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REVIEW

COMPUTER-AIDED MOBILE PHASE OPTIMIZATION AND CHROMATOGRAM SIMULATION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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1. INTRODUCTION

Several different methods of computer- or microprocessor-aided mobile phase optimization in high-performance liquid chromatography (HPLC) have recently become available. Some can be run off-line using programs designed for personal microcomputers and others are integral routines in microprocessor-controlled chromatographs. The purpose of this review is to outline some of the salient features of these different systems, and to compare them with the computer chromatogram simulation method (CCSM) developed and tested in our laboratories. The procedures under review are listed in Table 1. While this list will certainly not remain comprehensive for long, the methods it contains are broadly representative of the various semi-empirical and systematic strategies that can be adopted with the goal of multi-solvent mobile phase optimization in view.

2. STRATEGIES FOR OPTIMIZATION

Most strategies for the optimization of HPLC conditions are based on the well verified relationship [1]

$$R_s = \tfrac{1}{4} \cdot (\alpha - 1) \cdot (\sqrt{N}) \cdot \frac{k'}{k' + 1} \qquad (1)$$

where R_s = resolution factor, N = plate number, k' = solute capacity factor and α = selectivity factor (k'_2/k'_1).

At the present time approximately 100 000 theoretical plates per m represent the maximum efficiency attainable with conventional packed columns, and, as is well known, because resolution is a function of $\sqrt{N}$, any increase in plate number results in a proportionately smaller increase in effective resolution. Increases in resolution obtained by increasing solute capacity factors obviously compromise sensitivity of detection. Consequently, most efforts at enhanced resolution have been directed at changing the selectivity factor (α), either by altering the stationary phase or the mobile phase, or both. There are, however,

some difficulties in ensuring reproducibility of separations when different stationary phases are used for selectivity, particularly when reversed-phase (RP) packings are employed, and complex separations are attempted which may depend on some degree of mixed-mode chromatography for success. Selectivity through mobile phase modification is therefore preferable in principle, and in practice. Mobile phase modification includes, of course, the commonly adopted methods of gradient (i.e., non-isoeluotropic) elution. This is usually employed simply to reduce the time of analysis and increase sensitivity, but can also be used to generate retention data with which to predict retention times in corresponding isocratic systems (and vice versa), aspects of the theory and practice of which have been extensively discussed by Snyder and co-workers [2,3].

The ideal system of chromatographic optimization should enable the chromatographer to achieve the maximum attainable separation of all components of complex multi-component mixtures of solutes within the minimum time of analysis possible. The advent of the microcomputer has recently placed within the reach of all chromatographers a variety of possible computational methods for achieving this goal. Under ideal circumstances, the best method would involve the direct (a priori) prediction of chromatographic behaviour from the individual chemistry of each solute in relation to the mobile phase composition. While it is, for example, possible to predict the retentions of, for example, small peptides and polypeptides in reversed-phase chromatography from their amino acid composition [4, 5], the general utility of this approach is severely limited [4,6].

In more typical cases of smaller molecules the complexity of the interactions between such solutes and the mobile and stationary phases is such [7] that deterministic models of their chromatographic behaviour and solvent theory have been of limited value for optimizing complex multi-component separations, particularly with respect to the reversed-phase mode which has essentially come to dominate HPLC on account of its reproducibility and versatility. A deterministic procedure that has been adapted for microcomputer-aided mobile phase optimization in isocratic RP-HPLC has, nevertheless, been recently developed by Jinno and Kawasaki [8]. Their method (Retention Prediction System) is based on the type of quantitative structure—retention relationships (QSRR) that derive from principles elaborated in gas—liquid chromatography (GLC). It is, therefore, based on the assumption that the free energy of retention of a molecule can be derived from a linear combination of retention energies of its constituent functionalities. It is also assumed that there will be no changes in the stationary phase (due for example to column conditioning) that will modify QSRR. In Jinno and Kawasaki's [9] latest approach the correlations between log k' and physicochemical parameters such as π (hydrophobic parameter), P (partition coefficient), F (correlation factor), χ (molecular connectivity index), L/B (shape parameter) and V_W (Van der Waals volume) have been reduced to log P and F, enabling the calculations of these correlations by linear multi-regression analyses to be carried out on a 16-bit microcomputer. The database for such predictive procedures obviously requires a large number of experiments and compounds, and empirical correction factors have had to be included even with the closely related substituted polycyclic aromatic hydrocarbons studied by Jinno and Kawasaki [9] in order to obtain

a reasonably useful correlation between predicted and observed retentions as a function of mobile phase compositions. The application of this procedure to other less ideal solutes may, however, be less satisfactory.

In the majority of instances, trial and error methods of reversed-phase optimization have predominated with in general the selection of the best binary mobile phase on the basis of individual organic modifier selectivities [10,11]. However, as separations of increasing complexity have been required, multi-solvent mobile phases have been used with increasing frequency because of the different specific selectivities that can be obtained with individual organic modifiers [12]. Intuitive or non-systematic optimization of three-component mobile phases is both time-consuming and ineffective, for four components it is virtually impossible, and methods of sequential approximation and/or stochastic prediction employing computer-aided factor design or mixture design strategies become necessary. The former are often applied to discrete variables (e.g. pH, temperature) and the latter to related variables such as solvent concentrations. Table 1 includes examples of both of these optimization strategies.

TABLE 1

METHODS OF MICROCOMPUTER- OR MICROPROCESSOR-AIDED MOBILE PHASE OPTIMIZATION IN HPLC

Method	System	Type	Reference	
			Authors	No.
COF	Off-line	Isocratic	Glajch et al. (1980)	20
Window diagram	Off-line	Isocratic	Sachok et al. (1980)	32
ORM (SENTINEL)	Integral	Isocratic	Glajch et al. (1982)	35
ISOOPT	Integral	Isocratic	Berridge (1982)	25
TERNOPT	Integral	Isocratic	Berridge (1982)	25
GRADOPT	Integral	Gradient	Berridge (1982)	25
Search and stop	Off-line	Isocratic	Drouen et al. (1982)	19
OPTIM (I)	Integral	Isocratic	Bradley and Gillen (1983)	27
OPTIM (G)	Integral	Gradient	Bradley and Gillen (1984)	28
OPEX	Off-line	Gradient	Sabate et al. (1983)	26
IMGE	Off-line	Gradient	Kirkland and Glajch (1983)	21
SMGE	Off-line	Gradient	Kirkland and Glajch (1983)	21
PEAKIN/SAS	Off-line	Isocratic	Issaq (1984)	36
RPS	Off-line	Isocratic	Jinno and Kawasaki (1984)	8,9
CCSM-I	Off-line	Isocratic	D'Agostino et al. (1984)	39

3. CRITERIA FOR OPTIMIZATION AND PARAMETERS FOR EVALUATION OF CHROMATOGRAMS

In order to use some form of automated method for optimization it is necessary to derive an objective parameter to define the 'goodness' of chromatograms. There is, as yet however, no universally accepted measure. A variety of different quantitative measures of the quality of peak separation have been proposed. Those commonly used are usually defined either in terms of separation/resolution of either all peaks collectively, or alternatively the least well

resolved pair, together with, in some instances, a term which relates to the total time of analysis required to effect a given separation. The resultant parameter or function constitutes the response with which the quality of an experiment is evaluated when using, for example, a simplex method [13] as a multi-dimensional search procedure for localising the optimum conditions. From a stochastic point of view the use of only the least well resolved pair as the response has certain limitations for multi-component separation in HPLC. A criterion which operates on all solutes, either by summating individual peak pair resolution values, or by calculating their product, is, in our opinion, preferable.

3.1. Chromatographic response function

The chromatographic response function (CRF) is a criterion which has been used in several forms but which can be essentially defined as peak separation parameter (Fig. 1). It was originally suggested by Kaiser [14] and developed as a summated function for multi-component separations in GLC by Morgan and Deming [15], who introduced the use of a logarithmic as distinct from geometric peak separation factor to enhance the sensitivity of the CRF value to poorly separated peaks. It was applied to HPLC by Watson and Carr [16], who included a term to measure excess analysis time, as in the form given in eqn. 2.

$$\mathrm{CRF} = \Sigma \ln(P_i/P_d) + \alpha(T_m - T_l) \qquad (2)$$

where P_i is the experimental peak separation, P_d is the desired peak separation (common to all pairs of peaks), T_l is the actual analysis time, T_m is the acceptable analysis time and α is an arbitrary weighting factor adjusted to achieve an operationally satisfactory balance between resolution and analysis time (typical values are 0.1–0.01).

In calculating the CRF, only pairs of peaks that do not give effectively baseline resolution ($P<1$) will contribute to the overall value, and under ideal conditions, e.g. $P_i>P_d$ for all peaks and $T_l<T_m$, CRF is zero (see Fig. 1). However, this criterion may not provide a particularly robust separation, particularly when there are large differences in adjacent peak height. For this reason optimization criteria based on a peak resolution parameter in which separations greater than baseline may be optimized are generally to be preferred on

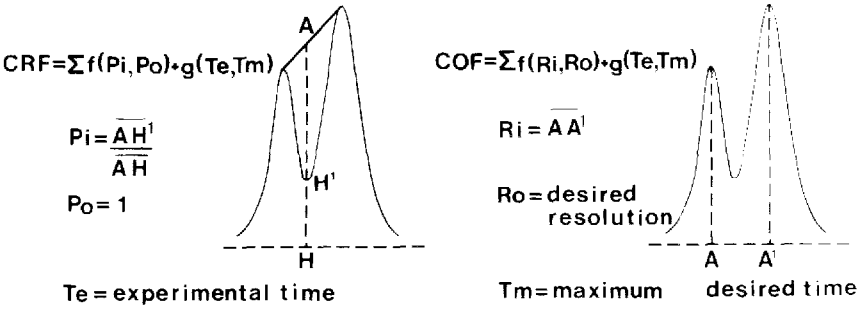

Fig. 1. Peak separation and peak resolution based parameters. CRF and COF are shown as examples.

account of their greater flexibility in an interactive context. Furthermore, although Wegscheider et al. [17] have introduced a 'noise' term into CRF calculations (which they calculate as a product rather than a sum), peak separations according to the CRF criterion may, in practice, be difficult to measure accurately because of baseline drift, baseline noise or large differences in peak heights. Most CRF-type calculations have involved the assumption that separations of all components are equally desirable, although this is often at variance with real chromatographic requirements.

3.2. Relative resolution product

The relative resolution product (RRP) defined by Schoenmakers et al. [18] and Drouen et al. [19] measures greater than baseline separations, and in its original form makes the assumption that all solutes are of equal importance, although weighting factors could be included. The latest form taken by this criterion is given in eqn. 3.

$$r = \prod_{i=1}^{n-1} R_{s_{i+1,i}} \Big/ \left[\left(\sum_{i=1}^{n-1} R_{s_{i+1,i}} \right) / (n-1)^{n-1} \right] \qquad (3)$$

where $R_{s_{i+1,i}}$ is the resolution factor for adjacent peaks i and $i+1$, defined as $R_s = (V_{i+1} - V_i)/(w_i + w_{i+1})$ where V is the elution volume and w the 2σ peak width.

The RRP (r) reaches a maximum of 1 (the optimum) when all R_s values (resolution factors) are equal, with the result that it describes a situation when all peaks are evenly distributed throughout the chromatogram. It is, however, reduced to a minimum (0) when any two peaks are co-eluted. This, together with the lack of a peak priority term whereby the separation of peaks of particular analytical importance can be protected, constitutes a serious limitation of this criterion. Furthermore, because it considers only the relative and not the absolute positions of peaks it does not, in its original form, distinguish between alternative solutions with the same r values but different analysis times. A modification of eqn. 3 was consequently proposed by Drouen et al. [19] to take into account the observed different overall analysis times encountered when using nominally isoeluotropic ternary mobile phases. This consists of incorporating a factor in the denominator of the equation representing an imaginary peak ($i = 0$) eluting at the desired starting point of the chromatogram (t_0) such that, if the first actual peak elutes later than this point (for example $k' = 1$), the value of r is reduced. In this form the RRP aims at an even distribution of peaks early in the chromatogram.

3.3. Chromatographic optimization function

The chromatographic optimization function (COF) [20], on the other hand, has interactive features which commend it for stochastic (i.e. statistical) predictive approaches to chromatographic optimization. Like the RRP it is a peak resolution rather than a peak separation parameter (Fig. 1), but includes in its original form weighting factors for both time of analysis (like CRF) and peak

resolution priority (eqn. 4):

$$\mathrm{COF} = \sum_{i=1}^{k} A_i \cdot \ln\left(\frac{R_{s_{i+1,i}}}{R_{s_{id}}}\right) + B(T_m - T_l) \quad (4)$$

where R_{s_i} = peak resolution, $R_{s_{id}}$ = desired peak resolution, T_m = maximum analysis time acceptable, T_l = actual analysis time and A_i and B are arbitrary weighting factors.

Under optimized conditions the COF approaches zero from a negative direction and only when peak priority weightings are all set to unity does it reduce to a form akin to CRF (eqn. 2). As discussed below, however, the full form of the COF has not been utilised in all applications for automated optimization.

3.4. Chromatographic optimization coefficient

In our own approach to computer-aided optimization by off-line methods we have used an extended version of the full COF formula which we have termed the chromatographic optimization coefficient (COC) (eqn. 5). As described below it is used as a coefficient to calculate quality of chromatograms, real and predicted, and not as a function whose variation is directly correlated with mobile phase composition as in the original method of Glajch et al. [20]. The COC is, like COF, a peak resolution parameter and includes weighting factors for peak priorities as well as a term reflecting the total analysis time.

$$\mathrm{COC} = \sum_{i=1}^{n} \; i \neq j \sum_{j=1}^{n-1} A_i \cdot A_j \cdot \ln\left(\frac{R_{s_{i,j}}}{R_{s_{i,jd}}}\right) + \sum_{j=1}^{n} B \cdot \sqrt{A_i} \cdot \frac{T_m - T_i}{T_m} \quad (5)$$

where n = number of compounds, A_i and A_j are weighting factors for each compound, $R_{s_{i,j}}$ = resolution between peaks i and j, $R_{s_{i,jd}}$ = desired resolution between i and j, T_m = desired total analysis time, T_i = retention time of peak i, and B is a weighting factor for analysis time (typically 0.1). If $T_i < T_m$ then $T_m - T_i = 0$; if $R_{s_{i,j}} > R_{s_{i,jd}}$ then $R_{s_{i,j}} = R_{s_{i,jd}}$.

Like COF, the COC is the sum and not the product of individual peak pair resolutions and is not unduly influenced by a single instance of eclipsed peaks, although as a logarithmic resolution function it remains sensitive to incompletely resolved solutes.

By insertion of appropriate parameters (A_i, $R_{s_{i,jd}}$, T_m, B) optimum conditions can be selected in terms of peak resolution, total analysis time or any combination of these parameters. When the COC is implemented as part of the automated computer chromatogram simulation method (CCSM), the chromatographer has interactive control over peak priority weightings and desired total analysis time, and must enter the desired resolution in the form of the experimentally determined peak width for each component of the mixture (i.e. as a function of the efficiency of the system with respect to each solute), or a greater value as he deems appropriate, depending on the robustness of the predicted separation.

4. METHODS OF OPTIMIZATION

4.1. Isoeluotropic versus non-isoeluotropic optimization

Optimization procedures can be operationally divided into those for isocratic separations and those for gradient elution conditions. Table 1 includes examples of both, as indicated. However, they are more logically divided into those which involve an empirical pre-selection of overall solvent strength and which then search for a local optimum involving an isoeluotropic multi-component mobile phase and those which systematically optimize a multi-component mobile phase without prior selection of solvent strength. Among the latter non-isoeluotropic (or multi-eluotropic) methods are those which allow systematic optimization of multi-solvent gradient elution (e.g. SMGE [21] and CCSM-Gradient, see below), as well as a fully systematic optimization of solvent strength for isocratic separations with mixtures of two (PEAKIN/SAS) or three (CCSM-I) organic modifiers in water.

4.2. Sequential approximation with factorial design versus statistical prediction with mixture design

In addition to discriminating between isoeluotropic and non-isoeluotropic optimization systems, two main design approaches can be distinguished among the procedures listed in Table 1. The first involves a method of successive approximation requiring an initially undefined number of experimental chromatograms before an optimum is found, and the second is a stochastic predictive approach based solely on data from a limited pre-defined number of chromatograms. The nature of the equations used to define the relationship between solute behaviour and chromatographic conditions is also significantly different in various methods with linear, quadratic and higher-order polynomial regressions used in different cases, with implications with regard to precision with which optima are defined, and the speed with which they are found.

4.3. Sequential approximation methods

4.3.1. Simplex optimization

Some optimization procedures have chosen sequential simplex methods for attaining the best response. A simplex is a geometric figure defined by the number of vertices equal to the number of dimensions of the factor space plus one [13]. Sequential simplex optimization is a geometric search pattern technique which evaluates the response of a system from a set of points forming a simplex in the factor space and tracks the optimum by continually forming new simplices by reflecting one point in the hyperplane of the remaining points, according to the rules proposed by Spendley et al. [22]. The latter have their origin in a consideration of how the evolutionary operation of the steep ascent optimization procedure of Box could be automated. The response is defined as a surface in $(n + 1)$-dimensional space, where n is the number of independently variable experimental factors (flow-rate, temperature, pH, solvent concentration, etc.). Thus, in a typical case of ternary solvent optimiza-

tion with pre-determined solvent strength there are two such variables and the response surface lies in three-dimensional space, with the simplex requiring three initial experimental points and mapping as a triangle in two-dimensional factor space (Fig. 2). With the aid of function minimization techniques [23], the simplex can be made to contract towards the optimum (provided this is unique). In a quaternary solvent system for RP-HPLC in which solvent strength (i.e. water concentration) is an independent variable the appropriate simplex is

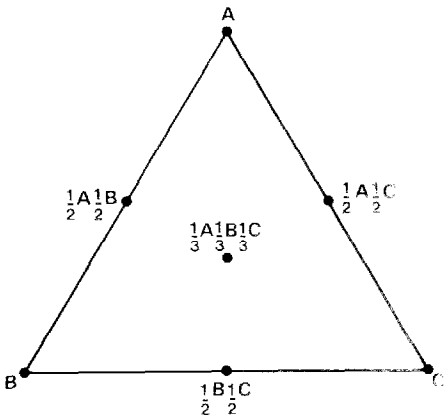

Fig. 2. Seven-point simplex lattice for mixture design optimization of a ternary mobile phase (after Glajch et al. [20]), where A, B and C are three solvents in normal phase or three binary organic modifier–water mixtures of equivalent solvent strengths in RP-HPLC.

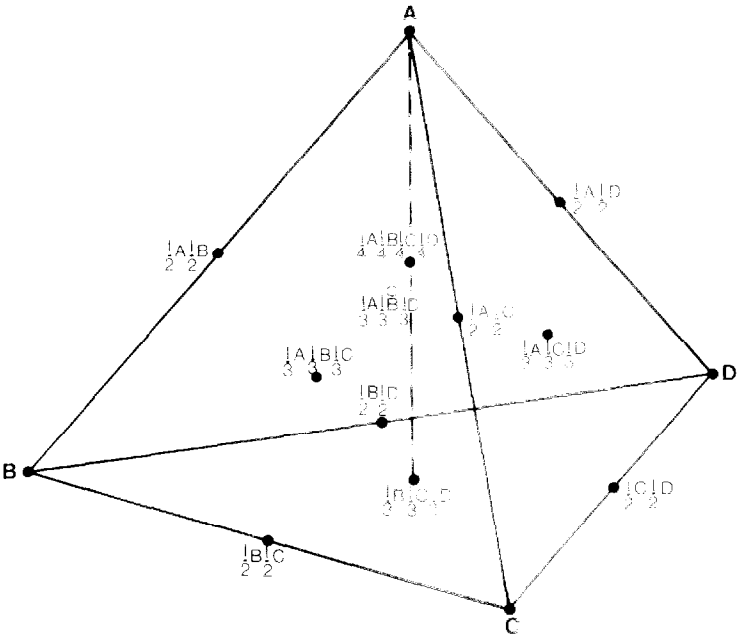

Fig. 3. Fifteen-point simplex lattice for mixture design optimization of a quaternary mobile phase in normal-phase HPLC showing the properties of the four different solvents in each experiment.

a tetrahedron (Fig. 3), with the response surface mapping in a four-dimensional hyperspace ($n + 1 = 4$). Function minimization techniques or sequential simplex optimization runs of different sizes are essential for localizing true optima in this situation. The problems associated with applying simplex algorithms for numerical optimization to experimental optimization have been extensively considered by Deming and Parker [13].

In their original experiments Morgan and Deming [15] used a 3^2 factorial design to optimize carrier gas flow-rate and temperature in GLC. As with most practical applications of simplex designs, it was necessary to place certain limits on these variables. This was achieved by assigning an undesirable arbitrary response value (CRF = −100) to any vertex of the simplex that violated this boundary. Anything up to 25—30 vertices (i.e. chromatograms) were generated in the search for the optimum, and the likely existence of multiple optima in the factor space domain, due to changes in peak retention order [24], means that these techniques cannot guarantee that the global optimum has been achieved. Nevertheless, sequential optimization is the basis of several methods for mobile phase optimization in HPLC, both in the form described here and in other semi-predictive techniques designed to limit the number of experiments required for full sequential optimization.

4.3.2. ISOOPT, TERNOPT and GRADOPT

These three closely related methods, devised by Berridge [25] for fully automated optimization are all based on a sequential 3^2 factorial simplex design. Response is measured by a CRF parameter, (eqn. 2), with the addition of a further term (L^x) in respect of peak number and dictated by the unattended on-line operation of these programs, controlling the chromatograph. The large step size simplex method of Yarbro and Deming (see ref. 13) is used with Nelder and Mead's [23] algorithm for function minimization. In ISOOPT flow-rate and binary composition are optimized, in GRADOPT solvent strength and gradient duration, while in TERNOPT a three-component isocratic mobile phase (two variables) is defined. The only computational difference is that in ISOOPT and TERNOPT boundary violations result in a rejection of corresponding experimental coordinates by assignment of CRF=−100, while in GRADOPT the simplex contracts to the relevant boundary to facilitate a more rapid convergence on the optimum. Despite this, however, up to fifteen sequential chromatograms are required for GRADOPT and up to thirty in ISOOPT and TERNOPT, even when optimizing conditions for a small number of solutes (e.g. four). None of the solute mixtures illustrated [25] involved crossovers, which as explained above can result in multiple local minima in factor space which these sequential simplex techniques are poorly equipped to handle.

OPEX [26] is another comparable multi-factorial sequential simplex method, based on resolution of only the least well resolved pair of solutes. These techniques are clearly of limited value for global optimization and are time-consuming even for simple problems in HPLC. This has lead to the development of techniques in which sequential approximation and stochastic prediction are combined to accelerate the process.

4.4. Sequential approximation with stochastic prediction

4.4.1. OPTIM

A simple example of this combined approach is the 'adaptive intelligence' ROM chip that constitutes the OPTIM system of Spectra-Physics. It is based on what is essentially a 2^2 simplex progress for binary optimization combined with a statistical predictive method for defining a ternary (three-component) optimum. Its criterion for optimization (XA) has not been precisely defined but is probably of the CRF type, being a function of separation times and the resolution of the worst resolved pair plus peak number term [27]. Having established a binary (A+B) optimum to within 1—2% in a step-wise manner it calculates the appropriate isoeluotropic A+C mixture, runs it and the 50:50 (A+B+C) mixture and by an unspecified computation predicts a ternary optimum. The binary mode is, however, time-consuming and total optimization times of 10—18 h for a twelve-component mixture are reported [27]. Its application to gradient elution has been recently described [28] and consists of substituting gradient slope for binary composition in the first stage of optimization, the starting point of the binary mobile phase being determined by the criterion that less than four peaks elute within a time equivalent to a k' of 4. It is capable of optimizing only a one-step linear gradient profile.

4.4.2. Window diagrams

Several of the semi-predictive techniques designed to overcome some of these problems are based on a window diagram approach. This originated in the studies of Laub and co-workers [29,30] on liquid phase component optimization in GLC, a problem which has obvious similarities with mobile phase optimization in HPLC, but deals with a simpler relationship between volatility and the effects of liquid phase composition on retentions than is the case for mobile phase composition and solute retention in the latter situation. Nevertheless, the window diagram is a more effective means of localising a global optimum if (and this is the key) an appropriate mathematical function can be found which accurately defines the relationship between retentions and the variable under consideration. In the experiments of Laub and co-workers [29,30] there was a single variable and a linear relationship was assumed. The window diagram is created from a graphical representation of all individual analyte pair relative retentions as a function of the variable, with intermediate values derived by linear interpolation from two experimental data sets for the limiting conditions. The intersections of the relative retention curves delimit a series of windows of accessibility. The highest point of the tallest window corresponds to the chromatographic conditions which should give the best possible separation of the two worst resolved pairs of analytes, with all other pairs separated better. This approach was transferred to HPLC with the substitution of the separation factor ($S = 2R_s/N^{\frac{1}{2}}$) for the relative volatility by Jones and Wellington [31], who also used it for mono-factorial optimization (pH). The window diagram method was also extended to non-simultaneous multifactorial analysis in RP-HPLC by Sachok et al. [32], when the windows become multi-dimensional in nature. Its limitations in this context include not only the greatly increased numbers of experimental chromatograms required,

but also a requirement for a more accurate correlation of chromatographic variables with predicted retentions for intermediate values than is possible with linear regression.

4.4.3. Search and stop

The method of Schoenmakers et al. [18] and Drouen et al. [19] for isoeluotropic mobile phase optimization is a mono-factorial sequential procedure but with certain modifications to the form of the regressions. The overall solvent strength in search and stop is chosen on the basis of a preliminary gradient elution chromatogram which establishes the range of polarities in the sample (equivalent to the SCOUT step in the SENTINEL procedure). Operationally search and stop starts with retention data from two pre-determined isocratic experiments (usually two different binary mobile phases chosen from three preliminary experiments) and linear regressions between solvent composition and ln k' values are calculated in the first instance. From these a so-called phase selection diagram (equivalent to a window diagram) is created using the relative resolution product (eqn. 3) as a function of solvent composition. The method continues with the generation of an experimental chromatogram under mobile phase conditions predicted as optimum (i.e. highest value of r) by this criterion. If the observed retentions differ significantly from the predicted values they are used to construct a corrected phase selection diagram from which a new, and hopefully improved, optimum is predicted. The iteration stops when the next optimum offers no further improvements. In an example of a five-component mixture a total of five to six chromatograms was required before this stage was achieved.

A significant disadvantage of search and stop is the fact that the RRP does not necessarily result in the most effective separation being chosen as optimal, as the results of Drouen et al. [19] themselves illustrate. Another major problem with this method is that if the variation of ln k' with solvent composition deviates significantly from linearity then the convergence to the optimum may be slow requiring many additional chromatograms. To compensate for this effect, in its latest form [19] search and stop runs these sequential chromatograms at a composition shifted from the linearly predicted optimum to a value which partially approximates a quadratic expression for ln k' versus mobile phase.

4.5. Stochastic prediction without sequential approximation

Mixture design experiments based on simplex designs can also be used for statistical predictive methods of mobile phase optimization that do not involve sequential approximation. The essential steps in this approach have been defined by Snee [33] as (1) generation of data using a pre-planned experimental design, (2) finding a mathematical model to fit this data using statistical curve-fitting techniques and (3) examining the response—surface contours to determine the best value. The advantages inherent in this approach are that they require a limited pre-defined number of experimental chromatograms and can be used for assembling a model of the chromatographic system in question that can, in principle, permit the behaviour of all solutes to be accurately pre-

dicted within the mobile phase envelope defined by the simplex, without recourse to further experiments, thus enabling the global optimum to be located. Their main disadvantage is that a relatively extensive series of polynomial regressions must be examined before coefficients that accurately describe the retentions as a function of solvent composition can be obtained.

4.5.1. Chromatographic optimization function (COF) method

The first attempt at this solely predictive approach was that of Glajch et al. [20]. The experimental design selected for isoeluotropic optimization was a ten-point design described by Snee [33], subsequently modified to a seven-point design as illustrated in Fig. 2, the other three points being relegated to a means of checking experimental error. These designs were based on the Snyder [2] solvent—selectivity triangle concept in which the combined specific selective effects of three different mobile phases are used to obtain the maximum resolution. Thus, unlike some of the ternary optimization methods described above [18,19,25,27], this scheme allows in the reversed-phase mode selection of a four-component mobile phase, i.e. three different organic modifiers and water, which obviously enhances its potential for resolving complex mixtures. The optimization criterion for the original COF method was based on eqn. 4, although A_i was generally set to unity for all peaks and B to zero so that the time term of the equation was eliminated. A single polynomial regression of a pre-determined quadratic form was used to describe the variation of the computed COF value as a function of mobile phase composition (i.e. solvents A, B, C versus COF). This procedure has, however, several distinct limitations, the most notable of which was that it only works if all peaks have the same relative retention order in all mobile phases, that is, if there is peak cross-over the COF value may not reflect this change. Furthermore, as no peak number term was included, solutions with co-eluting peaks may give better COF values than those in which more peaks are detected [20]. Nevertheless, in cases in which no peak order change was observed, useful prediction of optimum conditions was obtained, validating the general concept of mixture design statistical methods for optimization. Direct correlation of COF with solvent composition results, however, in a relatively high level of imprecision when applied to more than five to six solutes [20]. Attempts to extend the COF method to accommodate the problems were deemed cumbersome, however, and an alternative approach, the overlapping resolution map (ORM) was developed. This is essentially a simultaneous multi-factorial application of the window diagram approach with the use of quadratic regressions.

4.5.2. Overlapping resolution map (ORM)

This procedure also utilises the seven-point solvent selectivity triangle-based simplex design as its source of experimental data, and like COF is based on mathematical mixture design models (simplex—lattice) originally proposed by Scheffe [34]. It relies on comparing the resolution of every pair of peaks obtained for each solvent mixture by calculating the resolution surface of the solvent triangle by fitting the data to a second-order polynomial regression

equation [20,35]. A desired minimum resolution is chosen and the area of the triangle representing that separation for each pair delineated, the resultant overlapping resolution map thus comprising the intersection of Venn diagrams for acceptable resolution for all compounds. It extends the window diagram technique to the simultaneous analysis of ternary (four-component) mobile phases in the RP mode as distinct from binary solvent mixtures. In its original form [20] the ORM did not generate a unique optimum, but simply served to define an area corresponding to limits of mobile phase composition within which acceptable resolution of all peaks can be obtained, if this is possible, i.e. a window of accessibility. It handles peak cross-overs (provided that peaks are correctly identified in the experimental chromatograms) but assumes equivalence of priority for all peaks under consideration; minor peaks must be totally excluded if an improved separation of major components is required.

Subsequent modifications to the ORM procedure [35] relate essentially to its operation in the normal-phase mode and subsequent implementation as a fully automated optimization protocol (SENTINEL, DuPont) and include the definition of a unique optimum, by an unspecified procedure. This presumably involves the assignment of a numerical COF-type value to the sum of predicted solute pair resolutions. There remains, however, no facility for assignment of differing peak priorities and there is no systematic optimization of analysis time, other than that inherent in the selection of an overall solvent strength on the basis of a preliminary binary gradient chromatogram (SCOUT in SENTINEL terminology).

4.5.3. PEAKIN-SAS

This stochastic predictive procedure developed by Issaq [36] is based on a similar approach to ORM with peak interval as the criterion of optimum resolution and using a ten-point simplex lattice as the data base. If differs only in that it has been designed as a non-isoeluotropic system not requiring pre-selection of solvent strength and is limited in this form to mixtures of two organic modifiers and water, using a full cubic form of the regressions searched.

4.5.4. Computer chromatogram simulation method (CCSM-I)

It is clear that none of the above methods affords a complete solution to the problem of systematic optimization of the mobile phase. Each suffers from one or more defects, either in terms of assuming a specific and possibly inappropriate mathematical relationship between solute retentions and mobile phase composition, and/or lack of facilities to control optimization with respect to time of analysis or to the real analytical importance of individual peaks.

Our approach to this problem was to develop a coherent predictive statistical method which can cope with any change in retention order, with any number of solutes, which incorporates a peak priority weighting factor, and which can optimize analysis time in an interactive manner, according to the requirements of the individual chromatographer.

The CCSM is based on the use of the COC (eqn. 5) as the optimization criterion. It is a statistical mixture design method which also employed the seven-point simplex (Fig. 2) used by Glajch and co-workers [20,35] for ternary mobile phase optimization. It differs from the COF method (see *4.5.1.*) in that

the retention times for each component of the solute mixture are first transformed by the computer program into logarithmic retention indices ($\ln T_i/\ln T_0$) using one component of the mixture as an internal standard. Log retention indices give the best fit to a Gaussian distribution and reduce variance of data from replicate chromatograms obtained at different times. Furthermore, we cannot see any advantage in using peak interval ($\Delta T_0/4$) [20,35], a secondary variable which is geometrically increased in relation to peak number, as distinct from log retention indices, as the dependent variable. Calculation of the latter involves no loss of real chromatographic information and thus enables a complete simulation of a chromatogram to be assembled by the computer. The use of capacity factors does not improve the fit to a normal distribution and does not reduce variance due to time-related changes in, for example, column characteristics. Because calculation of capacity factors requires an additional step, it is not included in the CCSM procedure.

The next step is calculation by a basic algorithm of the best polynomial multiple regression for each solute (solvents A, B, C, D versus $\ln T_i/\ln T_0$). The a priori restriction of calculated polynomials to a pre-determined quadratic form by Glajch and co-workers [20,35] is not, in our view, justified. The complexity of solvent—solute—stationary phase interaction in HPLC may require even more complex mathematical models [37,38] for enhanced precision. In principle any continuous response surface can be represented by a polynomial, if enough terms are included. In our experience, treatment of water simply as a diluent of the organic modifiers is not valid with respect to a number of solutes. We have opted for selection from all possible incomplete 6th degree equations, with the best for each retention index selected by evaluation of correlation coefficients (r_{ij}). Analysis of data from experiments with mixtures of ten corticosteroid hormones [39] resulted in a significant improvement in precision, compared with other quadratic regressions calculated according to the COF method [39]. Provided that uncontrolled changes in column performance do not supervene, this must result in an increase in the likelihood that the predicted optimum will yield an improved separation in practice.

After insertion of desired separation parameters (A_i, $R_{s_{i,j\,\mathrm{d}}}$, T_m) by the chromatographer our program continues by simulating a large number of individual chromatograms at a pre-selected solvent composition interval until the entire mobile phase envelope defined as in Fig. 1 has been explored. COC values are thus calculated for chromatographic retentions predicted for all possible three- and four-component mobile phases, and the global optimum, together with any alternative local minima identified and displayed as well as the predicted retention times for these compositions (see Fig. 7). If the predicted separation is clearly inappropriate (for example because the most important solute is minimally resolved), then the simulations can be repeated with a new set of priority weightings, which will be reflected in the calculated COC values. In our experience not more than two or three such iterations are required to generate a satisfactory solution, and with prior experience of the chromatographic behaviour of the compounds in question, it is often possible to achieve a satisfactory optimum in a single run of the program, lasting approximately 2—3 h for mixtures of about fifteen compounds. If a good

separation is predicted, then attempts can be made to reduce analysis time by further simulations with a reduced value for T_m. The effective use of this method in optimizing the separation of a natural mixture of ten polar steroid hormones has recently been described [39].

Fig. 4 shows in a graphical form the solutions that the program found which yielded better separations than the best of the experimental chromatograms that provided the database. As can be seen, two local optima were discovered, one involving a four-component and another a three-component mobile phase, the latter giving the lower COC values and thus better overall separations. The actual chromatograms with this mobile phase and the predicted retentions are illustrated in Fig. 5. The error index calculated as the mean difference between all predicted and observed retentions was 6%.

We have also used this program in an attempt to optimize the reversed-phase separation of a group of naturally occurring catechol oestrogens, their metabolites and their precursors. The selectivity patterns obtained with four different binary mobile phases are illustrated in Fig. 6, and the experimental data for the seven chromatograms using acetonitrile, methanol and tetrahydrofuran as the organic modifiers (after Snyder [2]) are shown in Fig. 7, together with their computed COC values. With this mixture of solutes and solvents the program in fact identified a binary mixture (acetonitrile—water) as the optimum. As shown in Fig. 6, this failed to resolve only two compounds (Nos. 2 and 3) which were, however, resolved with tetrahydrofuran—water. When we weighted the priority values for 2 and 3, we could not, however, identify a solution in

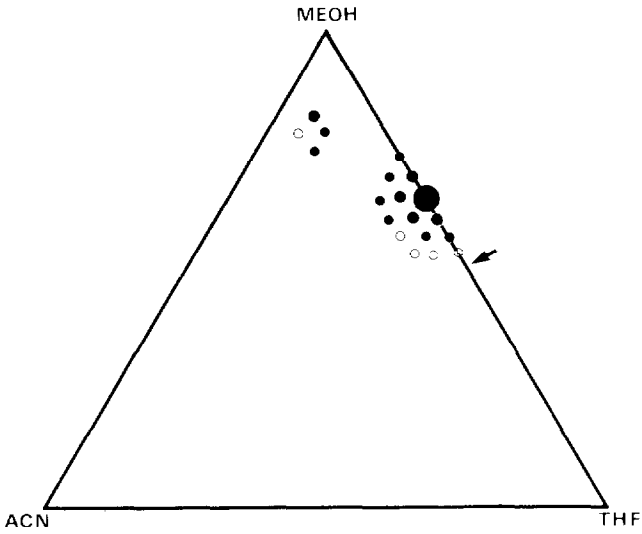

Fig. 4. Solutions afforded by CCSM-I for mixture of polar corticosteroids using a seven-point simplex. The points indicated and their size show the COC values for all mobile phases giving better predicted separations than the best of the experimental chromatograms (indicated by arrow). The best predicted solution corresponded to a three-component mobile phase comprising methanol—tetrahydrofuran—water (22 : 4 : 74) although note the four-component local minimum also found. The data for this optimization and the identities of the steroids are given in ref. 39.

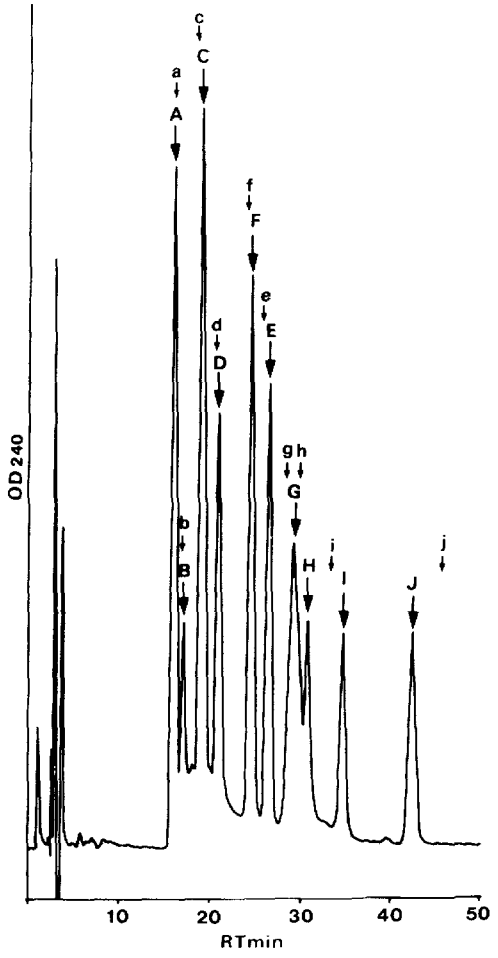

Fig. 5. Predicted and actual separations given by the mobile phase optimized in Fig. 4. The UV trace shows the actual separation with compounds (designated by upper case letters), the arrows with corresponding lower case letters show the retention times predicted by the program for each component of the mixture, with a mean error of 6%.

which complete resolution of all peaks was predicted when using the seven-point procedure with empirically chosen pre-defined overall solvent strenghts.

In the above form, the CCSM method still has important limitations; notably, there is an arbitrary selection of overall solvent strength for the iso-eluotropic optimization. Furthermore, because of non-additive solvent strength effects obtained with mixed organic modifiers [12] predicted absolute retentions may deviate significantly from those observed, if regressions are calculated on the basis of a seven-point design (Fig. 2), although relative retentions will be accurately predicted and in general the effective optimum will be found. Nevertheless, to encompass organic modifier—water interactions and their effects on absolute retention, we have extended the procedure, basing it on the same algorithm, but permitting a true optimization of a quaternary mobile phase with solvent strength as an independent variable (four components, three variables). In this modification a total of twelve pre-determined

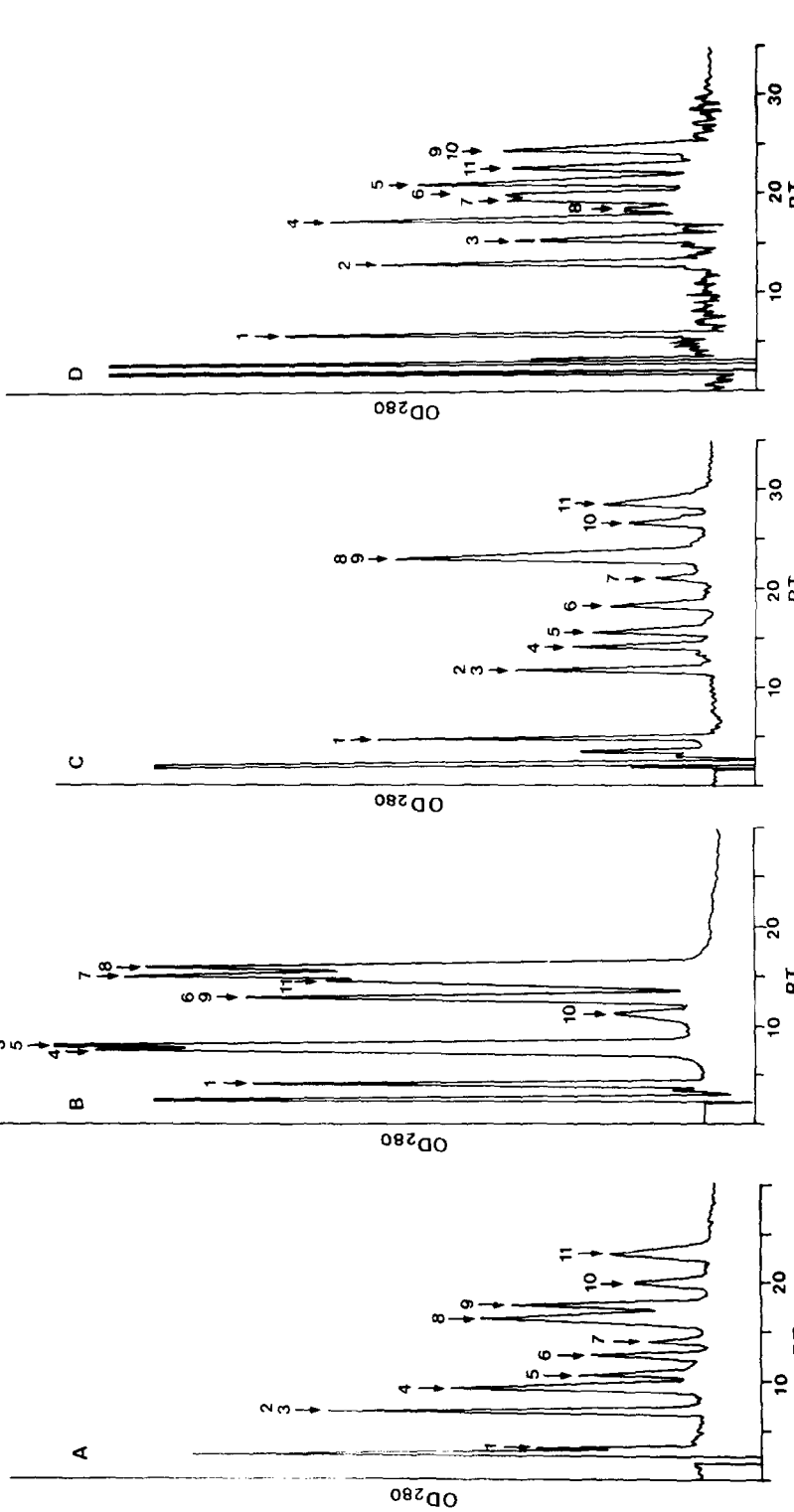

Fig. 6. Binary solvent selectivities for a mixture of oestrogens, catechol oestrogens and their metabolites. The chromatograms show, respectively, the isocratic separations obtained with (A) 40% acetonitrile; (B) 60% methanol; (C) 40% dioxane and (D) 30% tetrahydrofuran. The identities of the compounds are given in Fig. 7.

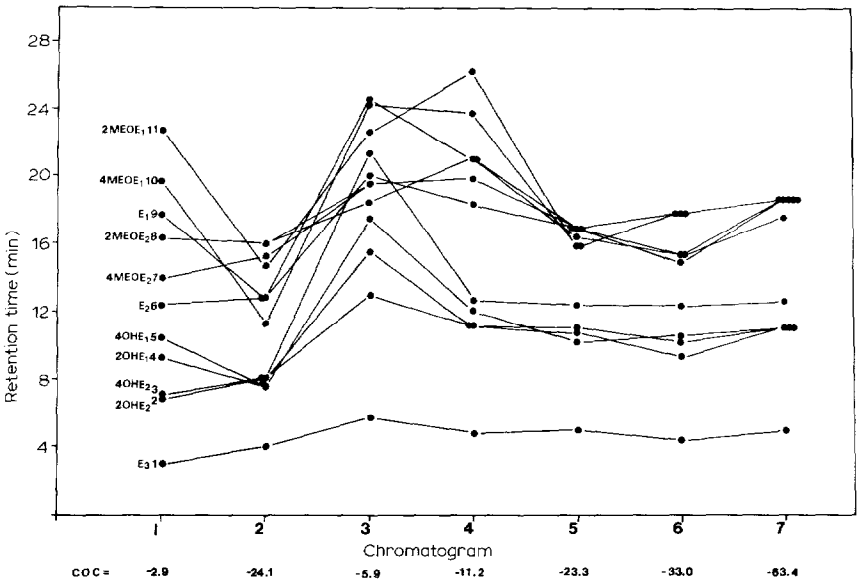

Fig. 7. Data from seven-point simplex for optimization of the isocratic separations of a mixture of oestrogens and catechol oestrogens and their metabolites shown in Fig. 6, together with corresponding COC values for each of the seven experimental chromatograms; with equal priority weightings and a desired analysis time of 25 min.

chromatograms (illustrated in Fig. 8) are required for RP-HPLC, in which solvent strengths bracketing the likely optimum are employed and very water-rich and water-poor mobile phases are excluded. Our program has, therefore, now been extended to this form of database. Introducing the water—solvent product as an independent variable in the regression calculations can result in up to a five-fold increase in the precision with which absolute retentions are predicted by CCSM.

The modified CCSM procedure can also be applied to normal-phase optimization in adsorption liquid chromatography (LC), in which it is possible that even greater specific selective effects may occur as a consequence of the more widely differing relevant properties of solvents used in this mode, i.e. non-localising, localising dipole and localising base solvents, compared with the mixtures of proton acceptor, proton donator and dipole solvents appropriate for bonded-phase LC [40]. As Glajch and Kirkland [41] have recently re-emphasized, the possibility of using quaternary solvent mixtures is a pre-requisite for utilizing the total range of potential selectivity effects in LC. For normal-phase isoeluotropic optimization with a mixture of four solvents a total of fifteen pre-determined chromatograms provides the data base in CCSM, corresponding to the full tetrahedral simplex lattice (Fig. 3).

The CCSM-1 program has, up to the present, been effected on an 8-bit Apple IIe computer, the program itself occupying about 12K of RAM in BASIC and computations of optimized mobile phases taking anything up to 4—6 h each using a non-compiled version. Significant improvements have, however, obtained with use of machine code routines and other time-saving manoeuvers. These have enabled different solutions involving different priority weightings, etc. to be generated more rapidly (50—80 min) for comparison.

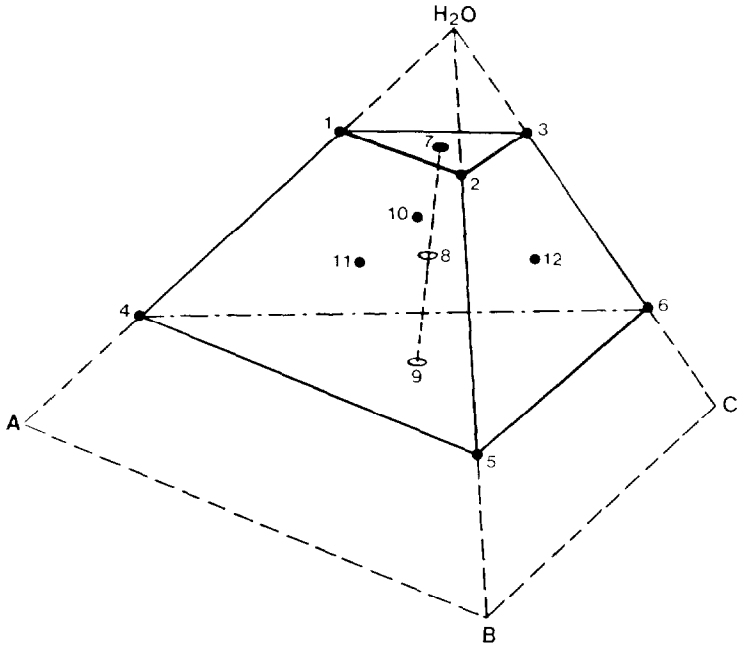

Fig. 8. Twelve-point design for systematic optimization of solvent strength in isocratic RP-HPLC without preselection of solvent strength, where A, B and C are three different organic modifiers, and the overall maximum and minimum solvent strengths are defined by the overall polarity of the solute mixture. This database can also be used for the optimization of a continuous two-pump gradient profile to separate the same solutes.

4.6. Optimization of gradient elution

The simplest form of automated gradient elution optimization has already been mentioned, viz. GRADOPT, OPEX and OPTIM [9] in which solvent strength and gradient duration are optimized without reference to solvent selectivity, which must be separately optimized beforehand in the isocratic mode. Simultaneous multi-factorial optimization by sequential simplex techniques and by window diagram methods is subject to various practical drawbacks, which have already been discussed. In principle, therefore, stochastic predictive methods based on an appropriate simplex lattice, combined with appropriate equations for describing solute relative retentions offer the most economical route to simultaneous optimization of gradient profiles and multi-solvent selectivities.

The most comprehensive approach to this goal of selective multi-solvent gradient elution (SMGE) optimization thus far has been that of Kirkland and Glajch [21,41]. Thus, although numerous studies of ternary gradient elution in practice have been carried out, notably by Jandera et al. [42], their theoretical treatment has been confined to linear gradients and has neglected mutual interactions between the two organic modifiers and water. The theoretical prediction of optimal ternary gradients from binary gradient data that they propose has not, as yet, been generally verified experimentally. Their system is, furthermore, implicitly confined to three-component mobile phases, thus limiting the range of potential specific selective effects that can be deployed.

Kirkland and Glajch [21], by contrast, have approached the problem from the standpoint of the solvent selectivity triangle with its four components. The first extension of the system was to develop a design for iso-selective multi-solvent gradient elution (IMGE) in which solvent strength but not separation selectivity (i.e. organic modifier ratios) is altered during the run [21]. To generate the requisite data for statistical optimization a series of seven iso-selective linear gradients are run, extending the selectivity triangle to a prism [41]. These data are used to calculate the coefficients of the quadratic equations that describe resolution contour plots for all peak pairs, exactly as in the ORM method of isoeluotropic optimization [35], except that peak pair resolution is defined in terms of absolute retention times, rather than on the basis of peak separations. This enables retention times for all peaks to be estimated through the complete solvent selectivity prism, and thus the global optimum with respect to linear iso-selective four-component gradients to be calculated, with a reported precision of 1—2% [41].

Thus far, however, the fully selective SMGE concept has not been realised as a systematic procedure. As an interim approach Glajch and Kirkland [41] have adopted a semi-empirical procedure in which different iso-selective gradients are selected for groups of compounds of similar polarity by inspection of iso-selective gradient runs obtained for IMGE, and these are then linked, empirically, in a discontinuous step-selective gradient with a linear solvent strength increase. A practical disadvantage of this method is that it can result in large fluctuations in detector baseline when abrupt changes of solvent composition are effected, and does not take into full account the effects of initial conditions on relative retentions of later eluting solutes. To overcome this problem and to attain the maximum flexibility it is necessary to incorporate continuous selectivity changes (preferably non-linear) in conjunction with non-linear changes in solvent strength. Kirkland and co-workers have not, as yet, provided details of how this is to be achieved.

It is clear, nevertheless, that when dealing with mixtures which contain several groups of solutes with similar selectivity factors and in which the overall polarity range is wide that a solution to systematic optimization must be sought in non-isoeluotropic designs. A program (CCSM-Gradient) has now been developed which uses the same twelve-point database illustrated in Fig. 8 to select the most appropriate binary (two-pump) gradient. The rationale of CCSM-Gradient is that a gradient can be divided into a series of instantaneous isocratic conditions for which the positions of each compound within the column can be calculated provided that certain physical characteristics of the system such as dead volume and column void volume are known. In this manner it is possible for the computer to systematically simulate the effect of various gradient profiles on elution volumes using data from a pre-defined limited number of isoeluotropic experiments. The result is an optimization with respect to a continuous gradient profile as distinct from a linked series of isoeluotropic conditions.

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6. SUMMARY

Systems for computer- and microprocessor-aided optimization of the mobile phase in high-performance liquid chromatography (HPLC) are reviewed and compared with the computer chromatogram simulation method (CCSM). CCSM was developed and tested in our laboratories for the off-line interactive optimization of four-component isoeluotropic mobile phases for HPLC. It is based on a statistical mixture design method which requires a limited predefined number of experimental chromatograms and it predicts solute retention times for systematically simulated chromatograms, selecting the best according to user-defined separation priorities. The application of similar procedures to gradient elution are discussed.

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