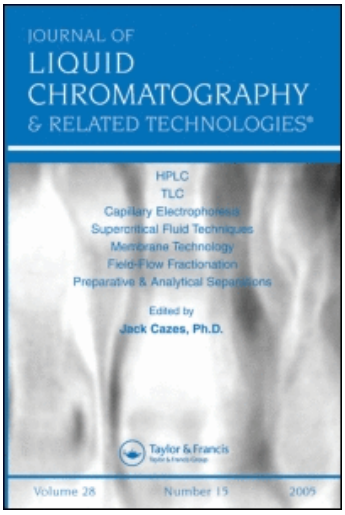

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Optimization Methods For Hplc

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OPTIMIZATION METHODS FOR HPLC

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Foreword

As almost every researcher in the field might be able to have a literature search done in the Chemical Abstracts on the keywords "HPLC" and "Optimization", no bare summing up of all papers that have been published in the last few years on this particular subject will be found in this review.

The scope of this paper is to give the reader some insight in the historical and theoretical background of optimization methods for HPLC and to make it possible for him/her to judge and use the methods published in the literature.

To achieve these goals the most important experimental optimization techniques used in HPLC are discussed thoroughly and some miscellaneous methods are mentioned and elucidated briefly. Finally optimization systems incorporated in commercially available HPLC instruments from several manufacturers will be evaluated.

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I. INTRODUCTION

Until the early 1920's, experiments investigating the effects of several factors(variables) on a response were designed using the "one-factor-at-a-time" method, neglecting possible interactions between the effects of the several variables. Already in 1935 Fischer (1) advocated the use of experimental designs in which all factors are varied simultaneously. During the second world war the discipline of Operations Research developed out of the efforts of scientists from several disciplines to tackle the military problems present that time. In the United Kingdom as well as in the United States the scientists working on this projects during the war turned their attention to the possibilities of applying similar approaches to civilian problems in the early 1950's. It was since then that the technique called Operations Research became widely known.

Although most Operations Research techniques deal with modelling of the response, some of the search techniques used do not need a well defined model to be able to optimize the response of a system. These search techniques are ideally suited for the optimization of systems in (Analytical) Chemistry, where it is often very difficult if not impossible to define a good descriptive model. This is possibly the reason why experimental optimization techniques became rather popular in Analytical Chemistry, while other Operations Research techniques are hardly used.

In 1951 Box and Wilson (2) published an important paper on how to attain optimal conditions in an experimental way. They proposed a sequential use of experimental designs in order to locate the optimum fast using relatively simple designs. This approach has been simplified by Spendley e.a. (3) in 1962, who defined the very well known sequential simplex algorithm. This sequential optimization technique is in fact nothing else than a subsequent use of (simplex)designs located nearer every step to the best response possible.

From these facts it might be concluded that the use of optimization techniques had become rather common in Analytical Chemistry too, but in the review of Currie e.a.(4) in 1972 it appeared that: "Although the area of statistics dealing with experimental design and optimization is extremely active and highly developed, there has been remarkably little use made of these techniques by English speaking chemists "(citation). A literature search under the heading "OPTIM" in Chemical Abstracts and Chemical Titles in 1973 covering the past eight years revealed that only few optimizations were statistically designed or otherwise systematically achieved. However during the 1970's especially the sequential simplex algorithm became more and more well known in Analytical Chemistry. This resulted in 1980 in the appearance of Chemometrics (from which experimental optimization is an important part) as a separate subject in the fundamental review section of Analytical Chemistry. In the last few years the sequential simplex algorithm is evermore replaced by other (more sophisticated) techniques from the field of Operations Research, which is promoted of course by the availability of computer hard and software in quantities that were not imaginable a few years ago.

The first papers wherein experimental optimization techniques were applied to chromatographic separations appeared around 1970. It were of course gas chromatographic separations that were considered. However, even at that time it was still common practice to use chromatographic theory to direct the steps in the optimization procedure. It lasted until the late 1970's before the pure experimental optimization techniques were applied to liquid chromatographic separation problems.

II. THEORY

- Sequential Experimental Optimization (Black Box Approach)

The reason why this kind of experimental optimization is called "black-box" approach is that it is assumed that no prior knowledge

of the system under consideration is available. In most cases the experimental parameters which influence the response are known, as is the response itself. But sometimes also these quantities have to be determined, before an experimental optimization procedure can even be started. The most commonly used technique in the past was the "one variable at a time" method, because most experimenters were afraid of getting to complex results when more than one variable(factor) at a time was varied. The fact that the interpretation of these results could lead to erroneous conclusions because of misgarded interactions between the several factors, was forgotten for convenience.

To solve the univariate(one factor at a time) problem several sequential optimization techniques are available.

One of the most well-known is the Fibonacci-search. This sequential search method is based on the Fibonacci series defined by the recursive relationship:

$$F_{n+2} = F_n + F_{n+1} \text{ , where } F_0=0 \text{ and } F_1=1$$

The experimenter has to start with the decision on the width of the optimal region, l, which is acceptable compared to the width of the original search region, L. The ratio L/l instantly indicates how many experiments are needed to reach the desired width of the optimal region, by comparing the value of L/l with the numbers in the Fibonacci series.

When the search is started using an optimal region width, l, given by:

$l = F_{n-2}/F_n * L$ where F_{n-2} and F_n are terms from the Fibonacci series,

then the last experiment will be situated exactly in the middle of the last search region, which is probably the optimum. For a detailed description of this method the books of Massart e.a.(5) and Beveridge and Schechter (6) are advised.

The often used "Golden Section" search is a special case of the Fibonacci search. The length of the sections into which the

original search area is divided is not determined by the ratio of two numbers of the Fibonacci series but is taken a constant value of 0.3820. This value originates from the very old knowledge of the Greeks that the best way to divide a segment into two unequal parts is to do it in such a way that the ratio of the whole to the larger part equals the ratio of the larger to the smaller part. This ratio is known to be $(1+\sqrt{5})/2 = 1.6180$ and is called the Golden Section or Golden Mean. Comparing this method with the Fibonacci search it appears that for large values of n (indicating the n^{th} term in the Fibonacci series) the ratio F_{n-2}/F_n almost equals 0.3820, making the two methods identical.

Another method to be mentioned here is the uniplex method, which is the one dimensional version of the (modified) sequential simplex algorithm, discussed later on. This means that contrary to the Fibonacci or Golden Section search this is an open ended search technique. A detailed description of the technique will not be given here, because it can be very easily deduced from the forthcoming vast description of the multidimensional simplex method. In the literature the method is described by King and Deming (7) and in the book of Massart e.a.(5).

Since the introduction of the simplex method by Spendley e.a.(3) and the useful modifications published by Nelder and Mead (8) this optimization technique has become very popular and widely used in analytical chemistry.

Reason enough to give a complete outline of the ordinary and the modified sequential simplex method in this review.

The ordinary simplex method:

Rule 1: An initial simplex is defined by the choice of k+1 vertices in the k-dimensional factor space.

Rule 2: A move is made after each observation of the response, once the responses of the initial simplex have been evaluated.

Rule 3: A move is made into that adjacent simplex which is obtained by discarding the point of the current simplex corresponding to the least desirable response and replace it with its mirror image across the (hyper)plane of the remaining points (see figure II-1).

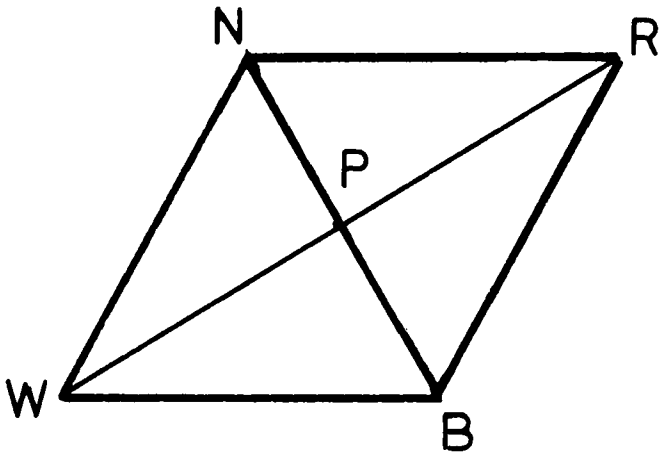

FIGURE II-1

Example of a move of a simplex in an ordinary simplex search; W indicates the point with the worst response, N the next to worst and B the best response; R is the reflected point.

Rule 4: If the reflected point has the least desirable response in the new simplex, do not reapply rule 3, but instead reject the second worst response in the new simplex and continue.

Rule 5: If a vertex has been retained in k+1 simplexes, before applying rule 2, reobserve the response at the persistent vertex (k is the number of factors(dimensions)).

Rule 6: If a new vertex lies outside the boundaries of the independent variables, do not make an experimental observation, but instead assign a very undesirable response to it.

In the modified simplex method rules 3 and 4 are substituted by the following ones:

- If the mirror image (R) of the point with the least desirable response (W) has a response which is more desirable than the best response (B) in the old simplex, an expansion is made to the point E in the following way:

$E = P + \gamma (P-W)$, where P is the centroid of the (hyper)plane to the opposite of W (see figure II-1)

$\gamma > 1$ (expansioncoefficient)

If the response in point E is more desirable than the response in R the new simplex is BNE, otherwise the new simplex is BNR.

- If the response in R is less desirable than the response in B, but more desirable than the response in N no expansion is made and the new simplex is BNR.
- If the response in R is less desirable than the one in N a contraction is made. Depending on whether the response in R is more or less desirable than the one in W a positive or negative contraction is made. This results in the point C_r if the response in R is more desirable than the one in W, otherwise the new point is C_w. For point C_r, γ lies between 0 and 1 ($0<\gamma<1$), for point C_w is negative ($\gamma<0$).

This whole procedure is best illustrated in figure II-2.

If none of the points R, C_r or C_w give more desirable results than the response in W, some corrective action has to be taken (for instance the application of the next to worst rule).

The simplex is halted when the step size becomes smaller than some predetermined value, or when the variance in the measured response becomes less than the measurement error. Of course other stop-criteria can be used.

Since 1974 very much attention has been paid by several authors to modifications of the simplex method in order to make it operate more effectively and efficiently. Routh e.a.(9) in 1977 introduced the Super Modified Simplex (SMS) in which the point with the most desirable response is determined by a second order polynomial fit through the points W, P and R in figure II-1. The new vertex is found by calculation of the extreme point of this second order polynomial. In 1980 vd Wiel (10) described some improvements of the SMS-method wherein he replaced the second order polynomial fit by a Gaussian fit. Finally in 1983 the last published modification of

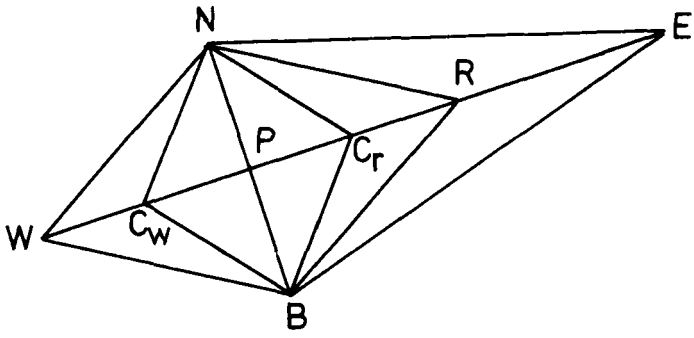

FIGURE II-2

Figure illustrating all possible moves in a modified simplex search; W, N, B and R as in figure 1; for explanation of the other symbols see text.

the simplex method appeared, also by vd Wiel (11). In this paper a symmetry controlled simplex is described, which is extremely useful when more than three factors are taken into account because in those cases it is not easily recognized whether the simplex is loosing one or even more dimensions by expansion or contraction. When a dimension is lost or nearly lost the simplex is not able to vary the factor which's dimension is lost anymore. Then the optimization might get stuck on a less desirable value of that factor. In spite of the development in the performance of the sequential simplex technique the most frequently used version in the optimization of HPLC separations is the modified simplex method, which will be illustrated in chapter III.

Other multidimensional sequential optimization methods like the steepest ascent or descent method and other gradient methods are described thoroughly in books on optimization (6,5), but as they are hardly used in the optimization of HPLC separations, they are not discussed here.

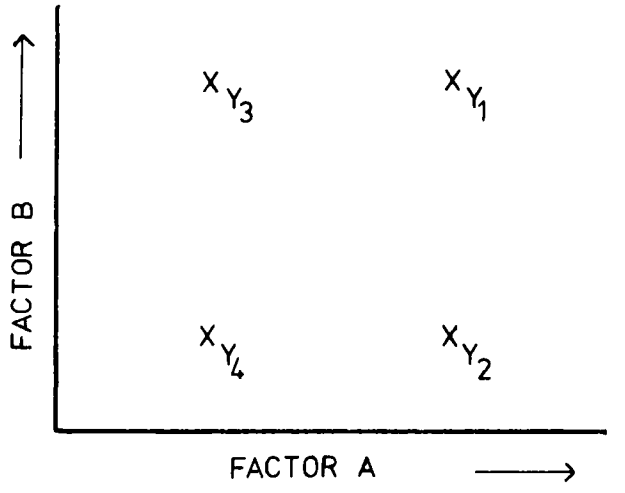

FIGURE II-3

The lay-out of a factorial design at two levels of the factors A and B; y_1, y_2, y_3 and y_4 indicate the measured responses according to table II-1.

- Simultaneous Experimental Optimization (Mathematical Approach)

Using this technique, one's goal is to be able to describe the reponse (dependent variable) that is to be optimized by an equation that is obtained using regression techniques. To be sure to get an equation, which succesfully describes the response all over the part of the factorspace under consideration, with a minimum number of measurements, one is obliged to spread out the measurements in a regular way over that factorspace. This is best done by using some kind of experimental design. The simplest form of a useful experimental design is the factorial design at two levels.

Let us asume that there are two factors (independent variables), which influence the observed response. A factorial design is then set up as shown in figure II-3.

TABLE II-1

A scheme for a factorial design at two levels of the factors A and B. y_i denotes the measured response in run i. The order of the runs should be randomized.

Run	A	B	result
1	+	+	y_1
2	+	-	y_2
3	-	+	y_3
4	-	-	y_4

The two values of each factor at which the response is measured are indicated by (-) and (+). So in this case four experiments have to be done.

The necessary results for optimization purposes can be calculated very easily, by gathering the results in a factorial scheme like the one shown in table II-1.

The mean effect of factor A on the measured response is given by:

$$E_a = ((y_1-y_3)+(y_2-y_4))/2$$

In the same way the mean effect of factor B can be calculated:

$$E_b = ((y_1-y_2)+(y_3-y_4))/2$$

Also the interaction effect between factor A and factor B on the response can be calculated:

$$E_{ab} = ((y_1+y_4)-(y_2+y_3))/2$$

That this term is indeed a measure for the interaction between the effects of factor A and factor B can be seen in the following way.

When there is no interaction, the influence of factor A and factor B on the response will be independent. This means that if a high level of factor A raises the value of the response, it will do that to the same extent whether factor B is at its high or at its low level. And the same holds of course for the response measured at a particular level of factor B. It can easily be seen that the term E_{ab} will be close to zero then. But if there excists an interaction between the effects of factor A and factor B, the term E_{ab} will have a positive or negative value depending on the way of interaction.

When conclusions about the effects of the varied factors have to be drawn from these experiments it is necessary to do a statistical significance test. This can be achieved by measuring some or all y-values more than once and calculation of the pure experimental error from the variances of the replicated measurements. Using simple statistical tests the significance of the effects can be estimated.

Of course it is possible to enlarge the number of factors which is taken into account, however this means that more measurements have to be done, as can be seen from the factorial scheme in table II-2.

TABLE II-2

A scheme for a factorial design at two levels of the factors A, B and C (see table II-1).

Run	A	B	C	Result
1	+	+	+	y_1
2	+	+	-	y_2
3	+	-	+	y_3
4	+	-	-	y_4
5	-	+	+	y_5
6	-	+	-	y_6
7	-	-	+	y_7
8	-	-	-	y_8

The number of measurements necessary in factorial designs at two levels can easily be calculated. Because every factor is established at two levels the number of measurements is 2^k for k factors. The extension to more factors does not influence the ease of calculation of the effects. Using the simple arithmetics explained in the two factor example, all first, second and higher order effects can be calculated. When higher order effects are not likely to occur the factorial design may be reduced to a so called fractional factorial design to diminish the number of experiments. Another possible use of the higher order effects is the calculation of the pure experimental error from it. This is statistically correct when it can be assumed that higher order effects are not likely to occur. A thorough theoretical treatment of factorial designs at two levels is given by Box e.a.(12).

However, factorial designs at two levels are unable to explore completely a wide region in a factor space and evenmore only first order regression equations (with interaction terms) can be estimated from its results. Therefore factorial designs at more than two levels for each factor have to be used when the feasible part of the factor space is to be explored more detailed.

This extension means that the more difficult Analysis of Variance calculations are necessary to calculate the effects of the varied factors on the measured response. This results in the need for computer capacity when the number of factors and levels increases. A clear and decent treatment of factorial designs, especially the ones used in analytical chemistry, is given by Massart e.a.(5).

To complete the so called mathematical (analytical) approach a model of the measured response depending on the level of the factors under consideration has to be fitted. When the design is laid out in a predetermined way simple arithmetics are sufficient to calculate the regression coefficients. What has to be kept in mind is that it is almost always possible to fit an arbitrary model to some measured responses offering a reasonably good coefficient of determination (r^2). However it is very questionable whether the fitted model really describes the behaviour of the system which is

monitored. It is therefore necessary that first a good theoretical understanding of the system in observation (an HPLC separation for instance) is achieved after which the regression model that is to be fitted can be formulated.

Another problem encountered using this technique is the interpretation of the estimated regression coefficients. For example let's consider the following regression equation:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2$$

When the terms X_1, X_2 or X_1X_2 are very different in order of magnitude, a simple comparison of the estimated regression coefficients b_i is not the correct way to determine the effects of the several factors on the response. Sometimes it is useful to scale the factors in such a way that their order of magnitude is almost the same.

In the application of this technique to the optimization of HPLC separations it is not usual to scale the factors. So care has to be taken especially when the effect of interaction terms on retention behaviour is considered. Because the second order terms in these equations are at least a ten-fold smaller in order of magnitude than the first order terms, while the third order terms are at least a hundred-fold smaller, regression coefficients which are a ten-fold or even a hundred-fold larger than the coefficients for the first order effects may be expected.

The use of simultaneous optimization techniques in the optimization of HPLC separations will be discussed in chapter III.

III. OPTIMIZATION METHODS FOR HPLC-SEPARATIONS

A. The Theoretical (Classical) Method

This approach is best illustrated by Scott(13), Guiochon(14) and more recently by Kaiser and Oelrich(15).

Already in 1970 Scott proposed, in his contribution to volume 9 of the series Advances in Chromatography, a systematic approach to the optimization of gas chromatographic determinations. Reasoning from the two basic ideas, firstly the necessity to move the solute bands apart and secondly the wish to maintain the bands sufficiently narrow, he discussed all aspects of chromatographic theory in order to reach a chromatographic system which was able to separate all components in a mixture in a minimal analysis time. Much attention was paid to the column and stationary phase design, which were optimized using schemes like the one shown in figure III-1. In 1980 Guiochon wrote his contribution to volume 2 of the series High Performance Liquid Chromatography(14), wherein he describes several fundamental and practical equations used for the optimization of experimental conditions in liquid chromatography. But as already noticed in Scott's contribution in 1970 main attention is paid to column and stationary phase design. However, the first sign of a new approach is noticeable. The formulation of equations for the maximum number of peaks to be separated on a given column and the proposal of logarithmic relations between the capacity factor k' and the molecular size of the solute to be retained may be seen as the early start of the recent optimization approaches where the more easy to vary parameters like mobile phase composition, column temperature, counter ion concentration, pH-value, etc. are related to the retention behaviour of the components to be separated.

In 1981 Kaiser and Oelrich (15) set the whole world of chromatographists in stir and commotion when they published their book "Optimization in HPLC". In this book the chromatographic theory of theoretical plate number, on which most classical optimization literature, including the two books mentioned before, was based is criticised heavily. The authors prefer to talk in terms of resolution, adequate separation and separation efficiency. For instance it is shown that depending on the experimental conditions the number of theoretical plates of one and the same chromatographic column may vary between 8000 and 25000, which

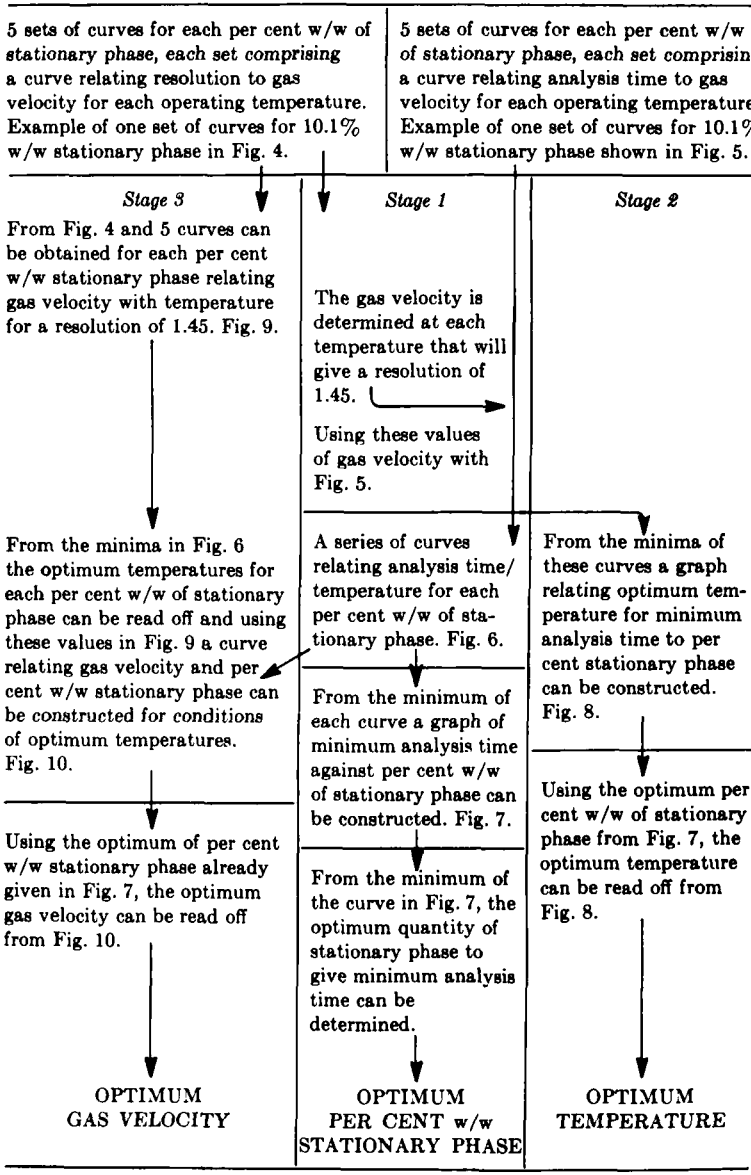

FIGURE III-1

Scheme for the optimization of chromatographic separations using information from chromatographic theory only.(figure from (13)).

contradicts heavily with classic chromatographic theory. From these results Kaiser and Oelrich make the step to a new pragmatic approach called "The abt-Concept". Although the usefulness of this new concept in the optimization of HPLC separations is not proven yet, the statement of the authors about optimization in HPLC is proven every time again in laboratory practice:
"Optimization is only possible if there is still latent potential present in a separation system !"

B. The "One-Variable-at-a-Time" (Univariate) Method

Although this method is mentioned very often in the introduction part of papers on the optimization of HPLC separations as being the most frequently used technique for method development or optimization of HPLC determinations, only few papers are found in which the optimization is carried out using a univariate method. This is probably caused by the fact that most researchers working in the field of experimental optimization of HPLC separations are well aware of the fact that univariate methods may lead to erroneous results. Most papers published under the header "Optimization" using a univariate method are from French scientists and are dealing with optimization based on chromatographic theory (16-21).
Other papers presenting a univariate approach are the numerous ones on "Factors influencing the retention behaviour of .....". Usually in these papers the several factors which are supposed to influence the retention behaviour are investigated succesively assuming no significant interactions between the effects of the several factors. Conclusions in these papers are also presented in such a way that it is insinuated that the optimal separation conditions are simply found by combination of the experimental values determined for each factor separately (22-25).

C. The Sequential Simplex (Multivariate) Method

This method was and still is probably the most simple and straightforward procedure for optimization purposes in analytical

chemistry. Rainey and Purdy (26) in 1977 were with the first to publish an article about the simplex optimization of an HPLC separation. They optimized a separation of phospholipids with respect to the composition of the mobile phase. The response used to direct the simplex search in the direction of optimal separation conditions was the resolution between the two peaks in the chromatogram. Another optimization of an HPLC separation described in the same paper clearly illuminated the problems arising when a multicomponent separation has to be optimized. A criterion for the quality of separation in the chromatogram has to be defined to generate the response necessary to direct the simplex search. Those quality-criteria had already been described for GLC separations by several authors (27-32) and new and sometimes better ones have been proposed during the past few years, but this will be discussed later on.

When a criterion for the quality of separation in a chromatogram has been chosen the optimization procedure can be started, once the factors influencing the response are established.

Most papers published in the last few years concentrate on the optimization of reversed or normal bonded phase separations with respect to the binary, ternary or even quaternary mobile phase, while sometimes column temperature, flow rate, gradient shape, buffer concentration and pH-value of the eluent are taken into account.

Watson and Carr (33) optimized the gradient elution of some PTH-amino acids using a simplex optimization with five factors to be varied. They already mentioned the problem of peak cross-over during the optimization run, which would lead to the occurence of local optima in the response surface. They suggested restarting of the simplex from a different region of the factor space. A similar problem has been described by Fast e.a. (34) leading to the same conclusions, with an extra remark that in the case of peak cross-over a simultaneous optimization method like the one proposed by Glajch e.a.(35) may be more succesful. Other workers during these years used mathematical modelling of capacity factor (k') and

reduced theoretical plate height (HETP) to establish the optimal composition of the eluent and the optimal column length using a simplex search with analysis time as the response (36).

In 1982, when micro-computers became more and more common equipment for the chromatographist, several researchers started to work at the automation of the simplex optimization method. Wegscheider e.a.(37) and especially Berridge (38,40) published several papers, in which they propose well suited approaches to automatic (unattended) simplex optimization of HPLC separations.

The automation of the optimization procedure puts some strong demands on the techniques used. The simplex algorithm itself (mostly the modified version) is rather easy to program in almost any available computer language (basic, fortran, etc.), but the difficulty lies in the judgement of the quality of separation in the chromatogram and in the use of constraints in the experimental factors. The quality-(performance-)criteria used have to be able to handle chromatograms of samples with an unknown number of components. Furthermore there have to be built-in assurances that the simplex search does not get stuck on local optima or ridges in the response surface. So it is not surprising that Wegscheider as well as Berridge propose new criteria for the judgement of the quality of separation in the chromatogram (see table III-1).

During 1983 and 1984 only a few papers on the optimization of HPLC separations using the sequential simplex method were published (42, 43) probably because more attention was paid to other optimization methods, more useful when peak cross-over can be expected.

Also papers on the comparison of several optimization methods (including the simplex method) appeared (44,45).

However, one very important aspect of a sequential optimization procedure is still subject of research in this field, and that is the judgement of the quality of separation in a chromatogram in such a way that one numerical value expresses the quality of overall separation.

As already mentioned before, this problem has for the first time been recognized when researchers started to optimize GLC

separations. In 1976 Morgan and Deming (28) published an excellent article on experimental optimization of chromatographic systems, in which an overview of the performance criteria for chromatographic separation published until then, was given. They noticed that although in many separation problems the primary response of interest is the overall separation, there are other measures of system performance that should be taken into account. Examples of these are analysis time, sensitivity of detection, cost, etc. A rather complete evaluation of those quality criteria has recently been given by Debets e.a. in 1983 (46). They discussed the performance of the quality criteria in several chromatographic situations and came to the following conclusions:

- all quality criteria give response surfaces with local optima when the elution order of peaks changes.
- all quality criteria need information about the number of peaks to be found in the chromatogram.
- all quality criteria need constraints or mathematical corrections in calculating the response either when peaks are baseline separated or strongly overlapping.

In the mean time Berridge (38) proposed a quality criterion which meets two of the three shortcomings mentioned by Debets e.a.. Using the criterion proposed by Berridge it is not necessary to know the number of peaks to be expected in the chromatogram and no constraints or corrections have to be considered when the response is calculated. Also the remarks of Morgan and Deming (28) concerning other system variables are taken into account. Using free to choose weighting factors for the several contributions to the total response, the performance of the quality criterion can be adapted to special demands put forward by the chromatographist.
Another quality criterion presented in 1982 is the one proposed by Wegscheider e.a.(37) in which the baseline noise of the chromatographic signal is taken into account. This is argumented by the authors by stating that this quality criterion prohibits the choice of experimental parameters whereby the signal to noise ratio of one of the peaks in the chromatogram is not good enough.

Knoll and Midgett (47) proposed an area overlap fraction as a criterion for the quality of separation which strongly resembles the fractional overlap S used by Smits e.a.(27) and Massart e.a.(30,48). But this criterion has never been used for optimization purposes. On the Symposium on Advances in Liquid Chromatography in Szeged, Hungary, 1982 Vajda e.a.(44) presented a quality criterion especially designed for automatic optimization of the separation of unknown samples. In this criterion the elution of more peaks is given more importance than baseline resolution. The authors state that a time constraint as used by Berridge (38), Watson e.a.(33) and Glajch e.a.(35) is not incorporated because it would contradict with the aim of finding the maximum number of peaks possible.

The last published quality criterion, which has already been mentioned by Debets e.a., is the Π_{Rs}-function of Schoenmakers and Drouen e.a.(49). This criterion is meant to give the highest response when the peaks in the chromatogram are as evenly spaced as possible, without taking into account the necessary analysis time. The authors remark that once the selectivity has been optimized the analysis time (or even the chromatographic resolution) can easily be adjusted by changing the column or the flow rate. This seems not very practical because most chromatographists will not be in the position to change columns whenever they want to. In another paper the authors refine the proposed optimization criterion in such a way that a shorter analysis time is preferred and that the resolution between pairs of adjacent peaks is more evenly distributed (50).

A complete overview of quality criteria for chromatographic separations is given in table III-1.

When the listed criteria are looked at a little closer it will be obvious that the chromatographist who wants to optimize a separation using one of the mentioned criteria is put in a dilemma. At a first glance it looks very attractive to use one of the well defined criteria without weighting factors that have to be given a specific value. On the other hand one of the more sophisticated

TABLE III-1

A list of published criteria for the quality of separation of two (1,2) or more (3-12) peaks in a chromatogram. For explanation of the symbols and more details see the references.

1. Valley to Peak ratio: $V = a/b$ (31)
2. Peak Separation factor: $P = f/g$ (32)
3. Total Overlap: $\Phi = \sum_{i=1}^{k} \exp(-2.R_i)$ (41)
4. Chrom. Resp. function: $CRF = \sum_{i=1}^{k} \ln(P_i)$ (28,29)
5. Chrom. Optim. function: $COF = \sum_{i=1}^{k} w_i \ln(R_i/R_d) + \beta(t_m - t_1)$ (35)
6. Informing Power: $P_{inf} = \sum_{i=1}^{k} {}^2\log S_i$ (27,30)
7. Separation Number: $SN = \sum_{n} {}^2\log p_n$ (39)
8. Product Resolution: $\Pi_{Rs} = \prod_{i=1}^{k} R_i$ (49)
9. Chrom. Resp. function: $CRF = 1/t \prod_{i=1}^{k} f_i/(g_i+2n_i)$ (37)
10. Area Overlap fraction: $A_o = A_c/(A_s\sqrt{2\pi\sigma_c^2}) \int_{B-2\sigma_s}^{B+2\sigma_s} \exp(-y^2/2\sigma_c^2)dy$ (47)
11. Chrom. Eval. function: $CEF = \sum_{i=1}^{k} A_i + 1/(n-1) \sum_{j=1}^{k=1} B_j P_j$ (44)
12. Chrom. Resp. function: $CRF = \sum_{i=1}^{k} R_i + L^x + a|T_m - T_1| - b(T_0 - T_1)$ (38)

criteria can be tuned in such a way that its performance is optimal in the optimization of the particular separation under consideration. However, the fine tuning of criteria can only be done when there is some prior information available about the solutes in the mixture that has to be analysed. If this information is not available at the first start some preliminary experiments have to be done from which the necessary information can be extracted, otherwise a sequential experimental optimization using one of the sophisticated criteria may lead to erroneous results. Weyland e.a.(51) showed, using retention data of separations of five sulfonamides, that some of the listed criteria indeed give response surfaces which are not suitable for sequential experimental optimization techniques. Very recently Berridge e.a.(52) claimed that a sequential simplex search using the same criterion as proposed in 1982 (see table III-1) and a preliminary gradient run to constrain the feasible part of the factor space, locates the global optimum for the separation of the five sulfonamides used by Weyland e.a. in only one run. So maybe this approach appears to be helpful when a sequential optimization technique is used.

D. The Window Diagram Technique

This technique has been introduced by Laub and Purnell in 1975 (53), who optimized the selectivity of GLC separations by mixing the solvents used as the stationary phase. They used a simple linear equation of which the validity had been shown earlier, to describe the retention behaviour of the solutes on a mixed solvent stationary phase. Using a binary stationary phase this approach gives straight lines when the infinite partition coefficient K is plotted against the composition of the binary stationary phase (see figure III-2). From this plot of K-values versus ϕ (fraction), α - values can be calculated. Using the convention that α remains greater than one ($\alpha>1$) a window diagram can be constructed as shown in figure III-3. The blank area in the plot represents those

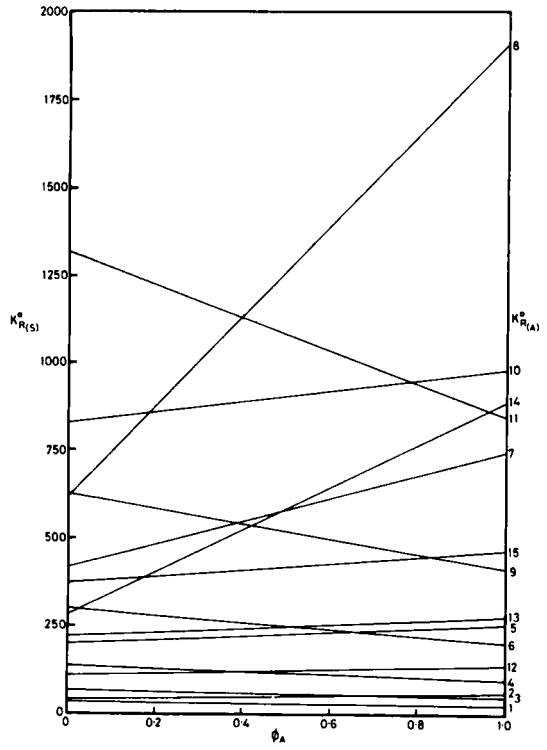

FIGURE III-2

Plots of K-values versus Φ_A for a gas chromatographic separation of 15 components; Φ_A indicates the fraction of A in the binary stationary phase.(figure from (53)).

minimum α-values that can be actually attained in practice, because each border-line between the blank and the shaded area in the plot represents the α -value of the worst separated pair of peaks as a function of the composition of the binary stationary phase.

A simple look at this window diagram reveals the composition of the stationary phase which gives the highest α-value for the worst separated pair of peaks. So all other pairs of adjacent peaks give higher α-values.

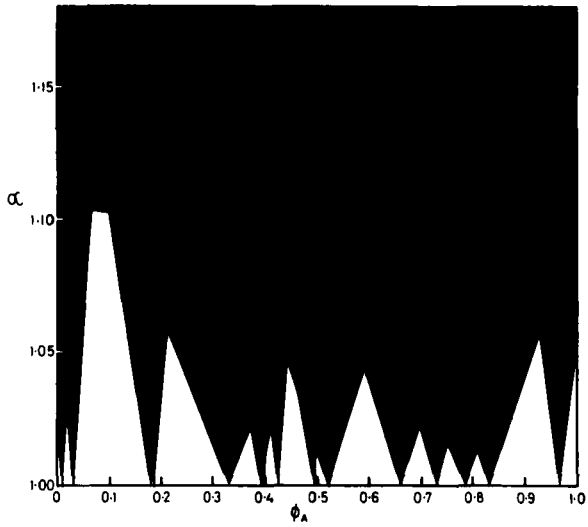

FIGURE III-3

Window diagram for the separation of the 15 components from figure III-2; optimum stationary phase composition occurs at $\Phi_A = 0.075$. (figure from (53)).

This technique has been used in GLC for the optimization of the composition of the binary stationary phase for the separation of samples of known composition (54) and also of samples with unknown composition (55). A computer program has been developed by the authors to automate the optimization procedure they had described earlier (56,57). In 1978 Laub and Purnell extend the technique to the optimization of column-temperature in GLC, GSC and HPLC (58), which makes them the first to use the window diagram technique in HPLC optimizations.

In the same year Deming e.a.(59) and Price e.a.(60) used the window diagram technique to optimize the pH-value of the mobile phase in the HPLC separation of several benzoic acids.

Sachok e.a.(61) presented a multifactor optimization based on the window diagram technique showing three dimensional diagrams wherein

the relative retention (selectivity factor) α is plotted as a function of pH-value and concentration of the ion-pairing reagent in the mobile phase.

In 1981 Sachok e.a.(62) introduced the name "minimum-alpha-plot" (MAP) for what Laub and Purnell had called "window-diagram". This definition of the plots obtained using this technique is more clearly in expressing what is really shown in the diagrams. What so ever, the use of window diagrams for the optimization of pH-value and concentration of the ion-pairing reagent seems succesful, as it is still used (63).

Already in 1983 the window diagram technique had been used by Issaq e.a.(64) to optimize the binary mobile phase composition of a reversed phase HPLC separation. In January 1984 another paper on the use of three dimensional minimum-alpha-plots was published by Weyland e.a.(65). They used this technique for the optimization of a ternary mobile phase in reversed phase HPLC. As may be noticed from part C of this chapter the optimization of mobile phase composition in RP-HPLC had already been studied for years using other optimization techniques. Weyland e.a. claim that the use of minimum-alpha-plots in the optimization of the mobile phase composition in RP-HPLC offers great advantages above other methods. Once the retention behaviour of the solutes under consideration is determined, all possible constrained optimization procedures can be applied without doing any chromatographic experiments. Recently Laub (66) published an article wherein he described a computer program ("window") for the optimization of mobile phase composition using the window diagram technique.

Overlooking the applications of the window diagram technique it appears that this technique is useful in very different kinds of chromatographic optimization. The great advantage is that the global optimum can be located either visually or with the aid of a computer in a very easy way. Disadvantages are in the first place that a description of the retention behaviour of the solutes as a function of the experimental variables is necessary and secondly that the calculated response is only a measure for chromatographic

resolution. The latter means that using this technique constraints (e.g. analysis time) have to be taken into account, which makes the problem of locating the global optimum a matter of non-linear programming, which is a technique that is not very familiar to chromatographists.

E. The Simultaneous Optimization Techniques (Experimental Designs)

As has already been indicated in part D of this chapter sometimes a definition of the retention behaviour of the solutes as a function of the experimental parameters is necessary. This definition can be achieved by the use of regression or regression-like techniques. When these techniques are to be used a number of experiments have to be done to collect the necessary data. These experiments have to be spread out as regularly as possible over the feasible part of the factor space. When the variables which influence the response are completely independent experimental designs can be used which were mentioned in chapter II and are more thoroughly discussed in several books on statistics or applied statistics (5,12). This technique has been used by Otto and Wegscheider (67,68), Berridge (69) and Lindberg e.a.(70).

If the variables are not independent the use of experimental designs becomes more complicated. Either the dependences have to be eliminated by substitution of equations defining the dependence or, in the case of mobile phase compositions for instance, mixture designs can be used. These mixture designs were introduced by Snee e.a.(71) based on statistical discussions by Scheffe (72) and Gorman and Hinman (73) (see figure III-4). The aim of these mixture designs is to measure the response at a few well-defined points in the design from which the coefficients in the regression equation can be calculated very easily. The degree (order) of the equations can easily be adapted to the complexity of the response surface that is to be described.

Snee already mentioned the possibility that the solvents on the extreme points of the mixture design are not pure components but

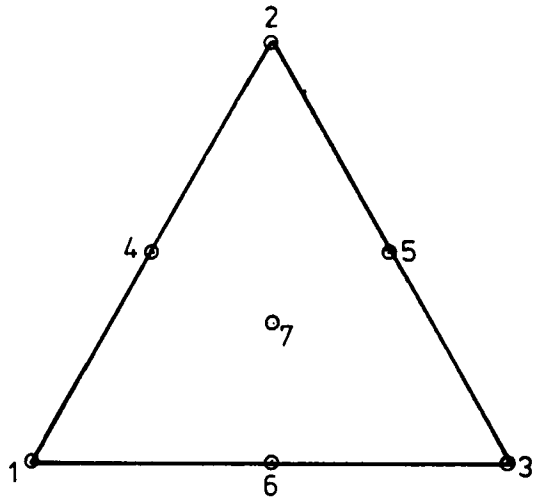

FIGURE III-4

The lay-out of a simplex lattice design for a three-component mixture; the points 1 to 6 are necessary for the description of the response-surface using a quadratic equation; point 7 has to be added when a special cubic equation is desired.

pseudocomponents (a fixed proportion mixture of several pure components). The composition of the pseudocomponents used in the optimization of HPLC separations is mostly determined by running a preliminary gradient run. This gradient run offers the information necessary to determine the elution strength of the mobile phase with which the solutes will be eluted in a desired k'-range. The theoretical basis for this approach is given by the solubility(-parameter) theory according to Tijssen e.a.(74), Bakalyar e.a.(75) and Schoenmakers (76). Once the composition of the pseudocomponents is determined in such a way that the elution strength of the binary mixtures is almost identical a mixture design is laid out. This is the approach introduced by Glajch e.a.(35,77-80), and used by Lehrer (81), Belinky (82), Antle (83), Waechter e.a.(84), D'Agostino e.a.(85), Landers e.a.(86) and Goldberg e.a.(87), which

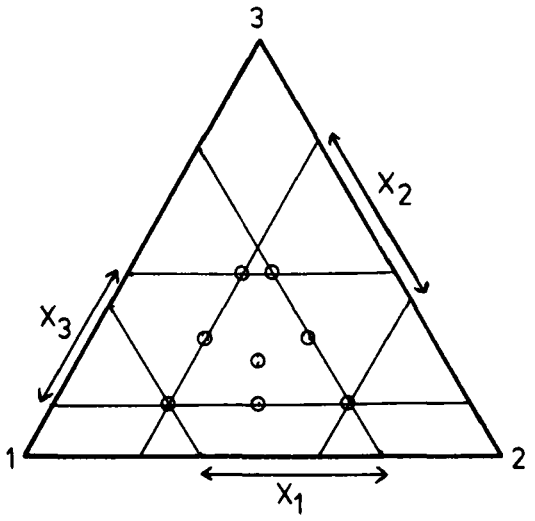

FIGURE III-5

Example of an "extreme vertices" design; component 1 is constrained to fractions indicated by x_1, component 2 to fractions indicated by x_2 and component 3 to fractions indicated by x_3.

are partly researchers working at Du Pont de Nemours Corporation. This manufacturer of HPLC equipment was the first to introduce an automated HPLC optimization system ("Sentinel"), but this will be discussed later.

The use of mixture designs with pure components at the extreme points has been shown by Weyland e.a.(51,65), who used ordinary regression statistics to calculate the coefficients in the regression equations. They used the so called "extreme vertices" design to constraint the feasible part of the factor space (see figure III-5).

Glajch e.a. claim that using the pseudocomponent-approach the selectivity of a separation is optimized best, because the k'-values of the last eluted solute will not change very much. Weyland e.a. argumented that this is not always true, because the choice of fixing the solvent strength of the pseudocomponents also restricts

the possibility of making full profit of selectivity effects, which can be caused by simply changing the composition of the water-organic modifier mixture (see Issaq e.a.(64)). That is why Weyland e.a. promote the use of pure components in a mixture design.

Once the data are collected, the retention behaviour of the solutes as a function of the mobile phase composition can be established. This is most simply done by using the general mixture design equations proposed by Gorman and Hinman (73) and Snee e.a.(71):

$$Resp = b_1\phi_1 + b_2\phi_2 + b_3\phi_3 + b_{12}\phi_1\phi_2 + b_{13}\phi_1\phi_3 + b_{23}\phi_2\phi_3$$

for the quadratic form, or:

$$Resp = b_1\phi_1 + b_2\phi_2 + b_3\phi_3 + b_{12}\phi_1\phi_2 + b_{13}\phi_1\phi_3 + b_{23}\phi_2\phi_3 + b_{123}\phi_1\phi_2\phi_3$$

for the special cubic form.

These equations are very general applicable, but Schoenmakers (76) showed that these equations are very well suited for the use in HPLC optimization when log k' or ln k' is taken as the response. This derivation is based on solvophobic theory, which is a widely accepted model for the description of retention behaviour in reversed phase HPLC.

The great advantage of this technique is that the relative retention (or selectivity factor) α or the chromatographic resolution R can be predicted over the whole feasible part of the factor space.

There are of course other equations that were used to describe the retention behaviour as a function of experimental parameters. Toon e.a.(88), Walters (89) and Jinno and Kawasaki(90-93) studied the optimization of HPLC separations using relations between retention behaviour and other parameters than the composition of the mobile phase. The results are similar to the ones described before and also in these approaches predictions concerning the quality of separation can be made all over the feasible part of the factor space.

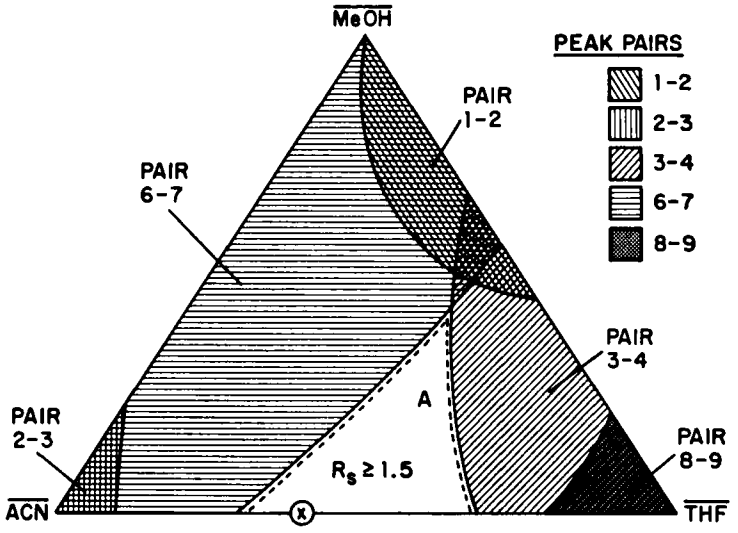

FIGURE III-6

Overlapping Resolution Map for a separation of nine substituted naphtalenes.(figure from (35)).

Once the retention behaviour of the solutes under consideration is defined in a satisfactory way, the optimal mobile phase composition can be determined in several ways.

Glajch e.a.(35) suggested the so called Overlapping Resolution Mapping (ORM) technique. Using this technique the predicted retention behaviour of the solutes is used to calculate the chromatographic resolution for every pair of peaks at every mobile phase composition within the solvent selectivity triangle. For every pair of peaks a triangle can be constructed in such a way that the areas in the triangle offering a bad resolution (R <1.5) are shaded. Then all triangles are overlaid to construct the overlapping resolution map which gives information about the area in the triangle where the resolution between all possible pairs of peaks is better than the desired value (R=1.5)(see figure III-6).

Another possible approach is the one suggested by Weyland e.a.(94).

They propose the use of non-linear programming techniques to locate the global optimum in the factor space, which is defined as the mobile phase composition offering the shortest possible analysis time while maintaining a desired resolution between all pairs of adjacent peaks. To be able to perform the necessary calculations mathematical relations describing analysis time and the resolution between all pairs of peaks as a function of the mobile phase composition (or other experimental parameters) have to be calculated The optimization can then be translated into the mathematical problem of minimizing a function under certain (equality and/or inequality) constraints. The results can be presented in a graphical way as is shown in figure III-7.

An optimization approach which differs a little from the ones described above is suggested by Schoenmakers and Drouen e.a.(49). The authors call it the iterative approach. The major difference is that at the start of the procedure a linear relationship between the response (ln k') and the experimental parameters is assumed. When the mobile phase composition is taken as the experimental variable, first the composition of the pseudocomponents is established using a gradient run. The sides of the solvent selectivity triangle are laid in a straight line to reduce the problem to a two dimensional one (see figure III-8), which means that only ternary compositions of the mobile phase are allowed. A search for the best value of the quality criterion proposed by the same authors (see table III-1) is started using the linear relations mentioned before. The next experiment is done at the mobile phase composition offering the best value of the quality criterion. The k'-values measured are used to correct the assumed linear relation and the quality criterion is calculated again for all possible ternary mobile phase compositions. This procedure continues until the predicted values of k' are close enough to the experimental values. Modifications in the determination of the mobile phase composition where the next experiment is to be done and in the calculation of the quality criterion have been made to optimize the performance of the method (50).

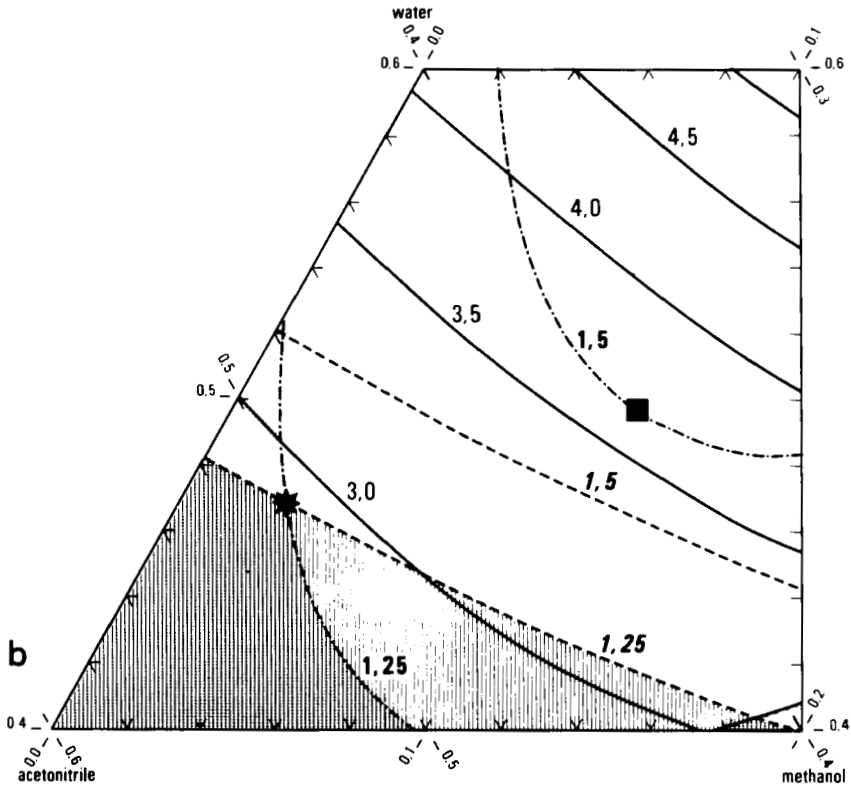

FIGURE III-7

Contour plot over a restricted region of the factor space for the HPLC separation of a three component mixture; (———) indicates the analysis time; (•–•–•–•–) indicates the resolution between component 1 and 2; (– – – –) indicates the resolution between component 2 and 3; the shaded areas are outside the constraints for resolution 1.25; optimum mobile phase compositions for minimal resolution 1.25 are indicated by ✸, and for 1.5 by ■.(figure from (94)).

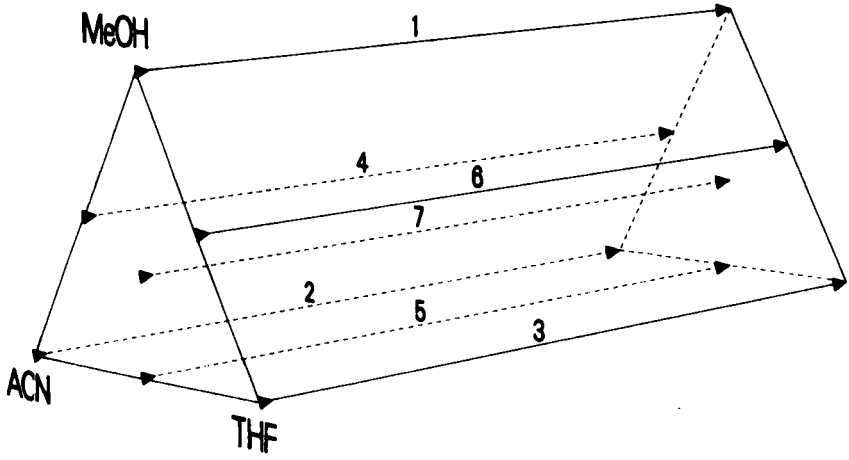

FIGURE III-8

Illustration of the "iterative" approach for a separation of five diphenylamines; the upper part shows ln k' versus mobile phase composition; the lower part shows the Π_{Rs} criterion and two modified versions versus mobile phase composition.(figure from (50)).

Besides the optimization of the mobile phase composition in reversed phase HPLC also the mobile phase pH (95) and the concentration of the ion pairing reagent in ion chromatography have been optimized using this approach (96,97)

Very recently the approach is extended to the three dimensional case (the use of quaternary mixtures as the mobile phase), which makes the necessary calculations a lot more complicated and time consuming (98).

Overlooking the vast amount of papers published on simultaneous experimental optimization of HPLC separations it seems that the analytical(mathematical) approach is the most popular one. Probably this is caused by the fact that most chromatographists abominate the idea of black box optimization, wherein no chromatographic knowledge is necessary most of the time.

From the optimization point of view the approach has some

disadvantages. In the first place the number of experiments is usually too small for a detailed description of the reponse surface, which can be very complex when the response of a quality criterion like the ones listed in table III-1 is to be described. Secondly the number of chromatograms that have to be recorded in each of the seven points in the triangle is rather large and depends on the number of solutes present in the mixture to be separated. This is caused by the fact that standards have to be run at every mobile phase composition to be able to identify the solutes in the chromatogram. And this is not even possible when the composition of the sample to be separated is not known.

To overcome this difficulty some authors investigated the use of dual wavelength detection (99,100) and multi-wavelength detection using a UV-diode-array detector (98,101). The dual wavelength detection seemed not very useful, and the results of the multi-wavelength detection are not completely published yet.

Issaq e.a.(102,103) also recognized the important problem of on-line peak identification in the optimization approach according to Glajch e.a.. They developed a computer program for the identification of peaks in a chromatogram based on the peak-area's. The identification is of course not perfect, but it might be a simple solution to analytical solutions in which the concentrations of the components can be chosen freely.

Another group of researchers, Detaevernier, De Smet and Massart e.a.(104,105), argumented that the Glajch, Kirkland, Snyder approach is too general and therefore too complex to be used in ordinary separation problems. That is why they propose a restricted version of the mentioned approach making use of only one preselected stationary phase and six possible mobile phase components. In this way it is possible to optimize and perform straight phase as well as reversed phase separations in one HPLC system.

IV. MISCELLANEOUS OPTIMIZATION METHODS

A. Gradient Optimization

In the experimental optimization part of this review some papers on the optimization of gradient elution in HPLC have already been mentioned (Watson and Carr (33), Fast e.a.(34)). But there have also been authors who studied the gradient elution technique itself with the aim to optimize it.

Between 1974 and 1981 Jandera and Churachek published a series of papers on Gradient Elution in Liquid Chromatography of which some parts were dedicated to the optimization of this technique (106-108). The optimization is based on equations describing the actual fraction of the stronger eluting component in the mobile phase at the place in the chromatographic column where a particular solute is situated at that moment. These concentration-time functions can be very complicated.

For example Borowko e.a.(109,110) discussed the optimization of stepwise gradient elution based on theoretical considerations published by Jandera and Churachek. During each step an isocratic elution was assumed during which the retention behaviour could be described using well known equations like:

$$\log k' = a + b\Phi$$

When a constant value for the theoretical plate number for all solutes is assumed, the expected chromatographic resolutions can be calculated. Jandera and Churacek described the optimization of a stepwise gradient elution of a mixture of six barbiturates and of a homologous series of alkyl-methyluracils in an eluent consisting of water and methanol (106). They make the remark that this kind of gradient elution is meant as a "tailor-made" technique for given particular separation problems and that the most commonly used gradient technique is the one with a continously changing modifier concentration.

In 1979 Snyder e.a.(111) and Dolan e.a.(112) published two papers on gradient elution in HPLC in which they state that Linear Solvent Strength (LSS) gradient elution is the optimal way of using the gradient elution technique in LSC as well as in reversed phase HPLC. They give several equations that can be used for the calculation of the optimal gradient steepness in varying experimental circumstances. It is shown that the later eluting bands are hardly affected by the choice of the initial modifier concentration, as long as this concentration is small compared to the modifier concentration present at the inlet of the column at the time the later eluted compounds are actually eluted. So the initial modifier concentration can be maximized, and at the same time the analysis time minimized, on the condition that the resolution of the early eluting compounds is maintained.

The steepness of the gradient is not really a suitable parameter for optimization purposes, because it is shown that for all solutes under consideration the optimal values for Φ' lie in the same range (0.05-0.2, i.e. 5%-20% per minute). The optimization, or fine tuning, of a gradient elution separation should, according to Dolan and Snyder, be done in the following way:

- increase the initial modifier concentration in order to minimize analysis time
- vary the gradient-steepness to achieve a better resolution
- decrease the flow-rate when the resolution is still not sufficient
- change the organic modifier when the selectivity is too low

Jandera and Churachek do not completely agree with Snyder e.a. and therefore they propose another optimization approach wherein three free to vary parameters are used: the initial modifier concentration, the steepness of the gradient and the shape of the gradient. However, in practice the shape of the gradient profile is often chosen to be linear. Taking this into account the two approaches are almost identical.

Characteristic for both approaches is that rather significant simplifications of the theory are necessary to come to a general

approach for the optimization of reversed phase gradient elution chromatography. The two major simplifications are:

- assume that the platenumber of the given column is independent of mobile phase composition and solute.
- assume that the selectivity, α, is not influenced by the composition of the mobile phase during isocratic elution.

Another striking point is that the authors, especially Jandera and Churachek, seem unaware of the possibilities for the simultaneous variation of three interdependent variables offered by experimental optimization techniques. It should be very easy, once the three important experimental parameters are determined, to perform a sequential search for that gradient elution profile which offers the best chromatographic performance for the particular separation problem under consideration. On the contrary the authors argument that the determination of the optimal values of the three parameters at a time is a very complex procedure and that therefore one of the three parameters should be given a fixed value in advance.

In 1981 the fourteenth part (the last one until now) of the series by Jandera and Churachek appeared (113) on the theoretical description of ternary gradient elution in liquid chromatography. Based on the theory developed in earlier parts of the series mathematical descriptions of solute behaviour in ternary gradient elution are postulated.

In 1983 Kirkland and Glajch (114) publish their paper on the systematic optimization of the mobile phase for multisolvent gradient elution liquid chromatography. They distinguish two types of gradient elution: Isoselective Multisolvent Gradient Elution (IMGE) and Selective Multisolvent Gradient Elution (SMGE).

The IMGE-optimization is based on the Kirkland-Snyder-Glajch-approach described in chapter III for isocratic separations. The solvent selectivity triangle used there is enlarged into the third dimension by adding an axis on which the solvent strength is displayed. The result is a so called solvent strength prism wherein all possible gradients can be represented by straight lines,

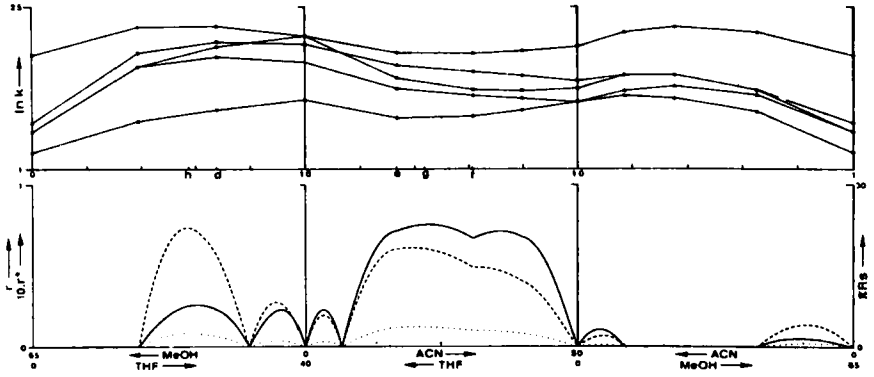

FIGURE IV-1

Experimental design for seven gradient elution runs to obtain basic data for optimization calculations using either the IMGE- or the SMGE-approach.(figure from (114)).

perpendicular on the front (solvent selectivity) triangle (see figure IV-1). So every possible gradient run can be represented by its initial mobile phase composition. Seven gradient runs are performed, from which the retention data are used to determine mathematical relations describing the retention behaviour of the solutes as a function of the initial composition of the mobile phase in the gradient run. Using these relations an ORM-plot can be calculated from which the optimal initial mobile phase composition, and thus the optimal gradient can be found. This kind of gradient optimization may be considered as a combination of the Linear Solvent Strength (LSS)-approach suggested by Dolan and Snyder and the isocratic optimization scheme using Snyders solvent selectivity triangle.

The SMGE-approach to gradient optimization is more general but also more complicated to perform. The exact composition of such a gradient run can be chosen by visual interpretation of the retention data obtained with the seven isoselective gradient runs, mentioned before. This implies that there will not be one exactly determined optimal gradient elution profile, but several profiles that will lead to satisfactory separation.

The authors remark that they feel that the IMGE-approach is suited to solve many separation problems involving mixtures of solutes having wide k'-ranges. The more powerful SMGE-approach wil only be needed for the most difficult separations in which the additional sophistication can be justified.

B. The use of Pilot-Techniques

The reason why experimenters have been searching for a pilot technique for HPLC-optimization is probably that they feel that HPLC is a too complicated technique and furthermore that it is too expensive for trial and error optimization. With the development of systematic optimization methods this reason does not hold anymore. Although, a number of papers have been published on TLC as a pilot-technique for HPLC-gradient elution, and that is not amazing regarding the complex theory for this kind of optimization as described in part A of this chapter.

As Golkiewicz e.a.(115-117) already stated the design of a gradient elution separation can be optimized much better using the simple and quick TLC-technique, when the same theoretical relationships hold for the retention behaviour of the solutes as in HPLC.

Jork e.a.(118) describe a practical example of the optimization of the HPLC-separation of a mixture of toxic compounds used as insecticids, and they discuss thoroughly the advantages and disadvantages of the use of TLC as a pilot technique for HPLC separations.

Other papers on this subject (119,120) use the same prediction technique based on mathematical description of the retention behaviour of the several solutes in isocratic elution.

Because of the simplicity and the relatively low costs of TLC it can be helpful to use it as a pilot technique for isocratic as well as gradient HPLC, however great care should be taken when retention behaviour in TLC is used to predict optimal separation conditions in HPLC. It seems much better to use TLC retention data instead of

the initial gradient run in HPLC optimization in order to determine the feasible part of the factor space in a very easy way, without the need for HPLC equipment capable of gradient formation.

C. Mathematical Resolution of Chromatographic Peaks

Although it could well be a subject for discussion whether mathematical resolution is a kind of optimization of chromatographic separations it is a fact that in the last few years, when computers became more common in HPLC practice, a lot of researchers felt that using mathematical techniques, like the ones described here, makes the optimization of chromatographic resolution superfluous. It might sound very strange in the ears of experienced chromatographists, but it is a fact that chromatographic resolution is not longer necessary to achieve, when mathematical resolution techniques can be used.

The basic step of these methods is the formulation of a peak model, and already at that moment the first problems arise. The ideal Gaussian peak shape is something that is always hoped for, but almost never achieved in practice. Therefore a number of other peakmodels has been described in the literature. In 1981 Lundeen and Juvet (121) published a paper in which they present a brief overview of the techniques and peak models used until then. They mention exponentially modified Gaussian, bi-Gaussian, Poisson, Gram-Charlier and combinations of Gaussian, exponential and hyperbolic tangent functions as peak shapes that have been succesfully described. Once the peak model has been chosen the proposed method involves the solution of a set of non-linear simultaneous equations having the form:

$$R_{Ti} = a_i x^2 + b_i x + c_i y^2 + d_i y + e_i \exp(f_i x^2) + g_i \exp(h_i y^2) + \ldots\ldots$$

This can be achieved by minimizing the sum of squares for the error function. Because approximations are necesarry the solution gained

will not be exactly correct and so a few iteration steps have to be performed. Quantitative determinations using this technique give results with errors in the range from 3% to 16% (for the smallest concentration). D'Allura and Juvet in 1982 (122) report similar work, but their application is the mathematical resolution of HPLC peaks rather than GLC peaks. The authors remark that the proposed method requires that parameters effecting peak position either be very closely controlled or else be accurately measured to make corrections possible.

In 1982 Grimalt e.a.(123) compared several functions of statistical distributions on their usefulness for mathematical resolution.

In 1983 Cela e.a.(124) presented a method which uses the sequential simplex optimization technique to achieve the mathematical resolution of overlapping chromatographic peaks, taking into account a number of different objective functions. So in this case the simplex search is used to find the best possible set of parameters which, by other authors, has been calculated using non-linear mathematical optimization techniques.

Very recently more sophisticated methods have been described using even more complex peak models and very complex mathematical optimization methods (125-127), but it is questionable whether these very special techniques are of any interest for chromatographists.

A very interesting development on the other hand is the use of multivariate data analysis on data sets achieved with the use of multichannel detection techniques coupled to an HPLC apparatus. This technique has been earlier described for GC-MS data (128,129) but in the last few years also LC-MS data and especially LC-UV-diode-array data have become available much easier than before. The data matrix achieved by using diode-array detection consists of rows representing absorbance spectra at given points in the chromatogram and columns representing chromatograms at given wavelengths. Factor analysis (Principal Component Analysis) allows the determination of the total number of peaks present in the overlapping profile. By transformation of the original data matrix

the identification and quantification of the components in the mixture is possible. This technique has been described by several authors (130-132) but it has not been worked out in such a way that the application in routine HPLC analysis is already possible. Because of problems with base line correction, non-negativity restrictions, etc. some research on this subject is still to be done.

Overlooking this approach to the enhancement of resolution in HPLC separations, it seems that no best way to tackle a particular separation problem can be given. Because of the large variety of useful peak models and the numerous mathematical optimization techniques that are available to solve the (non)linear equations there is no best approach. On the other hand, when a multichannel detection system is available the approach using multivariate data analysis is very promising.

V. INSTRUMENTATION FOR OPTIMIZATION METHODS IN HPLC

When the experimental optimization methods became more popular in liquid chromatography, manufacturers of HPLC equipment began to feel the need for instruments that would be able to perform the described optimization techniques. It is of course not surprising that Du-Pont was the first manufacturer to present an HPLC instrument equiped with four solvent capability and a built-in computer. An optimization method, according to the work published by Snyder, Kirkland and Glajch, was incorporated, the system was automated and it was introduced under the name "Sentinel". LDC, also associated with a researcher in the field (Berridge), was the next to introduce an HPLC apparatus capable of unattended optimization of separations. Other manufacturers followed these two predecessors and nowadays also Spectra-Physics, Bruker and Perkin-Elmer have instrumentation and software available for optimization purposes.

To make a comparison between the performances of the several systems possible, a brief description and evaluation of the different methods is given.

- Du-Pont

The Sentinel-system, although not commercially available anymore, is one of the most sophisticated optimization systems ever sold. It is based on the approach described by Snyder, Kirkland and Glajch (see chapter III) and operates in the following way:

- a preliminary gradient run is performed to determine the isocratic solvent strength necessary to achieve a specified k'-range.
- a number of experiments is done to establish binary mixtures of water/methanol, water/acetonitrile and water/tetrahydrofuran with solvent strenghts equal to the one calculated from the gradient run.
- four experiments are done to complete the simplex lattice design over the solvent selectivity triangle (see figure III-4).
- retention data of the seven experiments from the simplex lattice design are used to calculate either ORM-plots, from which an optimal composition of the mobile phase is determined mathematically, or an elution order table, from which the experienced chromatographist can choose himself the expected optimal mobile phase composition.
- verification of the predicted optimal mobile phase composition.

Some remarks can be made on the performance of this optimization system:

- it is assumed that all components present in the sample are known, because peak-identification is performed using standards.
- the number of experiments depends on the number of

components present in the sample under consideration, because all standards and the mixture have to be run at every mobile phase composition.

- the composition of the mobile phase is not truly quaternary but only semi-quaternary (or ternary) because the water content of the mobile phase cannot be varied over the whole range from 0% to 100%. This places a restriction on the optimization of the selectivity of the separation.
- when the ORM-technique is used, mathematical descriptions of retention behaviour as a function of the mobile phase composition have to be calculated.

It is disappointing that this optimization system did not get the opportunity to prove its value to a wide group of chromatographists, because this system had the potential to grow out to be a multifunctional HPLC development system useful for several kinds of method development and optimization.

- LDC/Bruker

The optimization approach chosen by LDC is the one developed by Berridge (see chapter III). It is a sequential simplex search using a response calculated from a quality criterion also described by Berridge (see table III-1). the experimental parameters which can be varied are:

- mobile phase composition (ternary)
- flow-rate
- column temperature
- pH of the mobile phase
- concentration of the ion-pairing reagent in the mobile phase

The quality criterion used is already mentioned in table III-1, and has the following general form:

$$CRF = \sum_{i=1}^{L-1} R_i + L^x + a.ABS(T_a - T_1) + b.(T_1 - T_0)$$

This quality criterion gives maximal values of its response when the maximal number of peaks is detected, the sum of the resolutions between pairs of adjacent peaks is as high as possible and when the time constraints are satisfied.
In its latest modification this optimization approach is improved by adding a preliminary gradient run, according to Snyder e.a.(111,112), to be able to restrict the feasible part of the factor space in order to avoid that the simplex search gets stuck on a local optimium (52). Using the results from the gradient run a maximal and a minimal water fraction of the mobile phase can be calculated using the desired T_a and T_0 values. Once this has been done the simplex search is started in the restricted part of the factor space.

Some remarks can be made on the performance of this optimization system:

- a simplex search, even in a restricted part of the factor space, might get stuck on a local optimum, caused by peak cross-over in the chromatogram.
- the response calculated using this quality criterion is influenced by the choice of the weighting factors. This choice assumes a considerable amount of chromatographic knowledge and knowledge about experimental optimization techniques.
- With the incorporation of the gradient run in the optimization procedure the time constraints in the quality criterion become rather useless, because the constraints in the mobile phase composition are chosen in such a way that the k'-values of the solutes lie in a specified region. This has been noticed by Berridge e.a.(52) in their latest paper.

The reason why the LDC-approach and the Bruker-approach are treated simultaneously is that they are completely identical, although the hardware-components are of course different. While LDC uses high-pressure mixing of the solvents in the mobile phase, Bruker uses, like most other manufacturers of optimization systems, low-pressure mixing of the eluent.

- Spectra-Physics

The optimization procedure called "Optim I", presented by this manufacturer in 1983, is based on a univariate search, which is not the most intelligent strategy possible. The response for the search algorithm is calculated using a similar criterion as the one used by LDC/Bruker, based on resolution, number of peaks and desired analysis time. Variable experimental parameters are the fractions of the modifiers in the ternary mobile phase and, using the gradient mode, the steepness of the gradient profile.
The procedure proceeds in the following way:

- an "optimal" binary composition of the mobile phase with the first chosen modifier has to be found using a simple sequential univariate search.
- from the best binary composition found a binary mobile phase composition with the second modifier is calculated, using semi-empirical rules according to Schoenmakers (76). The purpose is to calculate a binary mixture with the same elution strength as the one found in the first step.
- if the calculated mobile phase composition offers a better response, a univariate search is started to locate the best binary mobile phase composition with the second modifier.
- from the two "optimal" binary mobile phase compositions a ternary composition offering the same elution strength is determined (usually a 50/50 mixture of the two binary mobile phase compositions). If this ternary mobile phase composition offers a better response it is assumed to be the optimum, otherwise the binary composition offering the best response is chosen to be the optimum.

The system is also able to perform a binary or ternary gradient optimization, based on the same optimization strategy.
First the best binary initial composition is determined, then the "optimal" slope of the linear gradient has to be found. If a ternary gradient is desired a binary gradient using the second modifier is calculated in such a way that its elution performance

is comparable to the first gradient. Then the ternary gradient may be calculated from the two "optimal" binary gradients using the empirical rules mentioned before.

Some remarks can be made on the performance of this optimization system.

- the univariate search used in this approach posesses the same disadvantage as a simplex search and that is the possibility of getting stuck on a local optimum.
- by using a univariate method to optimize the mobile phase composition the manufacturer completely denies the existence of selectivity effects, as described in almost all papers on this subject. From the experimental optimization point of view a univariate search is useless when the effects of the experimental parameters are not independent, which is the case here.
- the ternary mobile phase optimization is in fact only semi-ternary (or binary), because the choice of the optimal ternary mobile phase is based on the retention behaviour of the solutes in binary mobile phases only.
- The binary gradient optimization is more promising because no large selectivity effects are to be expected. However, when the step to a ternary gradient is made the remarks on selectivity mentioned before hold.

Very recently an updated version of "Optim I", called "Optim II", became available. A brief study of the performance of "Optim II" did not reveal many improvements, except the way of calculating the "optimal" ternary isocratic mobile phase composition (or ternary gradient profile), which is now performed in a mathematical way. The response of the quality criterion is fitted, using a quadratic equation, along the straight line connecting the two "optimal" binary mobile phase compositions (or binary gradient profiles). The maximum of this quadratic function is assumed to be the optimum. However, the remark on the denying of selectivity effects still holds.

- Perkin-Elmer

In 1984 Perkin-Elmer introduced their optimization system "PESOS". This system is based on a solvent triangle, which is scanned in a brute force way. The response in each point can be represented by the minimal resolution in the chromatogram, the minimal peak-separation (see table III-1) or other quality criteria based on the worst separated pair of peaks in a chromatogram.
The distance between two measured points in the solvent triangle can be chosen in the range from 1% to 10%, however it should be noticed that a choice of 1%-steps over the whole triangle means a number of 5000 experiments that have to be done. The number of experiments can be reduced by scanning only the feasible part of the solvent triangle, which has to be determined first (by a preliminary gradient run for instance), using steps of 5% to 10%. The user can choose from four solvents, and the prior information necessary is restricted to the maximal analysis time and the step-width.
To reduce the time necessary for one optimization run the choice for High Speed LC is almost inevitable and even in that case the time needed will be close to 24 hours (in the case the step-width is chosen to be 10%).
The critical resolution map as well as all chromatograms recorded during the run can be inspected afterwards to allow the chromatographist to control the system.
Some remarks can be made on the performance of this optimization system.

- the method used is a so called "brute force" method, which is not the most efficient way of locating an optimum.
- to determine the critical resolution map it is necessary to know the number of components present in the sample, because the detection of less than the maximum number of peaks leads to a minimal resolution of zero(0).
- when the minimal resolution criterion is used in combination with a maximal analysis time the real optimum (a desired resolution within a minimal analysis time) will not be found.

- the determination of the chromatographic resolution of strongly overlapping or asymmetric peaks is complicated.

The PESOS (Perkin Elmer Solvent Optimization Software)-system has a very limited usefulness, because the only efficient way of locating the optimal mobile phase composition is the use of High Speed LC, which puts strong demands on the HPLC-equipment used.

Overlooking the optimization systems discussed in this chapter, it seems that the "Sentinel"-system of Du-Pont is the most sophisticated one. Unfortunately this system cannot be purchased anymore because Du-Pont stopped its activities on the instrumental HPLC market in autumn 1984.

For relatively simple separation problems, with no, or almost no, peak cross-over one of the simplex optimization systems might be useful.

If the separation problem is more difficult the "PESOS"-system may be a good choice, however its efficiency is very low.

The Spectra-Physics "Optim I/II" system is surely not the best choice for the optimization of an isocratic ternary mobile phase separation, but it might be a succesful approach to gradient optimization problems.

VI. EVALUATION

Although the vast amount of research on experimental optimization of HPLC separations performed in the last decade and described in this review sometimes makes chromatographists think that the developments in this area of research have reached their limits, it will be obvious that a multi-applicable technique, like HPLC, will never stop to chalenge researchers to solve separation problems in a systematic and efficient way. Especially the new developments, like multi-dimensional HPLC, high-speed LC, super critical fluid LC, post- and pre-column derivatization, trace analysis in enviromental samples, etc... will ask for new strategies and new

techniques for efficient method-development and optimization of separations.

So it is out of the question that within a few years, when the methods described in this rview will be common procedures in most HPLC laboratories, researchers in the field of experimental optimization will have focussed their attention on the optimization of the new techniques mentioned above, using more sophisticated (mathematical) techniques and bigger and faster (micro-)computers than ever before.

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