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SELECTION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS IN PHARMACEUTICAL ANALYSIS

I. OPTIMIZATION FOR SELECTIVITY IN REVERSED-PHASE CHROMATOGRAPHY

M. GAZDAG, G. SZEPESI* and E. SZELECZKI
Chemical Works of Gedeon Richter, Ltd., P.O. Box 27, 1475 Budapest 10 (Hungary)
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SUMMARY

The application of solvent optimization to the development of isocratic reversed-phase liquid chromatography has been reported in several publications. Two different approaches to solvent optimization for controlling band spacing for the maximum resolution of samples are "solvent strength" and "solvent type" optimization. To improve the separation selectivity further the combination of these two approaches was examined, as a (global) optimum mobile phase composition requires the optimization of the solvent strength by varying the percentage of organic component and of the solvent selectivity of methanol, acetonitrile, tetrahydrofuran and water. It was found that the combination of "solvent strength" and "solvent type" optimization provides a markedly better separation than either procedure alone.

INTRODUCTION

When a new analytical problem has to be solved and insufficient chromatographic information is available about the separation characteristics of the sample, the analyst is working "in the dark". As we must be sure that both unknown and known sample components are separated in the final procedure, two high-performance liquid chromatographic (HPLC) systems (*e.g.*, reversed-phase and normal-phase) should be developed in order to minimize the possibility of band overlap and failure to recognize the presence of unknown species. The initial eluent compositions in both systems can be chosen either on the basis of sample characterization (our work in this field will be published elsewhere) or by the application of gradient elution using one or more gradient runs to predict isocratic conditions from the retention data obtained by gradient elution. Several such appoaches have been published by Berridge[1], Schoenmakers *et al.*[2], Quarry and co-workers[3,4] and Molnár[5].

The next step in the experiments is the optimization of the mobile phase composition in order to improve the selectivity of separations in both reversed- and normal-phase systems. In this paper the optimization of reversed-phase systems is discussed, and Part II[6] will consider normal-phase chromatography.

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Several studies have been reported on the optimization of reversed-phase systems in which the organic solvents used in the mobile phase are varied, *e.g.*, methanol, acetonitrile, tetrahydrofuran (THF) and water. The methods have been well reviewed by Berridge[1] and Schoenmakers[7].

Among the methods applicable to the optimization of reversed-phase systems, two approaches can be distinguished. The first type is the "iterative lattice method", developed by Schoenmakers *et al.*[8], in which the sample resolution is expressed as a function of the composition of the mobile phase prepared from two isoelutropic mixtures from methanol–water, acetonitrile–water and THF–water. This procedure is a typical "solvent-type optimization" introduced originally Glajch and Kirkland[9], based on the almost constant elutropic strength of the mobile phase during an experiment. However, such an approach often requires a large number of experimental runs.

An alternative approach, introduced by Quarry *et al.*[4] for optimizing band spacing, is based on the variation of the solvent strength (organic solvent concentration in the mobile phase). Although this procedure is less powerful, it is simpler and faster than the iterative lattice method, requiring fewer experimental runs, and can lead to significant changes in band spacing for many samples[10].

In this paper, the combination of "solvent type" and "solvent strength" optimization is introduced, providing better separations than either procedure alone.

EXPERIMENTAL

A liquid chromatograph (HP1090A gradient system from Hewlett-Packard, F.R.G.) equipped with an autosampler, an HP 1040A photodiode array detector, an HP 85B personal computer, a disc drive and an HP 3392A electronic integrator (all from Hewlett-Packard) was used.

The separations were performed on a prepacked 250 × 4.6 mm I.D. column of Nucleosil C_{18} (10 μm). Eluents were prepared from HPLC-grade solvents (acetonitrile, methanol, THF) and used at a flow-rate of 1 ml/min. Compounds were detected at 240 nm.

Steroids were used as models for the experiments. They are listed in Table I and were prepared at the Chemical Works of Gedeon Richter (Budapest, Hungary) to USP XXI[11] quality.

TABLE I

COMPOUNDS USED IN THE EXPERIMENTS

Compound	*Abbreviation*
Norethindrone	N
Ethinylestradiol	E
Norgestrel	NG
Estrone	EO
Norethindrone acetate	NAC
Mestranol	M

RESULTS AND DISCUSSION

Optimization criteria

Based on the results obtained in the initial isocratic runs, the strategy for further investigation can be selected. As our subsequent experiments were focused on the improvement of the selectivity of separation, the following optimization criteria were established:

(a) Minimum value of the resolution ($R_{s,\min}$) obtained for the worst separated peak pairs appearing at any position on the chromatogram.

(b) Minimum value of the normalized resolution ($D_{\min}$). The term normalized resolution ($D_{\min}$) is similar to the meaning of "relative resolution" used by Quarry *et al.*[4] for calculating the resolution for a 10 000-plate column. Normalized resolution is calculated from the well known resolution equation:

$$R_s = 0.25 \left(\frac{k'_2}{k'_2 + 1}\right)\left(\frac{\alpha - 1}{\alpha}\right) N^{1/2} = 0.25 \cdot N^{1/2} D_{\min} \tag{1}$$

$$D_{\min} = \frac{k'_2}{k'_2 + 1} \cdot \frac{\alpha - 1}{\alpha} = \frac{k'_2 - k'_1}{k'_2 + 1} \tag{2}$$

The normalized resolution ($D_{\min}$) is directly proportional to $R_{s,\min}$; the only difference is its independence of the column efficiency (plate number, N). For this reason, $D_{\min}$ ignores differences between N values for different bands, and therefore $R_{s,\min}$ measures the actual resolution, corrected for the variation of N from band to band. (Further symbols in eqns. 1 and 2: k' = capacity factor; α = separation factor).

For the same mobile phase composition, R_s will be proportional to the column plate number and therefore it can be increased by increasing N (*e.g.*, by increasing the column length or decreasing the flow-rate).

The use of $D_{\min}$ as an optimization criterion leads to further information: when R and D do not show the same optimum, $D_{\min}$ shows an optimum with respect to the best solvent selectivity and $R_{s,\min}$ indicates where the best separation efficiency via increased column efficiency should be obtained. In this instance the next eluent composition is selected according to the optimum shown by the D value.

A knowledge of $D_{\min}$ also provides information when more optima are found, but both $R_{s,\min}$ and $D_{\min}$ indicate the same optima. When the $R_{s,\min}$ values are almost identical, the eluent composition for the next experiment can be selected on the basis of a higher $D_{\min}$ value. Naturally in this instance when the $R_{s,\min}$ values differ from each other the next eluent composition is selected at its highest value, independent of the actual values of D at the same optima.

Assuming an average column efficiency (16 000 plates/m), the optimal value of D is about 0.080–0.150, equivalent to a range of R_s of about 1.25–2.4.

(c) R_s values measured between the main component and compounds eluting most closely to it (R_{sb} and R_{sa}).

These criteria (their calculation can be seen in Fig. 1) are significant in trace analysis, because a value of $R_{s,\min}$ of 1.0 would be sufficient for the separation of two

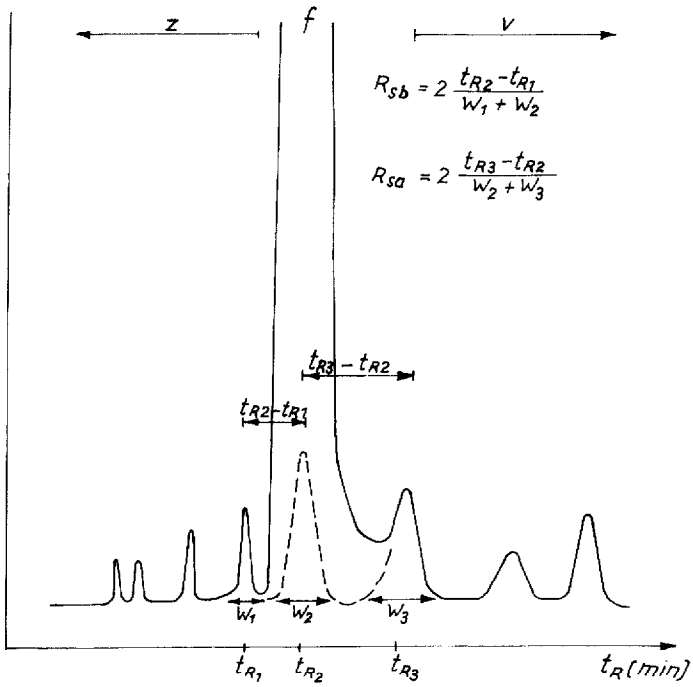

Fig. 1. Calculation of R_{sb} and R_{sa}. f = Main component; z = peaks eluting before the main component; v = peaks eluting after the main component. t_R = Retention time; W = peak width.

impurities present at similar concentrations, but would be unsuitable for separating compounds differing substantially in concentration.

R_{sb} and R_{sa} values are especially important when comparing HPLC systems with respect to their power and performance, as will be discussed in Part III[13].

Optimization for selectivity in reversed-phase systems

The combination of "solvent strength"[4] and "solvent type"[8] optimization was chosen in order to optimize the separation system using the model mixture of steroids indicated in Table I. For the calculation of R_{sb} and R_{sa}, norgestrel (NG) was selected as the main component and the others as trace components.

First, "solvent strength" optimization[4] was considered, studying the variation of resolution with percentage of organic component in methanol–water, acetonitrile–water and THF–water eluents. Retention data were plotted against volume fraction of organic solvent for the methanol–water eluent and from the window diagram its optimal composition was determined as shown in Fig. 2.

Fig. 2 also shows the chromatogram with methanol–water as eluent obtained for the model compounds. The values of R_{sb} and R_{sa} are adequate, but low values of $R_{s,min}$ and D_{min} were obtained, indicating the unsatisfactory selectivity with methanol as organic modifier in the eluent.

In the next experiment, the starting composition of acetonitrile–water was calculated according to Schoenmakers *et al.*[2] from the proportions of methanol and

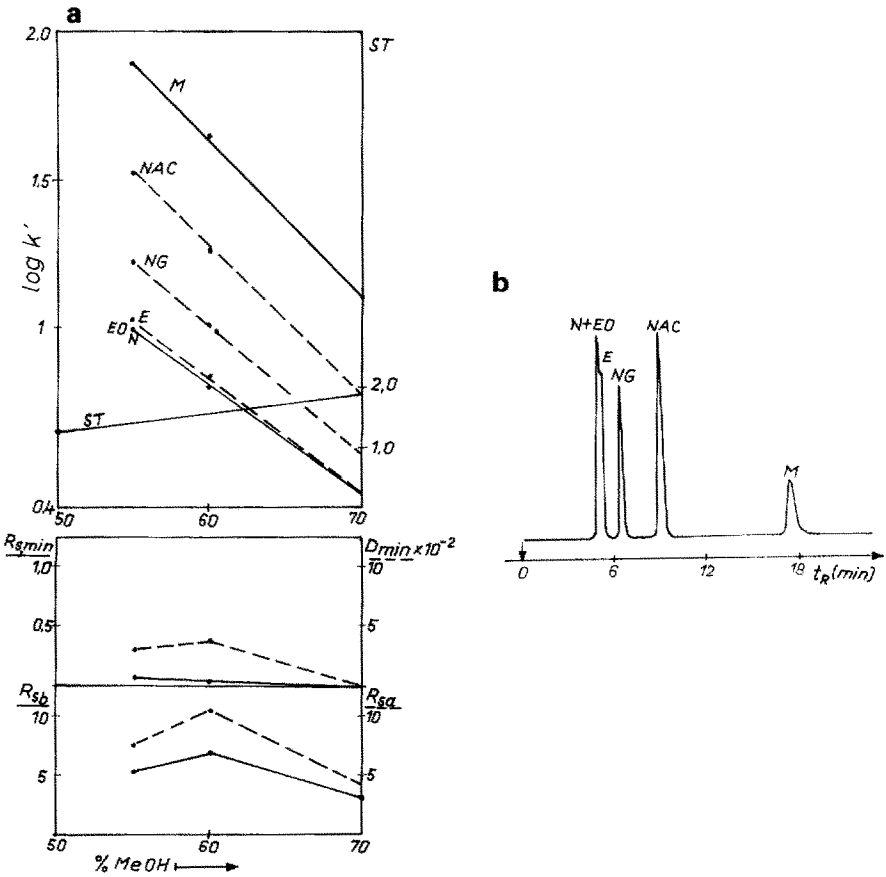

Fig. 2. Plots of log k' *vs.* volume fraction of methanol (MeOH) in water to illustrate the resolution of the steroid samples. (a) Window diagram; (b) chromatogram of model mixture (methanol–water, 7:3). Column, Nucleosil 10 C_{18} (250 × 4.6 mm I.D.); flow-rate, 1 ml/min; detection at 240 nm. Compounds as in Table I. Solid lines, $R_{s,min}/R_{sb}$; broken lines, D_{min}/R_{sa}.

water found to be optimal. (According to our findings, no significant differences in the eluent compositions were obtained when the calculations were performed using either the method published by Snyder *et al.*[12] or that of Schoenmaker *et al.*[2], but when using the iterative lattice method we wanted to follow the method described by Schoenmakers *et al.*[8] so all calculations were performed according to their procedure.)

The results of the experiments are shown in Fig. 3. The values of R_{sb} and R_{sa} are acceptable (NG is well separated from EO and NAC) and better values of $R_{s,min}$ and D_{min} were obtained than with methanol–water as the eluent, but the latter were not adequate for a perfect separation.

Similar experiments were carried out using THF–water eluents, the starting composition being calculated from the optimal acetonitrile–water ratio. The results are shown in Fig. 4.

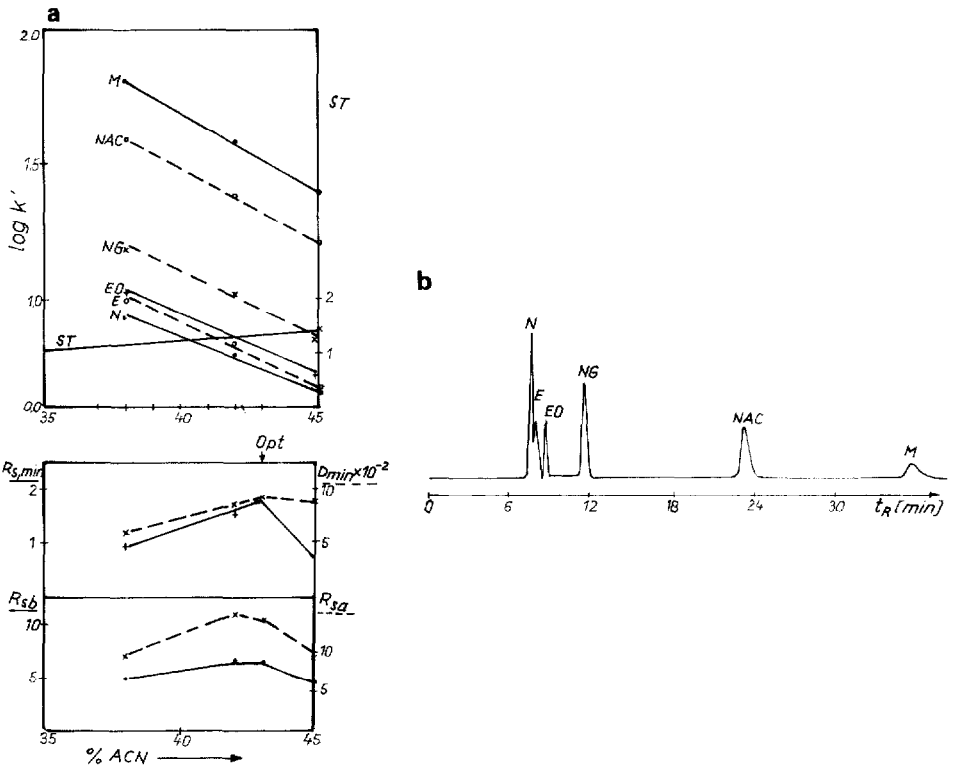

Fig. 3. Plots of log k' *vs.* volume fraction of acetonitrile (ACN) in water. (a) Window diagram; (b) chromatogram of model mixture (acetonitrile–water, 45:55). Details as in Fig. 2.

From the retention data and from the chromatogram obtained with the optimal THF–water system (Fig. 4), it can be concluded that this system provides the best separation of the steroid mixture, giving acceptable values of $R_{s,min}$ and D_{min}. However, a significant decrease in R_{sb} and R_{sa} was observed, resulting in a system suitable for the separation of steroid compounds present at similar concentrations but insufficient for trace analysis when the separation of impurities has to be performed in the presence of large amounts of the main component (NG).

From the data in Figs. 2–4 it can also be concluded that by using "solvent strength" optimization as described by Quarry *et al.*[4], variations in the solvent strength result in data points close to a straight line and, as a consequence of the different slopes obtained for the different steroid compounds, the relative band spacing can be altered and improved. The improvement in band spacing is a function of the type of solvents, and acetonitrile–water (43:57, system A) and THF–water (32:68, system B) were selected for further experiments as the best two-component eluents. With respect to suitable values of R_{sb} and R_{sa} system A and for $R_{s,min}$ and D_{min} system B provide the best possibilities.

The experiments were continued using the iterative lattice method but, in contrast to the original work of Schoenmakers *et al.*[8], "solvent type" optimization

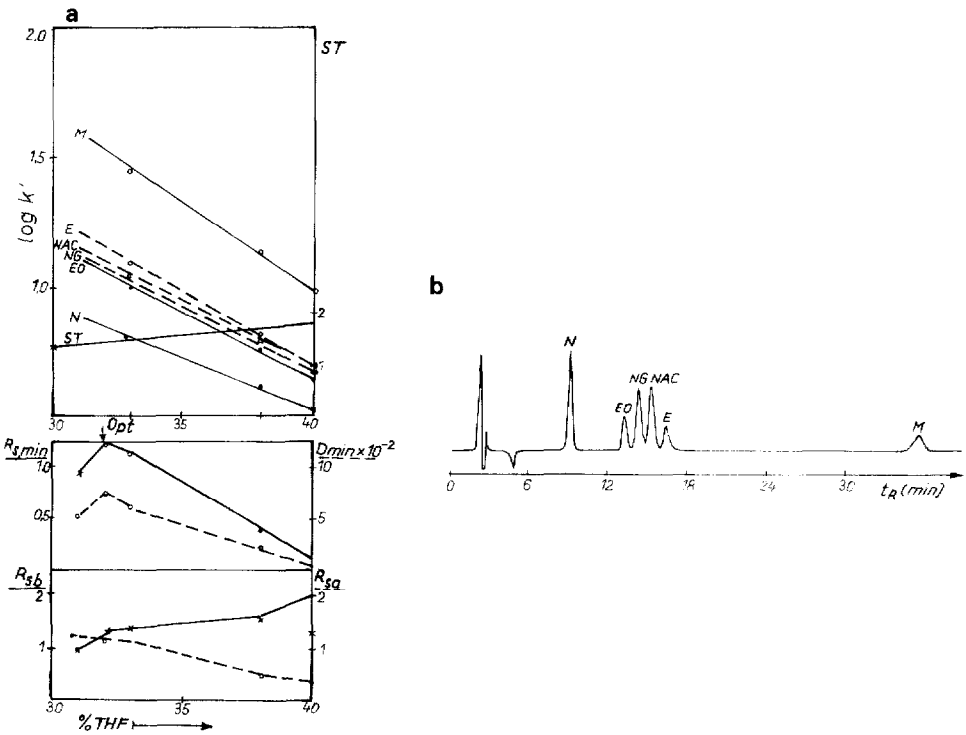

Fig. 4. Plots of log k' *vs.* volume fraction of tetrahydrofuran in water. (a) Window diagram; (b) chromatogram of model mixture (THF–water, 33:67). Details as in Fig. 2.

with mixtures of non-isoelutropic eluents was applied. The optimal composition for the initial three-component eluent mixture can be selected from the window diagram shown in Fig. 5 [A–B (45:55)].

The chromatogram obtained with this mobile phase composition is shown in Fig. 6. As can be seen, the separation is better but the R_{sa} value between NG and E is not satisfactory, that is, the predictions of Fig. 5 differ from the chromatogram shown in Fig. 6. The window diagram was corrected using the retention data obtained in this experiment and the corrected diagram is also shown in Fig. 6.

Based on the data for the corrected window diagram, the new optimum indicating the next eluent composition [A–B (25:75)] was determined. The chromatogram obtained with this eluent is shown in Fig. 7. The separation is further improved, acceptable values for R_{sb} and R_{sa} being obtained, and the system is suitable for purity testing. However, on re-correcting the window diagram a better optimum is indicated, corresponding to an eluent composition of A–B (30:70). When the separation was performed with this eluent composition an excellent separation of the Model compounds was obtained, as shown in Fig. 8. All criteria the show acceptable values, and the same optimum can be obtained again when the window diagram is re-corrected with the retention data from this experiment. The optimal composition of the mobile phase in this particular instance was found to be acetonitrile–THF–water (12.9:22.4:64.7).

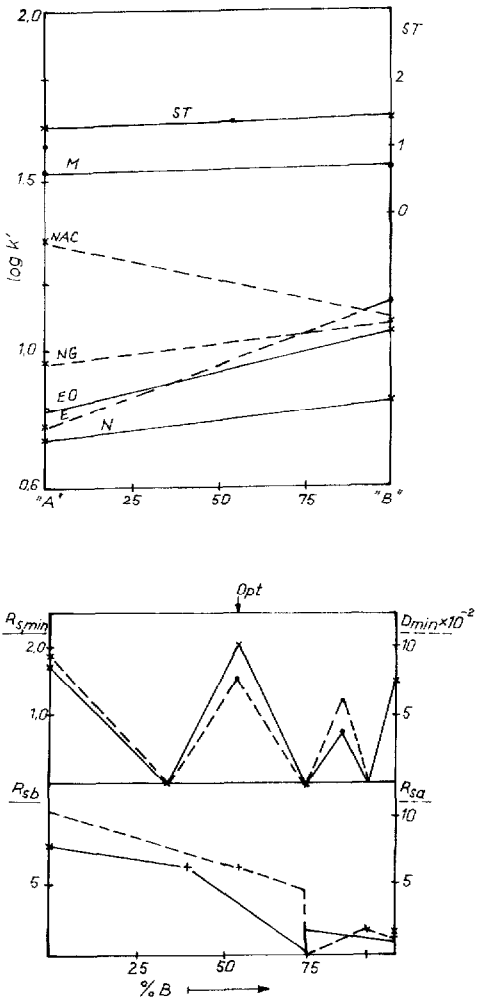

Fig. 5. Window diagram for the selection of initial three-component eluent mixture. System A, acetonitrile–water (43:57); system B, THF–water (32:68). Details as in Fig. 2.

The separation power of the combined "solvent strength"–"solvent type" optimization was compared with those of the individual optimization procedures using the conditions described in the original paper[4,8]. Figs. 2–4 show the chromatograms obtained with methanol–water, acetonitrile–water and THF–water eluents. Using "solvent strength" optimization a local optimum was obtained for the THF–water eluent (33% THF, Fig. 4b).

Initial eluent compositions for the iterative lattice method performed according to the original paper[8] were calculated according to ref. 2 [methanol–water (60:40); acetonitrile–water (45.7:54.3), system A; and THF–water (39.6:60.4), system B]. The initial optimum for three-component eluent mixture selecting system A (acetonitrile–

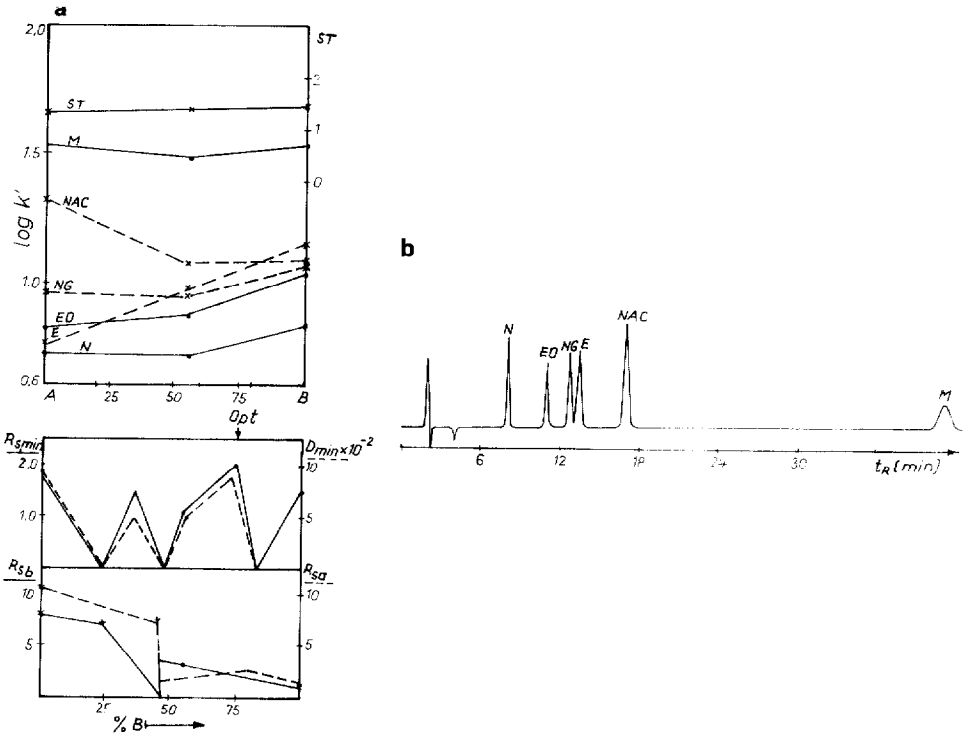

Fig. 6. First correction of window diagram (b) based on the retention data of the resulting chromatogram (a). Mobile phase composition in (a): acetonitrile–THF–water (19.4:17.6:63.0); ST (eluent strength) = 1.41. Details as in Fig. 2.

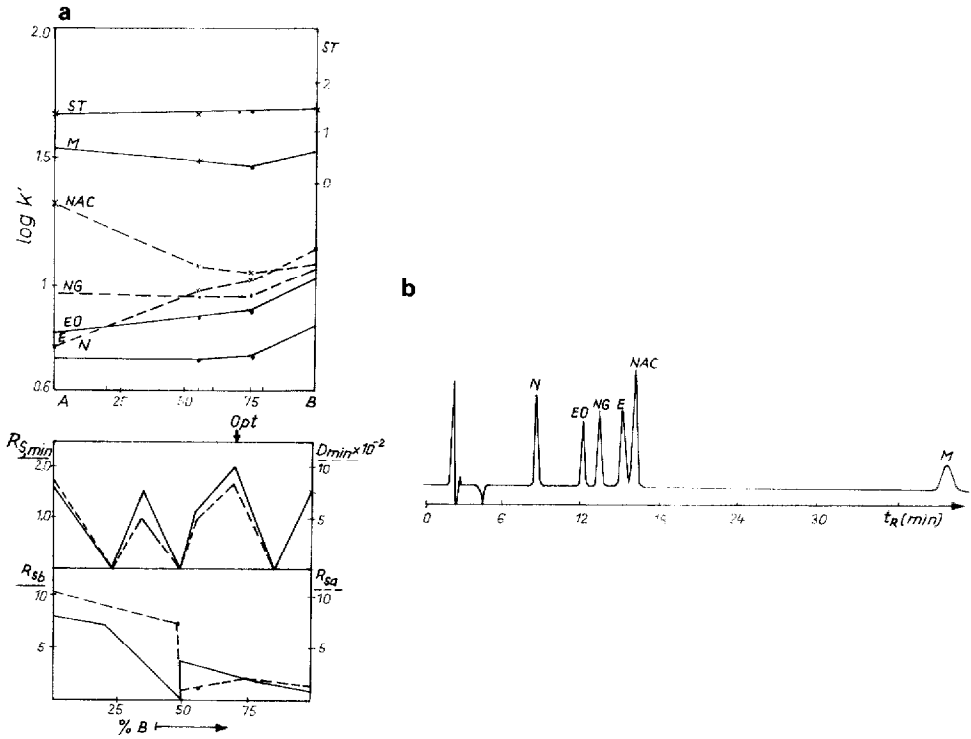

Fig. 7. Second correction of window diagram (b) based on the retention data of the resulting chromatogram (a). Mobile phase composition in (a): acetonitrile–THF–water (10.75:24.0:65.25); ST = 1.42. Details as in Fig. 2.

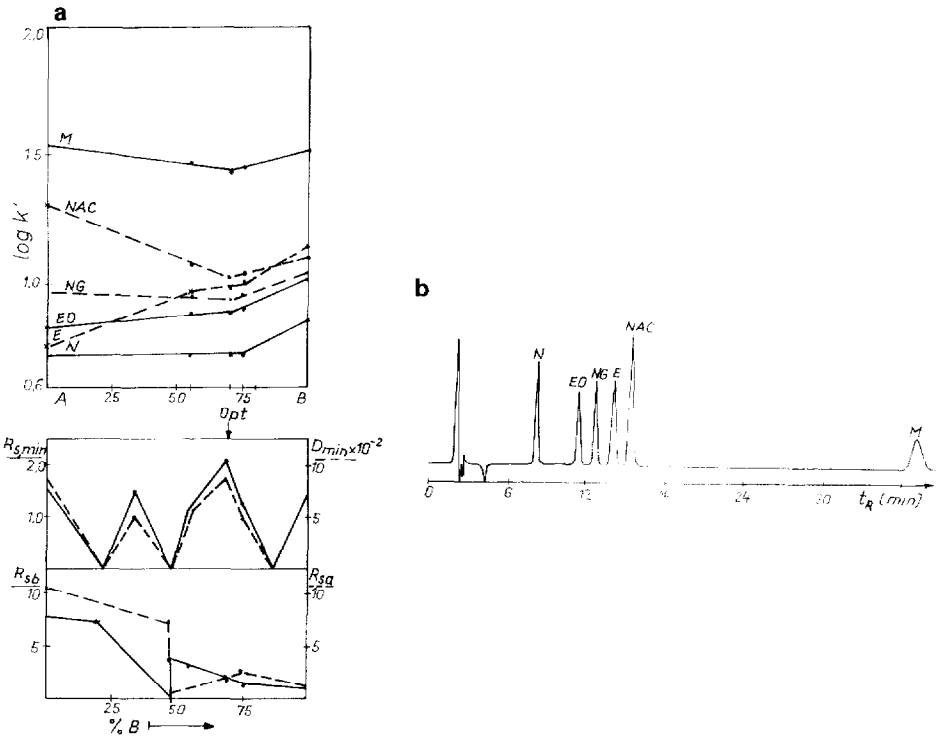

Fig. 8. Third correction of window diagram (b) based on the retention data of the resulting chromatogram (a). Mobile phase composition in (a): acetonitrile–THF–water (12.9:22.4:64.7); ST = 1.42. Details as in Fig. 2.

water) and system B (THF–water) as best two-component eluents was found to be A–B (29:71), corresponding to the eluent composition acetonitrile–THF–water (13.3:28.1:58.6). The chromatogram obtained with this eluent is shown in Fig. 9a.

The window diagram was corrected using the retention data from this experiment, indicating a new optimum of A–B (49:51) [corresponding to acetonitrile–THF–water (22.4:20.2:57.4)]. The resulting chromatogram is shown in Fig. 9b.

The experiments were not continued as the local optimum is far from the global optimum found in our experiments, and possibly cannot be reached owing to the imperfectly optimized starting conditions.

CONCLUSION

The advantages and limitations of combined "solvent strength"–"solvent type" optimization can be summarized as follows:

(a) It provides a markedly better separation (see Fig. 8) than either procedure alone (see Figs. 4 and 9b).

(b) A global optimum can be found with a significant ability to effect changes in band spacing.

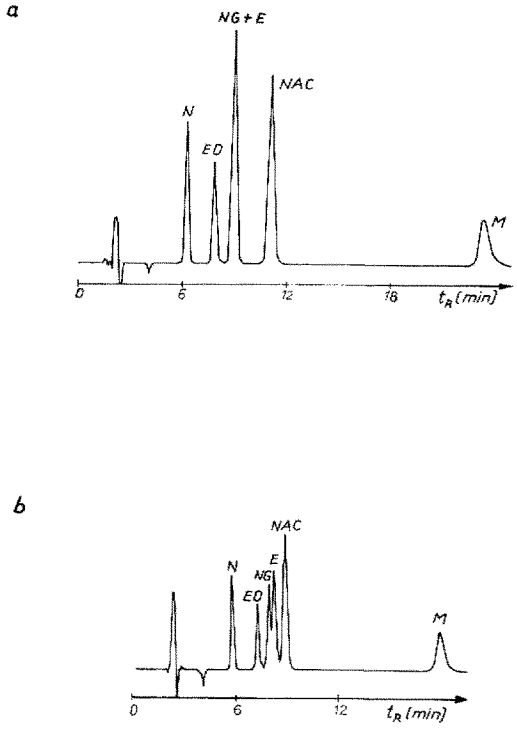

Fig. 9. Chromatograms obtained with the initial (a) and second (b) three-component eluents optimized according to the literature procedure[7]. Mobile phase compositions: (a) acetonitrile–THF–water (13.3:28.1:58.6); (b) acetonitrile–THF–water (22.4:20.2:57.6). Other conditions as in Fig. 2.

(c) The optimization procedure includes "solvent strength" optimization; when the samples do not require "solvent type" optimization, the experiments can be finished when the local optimum has been obtained. Several examples of HPLC method development based on "solvent strength" optimization were illustrated by Snyder *et al.*[10], which is a very practical approach.

(d) Large numbers of experimental runs are required to obtain the global optimum. The number of experiments can possibly be reduced (*e.g.*, only one experimental run with the solvent system resulting in a low separation efficiency, such as methanol–water here, and/or two runs with others, presuming a linear correlation between log k' and the percentage of organic component are carried out); however, there is a risk to failure to recognize the global optimum.

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