Measured retention*		Probability
Type	Concentration					
Methanol	69.1	0.85	3.76	1.14	2.57	0.101
	55.8	2.16	7.12	1.64	6.32	0.797
Acetonitrile	46.1	1.6	6.3	1.37	5.92	0.825
THF	38.2	1.6	6.3	1.85	7.44	0.832

* Capacity factors for first and last peaks.

gradient experiment predicts that the required peak capacity of 26 can be reached with an isocratic binary mobile phase containing 57.1% methanol, where the capacity factors of the first and the last peaks eluted are predicted to be 1.71 and 10.16, respectively. A column plate count of 8000 and a required resolution of 1.25 were used in these calculations. However, the measured data (k'_{first} = 1.66, k'_{last} = 6.0) deviate from the predicted, resulting in a much lower peak capacity and, hence, in a probability of successful separation of only 0.34 rather than the 0.75 originally required. Using the results of this isocratic and the gradient experiment, a new prediction is made at 48.5% methanol–water (for the details of this gradient isocratic procedure see ref. 5). This composition results in a high probability of 0.797, but much longer analysis time. It should be pointed out that the higher the probabilities required are (as a result of larger peak capacities), the longer the analysis time will become. Therefore, a compromise must be found between the probability of the separation success and a sensible length of the chromatographic analysis. It is not advisable to ask for a probability of >0.9 (for a maximum success) for any separation, since it can result in very long analysis times.

As a compromise between the probability of separation success and the time needed to record the chromatogram, a final prediction is made, based on both isocratically determined points, again for the probability of 0.75. Now, the result seems quite acceptable [k'_{last} = 11.2 (k' = capacity factor) and probability of 0.738] in the 49.9% methanol–water eluent.

The equivalent compositions of acetonitrile–water and tetrahydrofuran (THF)–water binaries (which are expected to elute the sample mixture within the same retention limits, *i.e.*, peak capacities, at k'_{first} = 2, k'_{last} = 11) are calculated from the empirical transfer rule equations, as described in ref. 5. Again, the original predictions for these binary eluent compositions are refined until the probabilities and the analysis time become acceptable. The results summarized in Table II for the 3-out-of-9 limited optimization problem were obtained in a similar manner, using the same gradient data as a starting point. Since the starting conditions for the isoeluotropic ternary eluent optimization offer reasonably high probabilities (see Tables I and II) of the separation success, no other more complex (*e.g.*, quaternary or variable-eluotropic-strength ternary) optimization parameter space is considered. Therefore, isoeluotropic ternary-eluent optimization is favoured for this sample, and this selection is highly justified by the optimization results discussed below.

Development of an "expert" algorithm

After defining the vector space of the optimization parameters, suitable optimization criteria must be selected which clearly reflect the goal of the analyst. In ref. 3 we suggested a sequential approach to the selection and use of the different optimization criteria, for both full and limited optimization. The goal of the optimization in this scheme is to find satisfactory resolution for the peaks of interest in a minimum analysis time.

First, the optimum resolution, $R_{s_{min}}$, must be found with confidence for the peaks of interest. Weighting factors with a value of 1 (important) or 0 (unimportant) are assigned to each component of the sample, and the resolution between two neighbouring peaks is taken as relevant when (w_i OR w_{i+1}) = 1. The analyst can also specify what resolution is required, $R_{s_{req}}$. When satisfactory minimum resolution is

found with confidence, *i.e.*, $R_{s_{min}} \geq R_{s_{req}}$, the optimization can be continued to find the minimum analysis time, using secondary criteria. These criteria can be adapted to the goals and (chromatographic hardware) abilities of the analyst to realize the shortest possible analysis time on the same column as that used for optimization or on another column of different length (preferably leaving the flow-rate and particle diameter unaltered). The threshold separation criterion, S_k, and the required analysis time criterion, $1/T_{nefd}$, suggested by Schoenmakers[7] can be used to optimize for these two goals in limited optimization as well[3].

However, for a given chromatographic system, the resolution at optimum conditions can still be lower than the required value. Most optimization procedures abandon the analyst in such a case without giving him any help on how to improve the current separation. Therefore, it seemed to be valuable to include a special algorithm in the solvent optimization program to combine two goals: (i) to guide the user through the selection of criteria and to help in the evaluation of the optimum found, (ii) to provide options and advice when the required resolution could not be reached in the actual chromatographic system. This "expert" algorithm is not a simple "on-line help" function, since it also reports the status of the optimization process and provides advice on how to continue the optimization procedure.

A simplified flow diagram of some major decision branches of the routine is shown in Fig. 2. The algorithm was integrated into the binary and ternary optimization procedures described elsewhere[3] for both full and limited optimization. When the maximum value of $R_{s_{min}}$ remains below the required level, $R_{s_{req}}$, the (righthand side of the) scheme is designed to accommodate a number of "backtracking" possibilities of revising (incorrect) decisions, made earlier during the optimization procedure. Meaningful suggestions are given in Fig. 2. Overall, a stopping point is provided, where the analyst may have to make even more trenchant decisions, *e.g.*, to change the phase system. Consulting (or interfacing the program) at this point with real expert systems may be a possibility for the future. However, the more elaborate evaluation of all the options and advice that can be given is outside the scope of this paper.

In the following discussion, the use of secondary criteria (left hand side of the scheme) will be evaluated in detail, with respect to the decisions based on the scheme in Fig. 2. All three criteria are calculated over the whole eluent composition range from the beginning of the optimization procedure. The "expert" algorithm keeps track of

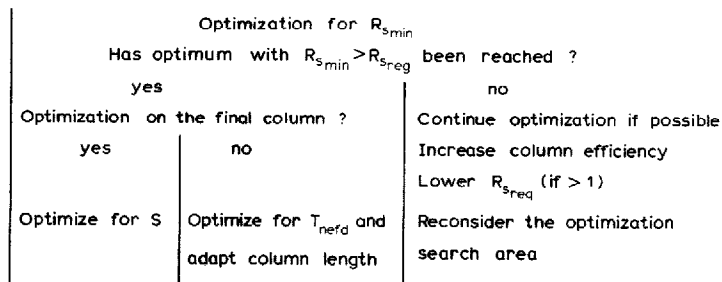| Optimization for $R_{s_{min}}$		
Has optimum with $R_{s_{min}} > R_{s_{req}}$ been reached ?		
yes		no
Optimization on the final column ?		Continue optimization if possible
yes	no	Increase column efficiency
		Lower $R_{s_{req}}$ (if > 1)
Optimize for S	Optimize for T_{nefd} and adapt column length	Reconsider the optimization search area

Fig. 2. Major decision branches used by the "expert" algorithm, guiding the analyst through the sequential use of the resolution-based optimization criteria.

the status of each criterion, and once the optimum is found, not only for the $R_{s_{min}}$ but also for (any of) the other two criteria, the option to realize any of these optima can become available without further experiments. This situation can occur when the predicted optimum composition for the analysis time criterion is within the confidence interval of a criterion determined earlier during the search. When a (satisfactory) optimum is found for both the minimum resolution, $R_{s_{min}}$, and the required analysis time criterion, $1/T_{nefd}$, the length of the column (or the flow-rate) may also be adapted in order to reduce the analysis time. The use of these options will be demonstrated on real experimental examples.

Comparison of the full and limited optimization

The last chromatograms determined in the start-up procedure provide the binary limits for the ternary eluent optimization procedure by means of the iterative regression method[2]. It is assumed that in the course of the optimization solute peaks are recognized in sequential chromatograms either on the basis of their UV spectra or by injecting known standards.

The nine-component aromatic solute mixture represents a typical example for ternary-eluent optimization. In each of the three binaries, different solute pairs are unresolved, while (small) variations of the solvent strength in the determination of the initial binaries did not reveal changes in selectivity. Larger variations of the solvent strength, *e.g.*, binary-eluent optimization may result in different band spacing; however, it would cause large changes in the analysis time as well.

Full optimization. The starting binary-eluent chromatograms of the nine-component aromatic mixture are shown in Fig. 3. All solutes are assigned to be of interest ($w_i = 1$, $i = 1 \ldots 9$) and to be separated from each other and the (imaginary) solvent peak at t_0 with a required resolution of $R_{s_{req}} = 1.25$. The logarithm of solute capacity factors is assumed to be a linear function of the ternary eluent composition (Fig. 4a). These plots are used for calculation of the minimum resolution criterion, $R_{s_{min}}$, in Fig. 4b. Clearly, separation selectivity is strongly influenced by the ternary-eluent composition (the elution order of the components varies) and a maximum value of $R_{s_{min}} = 1.64$ is predicted for a ternary mobile phase containing 24.4% methanol and 19.2% acetonitrile ($X = 1.51$); X is a parameter indicating binary and ternary compositions along the composition axis in the phase selection diagram[2]. After measuring two other chromatograms at "shifted" compositions[2] (see eluent compositions and retention data in Table III), a final optimum is predicted at $X = 1.70$ with a $R_{s_{min}} = 1.60$ (see phase-selection diagram in Fig. 5). The chromatogram verifying this optimum with an analysis time of 18.5 min is shown in Fig. 6a. If this analysis time is acceptable, the procedure stops at this point. However, according to the optimization scheme in Fig. 2, when $R_{s_{min}} \geqslant R_{s_{req}}$ at the optimum conditions, the analysis time can be minimized by using secondary criteria.

The response surfaces of the three criteria (relative plots) are shown in Fig. 5b. We point out that the optimization process can take different directions (eluent compositions), depending on the choice of the secondary (analysis time) criterion. The maximum of the threshold separation criterion, S_k, is in another (the acetonitrile–THF–water) ternary "window" at $X = 2.21$. However, before we continue the optimization, it is worthwhile to examine the possible gain in analysis time (on the given column) at this composition. From the retention plots, we estimate that the
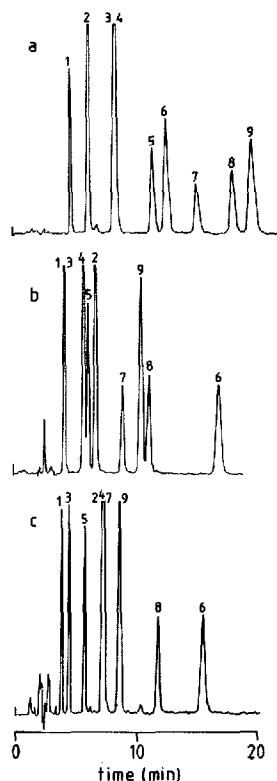
Fig. 3. Isocratic chromatograms of the nine-component mixture of aromatic solutes with the initial binary eluents for full optimization. Eluents: (a) 30.8%; (b) 49.9% methanol; (c) 37.5% acetonitrile. Detection: 260 nm. For other conditions see Fig. 1.
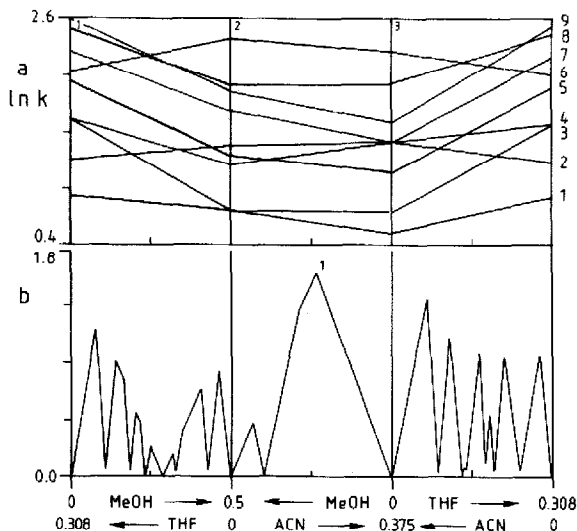
Fig. 4. Phase-selection diagram, constructed from the chromatograms shown in Fig. 3, for the full optimization. (a) Plots of ln k'; (b) response surface for the minimum resolution, $R_{s_{min}}$, criterion. MeOH = Methanol; ACN = acetonitrile; THF = tetrahydrofuran.

predicted optimum composition offers only minor reduction (less than 10%) of the analysis time compared to that already found for the $R_{s_{min}}$ criterion with confidence, since the retention of the last-eluted peak(s) is closely constant in the three isoeluotropic ternary eluent systems. Therefore the optimization is not continued in this direction. On the other hand, the optimum found for the required analysis time criterion, $1/T_{nefd}$, is identical with that for the $R_{s_{min}}$. Since $R_{s_{min}} = 1.6$ is obtained with 6000 plates (in the given ternary system) on a 20-cm column, a value of $R_{s_{req}} = 1.25$ can be realized with 3660 plates on a 12-cm column. The chromatogram obtained with a 12.5-cm column with the same flow-rate and particle diameter is shown in Fig. 6b. The observed $R_{s_{min}} = 1.3$ and analysis time of 12.2 min are in good agreement with the predictions.

When the analysis is to be performed on a routine basis, the saving in time and eluent consumption can be significant, and it pays to adapt the column length. However, if the analysis is to be performed only a few times and/or no shorter column

TABLE III

SOLUTE RETENTION DATA FROM SEQUENTIALLY MEASURED CHROMATOGRAMS, DURING THE FULL OPTIMIZATION PROCEDURE, USING TERNARY SOLVENT MIXTURES IN RP-HPLC

The value of w_i is 1 for the peaks of interest and 0 for the unimportant peaks.

| Solute	w_i	Retention times (min)					
		1*	2	3	4	5	6
1 Benzyl alcohol	1	4.77	4.33	3.77	4.17	3.95	4.02
2 Dimethyl phthalate	1	6.24	6.95	7.23	7.75	7.53	7.81
3 Phenol	1	8.53	4.33	4.35	4.49	4.30	4.49
4 Benzonitrile	1	8.53	5.95	7.23	6.63	6.85	7.04
5 p-Cresol	1	11.65	6.33	5.70	6.35	6.00	6.25
6 Diethyl pthalate	1	12.78	17.15	15.55	18.92	17.51	18.48
7 3,4-Dimethylphenol	1	15.30	9.23	7.23	8.87	8.11	8.54
8 Benzene	1	18.25	11.43	11.80	11.42	11.52	11.91
9 2,4-Dimethylphenol	1	19.83	10.80	8.58	10.41	9.50	10.07
Parameter X		0	1	2	1.51	1.736	1.70
Percentage of methanol		0	49.9	0	24.4	13.2	15
Percentage of acetonitrile		0	0	37.5	19.2	27.6	26.3
Percentage of THF		30.8	0	0	0	0	0
Percentage of water		69.2	50.1	62.5	56.4	59.2	58.7

* Number of chromatogram as indicated in Fig. 5.
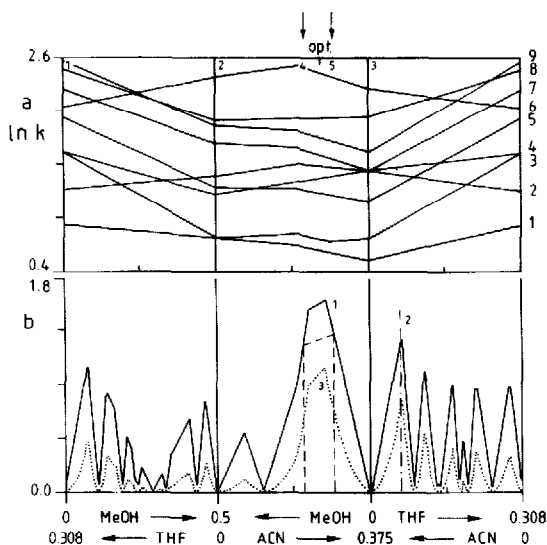
Fig. 5. Phase-selection diagram, showing the final result of the full optimization example. (a) Plots of ln k'; (b) optimization criteria $R_{s_{min}}$ (1), S_k (2) and T_{nefd} (3), normalized to give maximum values between 0.75 and 1.25.
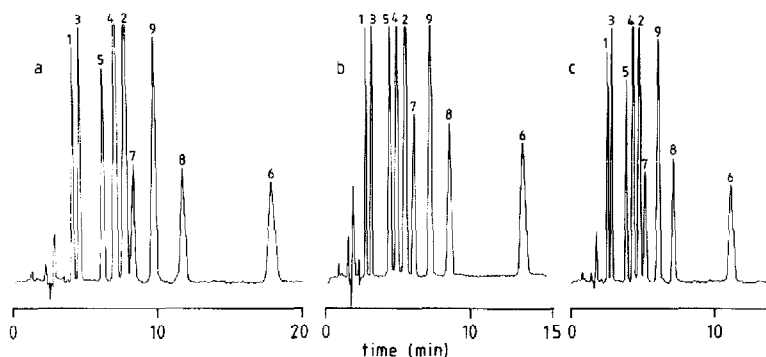
Fig. 6. Separation of all nine aromatic solutes at optimum conditions (15% methanol, 26.3% acetonitrile and 58.7% water) on (a) 20-cm column, flow-rate 1.0 ml/min, (B) a 12.5-cm column, flow-rate 1.0 ml/min and (c) a 20-cm column, flow-rate 1.66 ml/min; column pressure, 200 bar.

is at hand, as an alternative the flow-rate can be increased in order to shorten the analysis time at the expense of higher column pressure and eluent consumption. The limit of flow-rate increase is determined by the decrease in column efficiency (in our case it should not drop below 3660) and/or the increase in the column pressure drop. We decided to allow a pressure of 200 bar as the practical upper limit; the flow could be increased to 1.66 ml/min on the 20-cm column. As a result, the overall analysis time needed for the complete separation of our nine-component sample mixture decreased to 10.8 min (Fig. 6c).

   Limited optimization. Three phenolic compounds, phenol (3), 3,4-dimethyl-phenol (7) and 2,4-dimethylphenol (9), were designated as being of interest for limited optimization of the same nine-component sample mixture. The three initial chromatograms with the starting binary eluents are shown in Fig. 7. In these chromatograms the position of the important solutes is indicated by the symbol (*). Note the much
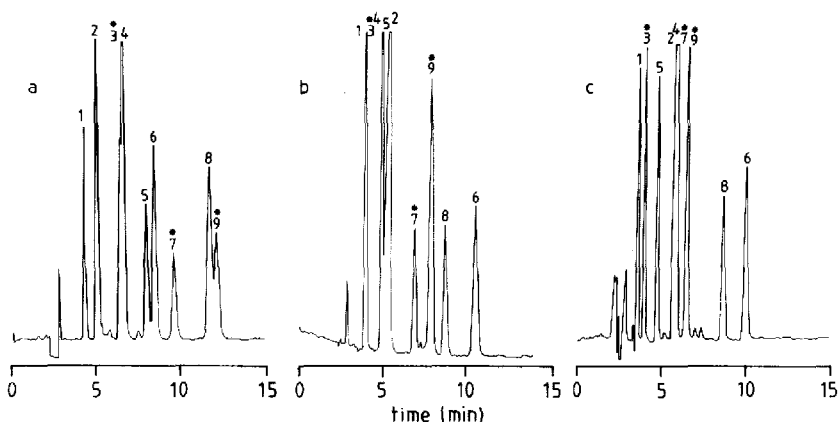

Fig. 7. Isocratic chromatograms of the nine-component mixture of aromatic solutes in the initial binary eluents for limited optimization. The three phenols of interest are marked with an asterisk. Eluents: (a) 38.2% THF; (b) 55.8% methanol; (c) 46.1% acetonitrile. Detection: 260 nm. For other conditions see Fig. 1.
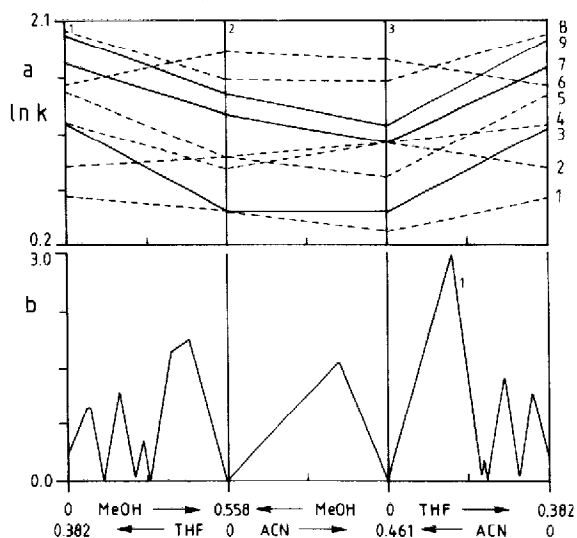
Fig. 8. Phase-selection diagram, constructed from the chromatograms shown in Fig. 7, for the limited optimization. (a) Plots of ln k'; (b) response surface for the minimum resolution, $R_{s_{min}}$, criterion.

smaller separation space, set by the statistical approach compared to the full optimization problem (*cf.*, Fig. 3).

The phase-selection diagram at the beginning of the optimization is shown in Fig. 8. The response surface of the minimum resolution criterion is also different from that of the full optimization and exhibits a maximum of 2.4 in another ternary system (acetonitrile–THF–water) at $X = 2.39$. In fact, after two additional measurements in this ternary system, the maximum value of $R_{s_{min}}$ for the three peaks of interest (3.0) is located at $X = 2.30$ (11.5% THF and 32.3% acetonitrile), as shown by the final phase-selection diagram in Fig. 9. The chromatogram (No. 6) verifying this optimum is shown in Fig. 10a, with an analysis time of 8.7 min. All retention data are given in Table IV. Comparison of the results of the full and limited optimizations on the same (20-cm) analytical column (see Figs. 6a and 10a, respectively) reveals that the separation of only three components out of nine can be accomplished in about half the analysis time, even before special time optimization or column length variation are invoked. This is achieved simply by (i) defining realistic starting conditions for the less complex (limited) optimization problem, and (ii) allowing other components, which are of no interest, *e.g.*, dimethyl phthalate, benzonitrile and *p*-cresol to remain unresolved.

Optima for the other two criteria have also been found (Fig. 9). Now the optimum composition ($X = 2.24$) predicted for the threshold separation criterion, S_k, is also within the confidence range of points measured earlier. In a way similar to the full optimization, the optimum for S_k offers only a minor decrease in the analysis time, since the overall solute retention is closely constant in this ternary system as well. However, this does not mean that the use of S_k is completely irrelevant in ternary-eluent optimization. When the eluents are less "isoeluotropic", the use of the S_k criterion can lead to significant savings in analysis time on the given column[3]. For instance, when similar peak capacities (probabilities) are set in the initial binary eluents
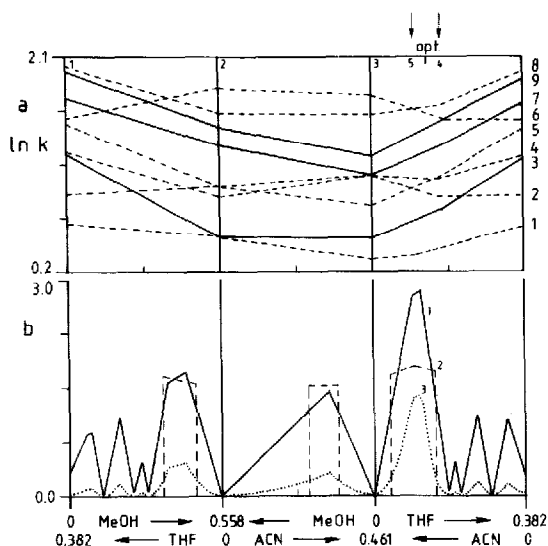
Fig. 9. Phase-selection diagram, showing the final result of the limited optimization example. (a) Plots of ln k'; (b) optimization criteria, $R_{s_{min}}$ (1), S_k (2) and T_{nefd} (3), normalized to give maximum values between 0.75 and 1.25.

or when the eluotropic strength is varied intentionally, different first and last peak retentions may result. On the other hand, the optimum found for the required analysis time criterion, $1/T_{nefd}$, is identical with that of the $R_{s_{min}}$ and allows a dramatic decrease

TABLE IV

SOLUTE RETENTION DATA FROM SEQUENTIAL CHROMATOGRAMS, IN THE LIMITED OPTIMIZATION PROCEDURE, WITH TERNARY SOLVENT MIXTURES IN RP-HPLC

The value of w_i is 1 for the peaks of interest and 0 for the unimportant peaks.

| Solute	w_i	Retention times (min)					
		1*	2	3	4	5	6
1 Benzyl alcohol	0	4.02	3.72	3.34	3.51	3.40	3.45
2 Dimethyl phthalate	0	4.79	5.09	5.49	4.75	5.03	4.94
3 Phenol	1	6.20	3.72	3.72	4.39	4.05	4.18
4 Benzonitrile	0	6.29	4.71	5.49	5.33	5.36	5.33
5 p-Cresol	0	7.71	5.05	4.51	5.40	5.00	5.33
6 Diethyl phthalate	0	8.19	10.32	9.76	8.16	8.87	8.73
7 3,4-Dimethylphenol	1	9.37	6.63	5.49	6.67	6.13	6.33
8 Benzene	0	11.91	8.45	8.42	9.07	8.80	8.73
9 2,4-Dimethylphenol	1	11.48	7.59	6.21	7.87	7.09	7.39
Parameter X		0	1	2	2.43	2.22	2.30
Percentage of methanol		0	55.8	0	0	0	0
Percentage of acetonitrile		0	0	46.1	26.1	35.7	32.3
Percentage of THF		38.2	0	0	16.6	8.6	11.5
Percentage of water		61.8	44.2	53.9	57.3	55.7	56.2

* Numberof chromatogram as indicated in Fig. 9.
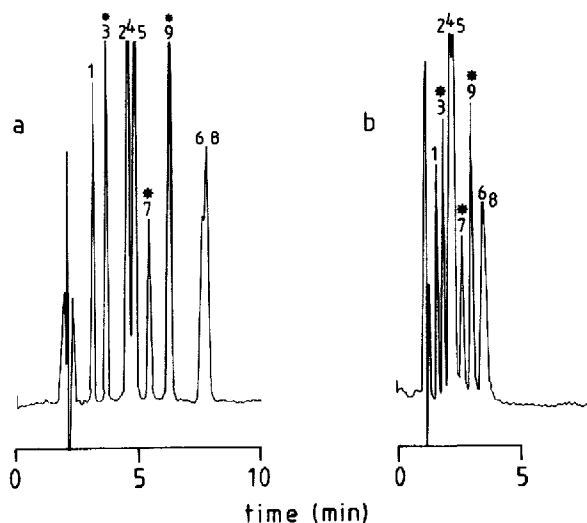
Fig. 10. Separation of the three phenolic compounds of interest (*) in the nine-component sample mixture at optimum conditions (11.5% THF, 32.3% acetonitrile and 56.2% water) on (a) a 20-cm column, flow-rate 1.0 ml/min, (b) a 7.5-cm column, flow-rate 1.0 ml/min.

in column length (plate count is 6500 in this ternary system). In principle, only 1105 theoretical plates (a 4-cm instead of 20-cm column) are needed to maintain a ($R_{s_{req}} = 1.25$) satisfactory resolution of the three peaks of interest within 2 min! Again, one must consider (i) the need for a shorter analysis time and (ii) the necessity of purchasing or packing a shorter column. Obviously, the reduction in column length should be considered carefully, since it is always better to "overscore" by using a column with a practical length than to use a very short (< 5-cm) column. In this study, this optimum was realized with a 7.5-cm (65% shorter) column; the chromatogram is shown in Fig. 10b. The analysis time was 4.0 min, and the three peaks of interest were baseline-separated. The results demonstrate the dramatic gain in analysis time between full and limited optimizations when the number of components of interest is much lower than the number of all solutes.

CONCLUSIONS

The combination of the statistical approach and the extended gradient isocratic scouting procedure results in rapid selection of realistic starting eluent conditions prior to systematic eluent optimization. The main advantage of the statistical approach is best demonstrated by comparing the starting conditions for the full and limited optimization of the same sample mixture. For limited optimization, the chromatograms with the starting binaries are proportionally shorter (in our case by *ca.* 50%) when assigning more realistic starting conditions and allowing a gain in analysis time due to the less complex (3-out-of-9) optimization problem.

The sequential selection and use of resolution-based criteria, which were adapted for limited optimization by assigning weighting factors to each solute in the sample

mixture, allows the efficient optimization of the separation of the peaks of interest when the iterative regression method is applied. In order to help the analyst to achieve the goal of satisfactory/required resolution in the shortest possible analysis time, an ("expert") algorithm has been developed and integrated into the eluent optimization program. The use of this algorithm is demonstrated in the optimization of the analysis time (while resolution is maintained at the required level) by applying suitable criteria and allowing column length variation. By comparing the full and limited optimization of the sample mixture, we have demonstrated that the degree of separation space required to resolve a limited subset of components (and hence the analysis time as well) can be reduced when unimportant solutes are allowed to remain unresolved and the number of the components of interest is much lower than that of all components of the sample.

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