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Complementary mobile-phase optimisation for resolution enhancement in high-performance liquid chromatography ☆

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Abstract

An optimisation methodology in high-performance liquid chromatography (HPLC) is presented for the selection of two or more mobile phases having an optimal complementary resolution. The complementary mobile phases (CMPs) are selected in such a way that each one resolves optimally only some compounds in the mixture, while the remainder, resolved by the other mobile phase(s), can overlap among them. The methodology is based on the computation of a peak purity measurement for each solute, using an asymmetrical peak model for peak simulation. Two global resolution criteria (product of elementary resolutions and worst elementary resolution) and two methods for solving the problem (a systematic examination of all possible solute arrangements, and the use of genetic algorithms to expedite the calculation time) were used to find the optimal CMPs. The CMP optimisation methodology was applied to the resolution of a mixture of 10 diuretics and β-blockers, which could not be resolved using a single mobile phase; virtual baseline resolution was achieved, however, with two CMPs.

Keywords: Optimization; Mobile-phase composition; Peak resolution; Complementary separation

1. Introduction

The separation power of one-dimensional chromatographic systems is limited. When a large number of compounds is involved in a separation problem, complete resolution can be very time-consuming and often unfeasible. The request to resolve complex mixtures has led to the development of several approaches to the idea of complementary situations, namely the use of two different separations, one of which resolves some of the solutes of interest, while the remaining solutes are resolved in the second separation. Analysis of the complete sample thus becomes possible. Complementary situations are based on the fact that the selectivity can be varied by changing the nature of the stationary phase, the mobile phase components or, alternatively, the chromatographic mode. These approaches can be used not only to enhance the probability of success of the separation, but also to confirm the identity of a suspected peak.

In most cases, complementary situations are created by the use of two, or more than two, columns in combination with different mobile phases, running independent experiments where specific compounds

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are resolved. This is not always the case: a single injection can lead to full resolution using two sequential columns, which is in fact a bi-dimensional separation. Two examples are the separation of phenoxy-acid herbicides using C_{18} and aminosilica phases with methanolic mobile phases [1], and 1,3-dimethyl-4-phenylpiperidine derivatives using two different chiral stationary phases [2]. The use of two parallel columns and a single mobile phase has been reported as another alternative [3].

Complementary gradients have been applied with an anion-exchange [4] column. An interesting approach has recently been developed where the solutes were optimally resolved by performing two runs at different temperatures and gradient times [5]. In that report, the optimisation criterion was the worst resolved pair of peaks measured according to the selectivity factor. The same column has also been used in two different chromatographic modes such as in the separation of tropane alkaloid stereoisomers with a cellulose-based chiral stationary phase in reversed- and normal-phase modes [6].

Complementary combinations of chromatographic techniques can produce a greater separation space (i.e., peak capacity). Examples include liquid chromatography–gas chromatography [7] or liquid chromatography–capillary zone electrophoresis [8] coupled systems. A comparison of the chromatographic properties in the individual one-dimensional systems is very useful for the selection of the combination of techniques that will separate a given mixture [9].

In any chromatographic system, one finds discrete factors (those that cannot arbitrarily be varied, at least in a practical way, such as column length, particle size, or packing and modifier nature), together with other factors that can easily be set at arbitrary levels (i.e., modifier(s) concentration, ionic strength, temperature, pH, or gradient time). Most approaches published in the literature for complementary situations concern the selection of discrete factors. Changes in these factors are often translated into drastic modifications in the separation system. In this way, the probability of a different interaction of the solutes to be isolated with the separation system increases, eventually obtaining full resolution. Complementary resolution can, however, be accomplished in a cheaper and easier way by selecting a specific set of levels in the continuous factors, which, in addition, can be optimised.

In this work, those factors affecting the eluent composition will be considered and, therefore, each complementary situation will correspond to a mobile phase with complementary behaviour, namely a 'complementary mobile phase' (CMP). Formally, CMPs should be chosen in such a way that each one tries to resolve fully only some compounds in the mixture, while the other compounds (resolved by other CMPs) are not optimised and can thus remain overlapped among them, which increases the separation space. However, when the whole set of CMPs is considered, all the compounds are resolved.

The problem is how to find these eluent compositions in order to achieve an optimal complementary situation. This is the aim of this work: to propose a new approach for obtaining an optimal resolution of complex mixtures, without the need of changing drastically the separation conditions (e.g., column, gradient elution, HPLC mode) when the separation in a single run fails. In situations like this, an important advantage is that the same information gathered for optimising the separation can be used for finding the optimal CMPs: no new experimental effort is necessary.

The proposed methodology is based on computer simulation using a small number of experimental runs to predict HPLC separation for a wide range of conditions. This reduces time and effort in the search for the best chromatographic conditions. Empirical or theoretical retention models have been used in computer simulation since the late 1970s [10]. Various optimisation software is available, such as DryLab [11], Osiris [12], or Michrom [13,14]. In all applications, computer predictions which are as accurate as possible are essential [15].

A separation problem studied in our laboratory is used to illustrate the CMP optimisation methodology proposed in this work: the isocratic separation of four diuretics (bendroflumethiazide, piretanide, amiloride and triamterene) and six β-blockers (atenolol, metoprolol tartrate, nadolol, propranolol chlorhydrate, acebutolol and labetalol chlorhydrate), with mobile phases containing sodium dodecyl sulphate (SDS) and 1-propanol as modifiers, which have been shown to be a good choice for the analysis of these drugs [16,17]. Simultaneous prescription of combinations of one diuretic and one β-blocker is made when appropriate control of the arterial pressure is not possible with each of them separately.

This mixture, not too complex, is a good example for explaining and checking the proposed methodology. The same procedure can be straightforwardly applied to more complex problems.

It should be noted that all that is explained below is applicable to any chromatographic or related technique, where an interpretive optimisation is possible. Similar treatments can be performed for separation problems with one, two or more experimental factors. Moreover, other continuous factors different from mobile-phase composition can be optimised.

2. Experimental

2.1. Apparatus

The equipment consisted of a Hewlett-Packard liquid chromatograph HP 1050 (Palo Alto, CA, USA), provided with an isocratic pump, an autosampler HP 1100, a fluorimetric detector HP 1046A, and an integrator HP 3396A. The injection volume was 20 μl and the flow-rate 1.0 ml/min. The dead volume was determined by the first deviation from the baseline after injection of the compound solutions. A Spherisorb ODS-2 column (5 μm particle size, 125 mm×4.6 mm I.D.) and pre-column (5 μm particle size, 35 mm×4.6 mm I.D.) were obtained from Scharlau (Barcelona, Spain). The mobile phase and the drug solutions were vacuum filtered through 0.45 and 0.22 μm Nylon membranes, respectively (Micron Separations, Westboro, MA, USA). Data acquisition was made through the PEAK-96 software from Hewlett-Packard.

2.2. Software

The CHROM software, available upon request, was used to model the chromatographic behaviour of sets of compounds under isocratic conditions, calculate the elementary resolution matrices of peak purities and, after processing the information, eventually simulate the optima found. CHROM consists of a set of MS-DOS programs, and is an evolution of a previous release (MICHROM) developed for micellar liquid chromatography [14], which was designed for an assisted optimisation of the resolution of arbitrary mixtures using one to three experimental factors (surfactant, organic solvent and proton concentrations). MICHROM is available through Marcel-Dekker [14]. Home-built routines, written in MATLAB 4.2c (The Mathworks Inc.), were developed for CMP optimisation. These routines, arranged in a Matlab toolbox, are also available upon request.

2.3. Reagents

The probe compounds were the diuretics bendroflumethiazide (Davur, Madrid, Spain), piretanide (Cusí, Barcelona, Spain), amiloride (ICI Farma, Madrid, Spain), and triamterene (Sigma, St. Louis, MO, USA), and the β-blockers atenolol (Zéneca Farma, Madrid, Spain), metoprolol tartrate (Ciba-Geigy, Barcelona, Spain), nadolol (Squibb, Barcelona, Spain), propranolol chlorhydrate (ICI Farma), acebutolol (Italfármaco, Madrid, Spain), and labetalol chlorhydrate (Glaxo, Madrid, Spain). The injected solutions contained 10 μg/ml of the drugs, and were prepared by dissolving the pure reagents in a few millilitres of ethanol with the aid of an ultrasonic bath. The excitation wavelength was 230 nm for all compounds. The emission wavelength was 300 nm for atenolol, metoprolol, nadolol and propranolol, and 440 nm for the remaining compounds.

The micellar mobile phases were prepared with sodium dodecyl sulphate (99% purity, Merck, Darmstadt, Germany) and 1-propanol (Scharlau). The concentration of 1-propanol is given as volumetric fractions (v/v). Triethylamine (99.5% purity, Fluka, Buchs, Switzerland) was also added (its concentration in the mobile phase was 0.5%), and the pH was buffered at 3 with sodium dihydrogenphosphate (Panreac, Barcelona, Spain), HCl and NaOH (Probus, Badalona, Barcelona, Spain) in order to enhance the efficiency. Nanopure water (Barnstead, Boston, MA, USA) was used throughout to prepare the solutions.

3. Results and discussion

3.1. Calculation of elementary response surfaces of resolution

The CMP methodology is based on the computa-

tion of a resolution parameter for each compound present in the mixture, which will be called 'peak purity'. Each response surface describes the separation of a given peak from the remaining peaks for a set of computer-simulated chromatograms predicted for a regular distribution of mobile phases, which were obtained by changing the concentrations of the modifiers (e.g., surfactant and organic solvent). The process for obtaining one of these surfaces for a given compound is given below.

In a first step, the retention is modelled for each compound using a small number of experiments. In the example shown, the data from five mobile phases (*M* SDS/(v/v) propanol: 0.05/0.05, 0.15/0.05, 0.10/0.10, 0.05/0.15, and 0.15/0.15) were fitted to the following model [18,19]:

$$t_R(c_M, c_S) = t_0\left(1 + \frac{K_{AS}[(1 + K_{SD}c_M)/(1 + K_{AD}c_M)]}{1 + K_{AM}[(1 + K_{MD}c_M)/(1 + K_{AD}c_M)]c_S}\right) \quad (1)$$

which further allows us to predict the elution behaviour at varying mobile-phase composition. In Eq. (1), t_R represents the retention time, t_0 is the dead time, c_S is the concentration of surfactant forming micelles (total concentration minus critical micellar concentration), c_M is the volumetric fraction of organic modifier in the mobile phase, K_{AS} and K_{AM} are constants that quantify the association of solute with the stationary phase and micelles, respectively, and K_{AD}, K_{MD} and K_{SD} measure the displacement of the partition equilibria with micelles and stationary phase produced by the modifier.

Performing only five experiments is risky, since there is no degree of freedom available, and validation of the model is not possible, except through eventual predictions. In experimental practice this risk is accepted, and when the predictions are not as good as expected, more experiments are added to the original design to obtain a better model, in order to enhance the predictions. Eq. (1) has been checked with solutes of very different nature (yielding errors usually in the 2–4% range), and the risk of using few experiments avoiding the validation step is, therefore, acceptable.

The calculation of resolution surfaces requires not only the prediction of the retention times, but also each peak profile. For the simulation of asymmetrical peaks, an equation with four parameters (a linear polynomially modified Gaussian model, PMG) was used [20]:

$$h(t) = H_0 \exp\left[-\frac{1}{2}\left(\frac{t - t_R}{s_0 + s_1(t - t_R)}\right)^2\right] \quad (2)$$

In this equation, H_0 represents peak height, a parameter linearly related to peak area. Areas were normalised throughout this work, but real values can eventually be used if required. The peak profile parameters in the PMG model (s_0 and s_1) are related to the efficiency (N) and asymmetry factor measured at 10% of peak height (B/A) as follows:

$$s_0 = 0.466\frac{\sqrt{[41.7(1 + t_R)^2]/[N(1.25 + B/A)]}}{1 + [1/(B/A)]} \times \left(1 - \frac{B/A - 1}{B/A + 1}\right) \quad (3)$$

$$s_1 = 0.466\frac{B/A - 1}{B/A + 1} \quad (4)$$

where s_0 is a measurement of peak width, and s_1 a parameter which quantifies peak distortion (note that it only depends on B/A). Due to the strong and often unpredictable variations in efficiencies and asymmetry factors (difficult to model), local linear models were applied as predictors of these properties. Since two experimental factors (surfactant and organic modifier) were used in this work, the data of N and B/A for the peaks obtained with the experimental mobile phases (i.e., those used to model the retention) closer to the simulated phase were fitted to a plane for predicting the N and B/A values.

The purity of the peak of solute s, at a given mobile phase composition defined by c_M and c_S, is calculated through the measurement of two areas in a simulated chromatogram. The first (w_s') is the area under the considered peak that is overlapped by the chromatogram yielded by the remaining peaks. The second is the total area of that peak (w_s). The ratio w_s/w_s' is the overlapped fraction. Since a value of 1 is required for fully resolved peaks, we will define 'elementary resolution' or 'peak purity' as the complementary value of the overlapped fraction, that is the fraction of peak free of any overlapping:

$$r_s(c_M, c_S) = 1 - \frac{w_s'(c_M, c_S)}{w_s(c_M, c_S)} \quad (5)$$

A value of r_s can be calculated for each solute in the simulated chromatogram predicted for a given mobile-phase composition. This process of simulation and calculation of peak purity is repeated for a regular distribution of mobile phases in the selected factor space, in order to obtain for each solute a vector (for one experimental factor), a matrix (for two factors as in the example shown) or, eventually, a tensor (for more than two factors). We will refer here only to matrices, but the same could be said for vectors and tensors. The final result is a set of elementary matrices of peak purities, a different matrix $\boldsymbol{R}_s$ for each solute. Each element of the matrix, $r_s(c_M, c_S)$, represents the resolution for a given solute at the mobile phase defined by the row and column indices in the matrix.

The peak purity criterion [21] was selected since it associates a resolution value with each compound in a mixture not affected by the identity of the neighbouring peaks (an essential feature for a CMP optimisation). Also, it has other additional advantages: peak purities are normalised values and have a straightforward meaning, which is useful for understanding the information obtained throughout the optimisation process. One should note that peak purity depends on the size of the overlapping peaks. This allows us to optimise in a realistic way a mixture where the concentration of each compound participates actively in the resolution measurements. For obtaining resolution measurements not affected by the concentration and sensitivity of each component, normalised areas should be used. Of course, the actual separation obtained in this way can be somewhat different from the predicted one. Peak purities also make some operations, such as the weighting or the exclusion of peaks, easily possible. The term weighting refers here to giving more importance to the separation achieved for a given compound or compounds. One accepts a certain decrease in the resolution for the accompanying compounds if that decision is translated into an enhanced resolution for the compound(s) of interest. This is done by just multiplying each peak purity value by a number between 0 and 1, which should be smaller as the importance of the compound is wished to increase. Weighting is independent of peak size.

All what follows is valid for other chromatographic modes or related techniques (e.g., capillary electrophoresis and capillary electrochromatography). It should be noted that the retention model (Eq. (1)) is the only element that should be adapted in order to apply the methodology to other techniques.

For the mixture of diuretics and β-blockers, chromatograms corresponding to 441 mobile phases, arranged in a regular distribution containing 21 levels in surfactant and 21 in organic solvent, were simulated. With this information, the peak purity matrix for each compound was calculated. Two peak purity surfaces, corresponding to triamterene and atenolol, are shown in Fig. 1a and b, respectively. The valley observed for triamterene corresponds to the peak crossing with acebutolol. For atenolol, the resolution decreases at low and high concentrations of both modifiers due to a change in elution order with piretanide and bendroflumethiazide, respectively. It should be remarked that elementary surfaces of peak purity are not affected by the identity of the interfering solutes that overlap the considered peak. Also, peak purities measure reliably the composition ranges yielding baseline separation. Both characteristics are fundamental for the success of the CMP approach.

Fig. 2 illustrates the dependence of peak purity on the relative peak areas, showing three peak purity surfaces for triamterene in a mixture of 10 compounds. In Fig. 2a, the peak area of triamterene is 10% of the peak areas of any of the remaining compounds (which are normalised). In Fig. 2b, the peak area of triamterene has been increased to obtain the same area for all the peaks. Finally, in Fig. 2c, the peak area of triamterene is 10-fold greater than for the other compounds. As can be seen, the peak purity surfaces are quite different. True peak areas can be used to enhance the reliability of the predictions when a difficult separation is expected (e.g., to separate an impurity at low concentration from a large peak). Alternatively, normalised areas can be used to obtain more general separation conditions.

3.2. Resolution of the mixture using a single mobile phase

Once the elementary matrices are obtained, these can be combined conventionally to achieve a single global resolution (or overall purity) matrix, including

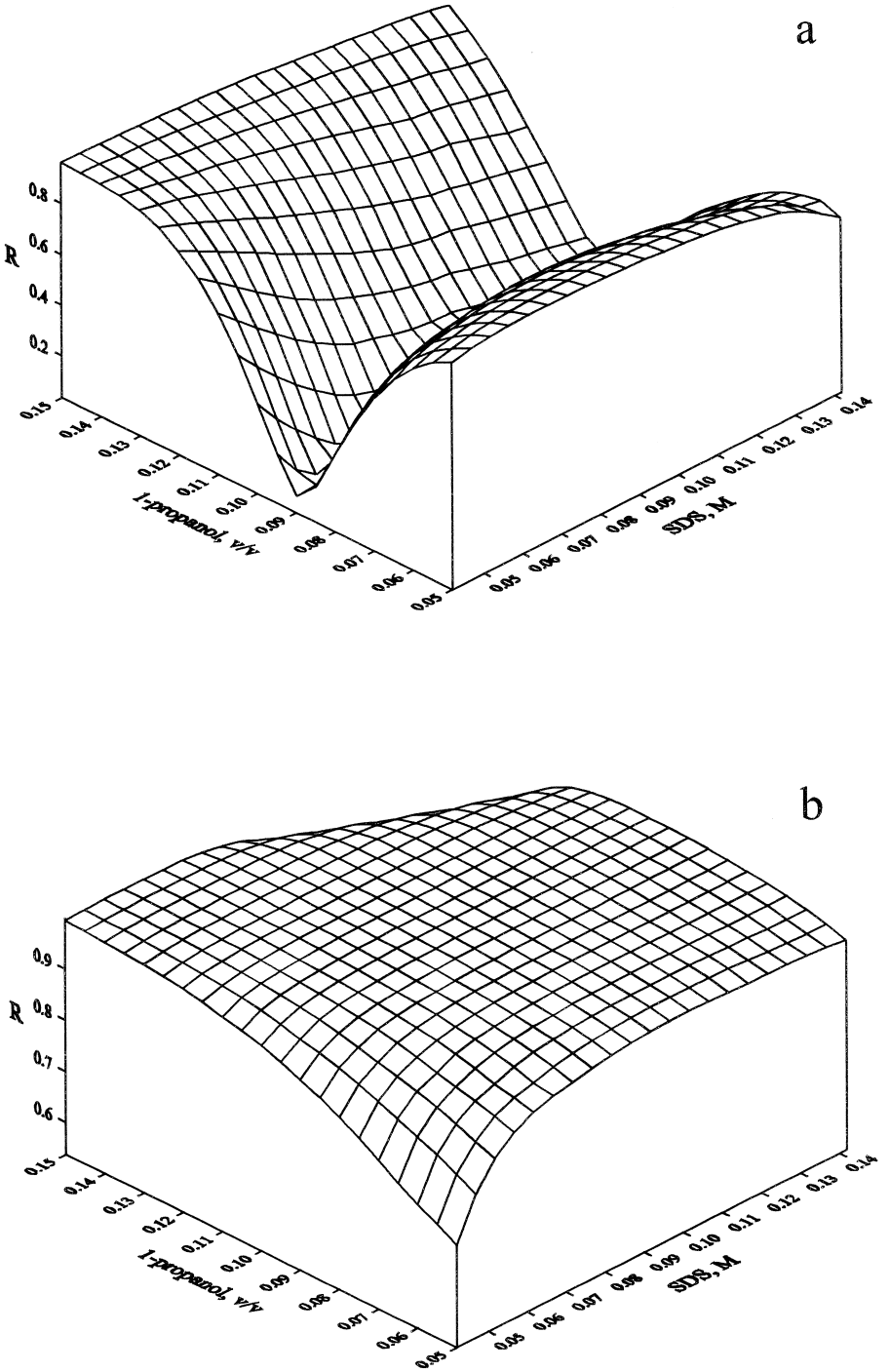


Fig. 1. Example of peak purity response surfaces. The plots shown correspond to a 21×21 matrix for (a) triamterene and (b) atenolol.

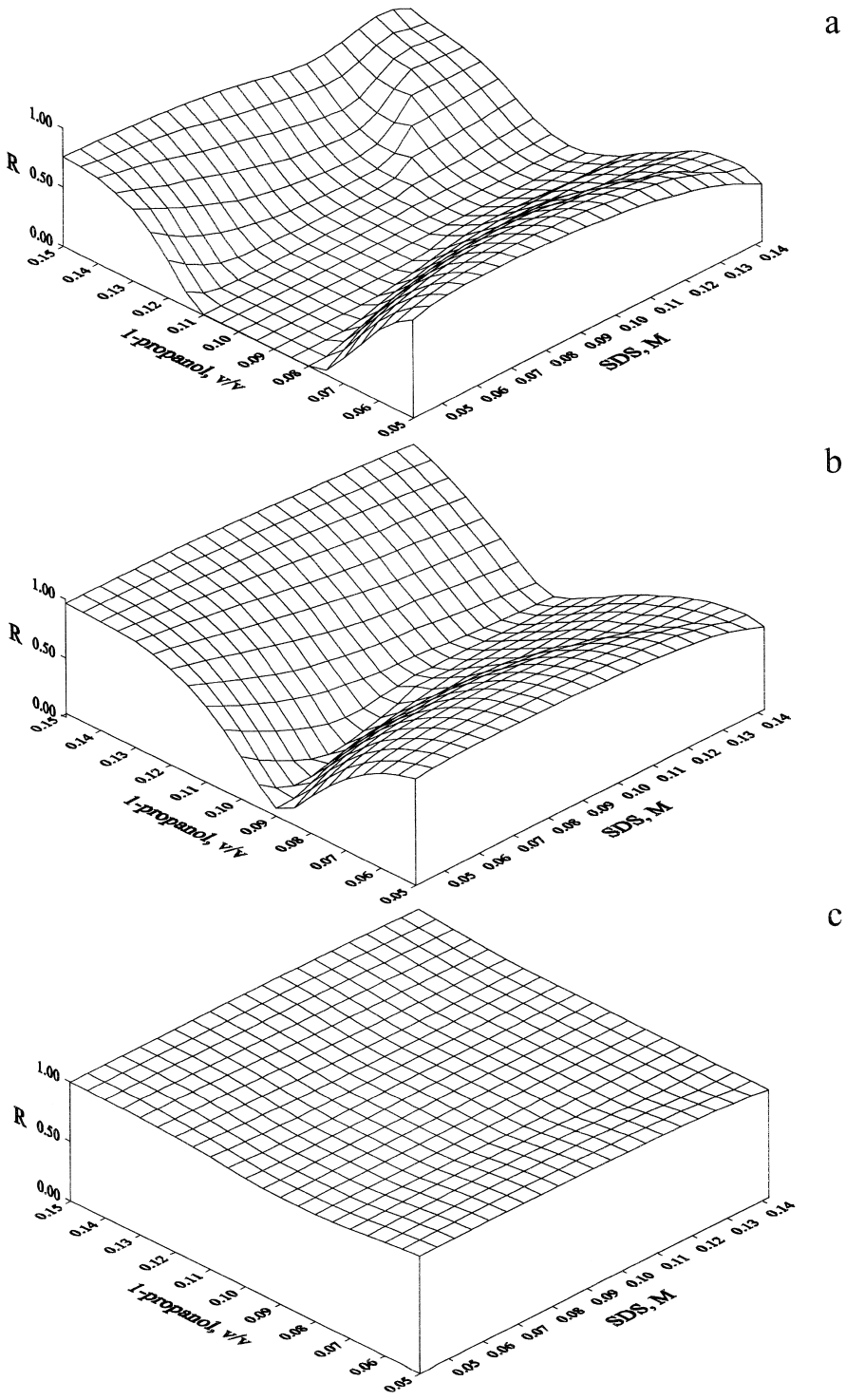


Fig. 2. Peak purity surfaces for the separation of triamterene at three concentrations. Peak area of triamterene relative to the peak areas of the other compounds in the mixture: (a) 0.1, (b) 1.0, and (c) 10.

the whole set of solutes, in order to search for the conditions that better resolve the mixture. In the literature, the worst elementary resolution is often taken as representative of the resolution achieved in a given chromatogram. An alternative way of measuring the global resolution is to obtain the product of peak purities as elementary resolution values:

$$\boldsymbol{R} = \prod_{s=1}^{ns} \boldsymbol{R}_s \tag{6}$$

The row and column of the element in the matrix having the maximal resolution indicates the optimal mobile-phase composition.

The outlined strategy constitutes the usual way of undertaking interpretive HPLC problems. However, if the mixture is too complex for the resolving power of the system, finding a single phase that resolves the whole mixture is often unfeasible. This is the case of the mixture of diuretics and β-blockers: the optimal mobile phase (0.0769 *M* SDS/0.055 v/v propanol) is unable to fully resolve the set of 10 compounds, as illustrated in Fig. 3. Atenolol, piretanide and amiloride, on the one hand, and triamterene and acebutolol, on the other, are partially overlapped. The peak purity values for each compound at the optimal mobile phase are: A (1.0000), B (0.9482), C (0.9891), D (0.9417), E (0.9594), F (0.9880), G (0.9944), H (0.9797), I (0.9358), and J (0.9842); the global resolution is 0.7512. The accuracy of peak simulation is limited for strongly asymmetrical peaks, which can lead to long tails that introduce errors in resolution measurements. This is the case for compounds F, J and H (see Fig. 3) at the optimal single phase, the peak purities of which do not reach $r_s = 1.00$ due to the tail of a preceding peak (**C**, with $B/A = 2.5$ for the optimal mobile phase) that produces a very low, but almost constant, signal which extends up to high retention times.

At this point, and according to these results, the chromatographist would probably decide to change the composition range, the nature of the modifiers, or even would try another HPLC mode. However, as demonstrated below, the mixture can be resolved without the need of changing the separation system drastically.

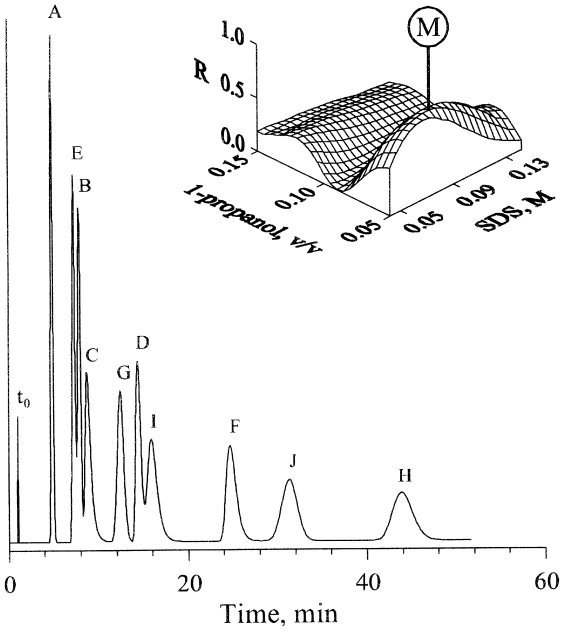


Fig. 3. Global resolution response surface and optimum found (0.0769 *M* SDS/0.055 v/v 1-propanol) when all solutes are resolved with a single mobile phase. Probe compounds: bendroflumethiazide (A), piretanide (B), amiloride (C), triamterene (D), atenolol (E), metoprolol (F), nadolol (G), propranolol (H), acebutolol (I), and labetalol (J). Encircled M indicates the mobile phase giving maximal resolution.

3.3. Limiting resolution

A useful concept that we propose for evaluating the capability of the chromatographic system is the 'limiting resolution', that is the maximal elementary resolution that can be obtained for each compound. This value can be used as a guide to determine whether the resolution of a given compound can be improved or, on the contrary, has reached its maximal value. Limiting resolutions are only meaningful for some resolution criteria, such as the criterion presented in this work, but not for others based on peak pair resolution, such as the selectivity and separation factor, which are affected by peak crossing.

The limiting resolutions for the 10 drugs in the SDS–propanol system are given in Table 1. Limiting elementary values indicate that baseline resolution of any of these compounds from the remaining com-

Table 1
Limiting resolutions and maximal elementary resolutions, for each solute, in the optimisation of the separation of the mixture of 10 diuretics and β-blockers[a]

Compound	Limiting resolution	Single mobile phase		Two CMPs		Three CMPs	
		*R*1	*R*2	*R*1	*R*2	*R*1	*R*2
A	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
B	1.0000	0.9482	0.9459	0.9970	0.9945	0.9993	0.9945
C	0.9999	0.9891	0.9955	0.9987	0.9977	0.9987	0.9999
D	0.9627	0.9417	0.9416	0.9613	0.9627	0.9627	0.9627
E	0.9991	0.9594	0.9521	0.9970	0.9947	0.9991	0.9947
F	0.9898	0.9880	0.9884	0.9898	0.9896	0.9898	0.9896
G	0.9953	0.9944	0.9946	0.9951	0.9947	0.9951	0.9949
H	0.9837	0.9797	0.9804	0.9834	0.9837	0.9834	0.9837
I	0.9700	0.9358	0.9371	0.9693	0.9700	0.9700	0.9700
J	0.9868	0.9842	0.9846	0.9866	0.9868	0.9866	0.9868

[a] Solutes: bendroflumethiazide (A), piretanide (B), amiloride (C), triamterene (D), atenolol (E), metoprolol (F), nadolol (G), propranolol (H), acebutolol (I), and labetalol (J).

pounds is feasible (except for triamterene and acebutolol, solutes D and I), finding a specific eluent composition, different and optimal, for each solute. However, one certainly does not wish to use 10 different mobile phases, but one if possible.

In the example, a single mobile phase is unable to resolve all solutes simultaneously. However, if the resolution of solutes E, B, C, G, D and I can be substantially improved finding two (or at maximum three) complementary mobile phases, the separation will have practical interest. The question now is to determine whether compatible groups of solutes (i.e., baseline resolved with the same CMP) is feasible or not.

3.4. Finding CMPs through the establishment of groups of solutes or the selection of mobile phases

From here, the idea of finding complementary mobile phases arises. In each one of these CMPs, only some solutes are resolved, whereas the remaining solutes can overlap among them. This enlarges the separation space. The problem of finding these phases can be treated in two ways, which lead to the same result.

3.4.1. Approach 1 — Defining groups of solutes for finding in a further step the CMPs

First divide the *ns* solutes into *ng* groups (i.e., $ng = 2$ or 3), and then find for each group the optimal mobile phase that resolves them. In this approach, ‘group’ means ‘subset of solutes’. The process consists of combining the peak purity matrices corresponding to the solutes belonging to group *g* (see Section 3.5) to obtain a combined resolution matrix ($\boldsymbol{RG}_g$). The row and column of the element having a maximum value in each $\boldsymbol{RG}_g$ matrix will indicate the composition of the best mobile phase for resolving the solutes in that group, whereas the numerical value will quantify the best separation achieved for that subset. The *ng* maximal resolution values thus obtained should now be reduced to a single value (see also Section 3.5) representative of the resolution of that solute arrangement in the *ng* mobile phases. Therefore, *ng* mobile phases, *ng* group resolution values, and a single measurement of the overall resolution will be associated with any distribution of solutes that could be established. We will use the term ‘combined resolution’ for the measurement of the separation of the solutes belonging to a given group, and ‘global resolution’ for the measurement of the separation of all solutes belonging to all the established groups.

3.4.2. Approach 2 — Selecting mobile phases for distributing the solutes optimally among them

First select *ng* mobile phases from the *np* available (in our example, *np* is 441 since 21 levels in

surfactant and 21 levels in organic modifier were defined), and then find how to distribute the solutes among the *ng* mobile phases in order to obtain a maximal resolution for each solute (i.e., select for each solute the mobile phase among the *ng* phases where it is better resolved). Once each solute has been assigned to the best mobile phase among the *ng* selected mobile phases, we arrive at the same situation as above. Since an arrangement of solutes has been established, the resolution can be measured similarly as when the solutes are divided into different groups. As observed, here the term 'group' means 'mobile phase', instead of 'subset of solutes', although an arrangement of solutes is associated with any possible selection of CMPs. Thus, in this approach, *ng* groups of solutes are unambiguously established when *ng* mobile phases are selected.

The search of the optimal complementary mobile phases involves the examination of all possible combinations of *ns* solutes in *ng* groups, or *np* mobile phases in *ng* groups, in order to maximise the resolution. Alternatively, other optimisation strategies can be used, as shown below.

3.5. Quantification of the resolution in CMPs

A main question is how to measure the separation quality achieved with the CMPs selected to resolve the mixture. This is essential since, given several combinations of solutes (or mobile phases), we must decide which of them is better. As explained, in order to find the CMPs, *ng* intermediate resolution values (combined resolutions) are obtained, which are further combined into a single measurement (global resolution). This final value should quantify the separation, independently of the way the solutes have been arranged. The reason is that, throughout the process, combinations including different numbers of solutes by group should be compared.

Several hierarchical approaches were checked. Two of them, based on the product of elementary resolutions ($R1$) and the worst elementary resolution value ($R2$) are proposed here. Let us consider first the case of forming groups of solutes (Approach 1).

3.5.1. Product of elementary resolutions (i.e., peak purities) (R1)

The process consists of building *ng* intermediate resolution matrices (or 'group matrices'), $\boldsymbol{RG}_g$ ($g = 1, 2, \ldots, ng$), each of them describing the resolution of a subset of $ns(g)$ solutes belonging to the associated group, g. These matrices store the element-by-element product of the elementary resolution matrices $\boldsymbol{R}(g, s)$ for each solute (s denotes here a given solute belonging to group g; the total number of solutes in the mixture is the sum of $ns(g)$ extended to the *ng* groups). The row and column (c_M, c_S) of each element in $\boldsymbol{R}(g, s)$ and $\boldsymbol{RG}_g$ are related to the composition of the mobile phase; the total number of elements in both matrices is *np* (21×21 in the example shown).

Next, the maximal value in each $\boldsymbol{RG}_g$, RG_g, is found; the row and column of this element give the optimal composition for resolving the solutes in group g. The optimal values for each group, RG_g, are finally multiplied. All these operations can be summarised as follows:

$$R1 = \prod_{g=1}^{ng} \underset{(c_M,\, c_S)=1}{\overset{(np)}{\mathrm{MAX}}} (\boldsymbol{RG}_g) = \prod_{g=1}^{ng} \underset{(c_M,\, c_S)=1}{\overset{(np)}{\mathrm{MAX}}} \left(\prod_{s=1}^{ns(g)} \boldsymbol{R}(g, s) \right) \quad (7)$$

This process is repeated for all arrangements of solutes that can be established, in order to find the one yielding the maximal global resolution value. Because all compounds take part in the final value, $R1$, the resolution of all of them is improved. Note that $R1$ does not depend on the way the solutes are arranged (i.e., number of solutes in the group and number of groups), since any arrangement leads to the same number of multiplied data in Eq. (7). Therefore, $R1$ values can be compared in situations where the number of groups differs (e.g., to determine the improvement achieved when three CMPs are selected instead of two), or when the arrangements include groups containing a different number of solutes by group. In contrast, the intermediate results, RG_g (the maxima of the $\boldsymbol{RG}_g$ matrices), are not comparable, since the RG_g values are products including different numbers of factors.

An extreme situation appears when one tries to resolve the mixture with a single mobile phase (that is, one undertakes the problem as a conventional single-phase optimisation). In this case, the optimal value in $\boldsymbol{R}(1, s)$ coincides with the optimum found in

$\boldsymbol{R}$ (Eq. (6)). This value will also be fully comparable to the global optimal resolution found using complementary mobile phases, independently of the number of mobile phases selected.

3.5.2. Worst elementary resolution (i.e., peak purity) value (R2)

The second hierarchical criterion that has been considered is the use of only one elementary resolution: that corresponding to the worst resolved solute. The first step consists of calculating matrices for each group, $\boldsymbol{RG}_g$, which now contain for each mobile phase the worst elementary resolution value found for the solutes in the group. This is made through a comparison element-by-element of the elementary resolution values, $r_s(c_M, c_S)$. Next, the element in each of these matrices yielding the maximum resolution (RG_g) is located. The location of this element in the matrix determines the optimal composition for the resolution of that group. The final resolution will be the worst value among the maximal group resolutions found, that is the minimum RG_g. Eq. (8) describes the whole process:

$$R2 = \text{Rearrange}\left[\min_{g=1}^{ng} RG_g\right] = \text{Rearrange}\left[\min_{g=1}^{ng}\left[\max_{(c_M, c_S)=1}^{(np)}\left(\min_{s=1}^{ns(g)} \boldsymbol{R}(\boldsymbol{g}, \boldsymbol{s})\right)\right]\right] \quad (8)$$

Since all the selected resolutions used to obtain $R2$ are directly related to one solute in all the steps of the process, all the results, even the intermediate ones, RG_g, are meaningful. $R2$, however, has an important limitation: only the resolution of one solute by group (the worst resolved solute) is considered. The method is thus blind to the resolution achieved in the separation of the other solutes. Therefore, a final rearrangement step is mandatory, where all the solutes are reassigned to that group in which they are better resolved. This is the purpose of the operator Rearrange in Eq. (8).

Another problem related to the $R2$ criterion is to decide what to do when two different combinations yield the same $R2$ value: which of them is better? In this case, the resolutions of all compounds are sorted from worst to best, and then compared element-by-element to find one solute whose resolution is different in both combinations. The combination that better resolves that critical solute is then selected. With this operation, the global resolution value will not change, although the separation will be enhanced.

In the case of selecting the mobile phases first, since the phases are initially decided, only the information about elementary resolutions at the selected CMPs is required. After examining the elementary resolutions for all solutes in the *ng* selected mobile phases, each solute is assigned to the phase where it is better resolved. The result of this operation is thus a combination of solutes as before. Next, the product of elementary resolutions (or the worst value) for each group is calculated and combined as when the solutes are directly arranged in groups. RG_g is, in this case, not a matrix but a scalar, since full intermediate group matrices ($\boldsymbol{RG}_g$) are not necessary.

3.6. The most economic CMP approach

As shown above, the CMP problem can be resolved through the establishment of groups of solutes (Approach 1) or the selection of mobile phases (Approach 2). The most simple way to find the optimal CMPs is by examining one by one all possible combinations that can be established. In order to calculate the number of combinations, one should consider that several combinations representing the same arrangement can exist, which can increase undesirably the calculation time. To illustrate this, let us consider the establishment of groups of solutes, and assume a combination of five solutes arranged in two groups, encoded as 11212, which means that solute numbers 1, 2 and 4 (order in the list of digits) are assigned to the first group, and solutes 3 and 5 to the second group. This combination represents exactly the same arrangement as 22121, since the labels ‘group 1’ and ‘group 2’ are meaningless and can be interchanged. For this reason, only one of these combinations should be examined in order to expedite the optimisation process.

When the problem is established according to Approach 2, the number of combinations to examine,

N_A, depends on the number of groups and available mobile phases, but not on the number of solutes:

$$N_A = \binom{np}{ng} = \frac{np!}{(np-ng)!ng!} \quad (9)$$

In contrast, when the solutes are arranged in groups (Approach 1), the number of possible combinations, N_B (after removing repetitions), is given by:

$$N_B = \frac{\beta_{ng}}{ng!} \quad (10)$$

$$\beta_2 = 2^{ns} - 2 \quad (11)$$

$$\beta_{ng} = ng^{ns} - \left[\sum_{i=2}^{ng-1} \beta_i \binom{ng}{i} \right] - ng \quad (12)$$

In Eq. (12), there is a term that should be determined recursively starting from two groups (β_2, Eq. (11)). As can be seen, N_B depends on the number of groups and solutes, but not on the number of mobile phases.

There will be situations where defining groups of solutes will imply the examination of a smaller number of combinations (i.e., less calculation time), and others where selecting mobile phases will be faster. Eqs. (9)–(12) can be used to decide the best strategy. An interesting rule of thumb to decide whether the first or second approaches (i.e., forming groups of solutes or mobile phases) is the most economic, consists of substituting the number of solutes, *ns*, in the following equations:

$$n = 0.9994 \cdot e^{0.3466 \cdot ns} \quad (13)$$

$$n = 0.9714 \cdot e^{0.3672 \cdot ns} \quad (14)$$

when two and three CMPs are searched, respectively. If *n* is smaller than the number of mobile phases that were considered in the resolution matrices, then the problem will be better faced defining groups of solutes (Approach 1). On the contrary, if the result is greater than the number of examined mobile phases, the problem should be faced selecting mobile phases (Approach 2).

For the mixture of diuretics and β-blockers, *np* is 441, and *ns* is 10. Therefore, when two CMPs are selected, $N_A = 97\,020$ and $N_B = 511$, whereas with three CMPs, $N_A = 14\,197\,260$ and $N_B = 9330$. In this case, it is evident that the problem is more efficiently faced defining groups of solutes, instead of mobile phases. When the corresponding rule is applied (Eq. (13)), $n = 31.99$. Since 441 mobile phases were examined, it is evident that Approach 1 is preferable.

3.7. Application of the CMP approach to the separation of diuretics and β-blockers

All possible combinations that can be established for the 10 solutes were first investigated systematically, arranging the solutes in two and three groups to obtain the optimal CMPs. The results are shown in Table 2. As commented, the optimal mobile phase found for $ng = 1$ is the same as in a conventional optimisation. In the other cases, two and three CMPs are found. The results according to $R1$ and $R2$ are given for each optimal CMP: solutes arrangement, CMP composition and combined resolution value, together with the global resolution for that arrangement.

The importance of the rearrangement step performed in the $R2$ criterion is now illustrated. When the three CMPs case was examined, the optimal combination found before performing the rearrangement was 3212221121 with global resolution values of $R1 = 0.8640$ and $R2 = 0.9627$. After the rearrangement, the optimal combination is 3232213121 (see Table 2). Comparing both solutions, it can be seen that solutes C and G have changed from the first group ($r_C = 0.9977$ and $r_G = 0.9947$) to the third group ($r_C = 0.9994$ and $r_G = 0.9953$), and solute F from the second group ($r_F = 0.9671$) to the first group ($r_F = 0.9896$). The global resolution according to $R2$ was not modified, as expected, but the resolution according to $R1$ was improved to 0.8850. The refined solution is better than the old one, since the resolution of three solutes (C, F and G) is improved.

The maximal elementary resolution values for the different solutes, obtained with one, two or three mobile phases, are given in Table 1. These values should be compared with the limiting values shown in the same table. As a convention to decide when the limiting values are reached with a given treatment, a decision limit of 0.1% below the limiting resolution value was taken. It can be seen that, using

Table 2
Results obtained dividing the solutes into one, two or three groups, according to the product of peak purities and the worst peak purity value[a]

ng	Criterion	Combination[b]		RG_1	RG_2	RG_3	Global resolution
1	Product	1111111111	*R*1	0.7512			0.7512
			c_S	0.0769			
			c_M	0.055			
	Worst	1111111111	*R*2	0.9371			0.9371
			c_S	0.0719			
			c_M	0.055			
2	Product	1212211121	*R*1	0.9543	0.9261		0.8838
			c_S	0.0519	0.0469		
			c_M	0.060	0.15		
	Worst	1212211121	*R*2	0.9837	0.9627		0.9627
			c_S	0.0569	0.0419		
			c_M	0.065	0.15		
3	Product	1312311121	*R*1	0.9543	0.9338	0.9985	0.8898
			c_S	0.0519	0.0419	0.0619	
			c_M	0.060	0.15	0.15	
	Worst	3232213121	*R*2	0.9837	0.9627	0.9953	0.9627
			c_S	0.0569	0.0419	0.0469	
			c_M	0.065	0.15	0.055	

[a] c_S is molar concentration of surfactant and c_M is the volumetric fraction of organic modifier (v/v); *R*1 and *R*2 are the different resolutions (combined and global) measured according to the product of peak purities and the worst peak purity value; RG_1, RG_2 and RG_3 are the combined resolutions in the complementary mobile phases.

[b] Each digit identifies the CMP (phases 1, 2 and 3), whereas the order in the list denotes the solute. For instance, 1212211121 means that solutes A, C, F, G, H and J are resolved with phase 1, and solutes B, D, E and I with phase 2 (see Table 1 for solute identification).

two CMPs, maximal resolution is reached for solutes A, F, G, H, I and J for *R*1, and for these solutes and solute D for *R*2. With three CMPs, all solutes are optimally resolved according to *R*1, but for *R*2 the resolution of solutes B and E can still be improved.

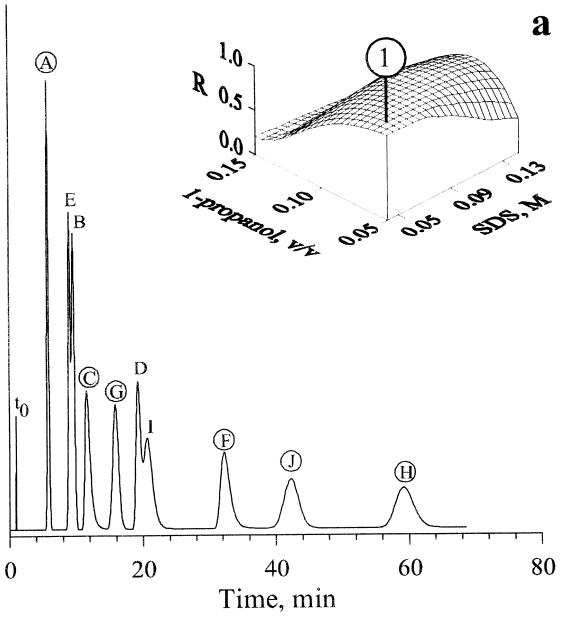
Using the CMP approach, the mixture can be fully resolved. Figs. 4 and 5 show the resolution surfaces for the $\boldsymbol{RG}_1$ and $\boldsymbol{RG}_2$ matrices and the optimal chromatograms for the *R*1 and *R*2 criteria, respectively. As expected, the CMP compositions are similar for both criteria. However, small differences are observed in the corresponding chromatograms. Note also the improvement in the resolution obtained with respect to the use of a single mobile phase (see Fig. 3), where no baseline resolution was found.

3.8. Robustness of the optimal CMPs

Knowledge of the CMPs that resolve the mixture better is not enough. It is also highly desirable to evaluate the degree to which the solution found is affected by changes in the distribution of the solutes in the groups, and by the errors in the preparation of the mobile phases. The robustness in CMP problems can be quantified, therefore, in two ways.

The reliability of solute assignment to each group can be studied by measuring the diminution in the elementary resolution when a given solute is changed from the optimal CMP to the complementary one. An example of two CMPs is considered. The results for the diuretics and β-blockers are the following (%): A (0.0), B (9.8), C (51.9), D (9.0), E (9.9), F (2.2), G (51.5), H (4.1), I (10.4), and J (3.2). As observed, solutes C and G are critical. An error in the assignment of solutes B, D, E and I produces smaller decreases, but still important, in the resolution. The assignment of A, F, H and J is almost indifferent, since these solutes are well resolved with both mobile phases.

Surfaces in Fig. 6a and b indicate the errors



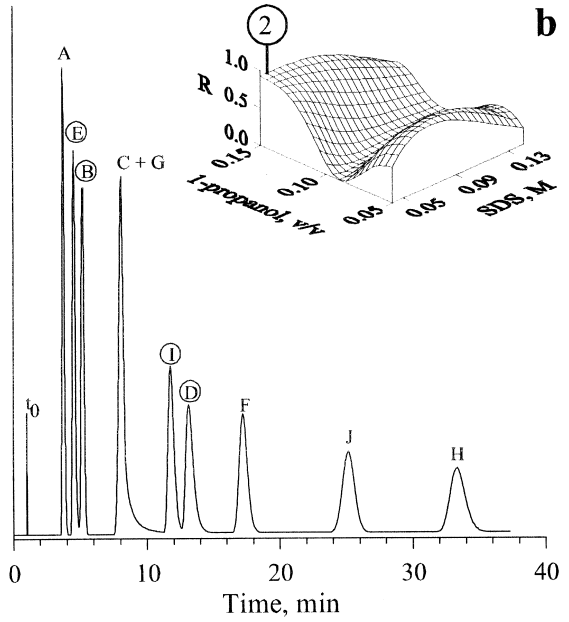
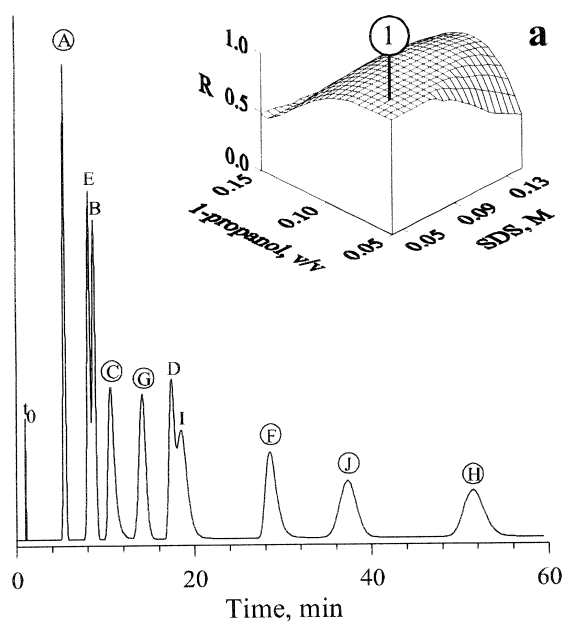


Fig. 4. Resolution surfaces and simulated chromatograms for the two best CMPs found, and solute arrangement after optimisation according to the *R*1 criterion. Composition for both CMPs was: (a) 0.0519 *M* SDS/0.060 v/v 1-propanol, and (b) 0.0469 *M* SDS/0.15 v/v 1-propanol. The circles in the chromatograms indicate the solutes resolved with each CMP. See Fig. 3 for peak identity.



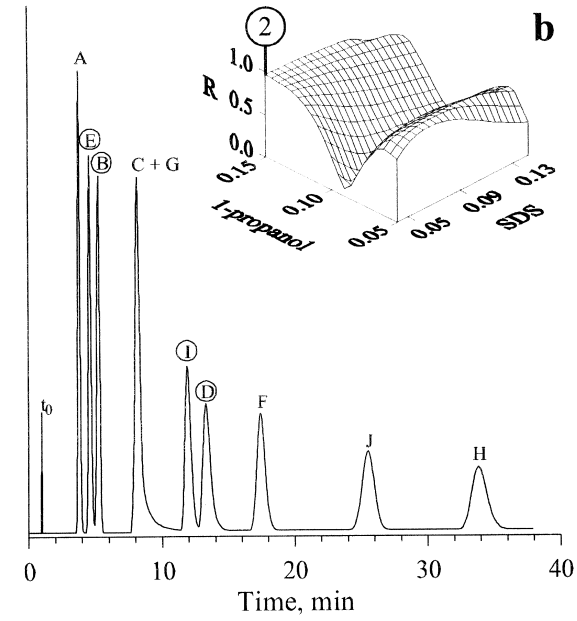


Fig. 5. Resolution surfaces and simulated chromatograms for the two best CMPs found, and solute arrangement after optimisation according to the *R*2 criterion. Composition for both CMPs was: (a) 0.0569 *M* SDS/0.065 v/v 1-propanol, and (b) 0.0419 *M* SDS/0.15 v/v 1-propanol. The circles in the chromatograms indicate the solutes resolved with each CMP. See Fig. 3 for peak identity.

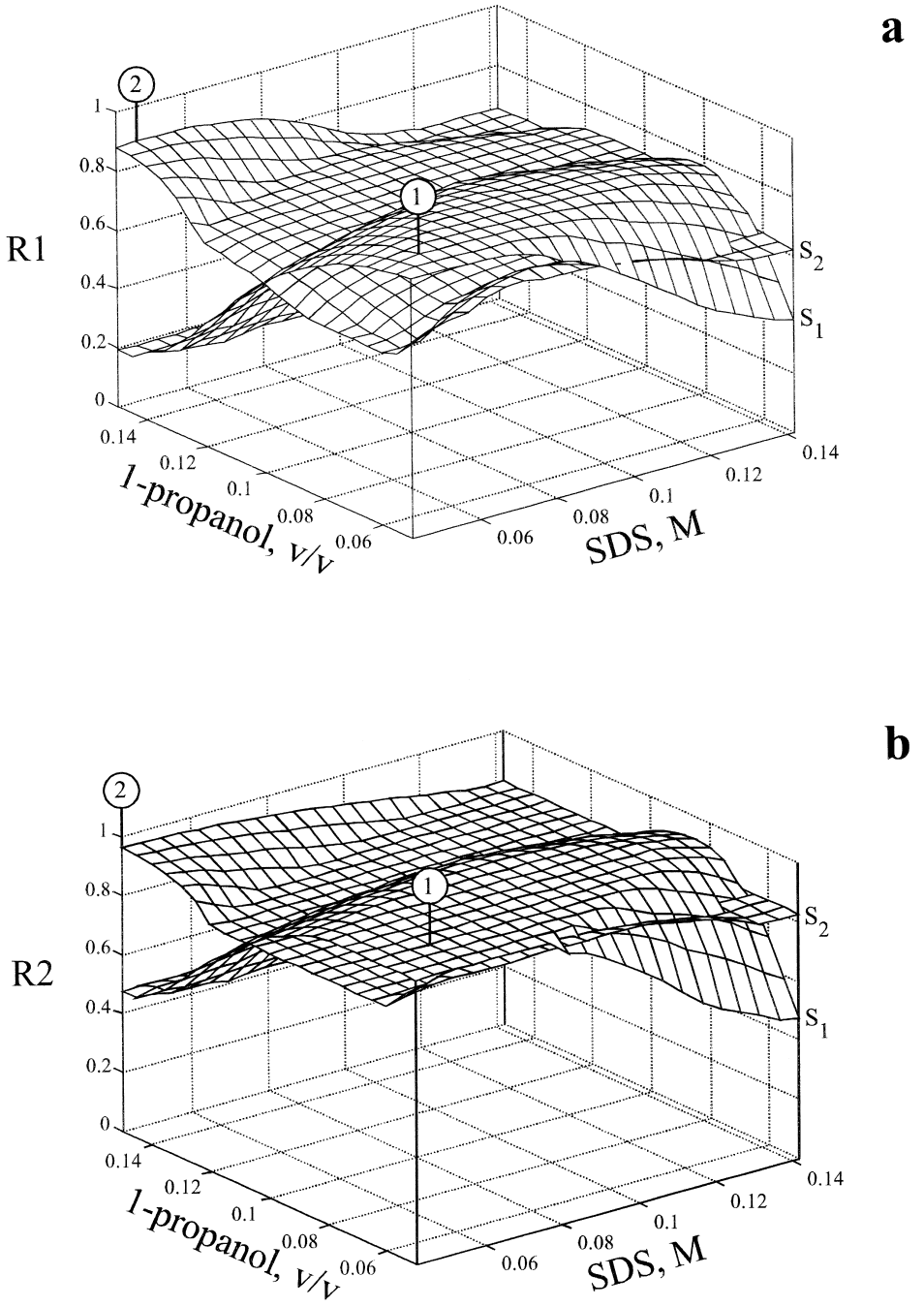


Fig. 6. Robustness measurement considering hypothetical errors in mobile phase preparation when two CMPs are selected according to the: (a) *R*1 and (b) *R*2 criterion. The location of the optimal complementary mobile phases 1 and 2 is encircled. See text for meaning of drawn surfaces.

obtained when one mobile phase is free of errors in its preparation, while the other is gradually misprepared. The surfaces drawn were obtained by fixing alternatively the composition of one of the two optimal CMPs and varying systematically the composition of the other (keeping, however, the solute assignment), in order to simulate errors throughout the whole factor space. Each point on the surface is a measurement of the global resolution according to $R1$ or $R2$, for the optimal combination of solutes. As can be seen, for both criteria, the robustness of phase 1 (0.0519 *M* SDS/0.060 v/v propanol for $R1$ and 0.0569 *M* SDS/0.065 v/v propanol for $R2$) is high in both surfactant and organic modifier (surface S_1), whereas phase 2 (0.0469 *M* SDS/0.15 v/v propanol for $R1$ and 0.0419 *M* SDS/0.15 v/v propanol for $R2$) is quite robust in the direction of the surfactant axis, but not so much in the direction of the modifier (surface S_2).

3.9. Use of genetic algorithms

The results obtained through the systematic search explained above are the true solutions, since all combinations were exhaustively examined, and can be taken as a reference to check whether other methods, faster but not so exhaustive, are able to find the same solutions. Genetic algorithms (GAs) [22] are constrained global optimisation methods and can be a good alternative in problems where a high number of local solutions exist. Several arguments justify the use of GAs in the optimisation of CMPs. First, the CMPs problem has a discrete nature: one solute can only be assigned to one mobile phase or vice versa; the numbers involved are thus necessarily integers, usually also small. Secondly, a large number of local maxima (defining different solutions to the same problem) exists. Thus, conventional optimisation methods (e.g., simplex, conjugate gradient, or metric variable) cannot be applied to find the optimal CMPs.

One mandatory step in any GA is to encode the information in discrete numbers, usually binary, which constitutes an entity called chromosome. Each chromosome contains a sequence of one or more encoded parameters, each having several associated integer numbers. For each parameter, the user must establish a lower and an upper limit between which the parameter can vary. GAs improve a population of solutions mimicking the operations made in Nature to obtain individuals optimally adapted to the environment. In the CMPs problems, the way of measuring the adaptation of the individuals (combinations) to the environment (or cost function) is straightforward: $R1$ or $R2$ should just be maximised.

The way of encoding the information is perhaps the most important question, in order to avoid meaningless solutions and make the search more efficient. For this reason, the performance of several encoding systems in both arranging strategies (i.e., Approach 1 and Approach 2) was checked. In the first case, it was found that the best encoding system was to make bits and parameters equal (the parameter here is the group to which a given solute belongs), converting directly a solute combination into a chromosome (e.g., 1212211121). In the second case, ordinary binary encoding should be applied.

Fig. 7 compares the results obtained in both systematic and genetic algorithm searches. The global limiting resolution (upper dashed line) was obtained by multiplying the limiting resolution values for each compound, and indicates the maximal $R1$ value that can be obtained using a different CMP for resolving each compound. True solutions for two and three CMPs (dashed lines), together with the GAs evolution for solving the corresponding problem, are shown overlayed. Since the population being improved involved 30 chromosomes, the numbers of combinations examined were 1830 and 3240 for two and three CMPs, respectively. This means that the calculation time was reduced to 1.8 and 0.02% with respect to the systematic search. As can be seen in the figure, GAs found the same solution in a few generations (or a very close solution in the case the process was stopped prematurely). The more complex the situation, the more advantageous the application of GAs. With the computer used, finding the optimal CMPs by GAs required only a few seconds.

4. Conclusions

In the complementary separation strategies reported in the literature, sufficiently different con-

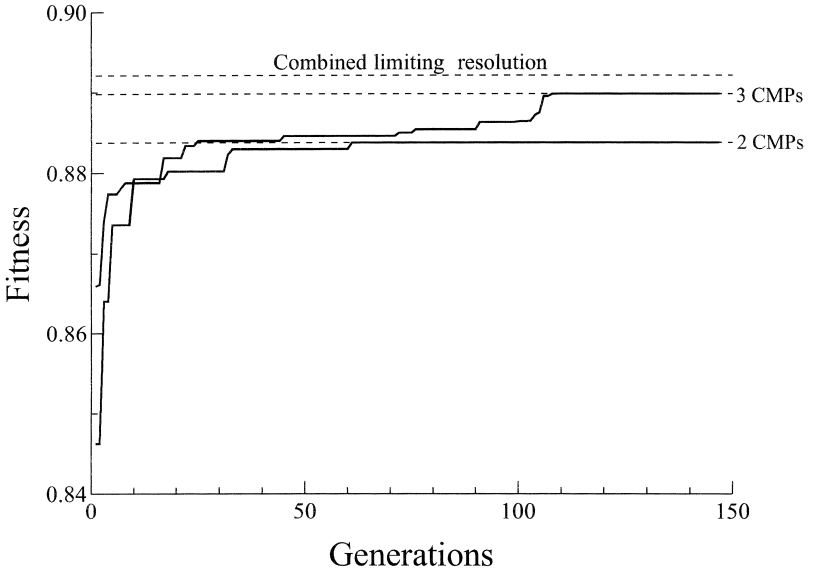


Fig. 7. Fitness plots in a genetic algorithm search showing the improvement in resolution versus number of generations, for (a) two CMPs and (b) three CMPs. The GAs cost function (i.e., fitness) that is maximised is $R1$. The global limiting resolution and the true solutions found in the systematic search for two and three CMPs are overlayed as dashed lines.

ditions are searched to obtain the desired resolution, but the diverse separation systems are usually optimised separately. The proposed CMPs approach implies a combined optimisation of different mobile phases. It is shown how this approach may have a considerable effect in the resolution of complex mixtures. The success of the separation depends on the variation in the selectivity of the eluted compounds.

An interesting point is that new experimental work is not required. If a separation with a single mobile phase fails, the CMPs search can be performed without carrying out any new experiment. Also, the limiting resolutions indicate whether or not it is feasible to resolve the mixture or, on the contrary, if the system is unable to do it. Several approaches were checked to optimise the calculation speed: the establishment of groups of solutes or the selection of mobile phases, a systematic search of all possible combinations or the use of GAs. In the case of considering two or three CMPs, the problem is usually better faced establishing combinations of solutes. When the number of mobile phases and solutes leads to excessive calculation time, GAs yield a faster solution than a systematic search.

The use of two CMPs instead of a single mobile phase is advantageous when the limiting resolutions are significantly higher than the elementary values reached in the single optimal phase found. If the limiting values are not satisfactorily reached using two CMPs, three CMPs can be selected, although the practical interest of the proposed approach is smaller. In the particular separation problem shown in this work, two optimal CMPs yielded good resolution, and three CMPs practically reached the expected limiting resolution values for each solute.

Although the results obtained with both criteria, product of resolutions ($R1$) and worst elementary resolution ($R2$), were satisfactory, the first criterion is preferable since it improves the resolutions of all the compounds instead of only some of them. The rearrangement step in $R2$ improves the reliability of this criterion, but further refinement is needed. The reason is that when the assignment of the solutes changes after the rearrangement, the optimal CMPs are not usually the same as those found before. Also,

other considerations can be taken into account. For instance, CMPs leading to long retention times can be penalised.

5. Nomenclature

B/A	asymmetry factor measured at 10% of peak height
c_M	volumetric fraction of organic modifier
c_S	concentration of surfactant forming micelles
CMPs	complementary mobile phases
g	group
GAs	Genetic algorithms
$K_{AD,}$ $K_{MD,}$ K_{SD}	displacement constants of the solute–micelle and solute–stationary phase partition equilibria
K_{AM}	association constant of solute with the micelles
K_{AS}	association constant of solute with the stationary phase
H_0	peak height
N	efficiency
N_A	number of combinations of mobile phases
N_B	number of combinations of solutes
ng	number of groups
np	number of mobile phases
ns	total number of solutes
$ns(g)$	number of solutes belonging to group g
PMG	polynomially modified Gaussian model
$\boldsymbol{R}$	global resolution matrix for a single-phase optimisation
r_s	peak purity for solute s
$\boldsymbol{R}_s$	peak purity matrix for solute s
$\boldsymbol{R}(g, s)$	elementary resolution matrix for solute s in group g
$\boldsymbol{R}(1, s)$	global resolution matrix for a single-phase optimisation
$R1$	product of elementary resolutions
$R2$	worst elementary resolution
$\boldsymbol{RG}_g$	combined resolution matrix for group g
RG_g	maximal value in each $\boldsymbol{RG}_g$ matrix
s	solute
s_0, s_1	peak profile parameters
SDS	sodium dodecyl sulphate
t_0	dead time
t_R	retention time
w_s	total peak area of solute s
w'_s	area under the peak of solute s overlapped by the chromatogram of the remaining peaks

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