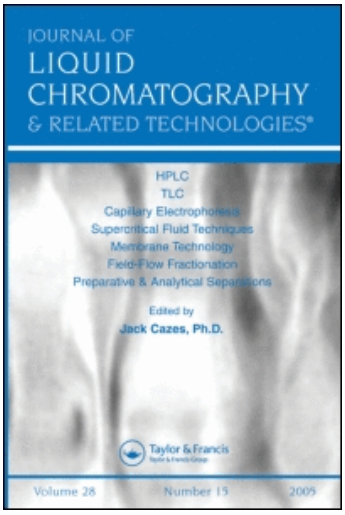

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Global Optimization of HPLC Separations

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GLOBAL OPTIMIZATION OF HPLC SEPARATIONS

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ABSTRACT

A method suitable for automated optimization of HPLC separations is developed. A sequential strategy based on a non-linear interpolation between measured retention data permits a stepwise approximation to a global optimum. This scheme provides a smooth representation of the elution behaviour from an unspecified number of regularly or irregularly spaced data points. The accuracy of the so defined curves can be gradually improved by successive addition of new experimental data as they become available. It is possible, therefore, to employ variables yielding multimodal response hypersurfaces in the optimization work. Further emphasis is given to an individual weighting of all detectable compounds with respect to their analytical relevance. Examples including an isocratic separation of fat soluble vitamins as well as gradient separations of dansylated amino acids and steroid hormones demonstrate some applications of the proposed procedure.

INTRODUCTION

The enormous increase of analytical techniques that involve liquid chromatographic separation methods has emphasized the demand

for systematic method development in high performance liquid chromatography (HPLC). The versatility of HPLC is mainly based on the big variety of chemical equilibria known to be useful for tuning separation selectivity. Consequently, any approach towards automated method development has to be capable of handling all types of variables employed in separation systems.

A number of different approaches have been proposed so far, either based on theory or on semiempirical functions that account for specific molecular interactions or on fully empirical search and mapping techniques. For retention modelling on theoretical or semiempirical basis all knowledge acquirable about the sample and/or the chromatographic process should be considered. Concepts developed from chromatographic theory are easiest to be generalized. Thus, well established algorithms are used to construct the framework of simulation of isocratic (1-3) as well as gradient (4,5) solvent strength conditions. Mechanistic and semiempirical models for description of chromatographic elution characteristics are valuable tools to achieve global solutions; they can, however, rarely replace optimization of a particular separation problem (6,7).

Owing to the complex nature of inter- and intramolecular interactions encountered in retention phenomena most commonly fully empirical procedures are used for computer assisted optimization of experimental separation conditions. Excellent reviews on this subject can be found in the literature (8,9). Global strategies require a true consideration of all variables that can be employed to tune separation selectivity, -capacity or -efficiency, even, when response hypersurfaces with high modality are to be expected. We have, therefore, developed a sequential procedure based on a non-linear interpolation between experimentally obtained data points. The algorithm employed for interpolative predictions is discussed in THEORY. Optimization runs illustrate the application of this scheme on chromatographic separation problems.

EXPERIMENTAL

HPLC was performed with Waters M6000 pumps, an automatic injection system Waters WISP 710A and either a Waters M440 UV-detector

or a Perkin Elmer LC-1000 fluorescence detector. For system automatization and data acquisition a Waters 840 chromatography data station with a Digital Equipment 350 personal computer was used.

All chromatographic separations were carried out on 150 x 4.61 mm i.d. HPLC columns filled with 5-μm Spherisorb ODS2. Flow rates were maintained at 1 mL/min throughout all experiments. Solvents and chemicals used for the experiments were analytical reagent grade and had been obtained from various sources.

The mobile phase and detection parameters for the optimization runs were for the

- separation of fat soluble vitamins: acetonitrile, methanol and water; UV-detection at 280 nm
- separation of dansyl-amino acids: A-solvent: citrate-phosphate buffer adjusted to several pH values; B-solvent: acetonitrile; fluorescence detection, exc 338 nm, em 520 nm
- separation of steroid hormones: A-solvent: water; B-solvent: methanol, acetonitrile; UV-detection at 254 nm.

The optimization software was written in compiled Basic-Plus-2 for use with the chromatographic data station or, alternatively, in compiled Microsoft QuickBasic for use with MS-DOS computers.

THEORY

The application of formal optimization strategies to chromatographic separations has quickly shown that direct search methods, like the Simplex search, are inferior to methods based on retention modelling. Although search methods have found applications, these seem to be centered around problems that do not involve large variations of retention data. Thus, by inverse reasoning, it follows that direct search methods are not optimal when potential gains from optimizations are largest.

The adaptation of modelling techniques to the optimization of chromatographic separations is based on an idea apparently due to Laub and Purnell (10-12) who have interpolated retention data between measured data points by a straight line. The crucial point is that the retention modelling of individual solutes gives smoother

functions than an overall chromatographic response function, so that little error is made by choosing a wide step width between experimental data and the chromatographic response function is subsequently assembled by computer methods.

A general approach to retention modelling should

(i) be very adaptable to different retention models as encountered for different experimental variables, and

(ii) be model free as it needs to operate on as little experimental data as possible.

The approach chosen in this work is derived from developments described in (13-15) and is termed 'moving least squares'. The technique was originally developed for interpolating irregularly spaced discrete bivariate data and is readily extended to higher dimensions (14). It is not a least squares approximation as it is expected to produce an interpolant (15).

A global interpolant function Gf(z) defined as moving least squares approximation is given in (15) as

$$Gf(z) = Sf(z) + \sum_{i=2}^{n} \alpha_{i-1}(z) \quad \{ b^{(i)}(z) - Sb^{(i)}(z) \} \qquad /1/$$

with

$$Sf(z) := \sum_{j=1}^{N} f_j \, v^{(j)} \qquad /2/$$

$b^{(i)}$ being simple functions, for instance z^i, and

$$v^{(j)}(z) = \frac{w^{(j)}(z)}{\sum_{k=1}^{N} w^{(k)}(z)} \qquad /3/$$

$w^{(j)}$ a weight corresponding to the j-th experimentally determined data point at the coordinate z with N being the number of data points; $\alpha_{i-1}(z)$ are regression coefficients determined from

$$U(z)\, W(z)\, U^T(z)\underline{\underline{\alpha}} = U(z)\, W(z)(\underline{\underline{f}} - Sf(z)\underline{\underline{b}}^{(1)})$$

U(z) is an (n-1) x N matrix with elements

$$u^{(i)}(z; z_k) = b^{(i)}(z_k) - Sb^{(i)}(z)$$

that have numerically different elements at each coordinate value z. The reader recognizes this as a form of weighted regression of U(z) on $(\underline{f} - Sf(z)\underline{b}^{(1)})$. As the matrix of weights W(z) is an N x N diagonal matrix, $W(z) = diag(w^{(1)}(z),\ldots, w^{(N)}(z))$, the solution vector $\underline{\alpha}$ is different for all z, therefore the full procedure is computationally expensive.

Tracing eqn. /1/ back to Shepard's interpolant by setting the term containing the α's equal zero, the procedure is much faster and can be executed as central part within a strategy for global optimization. In spite of this apodization sufficient flexibility remains to accommodate various functional dependencies of retention from chromatographic variables by adaptation of the weights $w^{(1)}(z)$.

The brevity of the above treatment requires of the reader interested in details of this procedure the study of the original literature (13-15). Some illustration of the operation of the proposed procedure is given in the following starting with simple hypothetical examples.

Some Illustrative Examples

With the simplification that sets all α's to zero eqns. /1/ to /3/ can be combined to give

$$Sf(z) = \sum_{j=1}^{N} f_j \frac{w^{(j)}(z)}{\sum_{k=1}^{N} w^{(k)}(z)}$$

From ref. (14), eqn. 3.6 and 3.7, a reasonable choice of weights is

$$w^{(k)}(z) = \prod_{j.ne.k} [(\sum_{i=1}^{m} |z-z_k|^{\delta})^{1/\delta}]^{\gamma_k},$$

with m the number of experimental variables involved in the optimization corresponding to the dimensionality of the optimization problem, and δ and γ's selected independently and a separate γ for each experimental data point k.

Let the simplest set of data for illustration consist of only two data points in one dimension (e.g. concentration of organic

modifier) at values of 10 and 80 with observed retentions at these points of 10 and 90 a.u. Fig. 1 shows the curves produced for a constant $\delta = 2$ (Euclidean distance) and γ's equal for both data points and varied between 0.5 and 10.0. It is clear that various experimental variables could be accommodated by changing γ. Fig. 2 shows the results for three data pairs at (10,10), (50,90) and (90,40) and a similar range of functional dependencies is accomplished.

For extending the problem to two experimental variables, an example is given in Figure 3 where the change of the position of isoresponse lines upon addition of an experimental point at (96,96,60) is clearly visible. As two (or more) experimental variables increase the dimensionality of the problem, the choice of δ gains in importance. For the same three points as in the last example a change from δ=1 to δ=4 narrows down the influence domain of the individual points for a more uniform (average) predicted response in regions not densely covered by experimental data (Figure 4). Although mathematically straightforward and chromatographically necessary, it is not attempted to extend the case beyond two dimensions at this point. Practical examples where this was done are found in RESULTS AND DISCUSSION.

RESULTS AND DISCUSSION

The Chromatographic Quality Criterion

In order to characterize chromatographic separations an adequate chromatographic response function (CRF) has to be selected. A detailed discussion on quality criteria is beyond the scope of this paper and can be found elsewhere (8,16). In the present study mainly a CRF defined by us (17) was used. The function

$$CRF = \frac{1}{t} \pi_j f_j/(g_j + 2 n_j)$$

is a generalization of Kaiser's peak separation f/g (18) and contains conceptual extensions for the total analysis time t and the baseline noise n. The estimation of peak separation factors was based on calculation of chromatographic resolution (19), linear solvent strength theory (4) was used to account for gradient systems

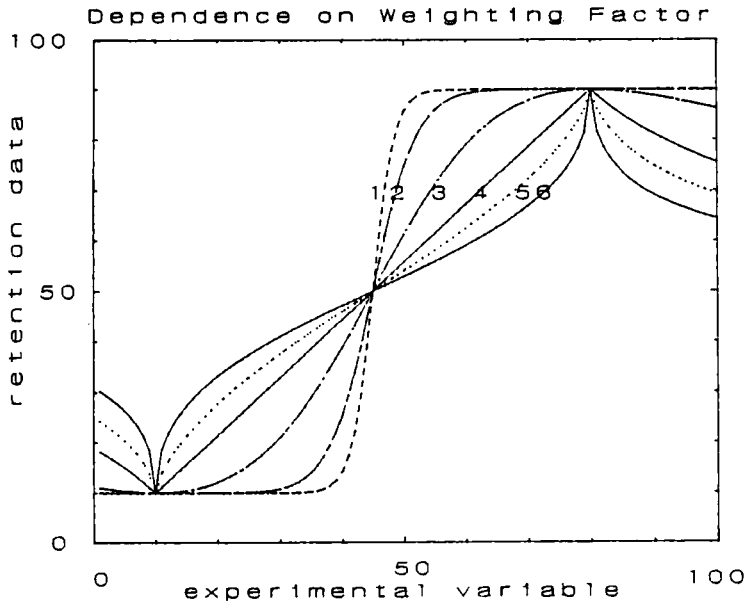

FIGURE 1: Functions produced for the one-dimensional case with 2 experimental data points as (10,10) and (80,90) for γ=10 (1), γ=5.0 (2), γ=2.0 (3), γ=1.0 (4), γ=0.7 (5), γ=0.6 (6).

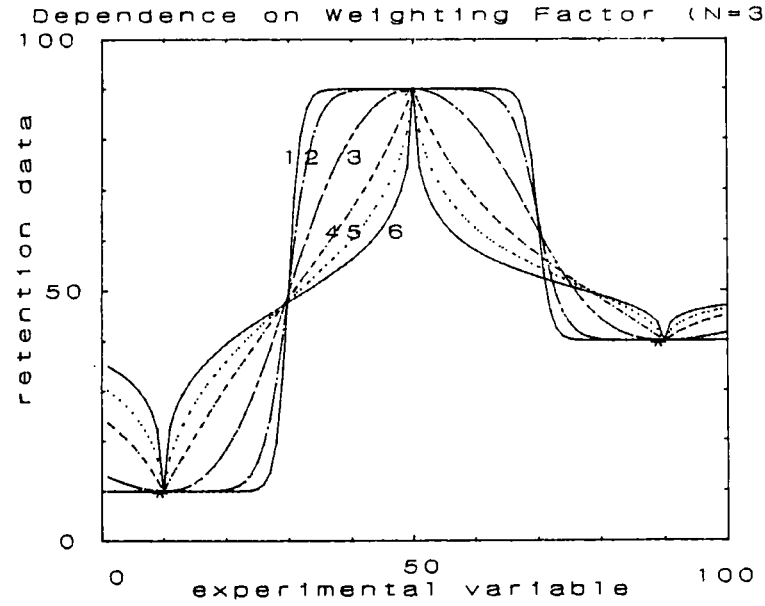

FIGURE 2: Same as in Fig. 1, but with three points: (10,10), (50,90), (90,40).

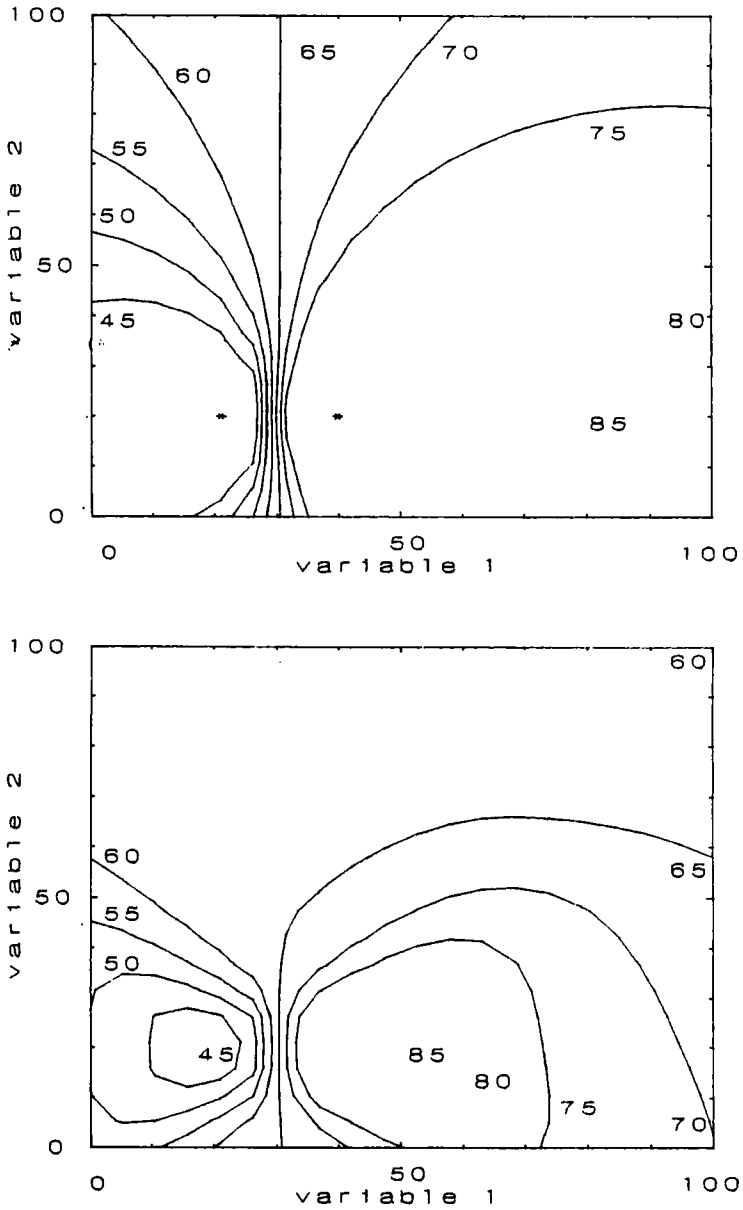

FIGURE 3: Predicted isoresponse lines for the two-dimensional case with γ=2.0 and δ=2.0
a) two points: (20,20,40), (40,20,90)
b) three points: (20,20,40), (40,20,90), (96,96,60)

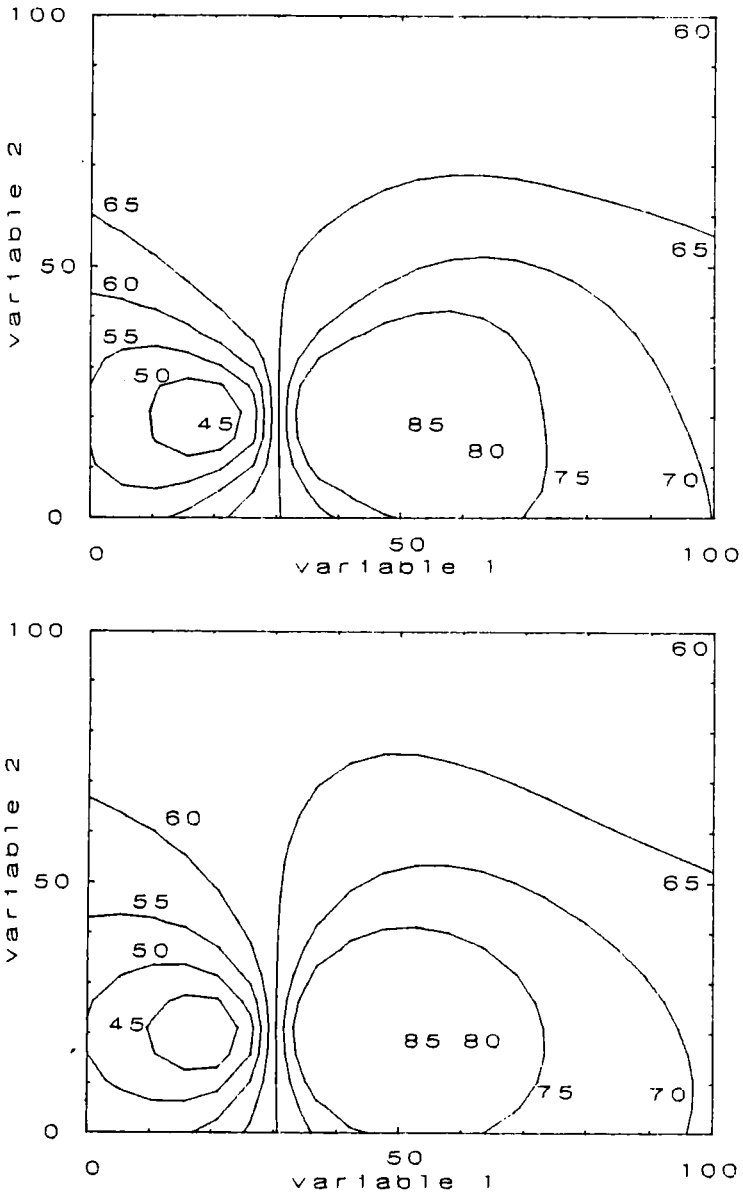

FIGURE 4: Predicted isoresponse lines for the two-dimensional case with a) δ=1.0; b) δ=3.0; c) δ=4.0 (For δ=2 see Fig. 3 b)

(continued)

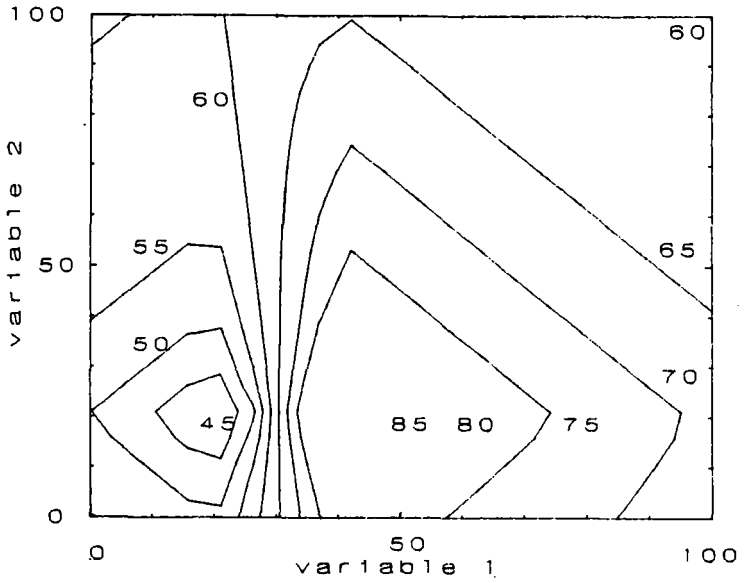

FIGURE 4c.

additionally. For the purpose of comparison the minimum-alpha criterion as proposed by Laub and Purnell (10) was used alternatively.

Optimization Strategy

A sequential strategy that acquires information step by step as the optimization run proceeds has been designed for localization of the best set(s) of experimental conditions in the space of variables. Interpolation between experimentally obtained data points is based on the above discussed moving least squares algorithm. In Figure 5 a simplified flow chart demonstrates in brief the course of the optimization. First, system input has to be provided, such as information on the number of components in the sample and also on number, range and grid width of the variables to be optimized. Furthermore, it is possible to decide on the analytical relevance of individual components in the sample mixture. Pertinent examples to that point are discussed below in some detail. Next, retention data obtained

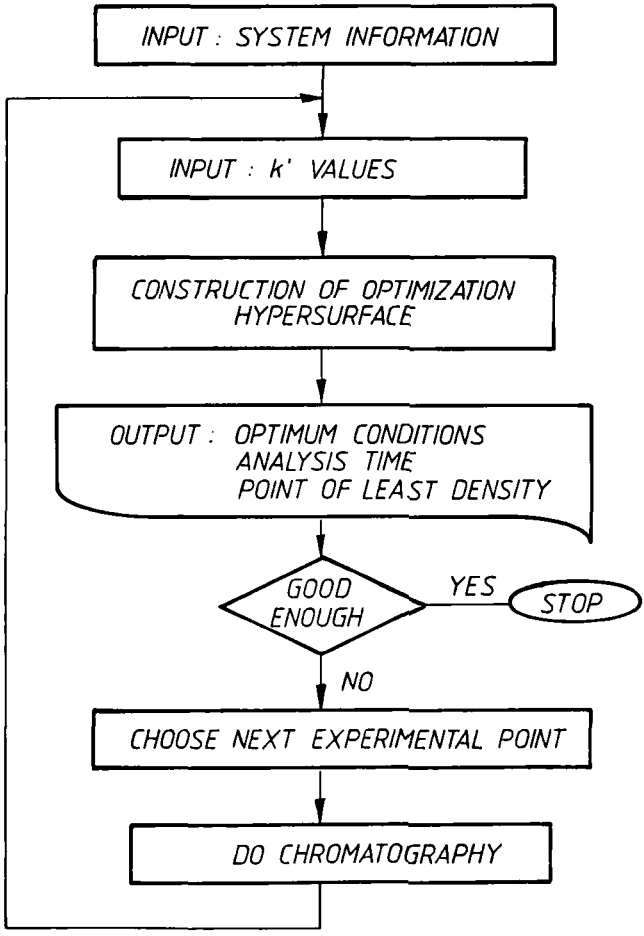

FIGURE 5: Simplified flow chart of the optimization scheme.

from some initial chromatographic runs are needed for a systematic computer construction of the optimization hypersurface. The program terminates with an output of the ten best experimental conditions along with their associated analysis times and finally, the one point in the space of variables with the least density of experiments. As long as the actual chromatograms do not meet the analytical expectations one is advised to perform further optimization runs indicated by a loop in Figure 5. In order to fill up the variable space evenly with data, the point with the least experimental density is recommended to be chosen next. It is, however, also possible to verify the proposed optimum at any time. Owing to the sequential nature of this strategy the overall number of experiments necessary depends very much on the complexity of the separation problem itself. Consequently, simple separation problems may be solved within a few runs, more complex problems require a higher effort.

Isocratic Separation of Fat-Soluble Vitamins

The mobile phase to be optimized for a reversed phase separation of these components consisted of methanol, acetonitrile and water as variables which were permitted to vary within the limits specified in TABLE 1. In order to prevent unreasonably long analysis times, the water content in the mobile phase could not exceed 5% v/v in total. Separation selectivity was intended to be achieved from the organic modifiers with different proton acceptor and -donor properties according to Snyder's solvent selectivity concept (20).

TABLE 1
Experimental Variables with Corresponding Boundaries and Computational Step Widths for the Separation of Vitamins

VARIABLE	LIMITS	STEP WIDTH
METHANOL	0 - 100 %	1.00 %
ACETONITRILE	0 - 100 %	1.00 %
WATER	0 - 5 %	0.25 %

Two optimization runs were started with plain methanol and acetonitrile, respectively, and were continued step by step with the point in the space of variables exhibiting the least experimental density. The third and fourth experiment were, therefore, carried out at the maximum value of water consistent with the boundary i.e. 5% water and 95% of the organic modifier. The retention data from these experiments and an additional fifth chromatographic run at conditions of 50% each methanol and acetonitrile as eluent were sufficient to predict an optimum at a mobile phase composition of 98% methanol, 1% acetonitrile and 1% water. The chromatographic verification of the computed optimum is shown in Figure 6. Two types of eluites are marked separately, the vitamins to be determined and peaks that need not be further evaluated. This accounts for those situations when the analyst is not interested in all signals that emerge from the detector of the chromatograph. As with many other examples the estimation of vitamins e.g. from pharmaceutical or industrial formulations can turn out to be a tedious task since coeluting degradation- and byproducts are frequently accompanied with the analytes. It is, therefore, desirable to have a means at hand that permits to differentiate between the eluites of interest and analytically non-relevant signals in a chromatogram. These two groups of signals may be weighted differently for an evaluation of optimum conditions. Signals derived from compounds of interest have to be resolved from all adjacent signals whereas peaks from solutes which have not to be assessed analytically need not necessarily be separated from each other. Thus, software controlled mutual overlap of e.g. byproducts can considerably help to cut down the overall effort needed for developing a separation of all compounds giving rise to a detector signal. As a consequence the analysis time from the optimized chromatographic conditions is also kept to a minimum. Hence a fundamental requirement of analytical operations in general, a consideration of analysis time as a basic analytical performance characteristic has been realized with this approach (16).

Linear Gradient Separation of Dansyl-Amino-Acids

It has been recognized that the transfer of methods from one laboratory to another is frequently hampered by small deviations of

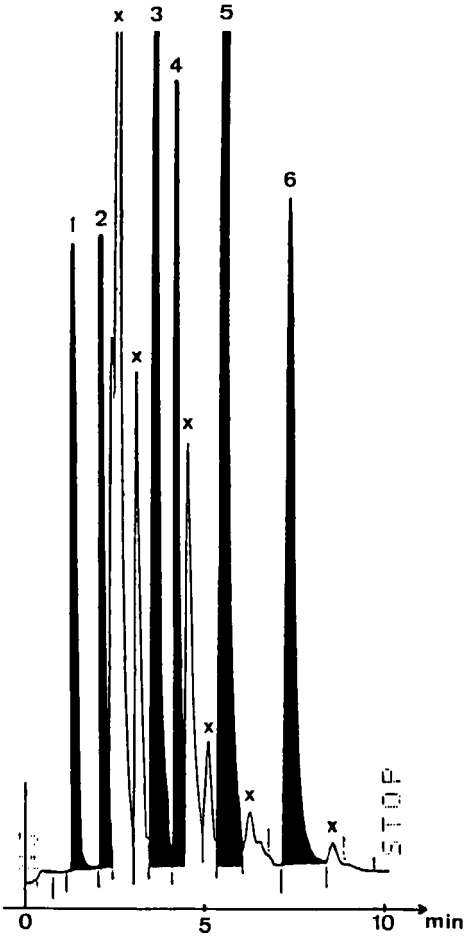

FIGURE 6: Separation of fat soluble vitamins at conditions of the predicted optimum (see text). 1 = vitamin K3 (menadione), 2 = vitamin A - aldehyde, 3 = vitamin D2 (ergocalciferol), 4 = vitamin A - acetate, 5 = vitamin A - palmitate, 6 = vitamin K1 (phytomenadione). x = decomposition- and byproducts.

TABLE 2
Experimental Variables with Corresponding Boundaries and Computational Step Widths for the Gradient Separation of Dansyl-Amino-Acids

VARIABLE	LIMITS	STEP WIDTH
pH VALUE OF SOLVENT A	2.5 - 7.5	0.25
SLOPE OF GRADIENT (%B/MIN)	1.0 - 2.0	0.20
ENDPOINT OF GRADIENT (%B)	50 - 70	5.00

mobile phase composition and depends to a good deal on the reproducibility of column parameters, like the surface chemistry of the stationary phase or column efficiency. This leads to the necessity in practice to reoptimize those experimental variables that effect the separation process most effectively. Separation of dansylated amino acids can be achieved on reversed phase columns, complex mixtures may advantageously be resolved with gradient elution. Since they are ionizable compounds, the pH value of the mobile phase is known to play an important role in their chromagraphic behaviour (21,22). For this reason, the pH value was chosen as a variable to be optimized. Given a constant elution strength for the starting conditions, a linear gradient can be characterized by the rate of increase of B-solvent (%B/min) and the endpoint of the gradient in terms of elution strength of the B-solvent (%B). An experimental setup as listed in TABLE 2 had been designed for this particular optimization example. Owing to the number of the variables as well as the nature and the widespread lower and upper limits of the variable 'pH value' the capacity factors of the dansyl-amino acids were evaluated for twelve different chromatographic conditions. This provided sufficient information on their retention behaviour in the space of variables in order to compute a global optimum for a separation of all sixteen amino acids. One experiment at either boundary value of slope and endpoint of the gradient (slope: 1 and 2% B/min, endpoint: 50 and 70% B) was performed at pH 2.5, the lower limit, pH 7.5, the upper limit and pH 5.0, an intermediate value of the pH of solvent A.

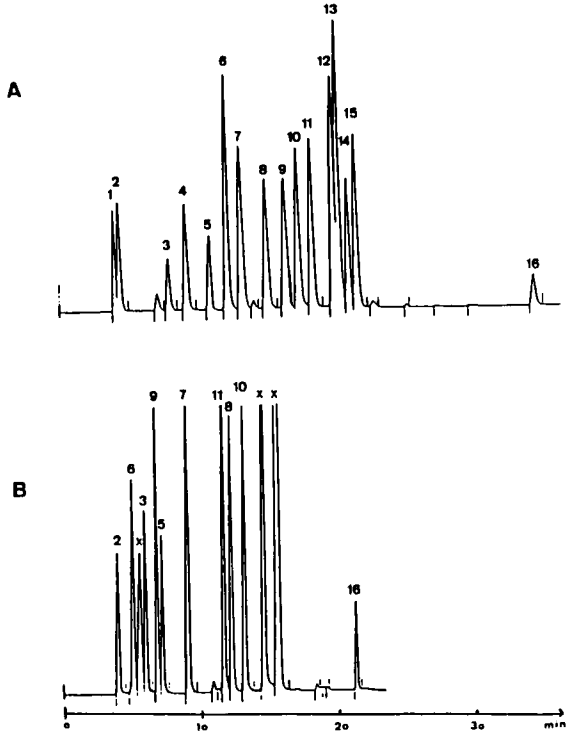

FIGURE 7: Separation of dansylated amino acids. A : Separation of all sample constituents. B : Estimation of a user specified subset of amino acids. 1 = aspartic acid, 2 = asparagine, 3 = glutamine, 4 = serine, 5 = threonine, 6 = arginine, 7 = alanine, 8 = proline, 9 = lysine, 10 = valine, 11 = methionine, 12 = i-leucine, 13 = leucine, 14 = tryptophane, 15 = phenylalanine, 16 = tyrosine, x = software-controlled overlap.

The systematic computer-aided construction of the optimization hypersurface obtained with the retention data from the twelve initial experiments predicts the optimum conditions for a separation of the entire set of derivatized amino acids to be at conditions of pH = 7.25 a gradient slope of 1%B/min and an endpoint of 50% B-solvent. The actual separation obtained from verification of the computed optimum is illustrated in Figure 7. The quality of prediction is obviously

satisfactory for most chromatographic applications. If not, one is advised to proceed with further optimization runs which could potentially lead to a refined optimum. This had, however, not been investigated in this study.

It is interesting to experience the capability to recompute an entirely new set of optimum conditions as the analyst's focus on certain amino acids changes. Without having to perform any additional experiment, the data from the previous thirteen runs - the twelve initial experiments and one verification run for the optimum according to Figure 7A - can be used to reconstruct a hypersurface for a separation of a subset of the analytes. Thus, for a determination of e.g. 10 amino acids the best conditions are predicted to be at pH = 3.75 with a gradient slope of 2%B/min and an endpoint of the gradient of 70% B-solvent. The signals marked with 'x' in Figure 7B indicate the purposedly permitted overlap of user specified components resulting in a significantly shorter analysis time as the analytical task changes. Following the line of argumentation in an earlier paper (17), reduction of analysis time can in general be approximated with a concurrent reduction of the cost of the analysis. The effort and the time needed for recomputation of new conditions is negligible compared to experimental work. This holds analogously true for altering column dimensions or decreasing column efficiency with time which can be easily accounted for with proven chromatographic theory.

Step Gradient Separation of Steroid Hormones

The task of optimization was to properly select mobile phase selectivity and to evaluate the best suited gradient conditions. The possibilities available include a simultaneous optimization of all parameters to be considered or, alternatively, to sequentially handle the sets of variables e.g. to first find a suitable solvent composition and to subsequently optimize the gradient. The approach presented in this paper allows an intuitive decision whether to proceed simultaneously or sequentially. The best choice will ultimately depend on quality and quantity of accessible preinformation on the analytical system and the variables selected for the optimization. Because of the ease of automatization in chromatography a simultaneous consideration of all parameters was applied in our experiments.

The ratio of methanol/acetonitrile fraction in the eluent which could vary from 0 to 1 with a computational step width of 0.05 was regarded as a variable for chemical selectivity. A scout gradient from pure water to 100% methanol was used to define a coarse frame of minimum and maximum elution strength necessary for a complete elution of all components. Arbitrarily a gradient design consisting of four evenly spaced step segments was exploited. Since steps are discontinuous *per definitionem* it is necessary to construct a special encoding logic for multidimensional grid formation. Thus, the gradient is assumed to proceed mandatorily from its start- to its final conditions with respect to the amount of organic modifier, it can, however, be formed from any possible combination of ascending or constant segments. Consequently a wide variety of gradient shapes can be interpolated including quasi convex, concave and linear designs. It is also possible to reach the final conditions of the gradient in less than the maximum number of segments. The variable set as actually used for the optimization work is shown in TABLE 3.

Due to the presence of five variables many runs were necessary to gain enough information for sufficiently accurate prediction. After 21 experiments the computed results and the corresponding chromatograms indicate clearly, that a separation of all components occurs with less respect to gradient shape at a narrow bandwidth of the concentration of methanol/acetonitrile only. In Figure 8 two gradient profiles and chromatograms are shown representing the optimized results from the same data set, however, computed for different

TABLE 3

Experimental Variables with Corresponding Boundaries and Computational Step Widths for the Gradient Separation of Steroid Hormones

VARIABLE	LIMITS	STEP WIDTH
MEOH / ACN	0 - 1	0.05
STEP 1 (%B)	0 - 100	5.00
STEP 2 (%B)	0 - 100	5.00
STEP 3 (%B)	0 - 100	5.00
STEP 4 (%B)	0 - 100	5.00

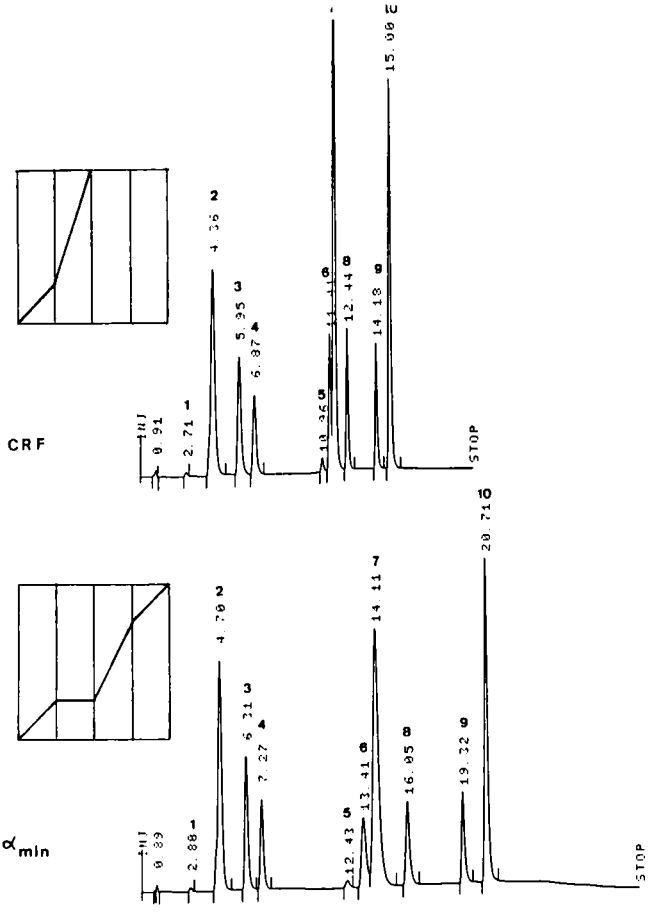

FIGURE 8: Predicted optimum step gradient separation of steroid hormones and corresponding gradient patterns for two different response functions. 1 = estriol, 2 = cortisone, 3 = adrenosterone, 4 = 4-androstene-11ß-ol- 3,17-dione, 5 = estrone, 6 = testosterone, 7 = 11-deoxy-corticosterone, 8 = 17-hydroxy-progesterone, 9 = dydrogesterone, 10 = 20-hydroxy-4-pregnene-3-one.

quality criteria. The time normalized chromatographic response function (CRF) favours obviously shorter analysis time than the minimum selectivity function.

The existence of a physically sound theory for gradient elution in HPLC (4,5) suggests, of course, to consider its use for chromatographic simulations which can potentially assist in reducing an unduely high number of experiments. We based our study on Snyder's model (4) and evaluated all parameters necessary for calculations with the following equation:

$$t_g = t_0 + t_d + d_g^* \log [(2.3^*p^*s^*k_0^*t_0/d_g)+1]/p^*s$$

Thus, gradient retention time can be predicted from parameters concerning instrumentation, gradient and solutes: chromatographic dead time (t_0), delay time of the gradient (t_d), duration of the gradient (d_g), range of the gradient (p), the negative slope of log k' vs. volume fraction of organic modifier plot (s) and the intercept (k_0). The individual values of s and k_0 estimated from three isocratic runs with fast, moderate and slow elution of the sample constituents in Figure 9 are reported in TABLE 4. A comparison of simulated and experimentally obtained chromatograms in Figure 9 of a linearly programmed gradient ranging from 50%B to 75%B within 10 minutes exhibits a match in the elution order and a comparable elution

TABLE 4
Slope and Intercept Values of Steroid Hormones on C-18 Reversed Phase with a Volume Fraction Methanol/Acetonitrile = 0.4 in the Eluent

COMPONENT	slope	intercept
adrenosterone	-0.0353	5.47
estriol	-0.0269	1.87
estrone	-0.0408	16.16
4-androstene-11β-ol-3,17-dione	-0.0355	6.92
testosterone	-0.0407	17.90
11-deoxycorticosterone	-0.0411	19.58
20α-hydroxy-4-pregnene-3-one	-0.0459	57.39
dydrogesterone	-0.0396	31.92
cortisone	-0.0349	3.61
17α-hydroxy-progesterone	-0.0454	29.02

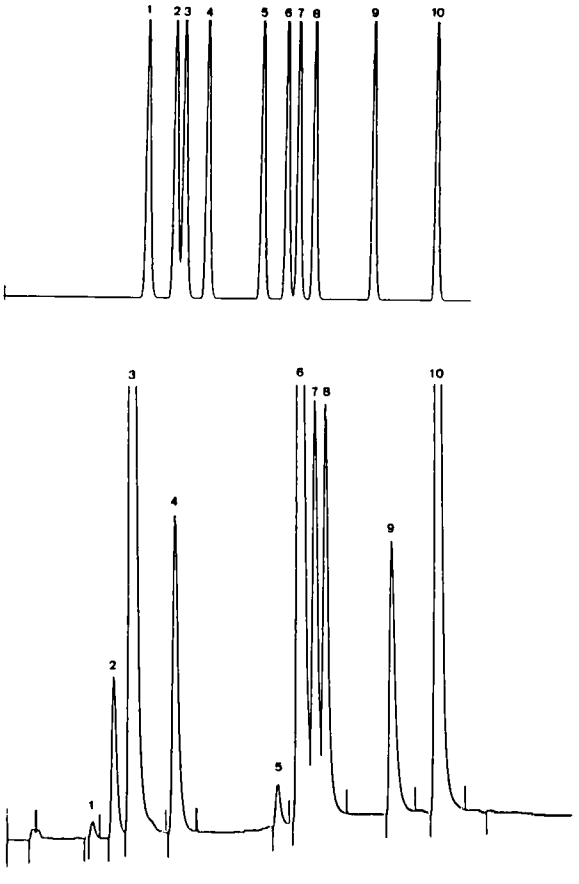

FIGURE 9: Comparison of simulated and experimentally obtained chromatograms for gradient separation of steroid hormones. Peak assignment see FIGURE 8.

pattern of the analytes. Correlation and relative error obtained from these data and and additional data from literature (23) are plotted in Figure 10. The overall correlation is quite acceptable, the relative error of prediction shows, however, a tendency to increase with decreasing retention time. This observation can be interpreted potenally by relatively constant absolute values of deviation between experiment and prediction for fast and moderately fast eluting compounds. The relative error and consequently, the quality of predic-

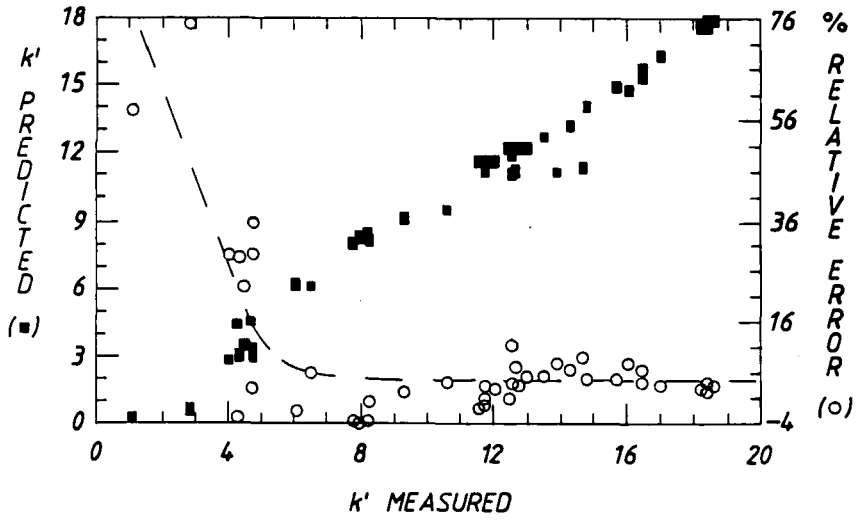

FIGURE 10: Plot of experimentally obtained vs. computed k' values (■) and the associated relative error of prediction (o).

tion will be dependent on elution time . This can be probably compensated for with improved precision of the evaluation of all parameters used for prediction. The higher number of experiments required for a reduction of the relative error of prediction has to be balanced against the effort needed with a fully empirical design. If precise information on system- and retention behaviour is available, optimization can be successfully assisted in this dimension.

CONCLUSIONS

The procedure described in this paper offers the possibility to predict retention behaviour in HPLC irrespective of the separation mode which may be adequate for a given separation problem. It is a global strategy that is well suited for optimization on multimodal response hypersurfaces. Improved optima can be sequentially approxi-

mated by incorporation of additional experimental data. Thus, the actual number of chromatograms required reflects the complexity of the separation problem. It is, however, instrumental that signals are correctly attributed to eluites at all experimental conditions. This presupposes the existence of a means to recognize the components. This very tedious task is currently done by a recognition routine based on fuzzy algebra (24).

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