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PRACTICAL PROCEDURE FOR THE OPTIMIZATION OF REVERSED-PHASE SEPARATIONS WITH QUATERNARY MOBILE PHASE MIXTURES

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SUMMARY

In this paper we describe an extension of the previously published one-parameter ternary mobile phase optimization to include quaternary mobile phases. Whereas in ternary optimization only mixtures of two from the three isoeluotropic binary phases of water with methanol, tetrahydrofuran and acetonitrile are optimized, with quaternary optimization a blend of all three binary phases is searched for the best separation conditions. This two-parameter optimization formulates first a linear estimate of the retention based on flat planes, fitted through a minimum number of three initial runs. The predicted optimum is verified and an observed difference is used to refine the retention surfaces. After a few such iterations, the true shape is found and the procedure can be stopped. Practical examples were run to demonstrate the possibilities of the procedure.

INTRODUCTION

In previous publications[1,2] we have described a systematic procedure for the optimization of ternary mobile phases for reversed-phase liquid chromatography. The basis of the procedure is the experimental observation that the retention, expressed as ln k, is a smooth function of the mixing ratio of two of the isoeluotropic binary compositions that constitute the ternary eluent. At the start of the optimization, the retention of all solutes is estimated from straight lines, connecting the retention data in the two binary compositions.

Linear retention functions are used to calculate an optimization criterion over the full range of ternary compositions. The highest value of the criterion defines the optimal composition. This prediction is verified and the resulting retentions are used to refine the linear approximation. As the procedure continues, the initial straight lines are segmented until the actual non-linear retention behaviour is adequately described and the procedure stops.

Linear segmentation has proven to yield a good approximation of the retention behaviour of solutes in ternary mobile-phase mixtures[1,2]. It has also been applied successfully to the optimization of mobile phase pH[3] and to the concentration of an ion-pairing reagent[4]. In all these cases, only a single parameter was optimized. The

present paper gives a description of the extension of ternary mobile phase optimization to quaternary solvent mixtures. Instead of a mixture of only two of the three limiting binary mobile phases (one parameter), a blend of all three is searched for the optimal mixing conditions. This can be considered as a two-parameter optimization in which the mixing ratio of two of the three ternary combinations can be changed simultaneously.

Ternary mixtures of two isoelutropic binary phases are expected to yield the same capacity factors, k, for a "hypothetical average solute"[5]. Of course, for real solutes, the different solvents cause peaks to shift either forward or backward in the chromatogram. These shifts are called specific effects or specificity[5,6] and make optimization of a ternary composition profitable. For example, consider a sample of three solutes, of which solutes 1 and 2 are unresolved by the binary mixture A, whereas solutes 2 and 3 are unresolved by a second mobile phase B. If we assume a linear behaviour of ln k vs. ternary composition, the centre of the composition axis, representing 50% mixture A and 50% mixture B, should yield a chromatogram with all solutes resolved.

Ternary optimization fails when a pair of solutes remains unresolved over the entire range of a ternary composition. This is usually the case when the two solutes are unresolved by both binary mixtures from which the ternary mixture is made. In this case, the addition of a third organic solvent to make a quaternary mixture might be beneficial. More generally, for an arbitrary sample, quaternary optimization may be profitable to enhance a (poor) separation that has been achieved with one or more of the three initial binary mobile phase compositions.

DESCRIPTION

Quaternary solvent mixtures can be graphically represented by a triangle, as shown in Fig. 1. Binary mixtures are positioned at the apices, ternary compositions
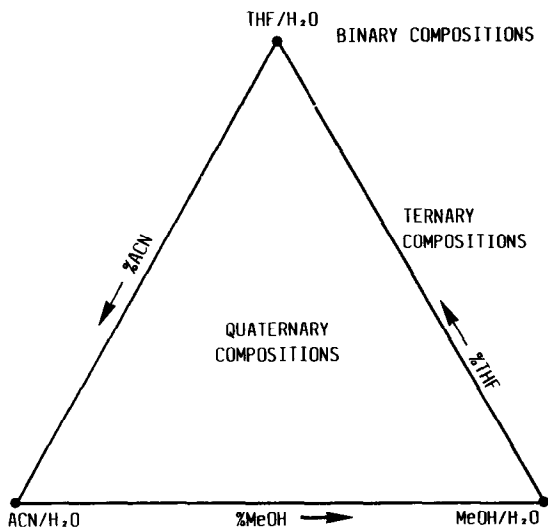

Fig. 1. The different types of mobile phase compositions in the isoeluotropic solvent triangle.

along the edges, and quaternary mixtures inside the triangle. Instead of the straight line used in the ternary scheme, the retention behaviour of a solute as a function of composition is now represented by a flat plane, fitted through three data points.

The four principles underlying the quaternary optimization procedure are similar to those used in the ternary procedure, i.e.: (i) consecutive data points are used for a better approximation of the real retention behaviour; (ii) the response surface, defined by the criterion, is calculated over the entire parameter space to locate optimal separation conditions; (iii) the concept of shifted compositions[2] is incorporated; (iv) a criterion to stop the optimization procedure is included.

These principles will be briefly elucidated.

Consecutive data points

Each additional data point is used to refine the retention planes for a better approximation of the real retention behaviour. A new point divides the initial triangle into three smaller triangles. If an additional point is situated on the edge of a triangle, it divides this triangle into two new triangles. The entire triangle is thus successively divided into a collection of smaller triangles.

Response surface

The response surface is represented by a lattice of criterion values for all binary, ternary and quaternary solvent mixtures over the entire parameter space. As criterion, we use the resolution product, which aims at an even distribution of solute peaks over the chromatogram. To correct for differences in length of the chromatogram, the criterion was made relative to the best possible peak distribution[2]. To calculate the relatative resolution product, defined by Drouen *et al.*[2] as

$$r = \prod_{i=1}^{n-1} Rs_{i+1,i} \bigg/ \left[\left(\sum_{i=1}^{n-1} Rs_{i+1,i} \right) \bigg/ (n - 1) \right]^{n-1} \tag{1a}$$

$$Rs_{i+1,i} = \sqrt{N}(t_{i+1} - t_i)/2(t_{i+1} + t_i) \tag{1b}$$

the retention times of all solutes for a particular solvent mixture must be available. These retention times are approximated by linear planes, fitted through the three data points on the apexes of the triangle in which the solvent mixture is located. This linear plane is defined by

$$\ln k_i = a_i x + b_i y + c_i \tag{2}$$

where k_i is the capacity factor of solute i; a, b and c are solute-dependent constants; and x and y are the two optimization parameters (mixing ratio of the three binary solvents). Thus, when constructing the lattice, the computer algorithm steps through the entire parameter space. For each composition, it first locates the triangle in which it is situated, then determines the three constants, a, b and c, for each solute, and finally calculates the retention times for all solutes and the criterion value according to eqn. 1a.

The distance between consecutive points in the lattice is derived from the error in preparing the solvent mixtures. As each reduction of the distance by a factor of 2 enlarges the calculation time by a factor of 4, it is important not to select steps that are too small. In the two examples discussed in this paper the response surface has been calculated over the entire triangle in steps of 1% increments of the mixing ratio. The response surface can be represented in a pseudo-isometric three-dimensional plot.

A clearer representation of the response surface is offered by a contour plot. This is a two-dimensional projection of the lines, representing points with equal criterion values (iso-response contour lines). Examples will be given below.

The quaternary optimization program needs up to 30 min of calculation time for the 5000 lattice points (increments of 1%), depending on the number of solutes and chromatograms measured. The construction of the pseudo-isometric three-dimensional plot and the iso-response contour plot uses an additional 20 min of computer time. This seems fairly large, but further reduction of the computing time is possible by using a faster computer or a faster language, such as machine code. Plotting time cannot be significantly diminished but plots are not essential for the progress of the procedure and could be limited to the final result. It is felt, therefore, that in terms of speed two-parameter optimization by the present procedure is quite feasible. However, extension to simultaneous three-parameter optimization would probably lead to excessive computing times.

Shift-and-stop procedure

After each new data point, the response surface is recalculated and an optimum composition is predicted. However, in analogy with ternary optimization[1,2], the procedure advises to measure not this optimum, but a shifted composition. The concept of shifted compositions is incorporated to speed up the procedure and to gather more information on the entire parameter space. Of course, the shift is more complex than in the ternary program. Three types of shifts have been incorporated: a shift towards a side of the triangle, a shift towards the centre of gravity of the triangle, and a shift along the side of the triangle. The optimization algorithm also contains a stop criterion, which is similar to the one used in the ternary optimization procedure[2]. It is clear that the procedure can be stopped when the retention data of the optimum, predicted on the basis of the triangular retention planes, correspond with the retention data actually measured. In this case, the retention planes, used for calculating the retention data, need no further refinement, and the position of the optimum remains the same. This offers a stop criterion: measure the retention times with the optimal mobile phase composition, compare with the predicted retention times and stop if the deviation is smaller than a certain preset limit. If not, continue and measure the shifted composition.

An alternative stop criterion is offered when the predicted optimum composition is close to a previously measured data point. Again, essentially the same approach is used as described by Drouen *et al.*[2]. Each data point is surrounded by a confidence area, for which the expected uncertainty in the predicted retention times is less than an acceptable threshold value (*e.g.* 0.01 units in ln k). The size of the confidence area depends on this accepted uncertainty in retention times and on the expected curvature of the retention planes. Initially, the confidence areas around the

three starting binary compositions (the apices of the solvent triangle) are very small (see Fig. 4, shaded area close to the apices). As further data points are entered, the areas stretch. If an optimum predicted in the course of the procedure falls within one of the confidence areas, the procedure advises to measure that optimum rather than a shifted composition. For example, in Fig. 4 S_5 falls inside the confidence range (shaded area) arising from the measurements S_1 to S_4.

EXPERIMENTAL

The liquid chromatograph consisted of two M6000A pumps together with an M660 gradient programmer, both from Waters Assoc. (Milford, MA, U.S.A.), a Rheodyne 7125 injector and an HP1040A photodiode array detector (Hewlett-Packard, Waldbronn, F.R.G.). We used a Novapak C_{18} column from Waters (15 cm × 3.9 mm I.D.). Methanol, acetonitrile and tetrahydrofuran (THF) were from Rathburn Chemical, Walkerburn, Scotland. The mobile phase was prepared by weight, using the density of methanol (0.791), acetonitrile (0.785), THF (0.889) and water (0.998). All mobile phases were ultrasonically degassed for 5 min.

The optimization program was developed in Fortran-77 on a Waters 840 Data Management System, equipped with 512 Kbyte of memory, dual diskette drive (2 × 400 Kbyte), integral 10-Mbyte Winchester disk drive, extended bit-map graphics with colour monitor, a Letterprinter 100 (all from Digital, Maynard, MA, U.S.A.) and a Hewlett-Packard HP7470A graphics plotter.

RESULTS AND DISCUSSION

The above-mentioned considerations will be exemplified by optimization of the separation of 11 aromatic solutes. The starting procedure of the optimization is the same as that described by Schoenmakers et al.[1]. A water–methanol gradient scan indicates that isocratic elution with 50% methanol yields capacity factors, k, ranging from 0.5 up to 10 for this sample. The transfer rules formulated by Schoenmakers et al.[5] indicate isoeluotropic binary mixtures of 33% THF and 37% acetonitrile. The resulting chromatograms are shown in Fig. 2.

The retention data of the three initial chromatograms are entered in the optimization program, and the response surface of the criterion (r) is plotted in Fig. 3A as a pseudo-isometric three-dimensional plot, together with the iso-response contour plot (Fig. 3B), and searched for the highest value. The two plots clearly indicate a quaternary optimum, located at 28.5% methanol, 5.9% THF and 9.3% acetonitrile. The procedure continues, because the optimum falls outside the confidence ranges, which are very small in the initial stage of the procedure. The optimum indicated by O_1 in Fig. 3B is shifted towards the centre of gravity. The chromatogram for this shifted composition (S_1, in Fig. 4, 25.9% methanol, 7.1% THF and 9.9% acetonitrile) is run and plotted in Fig. 5A.

The retention data, measured in Fig. 5A, are entered into the calculation routine, and a second quaternary optimum is calculated. This leads to a second advised composition, which happens to fall on the ternary methanol–acetonitrile–water edge of the triangle (S_2 in Fig. 4). Again, the chromatogram is measured (Fig. 5B), and the retention data are entered. Subsequently, a third and fourth refined
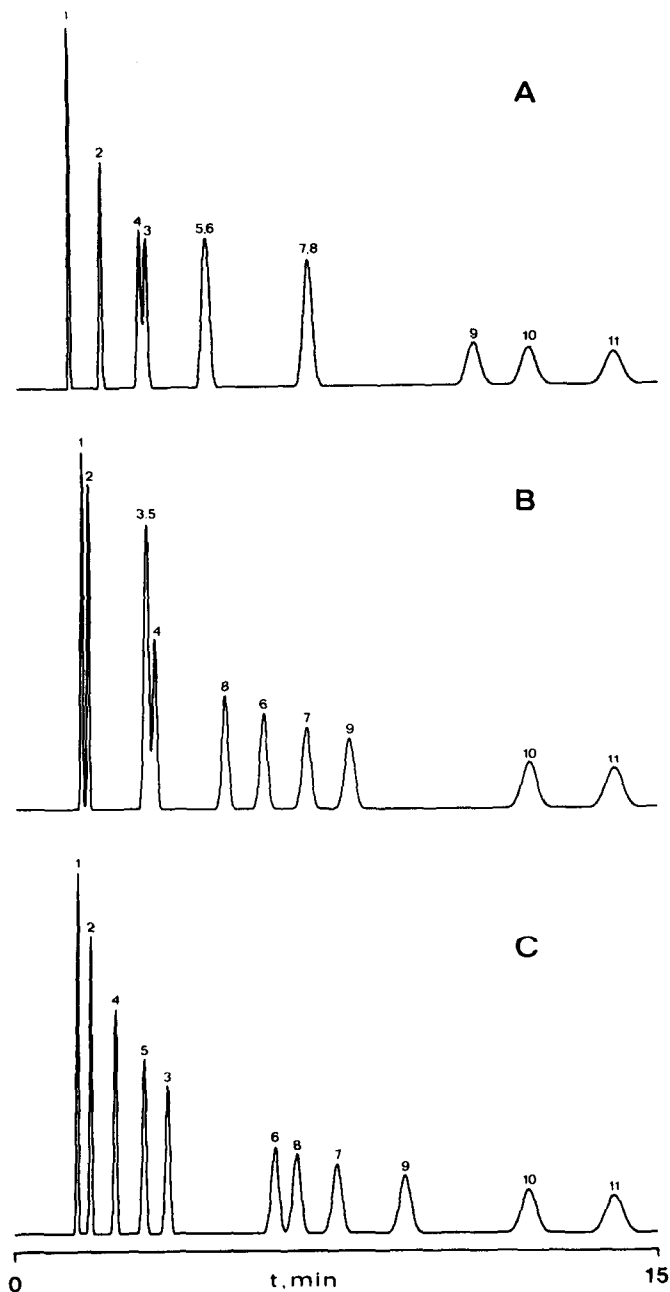
Fig. 2. The three initial chromatograms recorded in 50% methanol (A), 33% THF (B) and 37% aceto-nitrile (C) of a sample containing eleven aromatic solutes. Solutes: (1) sulfamerizine, (2) acetanilide, (3) benzaldehyde, (4) methylparaben, (5) cinnamyl alcohol, (6) nitrobenzene, (7) anisole, (8) methyl benzoate, (9) methyl salicylate, (10) 1-nitronaphthalene and (11) diphenylamine.
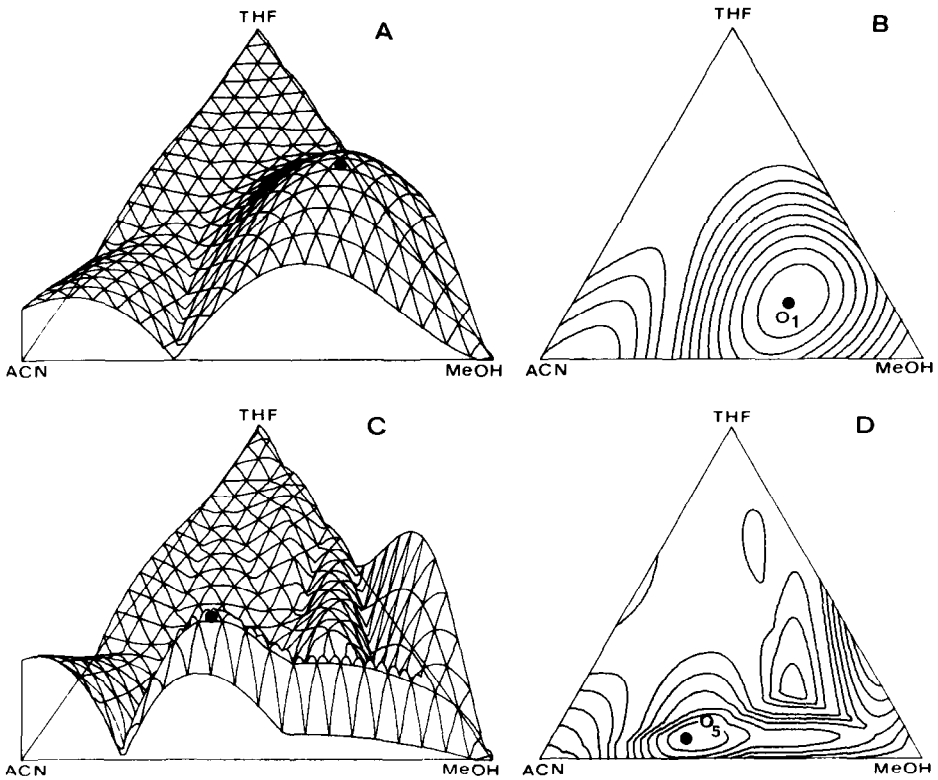
Fig. 3. The triangles A and B represent, respectively, the response surface and iso-response contour plot, calculated from the chromatographic data of the three initial chromatograms in Fig. 2. The lower part of the figure (C and D) represents the final plots, calculated after five additional steps in the optimization procedure.
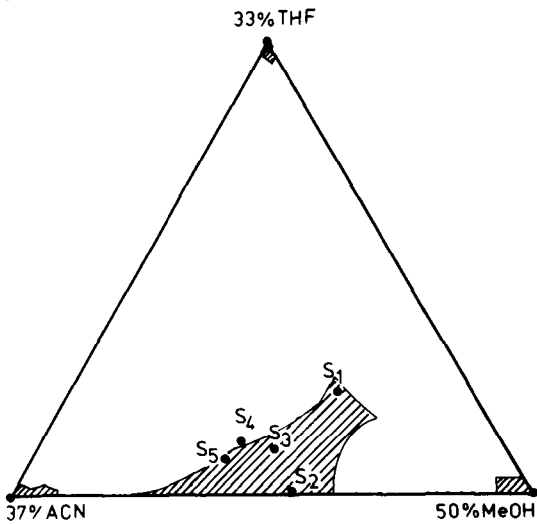

Fig. 4. S_1 to S_5 are consecutive, measured points in the composition triangle during the optimization procedure of eleven aromatic solutes (see Fig. 2).
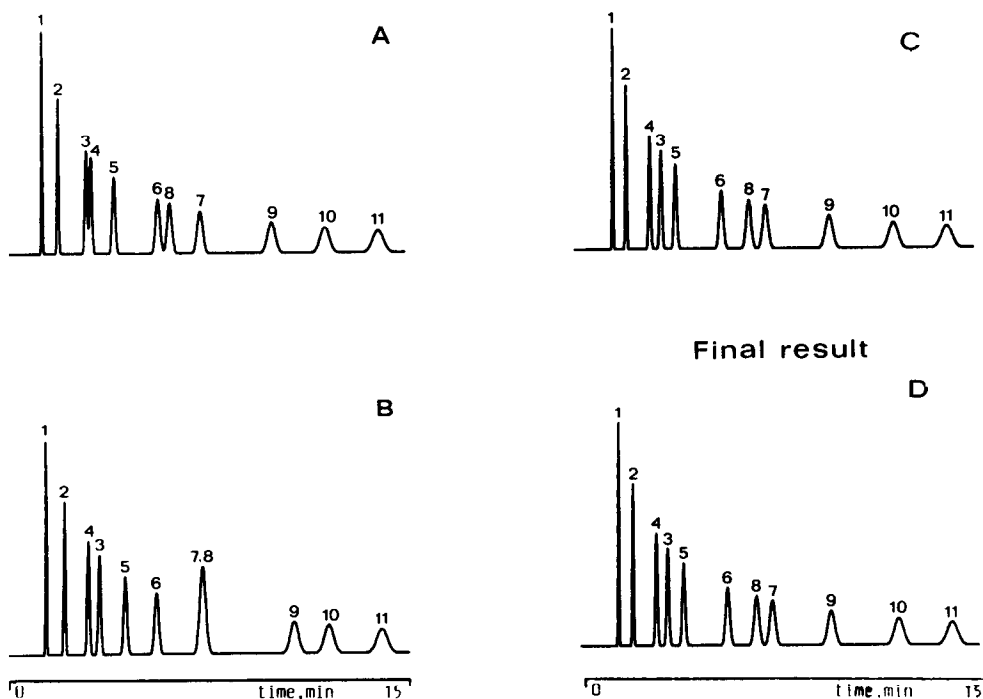
Fig. 5. Frame A shows the chromatogram of the solutes in Fig. 2, measured at the first predicted optimum, S_1 in Fig. 4 (25.6% methanol, 7.1% THF and 9.9% acetonitrile). Frame B gives the chromatogram at the second predicted optimum S_2 (28% methanol and 16.3% acetonitrile). Frames C and D show the recorded and the predicted chromatograms at the final optimum composition (S_5), respectively.

prediction is made (S_3 and S_4 in Fig., 4). When these retention data are again entered into the calculation, the fifth optimum falls within the confidence area of a previous data point and the procedure stops. Indeed, the chromatogram predicted for this final optimum (Fig. 5C) agrees with the experimental chromatogram measured at this composition (Fig. 5D). Fig. 3C and D represent the pseudo-three-dimensional and iso-response contour plots of the final result. These final plots (C and D) differ significantly from those derived from the initial conditions (A and B). This is due to the refinements of the retention planes during the evolution of the optimization procedure.

At this point, it is instructive to draw some conclusions. First, the procedure presented in this paper locates the optimum without any knowledge of the retention behaviour as a function of the parameter space. During the procedure, the continuous refinements of the retention planes result in a better approximation of the real retention behaviour. This can be seen by the difference between the predicted retention times and the actually measured times, which decrease as the procedure progresses.

Secondly, the shift rules and stop criterion are easy to use and yield a more efficient distribution of the measured data points over the parameter space. Indeed, the quaternary optimum has been reached after three initial binaries plus five consecutive steps, constituting a total of eight runs.

However, some limitations should also be indicated. As can be seen in Fig. 4,

a large area remains unsearched. This is not too serious, if the retention behaviour in that area can be approximated with a linear plane. If not, an optimum could be situated in this area without being recognized. The more the real retention differs from the approximated retention, the more serious this problem will be. A similar criticism applies to the ternary optimization procedure.

Perhaps the most important observation is that the result of quaternary optimization (Fig. 5D) is only marginally better than the best binary chromatogram (Fig. 2C). Indeed, the binary result is perfectly acceptable, and time could have been saved by making that decision at an early point in the optimization procedure.

This can be accomplished by an operator or a computer intervention, when, e.g., a minimal separation has been achieved (e.g. Rs 1.5). It is important to exhaust all the possibilities of the binary and ternary optimization, before the lengthy quaternary optimization is undertaken.

Calculation of the ternary optimum is realized within a minute plus a few additional minutes to draw the phase selection diagram, which provides all the necessary information. Calculation and construction of the diagrams of quaternary optimization requires almost 1 h.

To enlarge the scope of the optimization, we propose the strategy according to the flow-diagram presented in Fig. 6. After recording the chromatogram in the
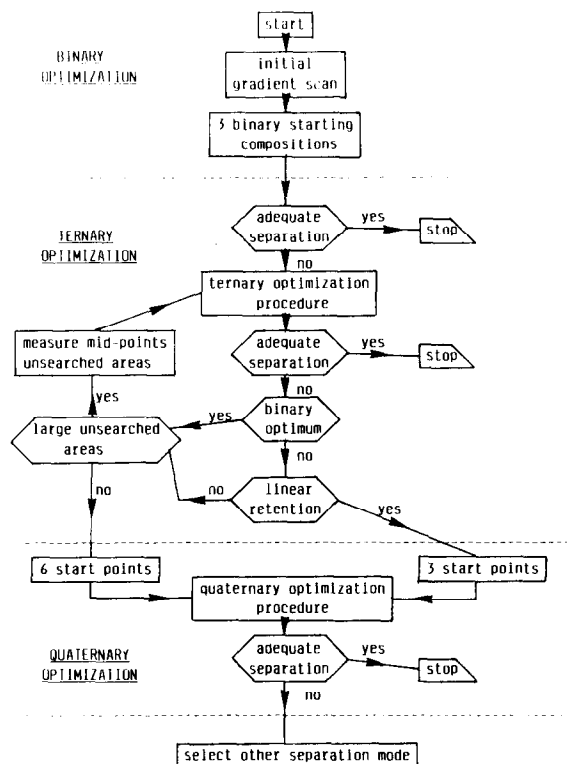

Fig. 6. Flow diagram for the successive approach of first optimizing the binary, then the ternary, and finally the quaternary mobile phase mixture. Discussion in the text.

three isoeluotropic binary mixtures, based on the initial gradient scan, a decision is made on whether the binary separation is adequate or the ternary optimization should be tried. Based on the final result of ternary optimization, one can again decide how to continue. If an adequate separation is achieved, the procedure can be stopped. If not, the procedure continues with the next step. Of course, the optimum can still be located at a binary composition.

When the solutes show a significant deviation from linear retention behaviour, large uncovered ternary solvent areas may contain a better solvent mixture without being recognized. This can be investigated by adding an additional point (located in the centre of these areas), and reinvestigating the computer phase-selection program. This situation is encountered, in particular, when the ternary program results in an inadequate binary optimum without additional ternary data points. In this case, no information is available on a possible curvature in the retention behaviour. Then, the mid-points of the composition axes (a solvent mixture of 50% of each of the two binary solvents) are run and entered in the computer program. In all other situations, the deviation from linearity is indicated by the additionally measured ternary data points. (This can visually be checked in the top portion of the phase-selection diagram.)
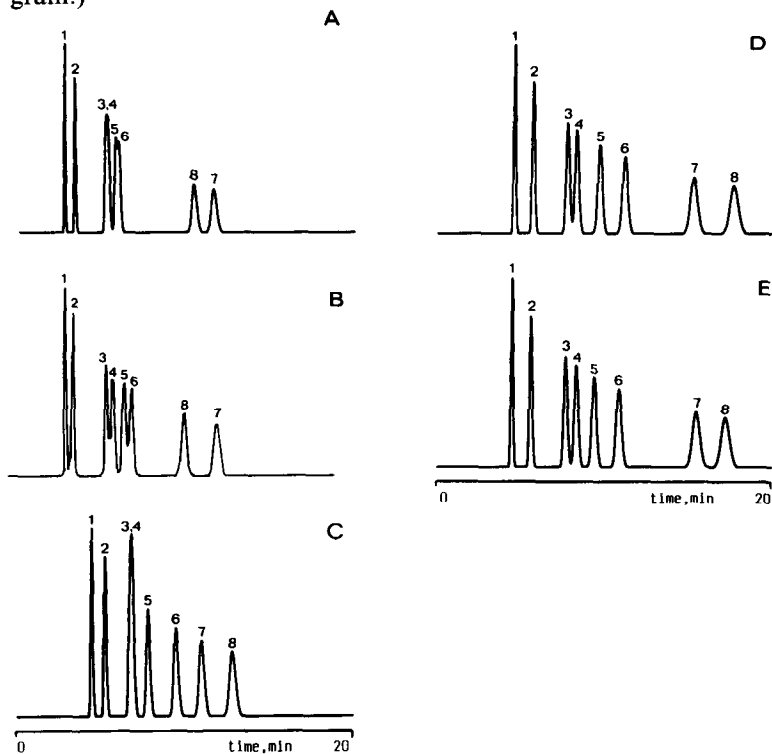

Fig. 7. Optimization of the separation of eight chlorophenols. The diagrams represent the three initial chromatograms, recorded in 50% methanol (A), 32% acetonitrile (B), 33% THF (C), the final ternary optimum (D, 11.5% methanol and 25.4% THF) and the final quaternary optimum (E, 8.4% methanol, 22.0% THF, and 5.3% acetonitrile). The sample contains the following polychlorinated phenols: (1) o-chlorophenol, (2) p-chlorophenol, (3) 4-chloro-3-methylphenol, (4) 2,3-dichlorophenol, (5) p-chloro-o-cresol, (6) 2,5-dichlorophenol, (7) 2,4-dichloro-5-methylphenol and (8) 3,5-dichlorophenol.
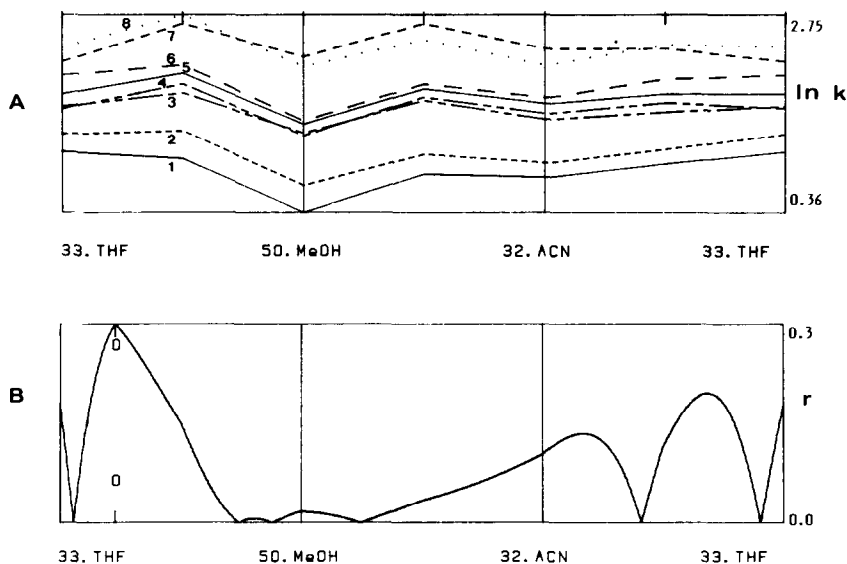
Fig. 8. Ternary optimization of eight chlorophenols. Frame A shows the variation of ln k data, recorded at the six mobile phase mixtures based on the three binaries and their midway compositions. Frame B shows the final phase selection diagram, calculated from the previous six data points plus one additional, measured point, derived from the first optimization step. O is the final optimum composition (Fig. 7D).

    If no further improvement is expected for ternary solvents, one can decide to continue with the quaternary optimization procedure, or stop if the result is satisfactory. When the retention behaviour differs significantly from linearity, indicated by the ternary data points in the previous step, six initial start points can be selected instead of three. These six include the three points positioned at the apexes and three more at the centres of the three edges.

    Optimization of the separation of eight polychlorinated phenols is illustrative for the proposed strategy. The initial three binary mobile phase compositions are 50% methanol, 32% acetonitrile, and 33% THF. The chromatograms for these three compositions are plotted in Fig. 7A, B and C. The best binary separation is achieved with 32% acetonitrile, but a better separation of solutes 3 and 4 and of solutes 5 and 6 would be desirable. It should be pointed out that the prospects for improvement are poor, because solutes 3 and 4, which are barely separated by acetonitrile, are unresolved by both methanol and THF binaries. Therefore, if the retention of these solutes varies linearly with mobile phase composition, no improvement will be observed. This was confirmed by the ternary and quarternary phase-selection diagrams calculated from the three initial binary results by using linear approximation. The optimal composition was always predicted at the binary acetonitrile composition.

    Only a deviation from linearity as a function of ternary or quaternary composition can offer an improvement in the separation. To this end, the chromatograms were recorded at the three mid-points of the three ternary composition axes. The data were entered into the ternary optimization program. The constructed plot of ln k vs. ternary solvent composition is given in Fig. 8A. This figure shows, indeed, a pronounced deviation from linearity. The highest criterion value indicates a ternary
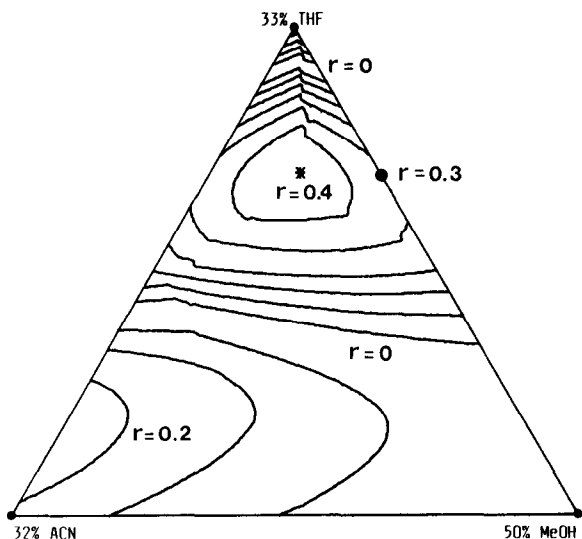
Fig. 9. Iso-response contour plot of the final result for the quaternary optimization of polychlorinated phenols. The asterisk denotes the final optimum composition (Fig. 7E).

mixture of methanol, THF and water. After an additional measurement of the shifted position, Fig. 8B shows the response curve for the relative resolution product (r) and the final optimum at 11.5% methanol, 25.4% THF and 63.1% water. The corresponding chromatogram is shown in Fig. 7D. As can clearly be seen, the separation of solutes 3–6 has improved, yielding an acceptable separation of all peaks in the chromatogram. As this ternary optimum does not contain acetonitrile, and because the binary chromatogram has shown that the separation of solutes 3 and 4 can be enhanced with acetonitrile, a quaternary optimization could be profitable.

The quaternary optimization program was initiated with the previously measured six data points, dividing the entire composition triangle into four new triangles. The program locates a quaternary optimal composition (asterisk in Fig. 9). This point almost coincides with the centre of gravity in the top triangle. After refinement of the retention planes based on the new data point, the calculated optimum remains unaltered and the procedure stops. The chromatogram (Fig. 7E) shows, as expected, a small improvement of the separation of solutes 3 and 4, compared to the optimal ternary composition (Fig. 7D). The iso-response contour plot of the final result is given in Fig. 9.

CONCLUSIONS

As these two examples show, the procedure offers a fast approach to the solvent composition for optimal separation, as defined by the selected criterion. The successive approach of first optimizing the binary, then the ternary, and finally the quaternary mobile phase mixture, makes it possible to interrupt or continue the procedure as long as it is profitable.

For the present purpose, localization of unsearched areas that should be in-

vestigated for an unexpected optimum is based on experience. The size of these areas depends, of course, on the deviation from linearity in the retention behaviour. If the curvature is pronounced, the size of the area is small, whereas for linear behaviour it is large. In the future, appropriate rules should be formulated to provide for a fully automated procedure, in the computer algorithm.

A conclusion, drawn by Schoenmakers *et al.*[1] and confirmed by the experience in this paper, is that increased complexity of mobile phase mixtures suffers from the law of diminishing returns. Thus, for many samples, one of the three isoeluotropic binaries already yields an adequate separation. Samples that are not well separated with binary solvents generally benefit only little from ternary (or quaternary) solvents, but there are a few samples for which ternary eluents provide a vastly superior separation.

However, the two-dimensional procedure developed in this paper has a much wider applicability than quaternary solvent optimization in reversed-phase liquid chromatography. This has already been demonstrated for the simultaneous optimization of modifier concentration and pH adjustment[3] and will be further used in ion-pair optimization.

ACKNOWLEDGEMENT

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