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COMBINED OPTIMIZATION OF MOBILE PHASE pH AND ORGANIC MODIFIER CONTENT IN THE SEPARATION OF SOME AROMATIC ACIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Using the separation of some closely related aromatic acids as an example, a mixture design approach is applied to the optimization of pH as a single parameter in aqueous mobile phases and also to the combined optimization of pH and modifier content in aqueous–organic mobile phases. The approach used has an advantage over factorial design experiments in that it requires substantially fewer retention data in order to locate the optimal mobile phase composition. A simple optimization criterion which considers both the separation characteristics and duration of a chromatogram is described and its use is illustrated.

INTRODUCTION

Selection of the optimal mobile phase composition in high-performance liquid chromatography (HPLC) is a problem which may be approached in a number of ways. One common approach is the intuitive method, wherein the chromatographer makes an initial selection of the mobile phase composition based on the nature of the solutes to be separated and then refines this selection on a trial and error basis. This method often fails when complex samples are encountered or when the nature of the solutes in the sample mixture is unknown. An alternative and very successful approach is a systematic search over a wide range of solvent compositions with the aid of predicted or extrapolated retention data and a subsequent assessment of the quality of the resultant chromatograms by a mathematical optimization criterion. This method generally requires the use of a computer.

Computerized procedures for selection of optimum mobile phase composition have become increasingly popular in recent years[1], and this can be attributed to three factors. First, the development of HPLC has reached the stage where the separation of very complex sample mixtures is being routinely studied, and this complexity pro-

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hibits the intuitive approach for mobile phase selection. Second, advances in the theoretical understanding of HPLC[2–4] have provided a sound basis for the prediction of retention behaviour when the mobile phase composition is changed. Third, the great diversity in available column packing materials means that a published separation procedure often cannot be reproduced in the laboratory without considerable further optimization.

In a recent review, Glajch and Kirkland[1] have examined a variety of mobile phase variables which can be optimized, and these are classified as 'related' variables (*e.g.*, the percentage of organic modifier and the percentage of water) or 'discrete' variables (*e.g.* temperature and pH). Optimization of related variables is generally achieved by using a mixture design approach in which retention times are predicted on the basis of a limited number of experimental retention data, together with a theoretical or empirical knowledge of retention behaviour under varying mobile phase conditions[5]. In contrast, discrete variables are optimized by using a factorial design approach wherein a relatively large number of initial experiments is required to determine retention behaviour, which is then used to select the optimum mobile phase composition. Most of the reported optimization procedures have been concerned with related mobile phase variables, particularly the percentages of organic modifiers in binary, ternary and quaternary solvent mixtures.

Selection of the optimum mobile phase pH is an extremely important consideration in view of the great effect of pH on solute retention in most forms of HPLC, and particularly in reversed-phase chromatography. Indeed, it is sometimes possible to resolve a mixture of acidic or basic solutes by pH manipulation alone, without the use of organic modifiers[6]. Optimization of pH has generally been approached by means of factorial design experiments in which the pH is varied alone or in conjunction with another discrete variable, such as the concentration of pairing ion in a reversed-phase ion-pairing chromatographic system[7–9].

The chief drawback of this approach is that a considerble amount of experimental data is required to enable the relationship between pH and solute retention to be accurately established. Typically, sixteen initial chromatograms are necessary in a two-factor optimization of, for example, pH and concentration of ion-pairing reagent[7]. In such cases, it may be questionable whether the required separation merits the use of an optimization procedure. An additional consideration is that optimization of the pH, independent of the mobile phase modifier concentration, may often be a fruitless exercise in view of the strong dependence of pH (and hence solute retention) on the modifier content of the mobile phase. In short, the pH optimum selected independently of modifier concentration will usually be inapplicable to mobile phases containing more than a small amount of modifier[10].

The purpose of this paper is to discuss the application of a mixture design optimization procedure, firstly to the selection of optimum mobile phase pH, and secondly to the simultaneous optimization of pH and modifier concentration in the mobile phase.

THEORY

Fundamental to all systematic approaches to mobile phase optimization is the use of a mathematical criterion to assess the quality of a chromatogram quantitatively

and to facilitate selection of the optimum separation. Laub and Purnell[11–13] have used the lowest value of the relative retention between two adjacent peaks as the optimization criterion, whereas Jones and Wellington[14] have employed the lowest value for the resolution (R_s). In either case, a so-called window diagram is obtained from which the phase system yielding the highest criterion value for the least resolved peak pair is readily derived. A similar approach is used by Glajch *et al.*[3] in their "resolution mapping" technique in which R_s values for several adjacent peaks are plotted to locate ternary or quaternary mobile phase mixtures that provide an R_s value larger than an accepted threshold for all peak pairs.

In a previous report[15] we have proposed an alternative criterion defined as the relative resolution product (r):

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

where $R_{s_{i+1,i}}$ is the resolution factor for the adjacent peaks i and $i + 1$. This criterion reaches its maximum value when all R_s values are equal, *i.e.* when all peaks are distributed evenly over the chromatogram. Use of this criterion enables selection of an optimum mobile phase, but not necessarily a satisfactory chromatogram, which can be achieved by variation of the plate number of the column, *e.g.*, by altering the column length or the flow-rate.

The above strategy was developed for the selection of ternary mobile phases by means of a mixture design approach in which iso-eluotropic mobile phase compositions were employed. This implied that the duration of the chromatogram remained essentially constant over the entire range of mobile phase mixtures examined. In the present study, however, variation of the mobile phase pH and modifier content produces large changes in the duration of the chromatogram. In view of this, we have examined ways in which the time required for the chromatogram can be incorporated into the optimization criterion.

In general, criteria based on the product of a time factor and a separation factor often encounter problems in the relative weighting assigned to each factor. Such weighting is necessary to prevent the time factor from exerting undue influence on the optimization criterion, which can result in the selection of fast chromatograms with poor separation[16]. In order to eliminate this problem, we have chosen to use a sequential optimization procedure in which peak separation is first examined to locate areas of mobile phase composition giving satisfactory resolution, and these compositions are subsequently searched to locate the fastest chromatogram. In this way it is possible to optimize a separation for the particular column and conditions in use.

In principle, any criterion based on peak separation can serve as the basis for the initial separation search. However, in this study we will use $R_{s,\min}$, which is the value of R_s for the least resolved peak pair in the chromatogram. A threshold value of $R_{s,\min}$ is selected (*e.g.*, $R_{s,\min} \geqslant 2$), and mobile phases providing the desired degree of separation can be located. A time search is then carried out over these mobile phase compositions, using $1/t$ (where t is the retention time of the most retained component) as the criterion.

EXPERIMENTAL

Materials and apparatus

All solvents used were of analytical reagent grade (Rathburn Chemicals, U.K.). The aromatic acids were obtained from E. Merck (Darmstadt, F.R.G.) and were used without further purification. Analytical reagent grade components were used for the preparation of the buffers. For the HPLC separations, 5-μm ODS-Hypersil (Shandon, U.K.) was used, packed in a stainless-steel column (150 × 4.6 mm I.D.) by a slurry technique.

The HPLC system consisted of a Waters (Milford, MA, U.S.A.) M6000 pump and M440 UV detector, interfaced with a PDP 11/45 computer (Digital Equipment, Marlboro, MA, U.S.A.) to enable accurate measurement of retention data. Samples were injected with a Rheodyne Model 7125 injector (Cotati, CA, U.S.A.), fitted with a 20-μl sample loop. A Plessey Micro II computer (Datacare, Zeist, The Netherlands) was used to calculate optimum mobile phase compositions.

Procedures

Buffer solutions of constant ionic strength were prepared by mixing appropriate amounts of 1.00 *M* acetic acid, 1.00 *M* sodium hydroxide, and 1.00 *M* sodium chloride, by using the procedure described by Deming and Turoff[6]. The pH of each buffer was checked with a calibrated pH-meter. When mixtures of organic modifiers with aqueous buffers were used, the stated pH of the solution refers to the aqueous buffer pH measured before addition of the modifier.

Separate solutions of the solutes benzoic acid, 2-aminobenzoic acid, 4-aminobenzoic acid, 4-hydroxybenzoic acid and 1,4-benzenedicarboxylic acid were prepared by dissolving 250 mg of the solute in 50 ml of water. These solutions were combined in suitable proportions so that each component of the mixture gave approximately the same response in the UV detector.

RESULTS AND DISCUSSION

Generalized optimization procedure

A strategy for the selection of optimum ternary mobile phases (*i.e.* those containing two organic modifiers and water) has been developed in this laboratory[5]. This procedure is based on the selectivity triangle approach, in which binary iso-eluotropic mobile phases of water with methanol, acetonitrile, and tetrahydrofuran, respectively, are used as the apexes of the triangle. Retention data are obtained for the above three binary mixtures, and linear plots are constructed of ln k' *versus* mobile phase composition over the entire range of ternary mixtures intermediate between the limiting binary mixtures. An appropriate criterion based on the product of resolution (R_s) of adjacent peaks[15] is then used to select the optimum mobile phase composition. The sample is chromatographed with the predicted optimal composition, the experimental retention data so obtained are used to refine the plots of ln k' *versus* mobile phase composition, and the optimization calculations are repeated. This process continues until there is little or no change in the optimal composition.

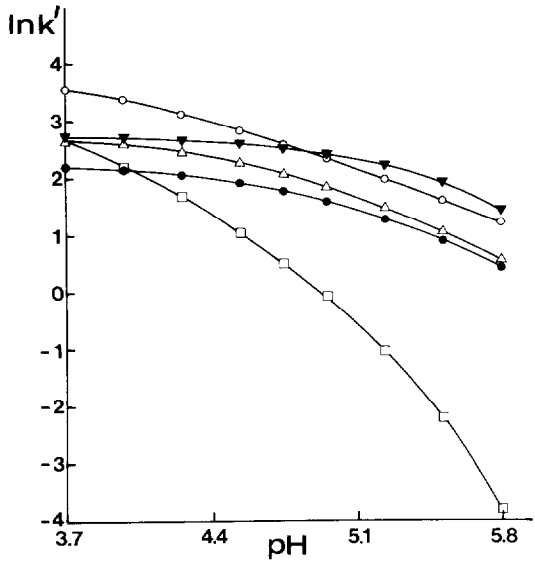

Fig. 1. Variation of ln k' for the solutes tested with changes in pH and with the use of an aqueous mobile phase. ○ = Benzoic acid; ▼ = 2-aminobenzoic acid; □ = 1,4-benzenedicarboxylic acid; △ = 4-hydroxybenzoic acid; ● = 4-aminobenzoic acid.

Optimization of pH in aqueous mobile phases

In order to apply the above-mentioned optimization procedure to pH optimization, it was necessary to establish whether linear plots of pH *versus* ln k' could be used to approximate initially the true retention behaviour. Clearly, in cases where the pH range studied encompasses the pK_a values of the acids (or bases) used, sigmoidal plots of pH *versus* ln k' can be expected. However, if the deviation of these plots from linearity is not great, it should be possible to initiate the optimization procedure on the basis of a linear relationship.

As an example, we discuss the separation of five aromatic acids by optimization of the pH of an aqueous mobile phase, as reported by Deming and Turoff[6]. Because all of the acids studied have similar pK_a values of *ca* 4.5, the separation of a mixture of these acids presents the greatest challenge to pH optimization. Fig. 1 presents the true variation of ln k' for each of the solutes studied over the range $3.7 \leqslant \text{pH} \leqslant 5.8$.

Using only the retention data measured at the two extreme pH values as initial input, the optimization procedure located on optimal pH of 4.18 (obtained by using the relative resolution product criterion, r). Retention data were measured at this pH value, and the optimization was repeated to give a new optimal pH of 4.31. Again, retention data were measured and, after recalculation, an unchanged optimal pH was indicated, thereby terminating the optimization procedure. Fig. 2 shows the final calculated variation of r with pH and the ln k' *versus* pH plots, used as the basis for calculation, together with a chromatogram obtained at pH 4.31.

The optimal mobile phase pH of 4.31 was found after only four experimental chromatograms, compared with at least nine used by Deming and Turoff for the same group of solutes[6]. These authors obtained a slightly different optimal pH (*i.e.* 4.48); however, this can be attributed to both the different optimization criterion and

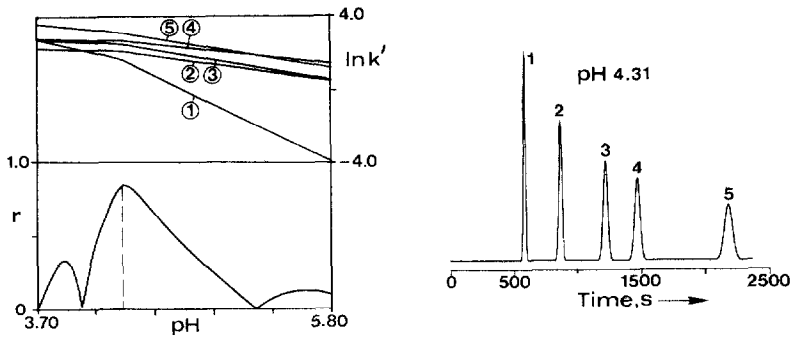

Fig. 2. Optimization of aqueous mobile phase pH with the aid of the relative resolution product criterion (r). The chromatogram obtained at the optimal pH of 4.31 is also shown. Peaks: 1 = 1,4-benzenedicarboxylic acid; 2 = 4-aminobenzoic acid; 3 = 4-hydroxybenzoic acid; 4 = 2-aminobenzoic acid; 5 = benzoic acid.

the different column used. Comparison of Fig. 1 with the ln k' *versus* pH plots in Fig. 2 shows that the true retention behaviour is correctly approximated in Fig. 2, thereby validating the predicted optimal pH value.

The procedure described above leads to an optimal mobile phase composition for which the solute peaks are evenly distributed over the length of the chromatogram. This does not necessarily imply a good separation. When the (approximately equal) resolutions are too low, the column may be lengthened to increase the number of plates. In the example of Fig. 2, the separation is actually too good and requires a long time. A shorter column would then provide a faster separation with acceptable resolution of all peak pairs. An alternative solution is to increase the pH, whereby the separation is considerably accelerated at the cost of a slight reduction in resolution.

In order to examine this aspect further, the $R_{s,min}$ criterion (*i.e.* the numerical value of the smallest R_s between adjacent peaks) was used as the basis for optimization. The same procedure as used previously was followed and a plot of $R_{s,min}$ *versus* pH was prepared. From this plot it is possible to locate pH ranges in which the resolution of the least resolved pair of peaks in the chromatogram exceeds a

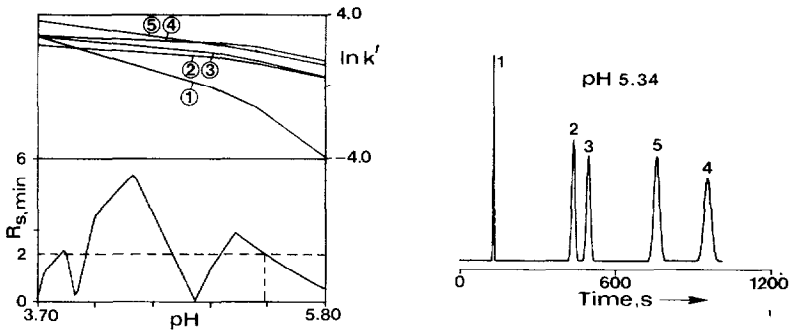

Fig. 3. Optimization of aqueous mobile phase pH with the aid of the minimum resolution criterion ($R_{s,min}$). The chromatogram obtained at the optimal pH of 5.34 is also shown. Identities of solutes are given in Fig. 2.

TABLE I

RETENTION TIMES OF AROMATIC ACIDS

Buffer of pH 4.31 with 20, 30 and 40% methanol and iso-eluotropic percentages of acetonitrile or tetrahydrofuran.

Organic modifier	*Retention times (sec)*				
	1,4-Benzenedicarboxylic acid	*4-Aminobenzoic acid*	*4-Hydroxybenzoic acid*	*2-Aminobenzoic acid*	*Benzoic acid*
MeOH (20%)	126	181	264	480	696
MeOH (30%)	97	132	175	298	401
MeOH (40%)	84	103	120	180	219
ACN (12.7%)	96	180	198	445	505
ACN (20%)	78	129	256	262	
ACN (28%)	72	105	105	133	139
THF (13.2%)	141	223	444	660	577
THF (19.8%)	126	193	330	501	415
THF (26.4%)	115	175	271	417	342

selected threshold value (presently, $R_s = 2$). These pH ranges can then be further examined to locate the pH value that produces the fastest chromatogram: information on the duration of the chromatogram is readily available from the plots of ln k' *versus* pH. Once again, the predicted optimum pH is used to measure experimental retention times, and the data are used to refine the ln k' *versus* pH plots and, ultimately, to locate a new optimum pH. Fig. 3 shows the result of this optimization process and also illustrates the chromatogram obtained at the selected optimal pH of 5.34. It can be seen that the separation achieved is entirely acceptable, whereas the chromatogram takes considerably less time than the one shown in Fig. 2.

Optimization of pH in aqueous–organic mobile phases

The discussion so far has been restricted to pH optimization in aqueous mobile phases. An attractive extension of this approach is the optimization of pH in mixed aqueous–organic mobile phases in order to exploit the additional selectivity effects

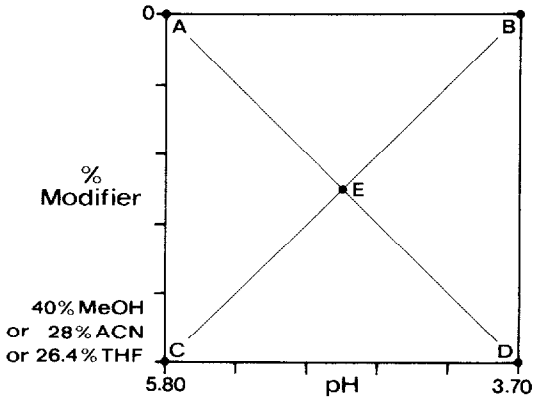

Fig. 4. Experimental design for the combined optimization of pH and organic modifier content of the mobile phase. The letters A–E represent the mobile phase compositions used to provide the experimental retention data necessary to initiate the optimization procedure.

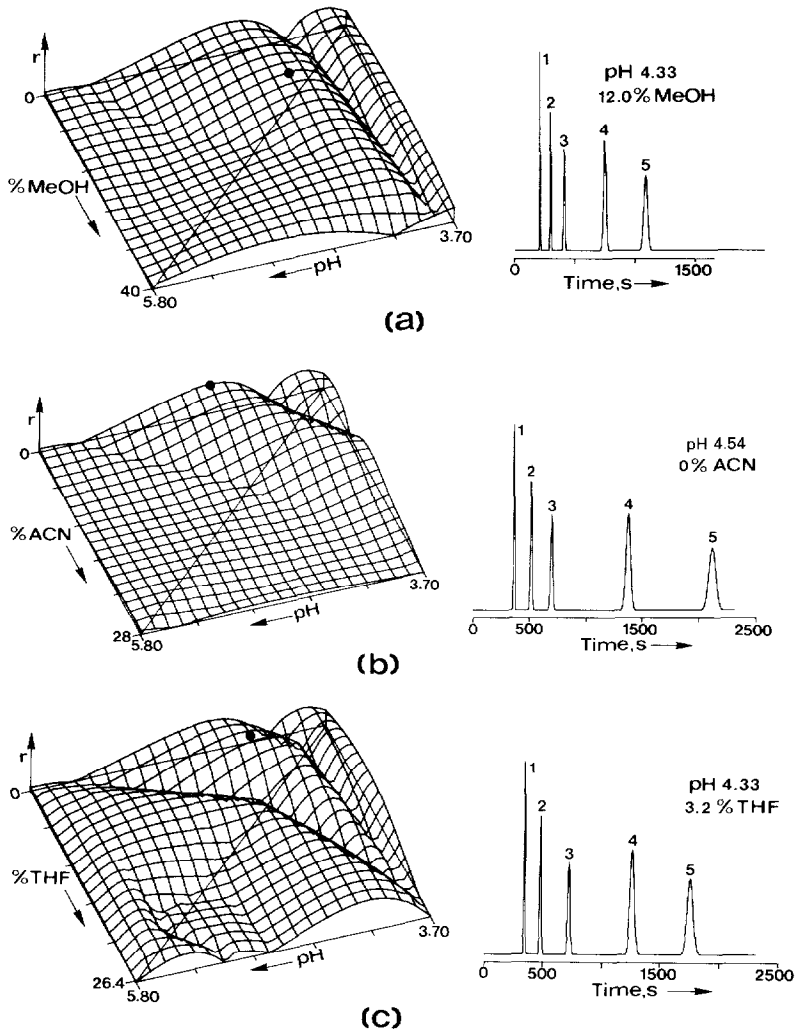

Fig. 5. Combined optimization of mobile phase pH and organic modifier content by means of the relative resolution product criterion (r). Each three-dimensional surface illustrates the variation of r over the indicated mobile phase compositions. Chromatograms obtained with the selected optimal mobile phases (indicated by ●) are also shown. The organic modifiers used were (a) methanol, 0–40.0%; (b) acetonitrile, 0–28.0%; (c) tetrahydrofuran, 0–26.4%. The identities of the solutes are given in Fig. 2.

that arise when an organic modifier is added to the mobile phase. For neutral solutes we have the selective interactions of the modifier with the solutes as is commonly observed in reversed-phase HPLC. For acidic or basic solutes there is also a second selectivity effect from the influence of the modifier on the pK_a values of the buffer and the solutes.

As an organic modifier is progressively added to an aqueous buffer, the apparent pH of the solution rises owing to an increase in the pK_a value of the buffer. This effect is particularly pronounced when the pH of the aqueous solution is close to the pK_a value of the buffer[10]. At the same time, the pK_a values of solutes also increase,

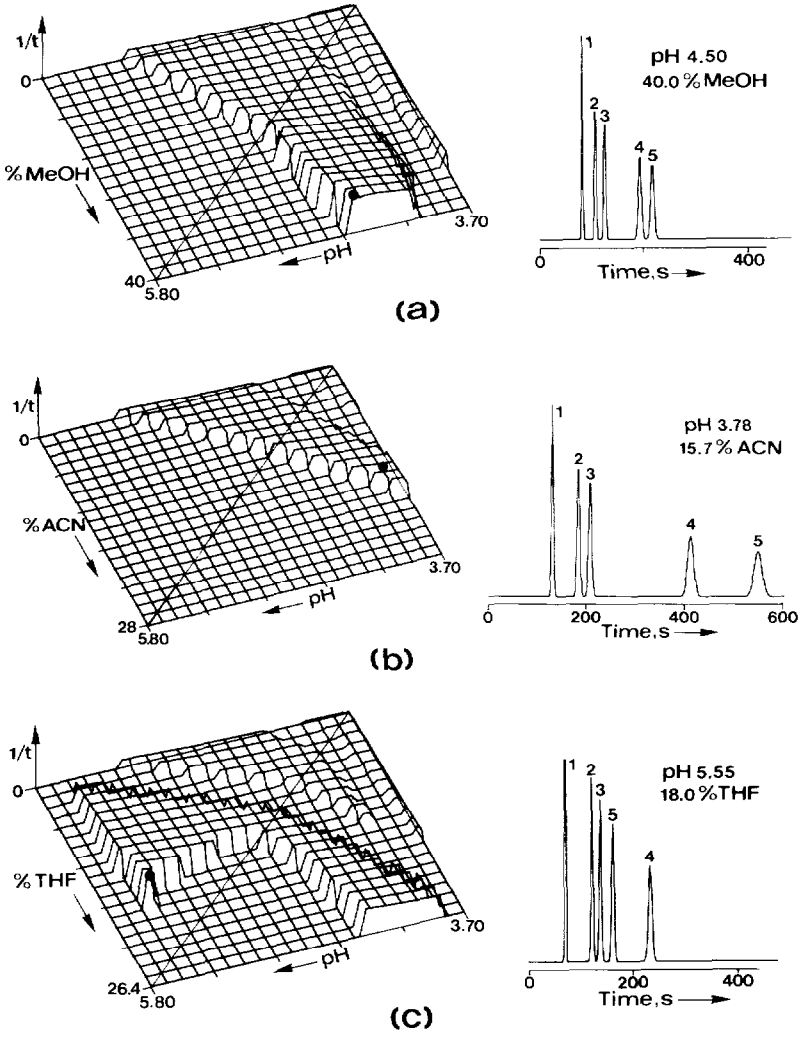

Fig. 6. As for Fig. 5, except that the sequential resolution–time procedure was used and the surface illustrate the variation of $1/t$ (where t is the retention time of the most retained peak). A value of $R_{s,min} \geqslant 2$ was used.

and concomitant changes in retention can be expected.

To illustrate these effects, the organic modifiers methanol (MeOH), acetonitrile (ACN) and tetrahydrofuran (THF) were added to the optimal aqueous mobile phase selected in Fig. 2. A buffer pH of 4.31 was used in conjunction with MeOH concentrations of 20, 30 and 40% (v/v) and the corresponding iso-eluotropic percentages of ACN and THF[17]. In each case, the same buffer was used, and the buffer pH was measured before addition of the modifier. The results are given in Table I.

Table I shows that for the group of aromatic acids and the modifier concentrations studied, the retention order and the resolution remain essentially constant with increasing percentages of MeOH. When MeOH is replaced by ACN, the reten-

tion order still remains the same, but the resolution varies. When THF is used as modifier, the retention order of benzoic acid and 2-aminobenzoic acid is seen to reverse. Selectivity effects are least evident in MeOH, which causes a reduction in the length of the chromatogram without any appreciable change in the resolution criterion used to assess the quality of the chromatogram. This can be attributed to the fact that the pK_a values of the buffer and the acidic solutes increase uniformly in MeOH. Similar behaviour is unlikely to occur for a mixture of solutes with more widely distributed pK_a values than those selected for this study. The modifiers ACN and, particularly, THF give rise to readily observable selectivity effects.

These results suggest that simultaneous optimization of pH and organic modifier concentration is necessary if aqueous organic mobile phases are to be used. This optimization can be accomplished with the factorial design approach with four different pH values, each of which is used with four modifier concentrations, giving a total of sixteen initial chromatograms. A possible alternative is the mixture design approach with the five initial compositions shown in Fig. 4. The basis of the optimization procedure is fundamentally the same as that described previously, except that planar surfaces of $\ln k'$ are constructed over the desired ranges of pH and organic modifier content. Each plane interconnects three experimentally measured retention data points, and these planes are used to calculate the values of selected optimization criteria. The midpoint E in Fig. 4 is included to enable the total optimization area to be subdivided into four initial retention planes, which are subsequently modified by the inclusion of more retention data in the course of optimization. The optimization criterion may be plotted as a three-dimensional surface, for example as shown in Fig. 5, which illustrates the variation of the relative resolution product, r, over the ranges of pH and modifier concentrations indicated in Fig. 4.

The chromatograms shown in Fig. 5 were obtained by using the predicted optimum mobile phase for each modifier. In each case, the optimal composition was finalized after three additional experimental chromatograms, giving a total requirement of eight chromatograms to complete the optimization process. This compares favourably with the requirement of at least sixteen chromatograms for a typical factorial design approach. However, the result obtained, is not entirely satisfactory, since it is clear from Table I that the sample mixture under study can be adequately resolved in a much shorter time than that given for the optimum mobile phase compositions suggested by Fig. 5, particularly when MeOH is used as modifier. It is therefore desirable to use an optimization criterion which considers the duration of the chromatograms as well as the separation characteristics.

This approach was investigated by using the separation criterion ($R_{s,\min} \geqslant 2$) and time criterion ($1/t$, where t is the retention time of the most retained peak) in sequence, as described in the theory section. The results obtained for the combined optimization of pH and organic modifier content are given in Fig. 6 for the modifiers MeOH, ACN and THF. In each diagram, the criterion ($1/t$) is assigned a value of zero over those areas of mobile phase compositions which do not give $R_{s,\min} \geqslant 2$, whereas the raised areas ($1/t \geqslant 0$) show where this condition is met. The height of the raised areas indicates the value of $1/t$, calculated for the chromatogram. The optimum composition is thus indicated by the highest point on each diagram.

Clearly, the optimum chromatograms obtained with each modifier (Fig. 6) are much faster than the equivalent optimum chromatograms obtained by using the

relative resolution product criterion (Fig. 5). The resolution is correspondingly lower, but still adequate for good separations. The three-dimensional surfaces in Fig. 6 indicate that there are relatively broad areas of mobile phase composition in which the time criterion varies only slightly, suggesting that the located optima are not critical. These illustrations are particularly valuable to the chromatographer in that they provide a clear picture of the separation and time characteristics of chromatograms obtained over a broad range of mobile phase compositions.

CONCLUSIONS

This study has demonstrated that mixture design optimization procedures can be successfully applied to both the optimization of mobile phase pH as a single parameter and the combined optimization of pH and organic modifier content. A considerable reduction in the required input of experimental retention data is achieved in the mixture design procedure, compared with the factorial design approach currently favoured for pH optimization. In view of the large changes in retention time that frequently accompany changes in mobile phase pH, it is desirable to include an appropriate time factor in the optimization. A sequential optimization procedure, which uses a separation criterion, followed by a time criterion is useful for this purpose. The methods discussed in this paper are currently being extended to mixtures of solutes with widely varying pK_a values.

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