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PRACTICAL USE OF AN OPTIMIZATION STRATEGY IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE SEPARATION OF A LIMITED SUBSET OF COMPONENTS IN A REACTION MIXTURE

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SUMMARY

A step-by-step development of a high-performance liquid chromatographic method for the optimized separation of a largely unknown sample is presented. A limited subset of two main components is separated from a ten-component reaction mixture of pyrroloquinoline quinone and cyclopropanol, using tetrahydrofuran–water binary eluents buffered to pH 2.5 with triethylamine phosphate on a reversed-phase column packed with 5-μm ODS-Hypersil. After the definition of the separation problem, the mobile phase parameters are selected rationally on the basis of a systematic gradient scouting procedure. The optimization search area is defined in accordance to the complexity of the sample mixture (two out of ten components are of interest) by utilizing a statistical approach, while the selectivity optimization is carried out using binary eluents of variable eluotropic strength.

INTRODUCTION

The separation of a few key components in sample mixtures containing a larger number of solutes has not received much attention. Although many samples are believed to fall into this category, most schemes described in the literature on experimental optimization address the separation of all components.

In a previous paper[1], we evaluated the merits of separating a limited subset of components by systematic optimization of the ternary mobile phase composition in reversed-phase high-performance liquid chromatography (HPLC). It was shown[1] that the analysis time can be reduced substantially by assigning more rigorous starting conditions for the optimization, when only few solutes are of analytical interest.

In this paper the other advantage of such "limited optimizations" is evaluated, *i.e.*, addressing the separation of only a limited subset of components (NI) will generally allow the analysis of sample mixtures containing large numbers of solutes, *M*.

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Schoenmakers and Mulholland[2] have pointed out the importance of the definition of the problem and the goal of the analysis as the first step of the chromatographic method development. We will also examine how these requirements can be translated into a meaningful design of the chromatographic experiments for the special case of limited optimization.

A reaction mixture of pyrroloquinoline quinone (PQQ) and cyclopropanol was selected as a typical example for limited optimization. The extent of conversion and mechanism of this reaction are of interest for studies in the inhibition of quinoprotein, *i.e.*, PQQ-containing methanol dehydrogenase (MDH)[3]. In solution the reaction of PQQ with cyclopropanol can occur only in the presence of a suitable catalyst. For studies of the mechanism of this reaction, the effect of different catalysts on the conversion of PQQ into the main product PQQ-M[3] (see Fig. 1) was also investigated. In order to monitor the extent of conversion, PQQ and PQQ-M must be separated from each other and from all the other (co)products in the sample mixture.

This reaction mixture represents an interesting example from another point of view. One of the most important steps in HPLC method development is the system selection, *e.g.*, reversed *versus* normal phase and the definition of the parameter space, *i.e.*, the combination and limits of chromatographic parameters which influence the separation selectivity of the relevant sample components. After the selection of the chromatographic system, initial scouting experiments should be carried out to limit the search for the optimum experimental conditions to a reduced parameter space (constrained from the relevant parameters). This parameter space was shown[4] to depend on the nature and complexity of the sample mixture.

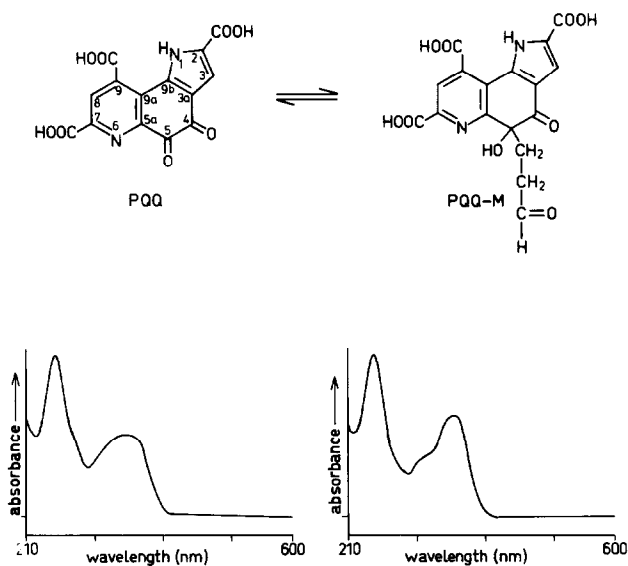In the case of our reaction mixture, little information was available on the

Fig. 1. The chemical structure and UV absorption spectra (taken in 50% methanol–triethylamine phosphate buffer at pH 2.5) of pyrroloquinoline quinone (PQQ) and its derivative pyrroloquinoline quinone (C₅)-3-propanal adduct (PQQ-M), formed in the reaction with cyclopropanol.

number and nature of the components in the sample. Pure standards were available only for the two compounds of interest, their UV absorption spectra are also shown in Fig. 1. The reaction products may also be ionizable solutes with (three or less) carboxyl groups like PQQ and PQQ-M and therefore different separation modes, *e.g.*, ion suppression and ion pairing might be applicable.

Recently, a systematic gradient scouting procedure has been developed by Low *et al.*[5] for reversed-phase HPLC, to track the nature of the solutes in such (relatively) unknown sample mixtures. The results of these scouting experiments can be used for the rational selection of the optimization parameters[4]. The application of this procedure is clearly required in the case of the present sample.

Once the mobile phase parameters have been selected, the statistical approach developed by Herman *et al.*[6] can be consulted to define retention limits between which the sample should be eluted in order to establish reasonably high probabilities (peak capacities) of separation success in the reversed-phase mode. Eluent compositions which provide the required retention limits will finally determine the optimization parameter space, where the systematic eluent optimization is carried out.

In this work, the step-by-step method development of an optimized separation of a relatively unknown reaction mixture is discussed in detail. We intend to demonstrate; (i) the importance of the correct definition of the separation problem and the goal of the analysis; (ii) the rational selection of the separation mode and the optimization parameters on the basis of systematic scouting experiments; (iii) the utilization of the statistical approach to determine starting eluent conditions (optimization search area) which are adapted to the complexity of the sample and (iv) the practical use of the limited optimization procedure using binary eluents of variable eluotropic strength, to locate the optimum separation of two (main) components of interest in a complex sample mixture.

EXPERIMENTAL

Chemicals and instrumentation

HPLC grade organic solvents were obtained from Rathburn (Walkerburn, U.K.). Distilled, deionized water was prepared in-house using a Milli-Q water purification system (Millipore, Molsheim, France). Buffer solutions contained 15 mM triethylamine (Gold Mark quality; Aldrich Chemie, Steinheim, F.R.G.) adjusted to pH 2.5 and 7.0 by the addition of phosporic acid (H_3PO_4). Tetrabutylammonium bromide (TBA) and sodium octanesulphonate ($OctSO_3$) were obtained from Janssen Chim. (Beerse, Belgium) and used as 1 M concentrated solutions in the pulse injection experiments. Pyrroloquinoline quinone (PQQ) was obtained from Sigma (St. Louis, MO, U.S.A.) and used as received.

ODS-Hypersil, 5 μm, (Shandon Southern Products, U.K.), was used as the stationary phase and slurry-packed into a 20 cm $\times$ 4.6 mm I.D. HPLC Valco column (Chrompack, Middelburg, The Netherlands).

The chromatographic system consisted of a Model 1090 chromatograph autoinjector and a Model 1040A linear photodiode array UV–VIS spectrometer (Hewlett-Packard, Waldbronn, F.R.G.).

The gradient scouting method used in this study was as described in ref. 5. All measurements were made at room temperature (25°C).

The computer program for limited optimization using binary eluent mixtures has been developed in PRO/BASIC and run on a Waters 840 data management system, equipped with 512 Kbyte of memory, a dual diskette drive (2×400 Kbyte), integral 10-Mbyte 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.).

Sample preparation

The reaction mixtures of PQQ and cyclopropanol were prepared as described[3], using ZnO as a catalyst. Prior to HPLC analysis the samples were diluted in 0.02 M HNO_3 and adsorbed on a Sep-Pak C_{18} cartridge (Millipore). After washing with 10 ml 0.002 M HNO_3, the components were eluted with 1 ml methanol and the solutions were stored in a refrigerator.

RESULTS AND DISCUSSION

Defining the problem

In the definition of this limited optimization problem we will follow some of the guidelines given by Schoenmakers and Mulholland[2]. All information collected about the sample is of importance later during the course of the optimization and method development.

The separation of the reaction mixture of PQQ and cyclopropanol had been performed earlier by a linear gradient of 28.5–53% (v/v) methanol and 0.4% H_3PO_4 on Novapak-C_{18} RCM cartridges (Millipore-Waters Assoc., Milford, MA, U.S.A.), as described in ref. 3. We were looking for an isocratic method to perform the quantitative separation of the two main components of this reaction mixture. The isocratic method was to be performed occasionally, not on a routine basis. It was intended to favour reduction of analysis time by changing from gradient conditions to isocratic, setting the highest acceptable limit to 30 min.

The samples were available in methanol after the sample preparation step (see Experimental for details). Standards were available only for the two components of interest (PQQ and PQQ-M), the total number of components in these samples being expected to vary between 9 and 16 (depending also on the reaction conditions applied and/or catalysts) according to the earlier gradient experiments.

The concentration range for the main components was expected to be 1–20 mg/ml, and UV absorption detection at 320 nm was applied to record the chromatograms.

Based on the chromatographic information above (reversed-phase gradient at acidic pH) the use of reversed-phase HPLC along with pH adjustment is a possible solution. Alternatively, ion-pairing reagents might also be used at neutral pH, *e.g.*, 7.0 for retention and selectivity control, where the weak carboxylic acid groups of PQQ will be dissociated. An additional problem is that the nature and number of the other (co)products in the reaction mixture are not known; such knowledge can help to reduce the possible range and number of mobile phase parameters. Therefore, the application of a systematic scouting procedure is in order here for the selection of the proper reversed-phase mode and set of mobile phase parameters.

Selection of the optimization parameters by systematic gradient scanning

One of the first steps in the development of an HPLC method is to decide which phase system would be used. The selection of the chromatographic method will depend heavily on the nature and complexity of the sample. When reversed phase seems appropriate, first usually a water to methanol gradient scan is performed in order to determine whether there is sufficient retention of the solutes. However, when the solutes are ionizable, variation of the eluent pH and/or the addition of an ion pairing agent might cause significant shifts in the retention of the solutes.

Recently a systematic gradient scanning strategy has been developed in our laboratory[5], to search for the relative hydrophobicity and charge type of the solutes in unknown sample mixtures. It consists of four linear gradients from 0 to 90% methanol: two at pH 2.5, one of which is performed with a pulse injection of an anionic ion pairing agent (sodium octanesulphonate), and the other two at pH 7.5, one of which is also performed with a pulse injection of a cationic ion pairing agent (tetrabutylammonium bromide) (see Fig. 2).

From the unique retention movement pattern of the (groups of) compounds, information can be deduced as to the nature of the solutes in the sample mixture. The nature and complexity of the sample has primary importance in the selection of the separation mode (ion suppression, ion pairing, etc.) and the optimization vector space. When the two gradients at pH 2.5 without (Fig. 2a) and with the pulse injection of octanesulphonate (Fig. 2b) are compared, no significant shifts in retention are seen, indicating that all solutes are in a uncharged form at this eluent pH. When the gradient elution is performed at pH 7.5, the retention times of all peaks are found to be shorter
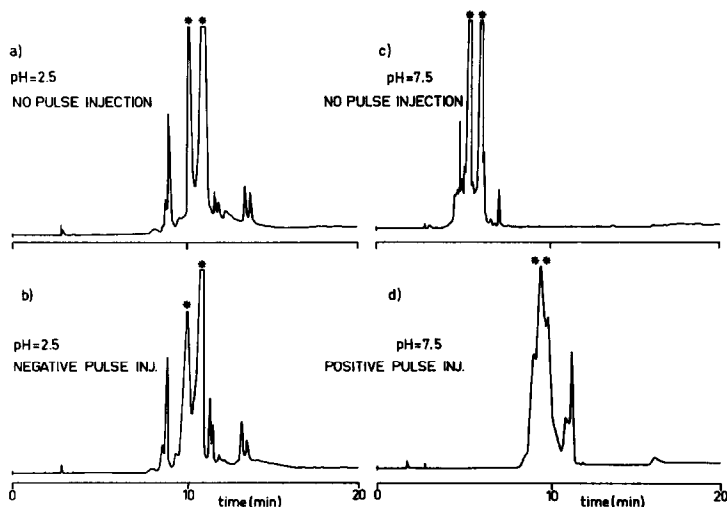

Fig. 2. Gradient elution chromatograms of the PQQ reaction mixture. Linear gradient of 0–90% methanol–triethylamine phosphate buffer in 15 min, at (a) pH 2.5 without pulse injection, (b) pH 2.5 with pulse injection of negatively charged octanesulphonate (as sodium salt), (c) pH 7.5 without pulse injection, (d) pH 7.5 with pulse injection of positively charged tetrabutylammonium (as bromide). Column: 20 cm × 4.6 mm I.D., packed with 5-μm ODS-Hypersil. Flow-rate: 1.0 ml/min ($t_0 = 2.05$ min). UV detection at 320 nm. Asterisks indicate positions of PQQ and PQQ-M.

(Fig. 2c) by about 50%, which is the result of the lower hydrophobic retention of the ionized (dissociated) weakly acidic groups.

At this point we know that all the solutes are weak acids. However, we do not know whether all contain the same number of charged groups, as one or two negative charges may cause an equally early elution in the gradient at pH 7.5. If the number of charged (carboxyl) groups is different for given solutes, the separation selectivity may be increased by the addition of a positively charged ion pairing agent. The fourth gradient, however, shows the entire collection of peaks moving in similar fashion (see Fig. 2d), indicating that all (co)products of the reaction have equal negative charge(s).

On the basis of the results of the scouting experiments we can select the eluent parameters (reversed-phase mode) to be optimized from the following three: the organic modifier concentration, the eluent pH and the concentration of a positively charged pairing ion.

From Fig. 2c we conclude that at pH 7.5 the isocratic retention of the sample is estimated to fall below $k' = 4$ even at 0% methanol, resulting in a very early elution of the (at least ten-component) sample mixture. Also, the UV spectrum of the weak acids may vary substantially with the eluent pH, which may cause problems in recognizing the (relevant) chromatographic peaks in the subsequently measured chromatograms. Thirdly, the larger the optimization vector space (range and number of parameters), the more experimental effort is needed to locate the optimum.

As a "procedural rule", we suggested that the most direct and simple approach should be taken in such cases[4]. Therefore, when it is possible to avoid the simultaneous variation of two or several parameters, the less complex mobile phase system is selected first. As a consequence, for our sample we select the reversed-phase ion-suppression mode at pH 2.5, where the hydrophobic retention of the sample allows the addition of organic modifiers. The optimization in the reversed-phase mode can advantageously be carried out using different organic modifier–water buffer combinations, while less variation is expected in the UV spectrum and in the solute retention.

Definition of the optimization search area

Once the separation parameters have been selected, the optimization search area, *i.e.*, the initial value of the parameters, must be defined. In reversed-phase chromatography (performed in this case at a constant pH of 2.5, *i.e.*, in ion-suppression mode for weak acids) binary, ternary or quaternary eluent mixtures (the last two with fixed or variable eluotropic strength) can be used for selectivity optimization.

Interest has only recently been focused on the selection of the optimization search area, and different approaches have been formulated by representative research groups[6–11]. Unfortunately, no simple rules are readily available for the analyst to decide on the scheme to be preferred.

The statistical approach suggested by Herman *et al.*[6] gives some guidelines on the choice of the optimization parameters in the reversed-phase chromatographic mode. For moderately complex mixtures where the (equivalent) number of components to be separated is less than seven, both binary and ternary solvent mixtures can be used to optimize the separation, with a reasonably high ($> 50\%$) probability of success. The lower limit of the maximum number of components in our sample mixture is estimated to be 10 on the basis of the gradient experiments shown in Fig. 2, while the

polarity range index is found to be 5 (see ref. 6 for details). The 2-out-of-10 limited optimization problem is calculated to be equivalent to a 6-out-of-6 full optimization problem, using the empirical relationships described in ref. 6.

Optionally, either isoeluotropic ternary or variable eluotropic strength binary (or ternary) eluent optimization can be applied. Optimized ternary eluents generally offer a better use of the separation space and shorter analysis time compared to binary systems[6], and this possibility is examined first.

Before starting the isoeluotropic ternary eluent optimization, chromatograms of the sample must be recorded in initial binary eluents which provide closely identical solute retention limits (peak capacities). Usually the results of the linear water–methanol gradient experiment and empirical transfer rules can be applied for the rapid determination of such mobile phase compositions[12]. Samples which deviate from the "average" behaviour may need further retention adjustment.

Our reaction mixture contained several homologues of PQQ-M, and predictions based on the average solute behaviour had to be corrected. Efficient experimental procedures described by Herman et al.[7], as well as by Sekulic and Haddad[8], can be applied to predict eluent compositions on the basis of isocratically measured retention data. Examples of the operation of such a correction procedure can be found in refs. 1, 7 and 8. In the present example, two or three isocratic measurements were needed to locate the methanol–, acetonitrile–, tetrahydrofuran (THF)–water buffer binaries where the retention of the PQQ reaction mixture was similar (see Fig. 3).

Before starting the optimization procedure it is always advisable to check the possible gain in selectivity by mixing these eluents. Comparison of the initial chromatograms reveals that the two main components (PQQ and PQQ-M) are eluted early and in the same order in these experiments. Retentions are especially short in the acetonitrile binary. The retention times and retention order of the two components of interest are closely identical for the binaries containing methanol and THF. Clearly, not much selectivity improvement is expected, when these binaries are mixed to obtain ternary eluents (likely behaviour is to be expected in a ternary methanol–THF–water mobile phase).
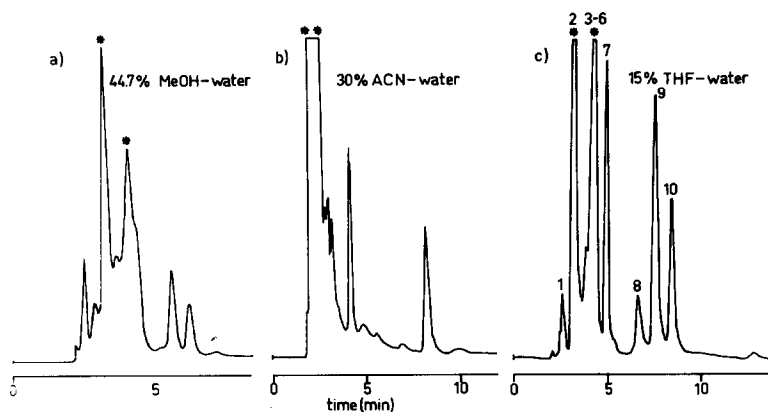

Fig. 3. Isocratic chromatograms of the reaction mixture of PQQ in binary eluents of organic modifier–water buffer (pH 2.5): (a) 0.447 methanol (MeOH); (b) 0.3 acetonitrile (ACN); (c) 0.15 THF. Asterisks indicate positions of PQQ and PQQ-M.
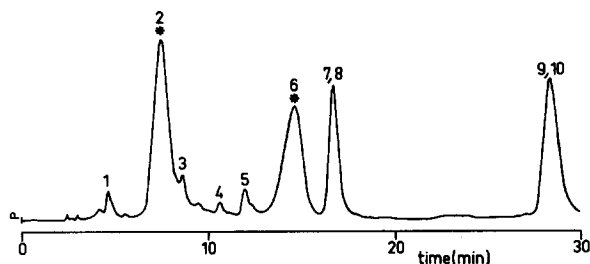
Fig. 4. Chromatogram of the PQQ reaction mixture in triethylamine phosphate buffer (pH 2.5) containing 7.5% THF.

Before (re)considering a more complex optimization vector space, *e.g.*, quaternary mixtures or pH variation it is worthwhile to examine the behaviour of the sample in the THF–water binary eluent system because the chromatogram shows more profiles then in methanol–water (see Fig. 3c). This system shows the best distribution of all peaks and the two main components of interest are separated from each other and from interfering coproducts, which is not the case in the other two systems. The variation of the concentration of the organic modifier in this binary eluent will significantly change the retention, but may alter the selectivity, too. The concentration of THF (0.15) in this eluent can be considered as an upper limit, as its further increase will result in even shorter retention. The lower limit of its concentration is determined by the maximum retention allowed for this sample, *i.e.*, the predetermined highest acceptable analysis time of 30 min.

Two additional isocratic experiments were needed to identify an eluent composition of 7.5% THF for eluting the last component at 28.2 min, $k' = 12.5$ (see Fig. 4). In the resulting chromatogram a better spread of all peaks is obtained, but the separation of PQQ from one of the reaction product is still not satisfactory. The retention limits for the first and last peaks eluted at 7.5% and 15% THF ($X = 0$ at 7.5% THF and $X = 1$ at 15% THF; X is a parameter running from 0 to 1, indicating the composition of the variable constituent of the mobile phase) define peak capacities of 9.5 and 14.6 for these chromatograms. A plate count of 3500 and a minimum required resolution of 2 (set to an high value to account for the unequal height of the neighbouring peaks) were used in these calculations. The corresponding probabilities of separation success are found to be 0.06 and 0.56. Therefore, the probability of optimizing the separation by mixing these two eluents containing 7.5 and 15% THF, respectively, is estimated to be about 0.50 (50%).

These starting conditions seemed to be acceptable to start the eluent optimization in the THF–water binary eluent system.

Limited optimization using binary eluent mixtures
The application of solvent optimization procedures to specific problem such as the separation of a limited subset of components requires the recognition of the two solutes of interest in subsequently measured chromatograms, special optimization criteria —which reflect only the separation of the peaks of interest— and a check on peak purity during the method validation procedure.

The "predictive" optimization procedures such as the iterative regression

TABLE I

SOLUTE RETENTION DATA FROM THE SEQUENTIALLY MEASURED CHROMATOGRAMS OF THE TEN-COMPONENT REACTION MIXTURE DURING THE LIMITED OPTIMIZATION PROCEDURE

Tetrahydrofuran–triethylamine phosphate buffer (pH 2.5) as binary eluent mixtures; 5-μm ODS-Hypersil as the stationary phase (column plate count 3500). The value of the weighting factor, w_i, is 1 for the peaks of interest and 0 for the unimportant peaks.

Solute	W_i	Retention times (min) in chromatograms				
		1	2	3	4	5
1	0	4.5	2.83	3.40	3.74	3.98
2 PQQ	1	7.27	3.45	4.69	5.38	5.92
3	0	8.42	4.21	5.78	6.57	7.15
4	0	10.42	4.30	6.64	7.77	8.65
5	0	11.75	4.40	7.00	8.71	9.74
6 PQQ-M	1	14.47	4.61	7.65	9.55	11.04
7	0	16.53	5.35	9.04	11.28	13.01
8	0	16.53	7.05	10.29	11.90	13.01
9	0	28.20	7.97	14.51	18.48	21.70
10	0	28.20	8.88	15.52	19.20	21.70
Parameter X		0	1	0.386	0.20	0.933
Fraction of THF		0.075	0.15	0.104	0.09	0.082
Fraction of buffer		0.925	0.85	0.896	0.91	0.918
Number of figure		4	3c	6a	6b	7

method[12,13] require the recognition of all peaks. Peak tracking in our case was mainly done on the basis of the UV spectra of the components. First the spectra of the PQQ, PQQ-M standards and the apparently pure peaks in the initial chromatograms (see Figs. 3c and 4) were collected. Coelution of peaks 9 and 10 in chromatogram 1 (see Table I) was found according to the peak areas (from chromatogram 2). Peaks 7 and 8 have very different spectra (taken from chromatogram 2) and their coelution in chromatogram 1 was easily recognizable. A number of small peaks (3–5) were recognized to be due to homologues of PQQ-M, and their retention was estimated to fall between those of PQQ and PQQ-M in chromatogram 2. All the assumptions on the identity and order of the peaks were justified by further measurements. Without giving all the details of peak tracking, the main steps of the limited optimization are discussed below.

Recently, we have adapted some resolution-based criteria in order to express the quality of separation in limited optimization[14]. Weighting factors, w_i, with values of 1 (important) and 0 (unimportant) are assigned to each component of the sample (see Table I). The resolution between two consecutive peaks is taken as relevant when (w_i OR w_{i+1}) = 1, $i = 0 \ldots (M-1)$, where OR means the logical OR function, $i = 0$ for the solvent peak and M is the total number of peaks. A sequential approach was suggested[14] and tested[1] for the selection of different optimization criteria, adjusted to the goal of the analyst. Our primary goal here is to reach a satisfactory (required $R_{s,req} = 2.0$) resolution for the two peaks of interest, within the time constraints set by the two initial chromatograms. For this purpose the minimum resolution criterion,
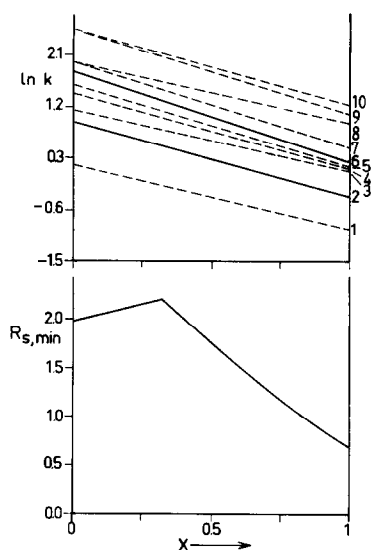
Fig. 5. Phase selection diagram in the THF–water buffer binary eluent system (0 = 7.5% THF and 1 = 15% THF), constructed from the chromatograms shown in Figs. 3c and 4. (top) Plots of ln k; solid lines refer to PQQ and PQQ-M; (bottom) response surface for the minimum resolution criterion, $R_{s,min}$.

$R_{s,min}$, can be used. The position of the two important solutes (PQQ and PQQ-M) is indicated by the symbol (★) in the chromatograms recorded in the THF–water buffer binary eluents.

Using the procedure applied for the optimization of ternary mobile phases, the logarithm of the solute capacity factors is assumed to be a linear function of the binary eluent composition (shown in Fig. 5a). These retention plots are used to calculate the minimum resolution criterion, over the concentration range of 0.075 to 0.15 THF in water (Fig. 5b). A maximum value of $R_{s,min} = 2.2$ is predicted for the eluent containing 9.9% THF.

After two additional measurements at "shifted" compositions (see Fig. 6) predicted by the iterative optimization method[12,13], a final optimum is found at 8.2% THF.

The chromatogram verifying this optimum is shown in Fig. 7, with an analysis time of 22 min. The two peaks of interest are separated from their (small) neighbouring peaks, while some pairs of peaks, which were assigned to be not of interest, are coeluted, e.g., 7–8, 9–10. The $R_{s,min}$ is calculated to be greater than 2 (using retention data and a constant plate count of 3500), a value representative of the extent of the separation for the large peaks (which are of interest in this case) as a result of different peak heights[15]. The resolution obtained between peaks 2 and 3 and 5 and 6 may be less than 2 due to tailing bands, but quantitation of the relevant peaks 2 and 6 should be no problem.

The optimum was considered to be adequate for the requirements formulated at the beginning of this discussion, and no attempt was made to optimize for higher R_s or shorter analysis time. If the analysis were performed on a routine basis, both R_s and the analysis time may need further adjustments. Nevertheless, a "better" optimum would
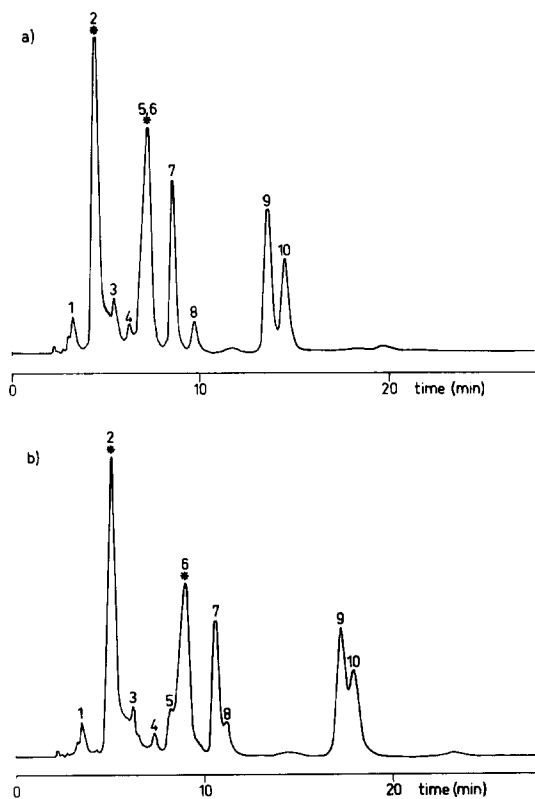
Fig. 6. Chromatograms measured during the iterative regression optimization procedure in the THF–water buffer (pH 2.5) binary eluent system. (a) 0.104 THF; (b) 0.091 THF.
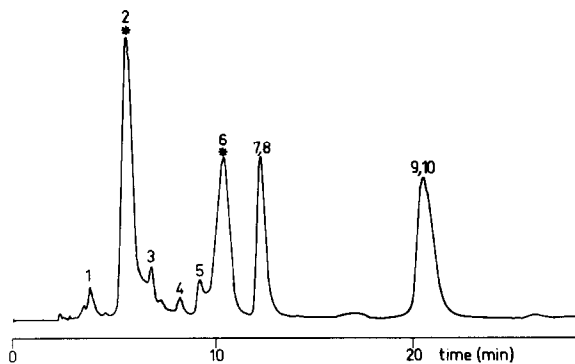
Fig. 7. Separation of the two solutes of interest (PQQ and PQQ-M) in the reaction mixture of PQQ and cyclopropanol under optimum conditions, in triethylamine phosphate buffer (pH 2.5) containing 0.082 THF.

need more effort, possibly reconsideration of the optimization search area, *e.g.*, variable eluotropic strength ternary eluent optimization or even the separation mode, *e.g.*, pH variation or ion pairing. Obviously, in that case one must perform some "backtracking" to an earlier stage in the method development scheme, as suggested by Schoenmakers and Mulholland[2].

When the optimum is accepted, a final step is the check on the purity of the peaks of interest. This is undoubtedly needed, as the peak of PQQ-M is much broader than the other peaks of the chromatogram. However, when the UV spectrum and chromatographic characteristics of the respective peaks are compared for the standards and the sample, no differences are found. Additional information on PQQ-M indicated[3], that a ring closure to a tetrahydrofuran structure between the $C(5)-CH_2-CH_2-CHO$ and the $C(5)-OH$ groups (see Fig. 1) can result in another possible configuration of this compound. The simultaneous existence of these two configurations both for the "standard" and the reaction mixture may be responsible for the broader sample peak with very similar spectral characteristics.

ACKNOWLEDGEMENTS

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