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COMPUTER-ASSISTED SELECTION OF MOBILE PHASE COMPOSITION IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

DEFINITION OF THE OPTIMISATION SEARCH AREA

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SUMMARY

In order to reduce the time required for successful implementation of computer-assisted methods for the selection of the optimal mobile phase composition in reversed-phase liquid chromatography, it is desirable to limit the range of the mobile phases that are to be considered. This is conventionally achieved by defining an optimisation search area bounded by isoeluotropic binary mobile phases of water with methanol, acetonitrile or tetrahydrofuran. This process requires the use of equations (or "transfer rules") relating the eluotropic strengths of these three organic modifiers.

By reference to the separation of seven amines by reversed-phase ion-interaction chromatography, published transfer rules are shown to be unreliable as a means of calculating isoeluotropic mobile phases. New transfer rules were derived for a small group of structurally related amines, and use of these rules to define the optimisation search area gave improved performance of the optimisation process. Nevertheless, these new transfer rules also showed considerable error in predicting isoeluotropic mobile phases. It is concluded that the optimisation search area is best established empirically, using transfer rules initially to estimate isoeluotropic mobile phase compositions, followed by experimentation to confirm that the mobile phases selected are isoeluotropic. When this approach was applied to the isocratic separation of ten fat-soluble vitamins, excellent results were obtained.

INTRODUCTION

Selection of the appropriate values for such chromatographic parameters as column type, mobile phase composition, flow-rate, temperature, etc., is fundamental to the development of a functional chromatographic method. Usually this selection

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is done on a trial-and-error basis, the success of which is strongly dependent on the experience of the chromatographer involved. An attractive alternative is to employ an interactive computer-based procedure for systematic screening of chromatographic parameters. If these procedures have a sound theoretical foundation, then enormous savings in time and effort can be achieved in the development of chromatographic methods.

Numerous computer-based optimisation procedures exist (see recent reviews[1,2]), and the majority of these seek to exploit selectivity effects resulting from variations in the mobile phase composition. The type of column used and the flow-rate, temperature, etc., are not varied and are arbitrarily selected at the commencement of the optimisation process. Thus for reversed-phase liquid chromatography (RPLC), an octadecylsilyl column operated at room temperature and a flow-rate of 1.0 ml/min are typical conditions. Moreover, optimisation procedures are often confined to selection of the *primary* mobile phase components, *i.e.* the concentrations of organic modifiers in the mobile phase. Secondary mobile phase parameters, such as pH[3], salt concentration and the nature and concentration of ion-interaction reagents[4], can also be optimised but require specialised computer programs.

When only primary phase components are considered, optimisation procedures generally follow the sequence of definition of a suitable range of mobile phase compositions to search, the acquisition of retention data for selected mobile phase compositions, the analysis of these data in terms of possible separations and finally a computer selection of the optimal mobile phase composition. Naturally, many variations to this general theme have been reported[1] and some of these are discussed below. In this paper we concentrate on the initial step of defining the optimisation search area and, by reference to two examples, show that this step is crucial to the success of the optimisation procedure.

THEORY

The range of possible primary mobile phase components is enormous; however, the classification scheme proposed by Snyder[5] enables solvents to be grouped according to such characteristics as proton transfer qualities and dipole interaction properties. In optimisation experiments, it is desirable to maximise selectivity effects resulting from the use of different solvents and, to achieve this, the solvents used as mobile phase additives should be those that show the greatest variation in the relevant characteristics. This consideration, combined with the need for solvents with suitable miscibility, viscosity, UV transparency and chemical stability, has led to the choice of methanol, acetonitrile and tetrahydrofuran as the most appropriate solvents for use as organic modifiers in RPLC. Water is then used to adjust the final eluotropic strength of the mobile phase.

The mobile phase domain created by combining water with any or all of these three organic modifiers can be conveniently represented as a tetrahedron (Fig. 1) where the apices indicate pure solvents. The edges of the figure represent binary solvent mixtures (*e.g.* water plus one modifier), with mobile phase strength decreasing as the lines approach the uppermost apex, that is, pure water. The faces of the tetrahedron represent ternary mobile phase combinations such that any point on these surfaces represents a precise combination of three of the solvents. Quaternary mobile

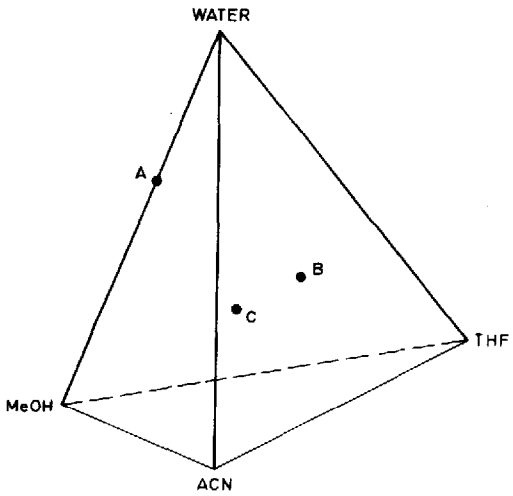

Fig. 1. The domain of mobile phases formed by mixing water, methanol (MeOH), acetonitrile (ACN) and tetrahydrofuran (THF). Examples of specific combinations of solvents are given by A (methanol–water, 40:60), B (acetonitrile–tetrahydrofuran–water, 33:33:33) and C (methanol–acetonitrile–tetrahydrofuran–water, 25:25:25:25). Note that point B lies on a face of the tetrahedron and point C lies within its body.

phases (water plus all three modifiers) are contained within the body of the tetrahedron. Examples of binary, ternary and quaternary mixtures of solvents are given in Fig. 1.

Two choices are available in implementing an optimisation procedure: the entire mobile phase domain can be searched for the optimal mobile phase composition or a smaller search area can be selected. The first approach has the disadvantage of requiring a prohibitive amount of time and experimentation, whereas the second approach succeeds only if the selected search area includes the optimal mobile phase (that is, the "global optimum") or at least a mobile phase that provides satifactory separation. Provided this disadvantage can be overcome, the second approach is very attractive.

Selection of a suitable search area can be initiated by determining the methanol–water binary mixture that gives an adequate degree of retention for all components of the mixture that is to be separated. Conventionally, this binary mobile phase is selected to give capacity factors in the range $1 \leqslant k' \leqslant 10$ for all components, which means that retention does not play a major role in the resolution of solutes[6]. This mobile phase is then converted into isoeluotropic binary mobile phases of ACN or THF with water. This conversion may be done with the aid of "solvent polarity values"[5,7,8] or "transfer rules"[9], with the end result that, on average, a group of solutes would be expected to exhibit similar retention times when each of the isoeluotropic binary mixtures was employed as the mobile phase. Whilst average retention is more or less constant, variations in selectivity can be expected to occur between the different binary mixtures and would result in changes in the elution order of the solutes.

Snyder[5,7] and Glajch *et al.*[8] have reported the following equation for calculating the strength of solvent mixtures:

$$P' = \varphi_A P_A + \varphi_B P_B \quad (1)$$

where P is the chromatographic solvent strength and φ_A and φ_B are the volume fractions of solvents A and B in the solvent mixture. A simple extension of this approach allows the calculation of isoeluotropic binary solvent mixtures.

Schoenmakers *et al.*[9] have reported alternative transfer rules, which they claim to be more reliable than eqn. 1: these are based on the retention behaviour of a large group of compounds with a wide variety of functional groups. These transfer rules are:

$$\varphi_{ACN} = 0.32\varphi_M^2 + 0.57\varphi_M \quad (2)$$

$$\varphi_{THF} = 0.66\varphi_M \quad (3)$$

where φ_M, φ_{ACN} and φ_{THF} are the volume fractions of methanol, acetonitrile and tetrahydrofuran, respectively.

Eqns. 1–3 are attempts to provide generalised values for the eluotropic strength of the three common organic modifiers used in RPLC. Some variation in eluotropic strength towards particular solutes must be expected; however, if these transfer rules are sufficiently reliable, they may be used to define the search area in computer optimisation methods. Eqns. 2 and 3 have been employed for this purpose[10].

Once the three isoeluotropic binary mobile phases have been calculated, they can be used to form the boundaries of the optimisation search area, as shown in Fig. 2. The lines connecting the binary solutions represent isoeluotropic ternary mixtures over which the optimisation procedure will search.

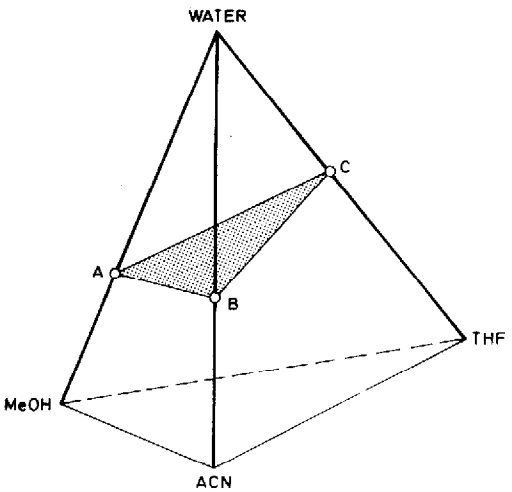

Fig. 2. Definition of the optimisation search area. In the example shown, point A is selected so that all solutes elute in the retention range $1 \leqslant k' \leqslant 10$. Points B and C are calculated to be isoeluotropic with point A. The search area of ternary mobile phases is defined by the lines AB, BC and CA, and quaternary mobile phases in the search area are indicated by the hatched portion of the diagram.

EXPERIMENTAL

Instrumentation

The liquid chromatograph used in this work consisted of a Waters Assoc. (Milford, MA, U.S.A.) Model M6000A pump, Model U6K injector, Model M441 UV detector operated at 254 nm and Model M730 data module. For the experiments on the fat-soluble vitamins, the detector was replaced by a Waters Assoc. Model M490 variable-wavelength detector. A Waters Assoc. C_{18} Nova-Pak column (150 × 3.9 mm I.D.) was employed.

The basic computing unit used was an Apple II-plus microcomputer (Apple Computer, Cupertino, CA, U.S.A.), fitted with twin floppy disk drives and containing a Digicard 80-column expansion card (Maclagan Wright and Associates, Eltham, Australia). This unit was interfaced to a BMC Model BX80 dot matrix printer (BMC International, Japan) and a Hewlett-Packard (San Diego, CA, U.S.A.) Model 7407A dual pen plotter.

The purity of the sympathomimetic amines was confirmed using a Finnigan Model 3200 gas chromatograph–chemical ionisation mass spectrometer interfaced to a 2300 Incos data system supplied by the same manufacturer. The column used was 1.5 m × 2 mm I.D., packed with 3% OV-1 or 3% OV-101 on Gas Chrom Q, 100–120 mesh.

Reagents

Chromatographic grade solvents were used for the preparation of mobile phases. The desired amounts of organic modifiers and water were measured by burette and the resulting solution mixed thoroughly, filtered through a 0.45-μm membrane filter and degassed in an ultrasonic bath before use. For the separation of sympathomimetic amines, the mobile phases contained 5 m*M* sodium heptanesulphonate (Ajax Chemicals, Sydney, Australia) and 1% acetic acid. All mobile phases were prepared freshly as required.

2-Phenethylamine hydrochloride, phenylpropanolamine hydrochloride and 2-hydroxy-2-phenethylamine hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.). Ephedrine hydrochloride, pseudoephedrine and N-methylephedrine were obtained from Fluka (Buchs, Switzerland), and amphetamine sulphate from U.S.V. Pharmaceuticals (Sydney, Australia). N-Methyl-2-phenethylamine hydrochloride, N,N-dimethyl-2-phenethylamine hydrochloride, N-*n*-butyl-2-phenethylamine hydrochloride and 2,2′-diphenylethylamine hydrochloride were synthesised using adaptations of previously reported methods[11–13]. The purity of these materials was confirmed using gas chromatography–mass spectrometry after appropriate derivatisation with pentafluoropropionic anhydride.

The fat-soluble vitamins were obtained as pure compounds from Fluka.

Optimisation procedure

The optimisation method used was based on the iterative procedure reported by Schoenmakers and co-workers[10,14]. In this procedure, retention data obtained for three isoeluotropic binary mobile phases are used to predict retention times for the ternary solvent mixtures formed from linear combinations of the binary mobile phases, *e.g.* those ternary mixtures represented by the edges of the triangle ABC in Fig.

TABLE I

RETENTION DATA FOR OPTIMISATION OF THE SEPARATION OF SEVEN AMINES USING A SEARCH AREA DEFINED BY EQNS. 2 AND 3.

The first three mobile phases shown are the binary compositions used to define the optimisation search area and the remaining mobile phases are those predicted in successive iterations of the optimisation procedure. The final mobile phase is optimal. All mobile phases contained 5 m*M* heptanesulphonate and 1% acetic acid. Solutes: PPA = phenylpropanolamine; PEA = phenethylamine; MePEA = N-methyl-2-phenethylamine; AMPH = amphetamine; diMePEA = N,N-dimethyl-2-phenethylamine; BuPEA = N-*n*-butyl-2-phenethylamine; diPEA = 2,2′-diphenylethylamine.

Mobile phase composition (%)*				*Retention time (min)*						
Methanol	*Aceto-nitrile*	*Tetra-hydrofuran*	*Water*	*PPA*	*PEA*	*MePEA*	*AMPH*	*diMePEA*	*BuPEA*	*diPEA*
50.0	0	0	50.0	2.13	2.23	2.20	2.70	2.10	4.10	7.25
0	36.5	0	63.5	1.03	1.10	1.16	1.14	1.23	1.86	1.84
0	0	33.0	67.0	1.19	1.19	1.13	1.24	1.10	1.50	1.92
22.5	18.1	0	59.4	2.18	2.30	2.42	2.86	2.46	5.35	8.96
15.0	23.9	0	60.1	1.67	1.81	1.89	2.06	1.98	4.08	5.33
17.5	21.9	0	60.6	1.78	1.94	2.04	2.26	2.08	4.52	6.20
15.0	23.9	0	60.1	1.67	1.81	1.89	2.06	1.98	4.08	5.33

2. All possible chromatograms within the mobile phase search area are then assessed on the basis of a relative resolution product criterion[13] and the optimal mobile phase is selected. Retention data for this mobile phase are then measured and added to the data file in the computer. The calculation of the resolution product criterion is then repeated and a new optimal mobile phase selected. This process continues until the same optimum is selected in successive calculations or a previously measured mobile phase composition is assigned to be the optimum. Full details of the operational procedure and theoretical basis of this method are given elsewhere[10,14,15].

RESULTS AND DISCUSSION

Separation of sympathomimetic amines

The primary aim of this study was to investigate the validity of general solvent transfer rules for definition of the range of mobile phase compositions comprising the search area in computer optimisation techniques. The first example chosen was the separation of a group of seven amines using ion-interaction chromatography. In addition to the organic modifiers employed, it should be noted that all mobile phases used in this example contained 5 m*M* heptanesulphonate as the ion-interaction reagent and 1% acetic acid. No attempt was made to optimise the nature or concentration of this ion-interaction reagent; rather, the optimisation procedure was directed solely towards selection of the types and concentrations of organic modifiers comprising ternary mobile phases. The optimisation procedure was initiated by adjusting the strength of a methanol–water binary mobile phase until all solutes eluted with capacity factors in the range $1 \leqslant k' \leqslant 10$. A methanol–water (50:50) mixture was found to be suitable for this purpose.

The transfer rules proposed by Schoenmakers *et al.*[9] (eqns. 2 and 3) were then used to calculate isoeluotropic binary mobile phases of acetonitrile and tetrahydrofuran with water, namely acetonitrile–water (35.5:63.5) and tetrahydrofuran–water (33:67). These three binary mixtures formed the boundaries of the search area over which the optimisation procedure was carried out. The retention data obtained at each stage of the optimisation are given in Table I, and a chromatogram recorded using the optimal mobile phase (determined after four iterations of the optimisation procedure) is shown in Fig. 3. It is clear that the optimal separation was unsatisfactory, which suggests either that the desired separation is unattainable with ternary mixtures of water and methanol, acetonitrile or tetrahydrofuran, or that the search area has been incorrectly defined. The latter situation can arise if the transfer rules used are not valid for the particular compounds used, and the disparity in retention times obtained with the three binary mobile phases in Table I shows that this is indeed the case. It is important to note that the inadequacy of the predicted optimal mobile phase does not result from any deficiency in the optimisation procedure itself, since experience in this laboratory has shown that this procedure gives excellent results. The problem lies in the search area selected, since it is evident from Table I that the retention times are too short to permit adequate separation of the mixture.

In order to test whether the use of alternative transfer rules would improve the performance of the optimisation procedure, extensive retention data were acquired for a series of amines using binary mobile phases of water with methanol, acetonitrile or tetrahydrofuran. These data were obtained using ten binary mobile phase com-

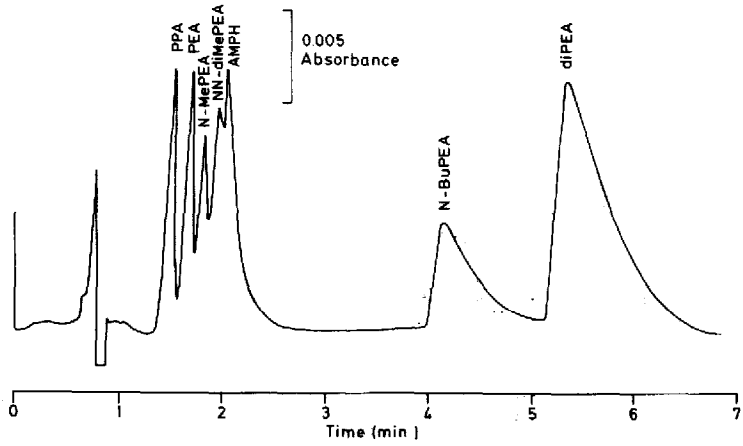

Fig. 3. Optimal chromatogram for seven amines when the optimisation search area was calculated from eqns. 2 and 3. Conditions: column, Waters Assoc. C_{18} Nova Pak (150 × 3.9 mm I.D.); mobile phase, methanol–acetonitrile–water (15.0:23.9:61.1) containing 5 m*M* heptanesulphonate and 1% acetic acid; flow-rate, 1.3 ml/min; detection, UV absorption at 254 nm. Solutes: PPA = phenylpropanolamine; PEA = phenethylamine; N-MePEA = N-methylphenethylamine; N,N-diMePEA = N,N-dimethylphenethylamine; AMPH = amphetamine; N-BuPEA = N-butylphenethylamine; diPEA = 2,2-diphenylethylamine.

positions for each modifier, covering the range 10–90% modifier. For each solute, the range of mobile phase compositions giving capacity factors between one and ten were fitted to a second-order polynomial equation, and these equations were then used to derive transfer rules for the solutes studied. The amines chosen included those listed in Table I, together with several structural analogues, namely 2-hydroxy-2-phenethylamine, ephedrine, pseudoephedrine and N-methylephedrine. The derived transfer rules are shown below:

$$\varphi_{ACN} = 0.90\varphi_M^2 - 0.21\varphi_M + 0.126 \quad (4)$$

$$\varphi_{THF} = 0.66\varphi_M - 0.15 \quad (5)$$

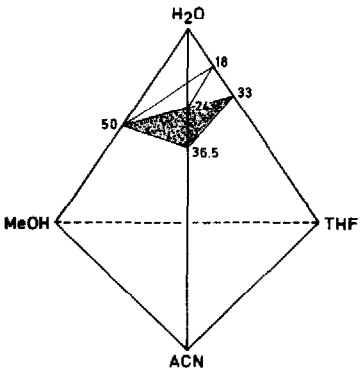

Fig. 4. Illustration of the search areas calculated from eqns. 2 and 3 (shaded triangle) and eqns. 4 and 5 (open triangle) in the optimisation of the separation of seven amines.

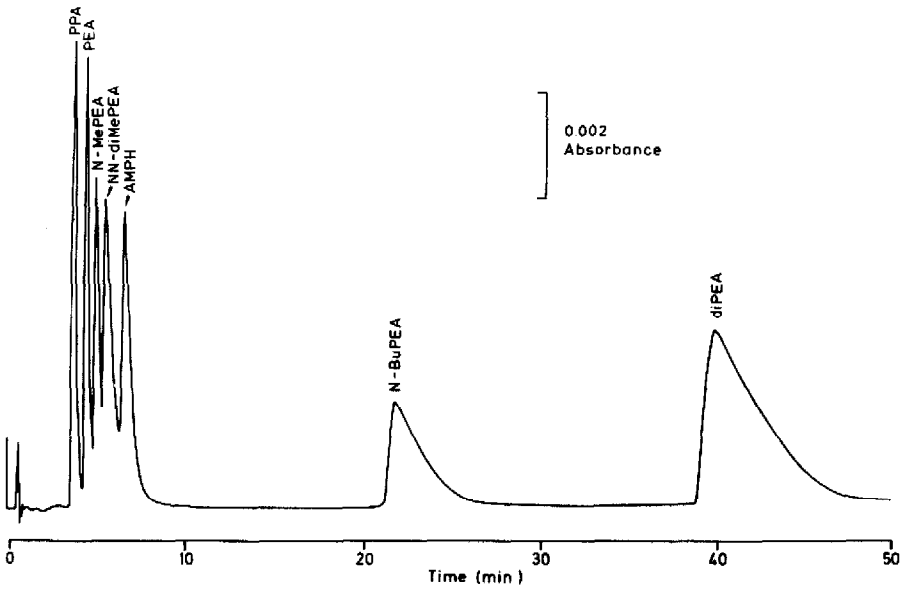

Fig. 5. Optimal chromatogram for seven amines when the optimisation search area was calculated from eqns. 4 and 5. Conditions: mobile phase, methanol–acetonitrile–water (12.5:17.4:70.1) containing 5 m*M* heptanesulphonate and 1% acetic acid. Other conditions as for Fig. 3.

Using eqns. 4 and 5, the optimisation search area was calculated to be defined by the binary solvent mixtures methanol–water (50:50), acetonitrile–water (24:76) and tetrahydrofuran–water (18:82). Fig. 4 illustrates this search area and, for comparison, also shows the search area calculated from eqns. 2 and 3 that was used for the optimisation above. Retention data for the binary mobile phases that define the new search area are given in Table II, together with those obtained for subsequent stages

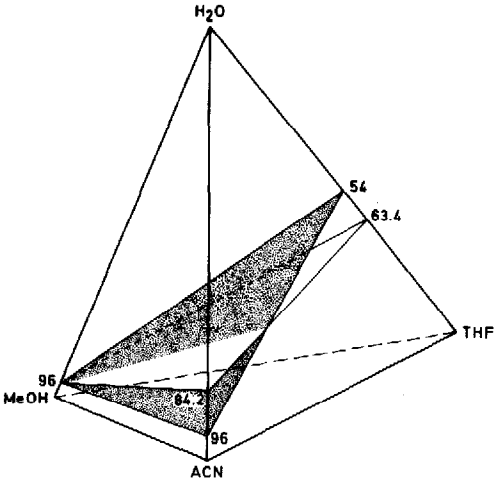

Fig. 6. Illustration of the search areas calculated from eqns. 2 and 3 (open triangle) and that obtained empirically (shaded triangle) in the optimisation of the separation of ten fat-soluble vitamins.

TABLE II

RETENTION DATA FOR OPTIMISATION OF THE SEPARATION OF SEVEN AMINES USING A SEARCH AREA DEFINED BY EQNS. 4 AND 5

All conditions as for Table I.

Mobile phase composition (%)				*Retention time (min)*						
Methanol	*Acetonitrile*	*Tetra-hydrofuran*	*Water*	*PPA*	*PEA*	*MePEA*	*AMPH*	*diMePEA*	*BuPEA*	*diPEA*
50.0	0	0	50.0	2.13	2.23	2.20	2.70	2.10	4.10	7.25
0	24.0	0	76.0	2.30	2.66	2.96	3.30	3.40	10.03	15.76
0	0	18.0	82.0	3.34	3.40	3.14	4.19	2.91	6.42	20.75
17.5	15.3	0	67.2	3.70	4.16	4.45	5.63	4.71	16.19	29.24
12.5	17.4	0	70.1	4.23	4.90	5.37	6.84	5.83	21.80	39.95
10.0	18.6	0	71.4	3.50	3.92	4.32	5.36	4.75	16.58	29.30
12.5	17.4	0	70.1	4.23	4.90	5.37	6.84	5.83	21.80	39.95

TABLE III

RETENTION DATA FOR OPTIMISATION OF THE SEPARATION OF TEN FAT-SOLUBLE VITAMINS

Mobile phases A, B and C are the binary compositions for the optimisation search area defined by eqns. 2 and 3. Mobile phases D, E and F are experimentally determined isoeluotropic binary compositions used to define the optimisation search area.

Solute	*Retention time (min)*					
	Methanol–water (96:4) (A)	*Acetonitrile–water (84.2:15.8) (B)*	*Tetrahydrofuran–water (63.4:36.6) (C)*	*Methanol–water (96:4) (D)*	*Acetonitrile–water (96:4) (E)*	*Tetrahydrofuran–water (54:46) (F)*
Vitamin K_3	0.96	1.20	0.80	0.96	0.92	1.16
Vitamin A alcohol	1.57	2.32	1.17	1.57	1.67	2.00
Vitamin A acetate	2.30	3.76	1.57	2.30	2.10	3.68
δ-Tocopherol	3.80	12.31	1.87	3.80	5.15	5.94
Vitamin D_2	4.15	13.65	1.58	4.15	6.60	3.95
Vitamin D_3	4.38	14.47	1.58	4.38	7.00	3.95
γ-Tocopherol	4.56	16.07	2.09	4.56	6.22	7.19
Vitamin E	5.44	19.29	2.34	5.44	7.69	8.62
Vitamin E acetate	8.17	32.60	2.68	8.17	9.79	10.59
Vitamin K_1	11.15	38.26	2.82	11.15	11.87	11.64

of the optimisation procedure. The chromatogram resulting when the predicted optimal mobile phase was used is shown in Fig. 5.

Comparison of Figs. 3 and 5 shows that the new search area yielded a better optimal mobile phase (Fig. 5), and this is directly attributable to the fact that this search area contained mobile phase compositions that provided a sufficient degree of retention to permit resolution of the early eluting group of solutes. This result attests to the importance of correct selection of the optimisation search area. Nevertheless, close scrutiny of Table II shows that the binary mobile phases used to define the search area were not isoeluotropic, despite the fact that the transfer rules used for their calculation were derived from retention data for a small group of structurally related compounds, which actually included the solutes under study. This highlights the difficulty in deriving transfer rules that are in any way generally applicable.

Some comment on the peak shapes evident in Figs. 3 and 5 is pertinent. The peaks are very tailed owing to the well-documented interaction between the amine functionality of the solutes and the residual silanol groups on the reversed-phase column. If normal procedures had been followed, this tailing would have been reduced prior to the commencement of the optimisation process through the use of an amine modifier (such as triethylamine) in the mobile phase. However, this was not done in the present study because the results obtained were intended for future use in an examination of the effect of peak distortion on the efficacy of optimisation procedures.

Separation of fat-soluble vitamins

The preceding discussion has indicated the importance of correct definition of the optimisation search area; however, the approach of calculating valid transfer rules for each type of solute is clearly impractical. This suggests that the search area would best be defined in an empirical manner, so that only truly isoeluotropic mobile phases are evaluated in the optimisation procedure. This, in turn, would ensure that the optimisation was based only on selectivity effects and was not influenced by significant changes in retention between mobile phases.

To investigate this approach, the separation of ten fat-soluble vitamins was studied. A methanol–water (96:4) binary mixture was found to give suitable retention behaviour for all solutes, and after additional trial-and-error experimentation, isoeluotropic binary mixtures of water with acetonitrile or tetrahydrofuran were found to be acetonitrile–water (96:4) and tetrahydrofuran–water (54:46). The search area defined by these binary mixtures is shown in Fig. 6, which also shows the search area that would have resulted had eqns. 2 and 3 been applied to the methanol–water (96:4) mixture. Table III lists retention data obtained with each of these binary mobile phases and, for comparison, also shows retention data for mobile phases calculated from eqns. 2 and 3 (*i.e.* mobile phases A, B and C in Table III). Once again it can be seen that these transfer rules have not been successful in identifying isoeluotropic binary mobile phases and that an optimisation using a search area defined by these transfer rules would be unlikely to succeed.

When the empirically determined isoeluotropic binary mixtures (*i.e.* D, E and F in Table III) were used to define the optimisation search area, four iterations of the optimisation procedure were required to locate the optimal mobile phase; the chromatogram obtained is shown in Fig. 7.

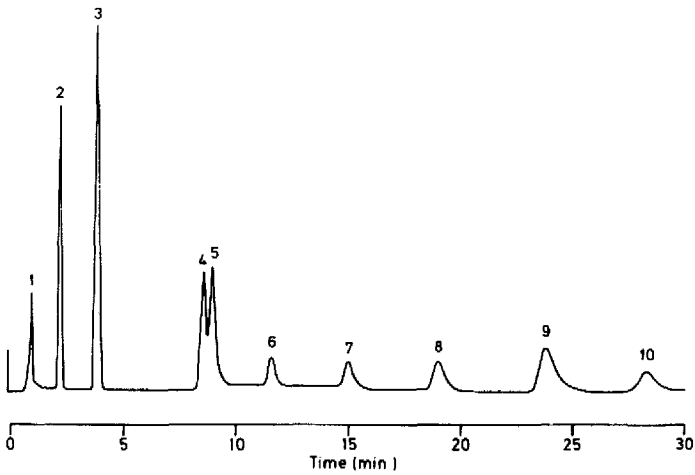

Fig. 7. Optimal chromatogram for ten fat-soluble vitamins when the optimisation search area was defined empirically. Conditions: mobile phase, acetonitrile–tetrahydrofuran–water (43.2:29.7:27.1) flow-rate, 1.5 ml/min; column oven temperature, 40°C. Peaks: 1 = vitamin K_3; 2 = vitamin A alcohol; 3 = vitamin A acetate; 4 = vitamin D_2; 5 = vitamin D_3; 6 = δ-tocopherol; 7 = γ-tocopherol; 8 = vitamin E; 9 = vitamin E acetate; 10 = vitamin K_1.

CONCLUSIONS

This study has shown that when a limited range of mobile phase compositions is to be defined in order to reduce the time and experimentation required to optimise a separation, the success of the optimisation procedure depends strongly on the correct choice of the search area. Transfer rules derived from retention behaviour of a limited set of solutes are not generally applicable for this task because of their inability to reliably convert a given methanol–water binary mixture into isoeluotropic acetonitrile–water and tetrahydrofuran–water binary mixtures. Rather, this conversion is best performed empirically, with transfer rules being used initially to estimate the compositions of isoeluotropic binary mobile phases, followed by subsequent experimentation to finalise the correct mobile phase compositions. Such an approach has been suggested previously[16], and the present study confirms the validity of this method. When the search area comprises only isoeluotropic mobile phases, retention changes do not play a major part in the optimisation process and only selectivity effects are exploited.

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