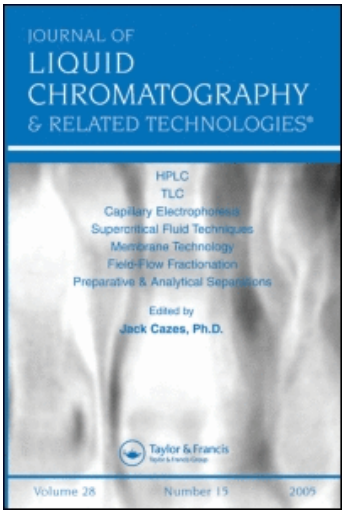

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A REVIEW OF CRITERIA FUNCTIONS AND RESPONSE SURFACE METHODOLOGY FOR THE OPTIMIZATION OF ANALYTICAL SCALE HPLC SEPARATIONS

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A REVIEW OF CRITERIA FUNCTIONS AND RESPONSE SURFACE METHODOLOGY FOR THE OPTIMIZATION OF ANALYTICAL SCALE HPLC SEPARATIONS

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ABSTRACT

Selecting optimum HPLC operating conditions during the development of new analytical separations is difficult due to the high degree of process variable interaction and the lack of robust process models. Traditionally, the methods development strategy in analytical applications involves a trial-and-error grid search method that is both inefficient and costly. Several researchers have investigated more practical and efficient methods for designing optimal HPLC separations at the analytical stage. These strategies typically utilize an efficient design of experiments and response surface optimization techniques. Response or criteria functions are employed to numerically quantify chromatograms and rank them in order of desirability.

A crucial step in the optimization problem is the selection of a proper response function. Several such response functions exist and the choice of a proper function is dependent on the overall goal of the separation at hand. The intent of this review is to present and comment on the strengths and shortcomings of several of the more commonly used criteria functions as well as to illustrate basic response surface optimization strategies and techniques as applied to analytical scale HPLC separations.

INTRODUCTION

Despite the advances that have been made in the field of liquid chromatography, the question of how to optimally develop a chromatographic process for new separations of multi-component mixtures still exists. The selection of optimal HPLC operating conditions is difficult and complex for multi-component mixtures due to the high degree of process variable interaction. Several process variables must be investigated in the optimization problem. Some of these variables, such as mobile phase composition, flow rate, temperature, and pH can be optimized on-line. Other factors such as the selection of chromatographic mode, stationary phase, and column size must be selected *a priori* based on sample composition and the experience of the chromatographer.

In preparative applications where the feed mixture is well identified and it is desirable to purify one or more target compounds, fundamental models may be employed to optimize the system by numerically simulating chromatograms.[1-3] However, these fundamental models, which require knowledge of the composition and physical properties of the feed mixture, are of little use in analytical applications where analysis and quantification of a sample is desired and the chromatographer has little, if any, *a priori* knowledge of the feed mixture composition.

HPLC methods development at the analytical stage is traditionally accomplished through an exhaustive grid search experimental method.[4] Though this method usually results in an acceptable separation of the feed mixture, it is an extremely inefficient trial-and-error process. It is overly expensive due to both the time involved and the potential product wasted in the many experiments that must be performed to find acceptable, though not necessarily optimal, operating conditions.

Several researchers have applied response surface optimization methods to the design of analytical scale HPLC separations. These empirical methods generally utilize a statistical design of experiments, which uniformly samples the experimental domain to generate an experimental test matrix. Response or criteria functions are employed to quantify results numerically and rank chromatograms in order of their desirability. These criteria functions are mapped to response surface models, which are then optimized within the constraints of the experimental domain with respect to the process variables.

Selection of a proper response function is a crucial step in the optimization process. Several response functions exist, and each is designed to quantify the resulting chromatograms based on the overall goal of the separation. For instance, a criteria function which is designed to quantify chromatograms resulting from separations in which the purification of one target compound is

desired do not perform well when used to quantify a separation in which baseline resolution of all solutes in the feed slug is required.

This paper reviews reported case studies in which these empirical approaches to the optimization problem have provided for the optimal design of HPLC separations with a minimum of experimentation. Of particular interest are the multitudes of criteria or response functions that have been designed to quantify chromatograms for the optimization of various systems. This paper contains a compilation of several commonly used criteria functions and a discussion of their strengths and weaknesses in quantifying HPLC separations, as well as detailed discussions on design of experiments and system optimization through response surface modeling. While the majority of the case studies reviewed illustrate the optimization of reversed-phase separations, it is a straightforward task to apply the same techniques to other modes of HPLC including normal-phase and ion-exchange chromatography.

CRITERIA FUNCTIONS AND NUMERICAL RANKING OF CHROMATOGRAMS

In order to rank chromatograms in order of desirability during the optimization process, it is necessary to quantify each chromatogram with a numerical value using a criteria function. Several criteria functions, also known as CRFs (chromatographic response functions), exist. The choice of a proper criteria function is crucial to the outcome of the optimization process. Selection of a criteria function should be made based on the ultimate goal of the separation such that the presence of unfavorable attributes on a chromatogram penalizes its criteria function value. Several commonly used criteria functions are listed as Equations 1-14 in Table 1. Definitions of the symbols present in Equations 1-14 can be found in the nomenclature section of this paper.

Several researchers discuss the use of capacity factors and the theoretical number of plates as optimization criteria in HPLC methods development. The capacity factor and number of plates are based on fundamental chromatographic theory and describe the degree to which each solute of the feed slug is resolved by the chromatography system. The capacity factor, k_i' (Equation 1), for a given solute i is defined as the ratio of the amount of solute i bound to the stationary phase to the amount of solute i in the mobile phase.[5] Lindberg et al.[6] report calculating capacity factors for each of the solutes in a multi-component feed mixture and selecting operating conditions at which these capacity factors are simultaneously maximized for all solutes. Note that capacity factors account only for the retention times of the various solutes and do not include a penalty for excessively wide peaks or for overlapping peaks which are both highly undesirable. Thus, the use of the capacity factor alone does not supply enough information to allow for adequate optimization of the system parameters.

Table 1

Compilation of Chromatographic Criteria Functions

$$k_i' = \frac{t_i - t_o}{t_o} \tag{1}$$

$$N_i = 16\left(\frac{t_i}{w_i}\right)^2 \tag{2}$$

$$R_{i,j} = 2\left(\frac{t_j - t_i}{w_i + w_j}\right) \tag{3}$$

$$R_p = \prod_{i=1}^{np} R_{i,i+1} \tag{4}$$

$$R_s = \sum_{i=1}^{np} R_{i,i+1} \tag{5}$$

$$CRF = \sum_{i=1}^{np} \ln\left(\frac{f_i}{g_i}\right) \tag{6}$$

$$D_1 = 1 + \frac{CRF}{1.2}$$

$$D_2 = \begin{cases} 1 & t \le t_{max} \\ 1 + (t_{max} - t_N) & t > t_{max} \end{cases} \tag{7}$$

$$D = \sqrt{D_1 D_2}$$

$$CRF = \prod_{i=1}^{np} \frac{f_i}{g_i + 2n} \tag{8}$$

$$F_{obj} = \sum_{i=1}^{np} \ln(1 + d_i)$$

$$d_i = \frac{h_{p,i} - h_{v,i}}{h_{v,i}} \tag{9}$$

$$F = \sum_{k=1}^{np} I_k + \frac{c_{max} - c}{c_{max}} \tag{10}$$

$$CRS = \left(\sum_{i=1}^{np}\left(\frac{R_{i,i+1} - R_{opt}}{R_{i,i+1} - R_{min}}\right)^2 \frac{1}{R_{i,i+1}} + \sum_{i=1}^{np} \frac{R_{i,i+1}^2}{np R_{avg}^2}\right)\frac{t_f}{N} \tag{11}$$

$$CEF = \left(\sum_{i=1}^{np}\left(1 - e^{\delta_1 (R_{opt} - R_{i,i+1})}\right)^2 + 1\right)\left(1 + \frac{t_N}{t_{max}}\right) \tag{12}$$

$$CRF = \sum_{i=1}^{np} R_{i,i+1} + \delta_1 N + \delta_2 (t_{max} - t_N) - \delta_3 (t_{min} - t_1) \tag{13}$$

$$COF = \sum_{i=1}^{np} \ln\left(\frac{f_i}{g_i}\right) - \delta_1 (M - N) + \delta_2 (t_{max} - t_N) + \sum_{i=1}^{N} K_i \tag{14}$$

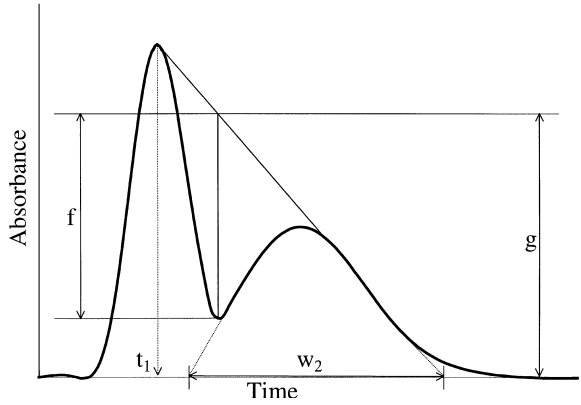

Figure 1. Description of CRF parameters *f*, *g*, *w* and *t*.

The theoretical number of plates for a given solute *i*, N_i, is a measure of column efficiency (Equation 2, w_i defined in Figure 1).[5] Cotton and Down[7] present the use of the theoretical number of plates as a criterion in the optimization of reversed-phase chromatography for the separation of sulindac from related compounds. They focus on maximizing the plate number for sulindac, the target product. In applications such as this example where purification of a single target product is the goal of the separation process, the use of plate number as the optimization criteria results in a sharp peak with a fairly long retention time. Like the capacity factor, however, the plate number is a measure of the systems capability for resolving a single peak and gives no information about surrounding peaks. Implementing the plate number as an optimization criteria frequently leads to unresolved peaks in multi-component systems. We, therefore, discourage the use of plate number as the optimization criteria.

In addition to these fundamentally based optimization criteria, several empirical criteria functions have been developed. Perhaps the most widely used criteria function is the resolution between peak pairs. Examination of Equation 3 shows that the resolution of a given peak pair will be highest when neighboring peaks are well separated (i.e. when retention times are far apart and peaks are relatively narrow). However, the resolution function contains no information about either the number of peaks eluted or the total time required for the separation, though it is desirable to account for both of these factors in the analysis. It is important that the number of peaks eluted somehow be included in the optimization criteria so as to account for co-eluting peaks (unresolved solutes), and it is desirable that the total analysis time be minimized to decrease optimization costs. Nevertheless, due to its simplicity, the resolution function is perhaps the most popular of the empirical criteria functions.

Researchers make use of the resolution function as an optimization criteria in several different ways. Wester et al.[8] report setting a threshold value for an acceptable resolution between peak pairs. They then discard from the optimization scheme operating conditions that lead to chromatograms in which any peak pair exhibits a resolution below this threshold. In this manner, they successfully optimize the composition and pH of the mobile phase for the reversed-phase separation of seventeen monoamine neurotransmitters.

Jandera and Prokes[9] use the concept of resolution mapping to optimize a ternary mobile phase gradient for the reversed-phase separation of phenylurea pesticides. In resolution mapping, the resolutions of each peak pair are plotted as a function of the optimization variables. An appropriate optimum set of conditions is found by manually selecting values of the variables at which each peak pair of interest exhibits an acceptable resolution value.

Another application of the resolution criteria involves maximizing the resolution between a given target peak and its immediate neighbors. This method is used when complete separation of only one of the components in the feed mixture is desired and frequently leads to the overlapping of other peak pairs. Dimov and Simeonov[10] report optimizing the separation of insulin from a complex feed mixture in reversed-phase liquid chromatography using this technique. Lundell and Markides[11] also employ this use of the resolution criteria to separate key peptides from complex mixtures using reversed-phase liquid chromatography.

Several researchers report maximizing the resolution of the least separated peak pair as the optimization objective. Guillaume and Guinchard[12] employ this technique to successfully optimize the mobile phase composition, mobile phase flow rate, and column temperature in reversed-phase liquid chromatography for the separation of a complex feed mixture. Bourguignon et al.[4] use this criteria to optimize mobile phase pH and organic modifier content for the separation of chlorophenols.

In addition, Wang et al.[13] report maximizing the minimum resolution to optimize initial solvent strength and gradient time in gradient elution liquid chromatography for the separation of common pesticides. Hu and Massart[14] also describe the use of this criterion for the optimization of a multi-component mixture containing paracetamol, acetylsalicylic acid, caffeine, benzocaine, carbamazepine, and propyphenazone using reversed-phase HPLC.

It should be noted that the resolution function itself accounts only for the separation of a given pair of neighboring peaks. In order to be useful as an overall criteria function for analytical separations in which baseline separation of all solutes is desirable, the resolutions of each peak pair in the chromatogram must somehow be combined to give an overall resolution value for the entire

chromatogram. This overall resolution can then be compared with the overall resolutions of chromatograms run at different operating conditions during the optimization process.

One way in which the resolutions may be combined is by forming a resolution product as in Equation 4. Wang et al.[15] report the successful use of the resolution product in optimizing the mobile phase pH and ion concentration in reversed-phase liquid chromatography. They conclude that the resolution product is useful for obtaining peaks which are evenly distributed over the chromatogram, but note that a serious drawback of using the resolution product as an optimization criteria is the loss of individual peak information.

Another drawback of combining the individual resolutions into a resolution product should be noted. In cases in which peaks are co-eluting in one experiment and marginally separated in another, the resolution product will be higher for the chromatogram in which the peaks co-eluted if the resolution for the marginally separated peak pair is less than unity. This is in contrast to the fact that partially overlapping peaks are more desirable than completely overlapping peaks in most applications. Adding the individual resolutions to form a resolution sum (Equation 5) rectifies this inconsistency.

Recently, more advanced empirical criteria functions have been developed to address the shortcomings of the standard resolution function. Palasota et al.[16] discuss the use of a CRF (chromatographic response function, Equation 6, f_i and g_i defined in Figure 1) in the optimization of the mobile phase composition for the separation of five neutral organic solutes. The logarithm of the separation measurement is employed so that the CRF will be more sensitive to highly overlapped peaks than to those that exhibit better separation.

In addition, Palasota et al.[16] introduce the use of desirability functions to combine the use of this CRF with a penalty for longer-than-desirable separation times (Equation 7). As can be seen, D_1 is maximized when the CRF is maximized, or when optimum separation of the feed mixture occurs. D_2 is maximized when the total analysis time is minimized. These functions are then combined to yield the overall desirability function, D, which becomes the optimization criteria function. Thus, it can be seen that any set of several different chromatographic criteria functions can be combined in a similar manner to yield an overall performance function suited for a given optimization task.

Djordjevic et al.[17] report using a CRF of the form found in Equation 8 where n is a measure of baseline noise. By accounting for baseline noise this statistic provides a more accurate analysis of the actual degree of peak separation than does its counterpart found in Equation 6. Although this CRF contains only information on separation between adjacent peak pairs, Djordjevic found this CRF useful for the optimization of the mobile phase in reversed-phase

chromatography. In addition, Guillaume and Guinchard[18] discuss yet another variation of the chromatographic response function (CRF). They report the use of a CRF of the form found in Equation 9 for the optimization of the mobile phase in the reversed-phase separation of benzodiazepines. It is desirable to maximize the objective function (F_{obj}) of Equation 9. As can be seen, this form of the CRF takes into account peak separation but fails to account for total separation time and the number of peaks eluted, which may be important parameters in the optimization problem.

Hatrik et al.[19] report the use of a threshold criteria function in the optimization of a binary mobile phase for the separation of six phenylurea herbicides using reversed-phase chromatography. In this threshold criteria (Equation 10), I_k is a Boolean expression and equals one if the degree of separation between peaks k and $k+1$ is above the set threshold value. This criteria function accounts for peak separation, as well as the cost of experimentation, which may become important if several experiments are run in the course of the optimization.

Lukulay and McGuffin[20] discuss the use of a modified chromatographic resolution statistic (CRS, Equation 11) for the simultaneous optimization of mobile phase composition and temperature in reversed phase liquid chromatography. The first term of the CRS is a measure of the extent of separation between adjacent peak pairs and approaches zero when all peaks approach the defined optimum resolution value. The second term of the CRS measures the uniformity of the spacing between peaks and approaches unity when sum of the individual peak pair resolutions is equal to the average value. Finally, the last term of the CRS accounts for total analysis time. Minimization of the CRS leads to peaks that are well resolved and uniformly spaced on the chromatogram.

Furthermore, Morris et al.[21] discuss the design of a new criteria function known as the chromatographic exponential function (CEF, Equation 12) which deals with the shortcomings of the CRS. The CEF improves upon the CRS by decreasing the sensitivity of the CRS to peak pairs that exhibit greater than the desired resolution and by placing minimal emphasis on overall analysis time unless the maximum acceptable time for the separation is exceeded. The CEF, like the CRS, is minimized during the optimization. Though Morris et al. illustrate the use of the CEF through the optimization of a gas chromatography separation, it is straightforward to implement the CEF as a valid criteria function for HPLC separations as well.

Nyiredy et al.[22] report the use of a chromatographic response function (CRF, Equation 13) to optimize the mobile phase for the reversed-phase separation of furocoumarin isomers. The first term in Equation 13 accounts for the resolution between peak pairs, with resolution given by Equation 3. The sec-

ond term assigns a bonus to chromatograms which exhibit more peaks than others, since co-eluting peaks are undesirable. The final terms assign a penalty if the elution time of any peak on the chromatogram is longer or shorter than the user-defined desirable maximum and minimum elution times. It is desirable that each of the individual terms, and thus the CRF itself, be maximized during the optimization. By accounting for the number of peaks and the total analysis time, the CRF of Equation 13 contains much more information than does the standard resolution function.

Klein and Rivera[23] describe the implementation of a chromatographic optimization function (COF) that was developed for use in the optimization of protein separations using ion-exchange chromatography (Equation 14). The first term of the COF accounts for peak separation and penalizes peak pairs that do not exhibit baseline resolution. The second term accounts for the number of peaks eluted in a given chromatographic run so that those chromatograms exhibiting fewer peaks than others are penalized. The third term of the COF accounts for total analysis time, which it is desirable to minimize. Finally, the fourth term of Equation 14 accounts for peak shape. A vector quantizing neural network is employed as a pattern recognition tool to automatically classify peaks based on peak shape. Classification results are used to penalize undesirable peak geometries in the COF and force the system towards conditions which produce symmetrical peaks during the optimization.[24]

The response criteria discussed above represent some of the more frequently used criteria functions in chromatographic optimizations. The choice of a criteria function should be made carefully, and should be based on the ultimate goal of the separation at hand. It is our opinion that neither the capacity factor nor the plate number is a good indicator of product purification and that neither should be applied as an optimization criteria. If the goal of a given separation is to purify one key product, the minimum resolution technique is an adequate choice. However, for more complicated separations in which baseline resolution is desired for all solutes, the total number of solutes in the feed slug is unknown, and/or where analysis time is a key factor, the chromatographer should select one of the more sophisticated chromatographic response functions (CRFs) discussed above. The chromatographer should also realize that several different criteria functions could be coupled to form an overall objective function as is illustrated by Palasota et al.[16]

RESPONSE SURFACE MODELING

Response surface modeling is a widely used empirical optimization strategy. In contrast with fundamental modeling techniques, no prior knowledge of the properties of the feed mixture or the chromatographic system is necessary when the response surface methodology is applied to the optimization of HPLC separations. Whereas, with a fundamental model, dozens of experiments may

be necessary to evaluate the thermodynamic and kinetic properties of the given system, the parameters of most response surface models can be determined with very few experiments, thereby significantly lessening development time and optimization costs.

The main drawback of response surface modeling is that instead of predicting actual chromatograms (eluent concentration versus time) these models predict the value of a chosen criteria function for a given set of operating conditions. However, these models have been shown to adequately represent the criteria functions inside of the defined experimental domains, provided a uniform sampling of the domains exists in the design of experiments. Several examples of response surface modeling, as applied to the optimization of HPLC separations, are present in the literature and are summarized here.

Design of Experiments

Due to the reduced number of experiments that are typically used to fit response surface models, it is important that these experiments be selected so that they adequately sample the entire experimental domain. This is usually accomplished through the use of a statistical design of experiments. Several different types of designs are reported in the HPLC optimization literature.

Often, a preliminary factorial design is performed to evaluate the extent of the effect of each potential optimization variable on the separation.[6,8] These initial factorial designs or screening experiments, usually sample only the extreme bounds of each experimental variable. Lindberg et al.[6] discuss the use of a full factorial design which samples the corner points of a cube formed by a three-dimensional representation of the experimental domain for three variables (shown in Figure 2a for two dimensions). Factorial designs of this type result in 2^d points, where d is the number of experimental variables. Full factorial designs are useful for determining which of the process variables have a significant effect on system performance.

While full factorial designs are useful for the determination of optimization parameters, they are not, in general, useful for the determination of response model coefficients due to an absence of information about the interior portion of the experimental domain. More sophisticated experimental designs are employed for this purpose.

The use of a full factorial design with added center points has been reported in the HPLC optimization literature. In this design of experiments, sampling is performed at the bounds and the center point of each variable's range, generating 3^d points (Figure 2b). Thus, for a two-factor design there will be *9* experimental points. Wang et al[15] report using a full factorial design with

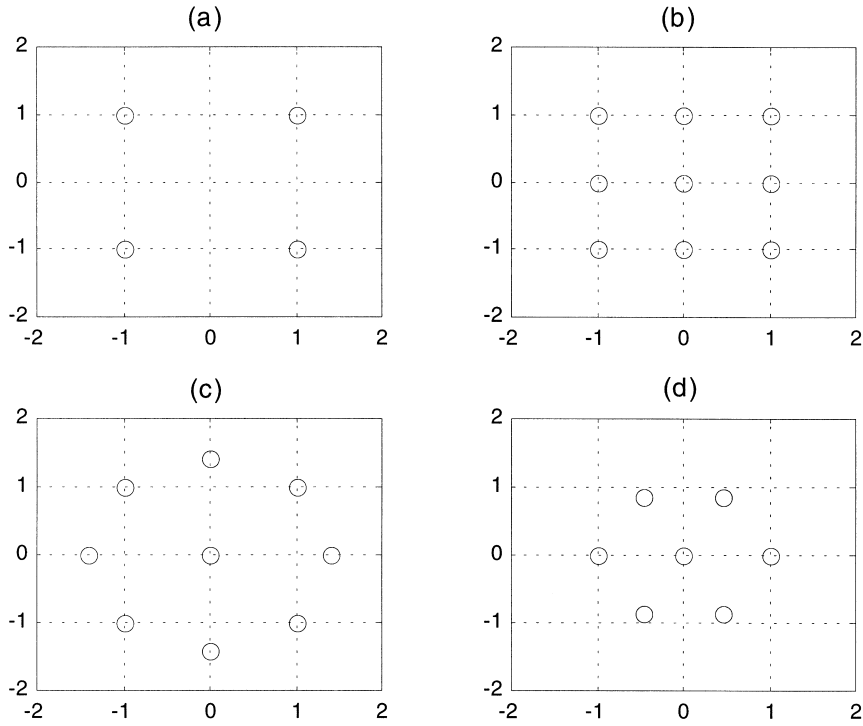

Figure 2. Full factorial design (a), full factorial design with center points (b), central composite design (c), and Doehlert uniform shell design (d) for two variables coded between -1.0 and 1.0.

added center points to design experiments for the optimization of mobile phase pH and ion concentration in reversed-phase chromatography.

Otto and Wegscheider[25] discuss the use of a variation of a full factorial design for the optimization of reversed-phase chromatography. Based on chromatographic theory, it is determined for their system that the main optimization variables in order of importance are the pH, methanol content, and ionic concentration of the mobile phase. Therefore, they select six levels for the pH, three for the methanol content, and two for the ionic concentration as a basis for their factorial design. The resulting experimental matrix thus contains *6 x 3 x 2*, or *36*, experiments. These types of designs which include an excessive number of experiments can be avoided if one of the following, more sophisticated, design of experiments strategies is employed.

The central composite design is an experimental design in which the points lie on the circumference and center of a circle in two dimensions. This type of design generates 2^d+2d+1 experiments. In a central composite design, as in a factorial design, each experimental variable is subjected to the same number of levels. The center point of a central composite design is often repeated so as to estimate experimental error (Figure 2c).[14] Cotton and Down[7] report the use of a central composite design of experiments for the optimization of organic modifier concentration, acetonitrile concentration in the organic modifier, and the buffer concentration in the mobile phase as well as column temperature for the reversed-phase separation of sulindac.

Two more advanced forms of factorial designs are also used to design experiments for the optimization of chromatography separations. The Doehlert design, also known as the uniform shell design, and the modified Box and Benhken design both generate d^2+d+1 points. The main difference between these two designs is that while all variables are subjected to the same number of levels in the Box and Benhken design, the Doehlert design has the added advantage of being multi-level. In a Doehlert design, variables of more importance may be subjected to more variations than other less significant variables are (Figure 2d).[26] These designs contain considerably fewer points than the central composite design, especially when a large number of variables are involved. Thus, a trade-off exists in the design of experiments. More experimental points may lead to a better sampling and a more accurate response model of the system. However, optimization time and costs also increase with the number of experiments, which is undesirable.

Guillaume and Guinchard[12,18] employ the modified Box and Benhken design for the simultaneous optimization of the mobile phase composition, mobile phase flow rate, and column temperature. They successfully optimize the separation of ten benzodiazepines using reversed-phase HPLC.

Bourguignon et al.[4] discuss the use of a Doehlert design in the optimization of mobile phase pH and organic modifier content for the isocratic separation of chlorophenol isomers using reversed-phase HPLC. They find the response model determined through these experiments to be accurate within the experimental domain, and they conclude that the Doehlert shell design is useful for uniformly sampling the experimental domain.

In addition, Hu and Massart[14] compare and contrast several of the factorial design strategies described above. They report finding that while the Doehlert design requires significantly fewer experiments than the central composite design, the response models determined by the Doehlert experiments are as accurate as those generated by the central composite design. Therefore, they conclude that the Doehlert design is the more economical choice. Klein and Rivera[23] also find the Doehlert design to be useful and efficient during the optimization of protein separations using ion-exchange chromatography.

Finally, it should be noted that while each factorial design has advantages and drawbacks, the ultimate choice of an appropriate factorial design also must be somewhat based upon the anticipated form of the response model. If from past experience the chromatographer knows *a priori* that quadratic effects and variable interactions will be statistically significant in the response model, one of the more sophisticated design of experiments should be chosen. For example, a second-order polynomial model complete with quadratic effects and variable interaction has six coefficients that must be determined for a two-factor optimization. A Doehlert design generates seven points in two-dimensional factor space, while a full factorial design generates only four points. Thus, a Doehlert design will provide enough information to confidently fit the coefficients of this model while a full factorial design will not.

Response Model Structures

After choosing an appropriate design of experiments, performing the experimentation, and quantifying the results with an appropriate criteria function, the final steps in the optimization process are to define and optimize an objective function. While some researchers report the use of the previously discussed criteria functions as objective functions which are then optimized by performing additional experiments, some researchers recently discuss the application of numerical optimization techniques which reduce experimental burden.

In order to numerically optimize the system, an appropriate objective function, or response model, must be developed. Once a model exists which maps the criteria functions to the experimental variables, the model can be optimized in the experimental variables using an appropriate optimization algorithm.

Many researchers report success in mapping the resolution criteria and various chromatographic response functions (CRFs) to second-order polynomial models. These models have the following form when two experimental variables are involved.[4]

$$y = b_o + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2 \qquad (15)$$

where y is the value of the criteria function (dependent variable), x_1 and x_2 are the two independent variables, and the b_i's are the model coefficients which are determined through a least-squares regression of the experimental data. Notice that third- and higher-order variable interactions are usually omitted since they have been shown to be statistically unimportant when modeling most criteria functions.[7]

It should also be noted that since higher-order interactions are omitted there still are more experimental points than model coefficients when either the

Doehlert or the central composite factorial designs are employed. Thus, the model coefficients can still be found with sufficient statistical accuracy. If higher-order interactions are included in the model, the central composite and Doehlert designs do not generate enough data to confidently fit the model and additional experimentation becomes necessary.

Bourguignon et al.[4] employ models of the type found in Equation 15 to map values of the minimum resolution to the pH and acetonitrile concentration of the mobile phase in the optimization of a chlorophenol separation. They find that the quadratic response model is sufficient for modeling the minimum resolution and that the addition of higher order terms to the model is not necessary. In addition, Cotton and Down[7] discuss using a second-order polynomial to model capacity factor and peak width responses. In their optimization scheme, these models are employed to predict the capacity factors and peak widths for each peak in the chromatogram at hypothetical experimental conditions. With this information, the theoretical number of plates is calculated using Equation 2 and the minimum plate number is maximized.

In addition, Hu and Massart[14] investigate the efficiency of several factorial design strategies combined with several possible response models, where efficiency is defined as the number of coefficients in the response model divided by the number of experimental points in the factorial design. They report finding that the Doehlert design is the most efficient factorial design for fitting a second-order polynomial model to the solute capacity factors during the optimization of reversed-phase chromatography.

Several other researchers have also reported the use of second-order polynomial response models. Nyiredy et al.[22] discuss the use of a quadratic response model for predicting CRF values as a function of experimental variables for the separation of furocoumarin isomers. Wang et al.[13,15,27] also discuss the usefulness of quadratic response models for a variety of optimization problems.

In cases where full factorial designs are employed to reduce experimental burden, it becomes necessary to drop the quadratic terms in the response model due to the lack of experimental data. Wester et al.[8] employ a full factorial design to generate an experimental matrix in three variables. Since this factorial design strategy results in only eight experiments, it is not possible to confidently fit a model of the form shown above for three variables (Equation 15). Therefore, they neglect the quadratic dependence of the variables but still account for first-order variable interaction with the following model:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3 \qquad (16)$$

Although quadratic dependency has been shown by other researchers to be important when modeling some of the more sophisticated CRFs, Wester et al. report finding Equation 16 to be useful in mapping the solute retention times to the optimization variables of their system.

In addition, Guillaume and Guinchard[12,18] discuss the use of a logarithmic variation of Equation 15 to increase the non-linearity of the response model when modeling the minimum resolution criteria. Their model has the form:

$$\ln(1+R_{min})=b_0 + b_1\ln(x_1)+b_2\ln(x_2)+b_3\ln(x_3)+b_{12}\ln(x_1)\ln(x_2)+b_{13}\ln(x_1)\ln(x_3)$$
$$b_{23}\ln(x_2)\ln(x_3)+b_{22}\ln^2(x_2)+b_{33}\ln^2(x_3) \qquad (17)$$

where R_{min} is the minimum resolution, x_1 is the mobile phase composition, x_2 is the mobile phase flow rate, and x_3 is the column temperature. While it is not obvious from their results whether or not the logarithmic dependencies of Equation 17 provide a substantial increase in model accuracy, they claim to have found it useful in their application.

To summarize, quadratic response models of the type found in Equation 15 are frequently used to map criteria function values to experimental variables with satisfactory results. It has been shown that in most cases third and higher order model terms are statistically unimportant. The chromatographer should be aware of how many model coefficients are going to need to be determined, through either previous experience or the use of screening experiments, before selecting an experimental design. This will ensure that enough data points exist to fit the model coefficients with a high level of statistical confidence.

Optimization Techniques

Having chosen an appropriate model and determined its coefficients through experimentation, the next and final step in the optimization procedure is to optimize the model with respect to the experimental variables so as to optimize the selected criteria function within the experimental domain. This can be done either graphically by plotting the response surface (only practical when two or fewer experimental variables are involved), or numerically by using an appropriate optimization algorithm. While both schemes are valid, numerical optimization has the advantage of being able to be implemented with a computer program, thus allowing for the possible automation of the optimization process.

The most basic and straightforward of the optimization techniques reported in the literature is response surface modeling. In response surface modeling, a plot of the criteria function versus the experimental variables is generated. This is usually accomplished by plotting a model of the form found

in Equation 15. The optimum operating conditions are those that correspond to the optimum value of the criteria function found on the plot. While this method produces good results, it should be noted that this technique can only be applied to optimization problems in which two or fewer factors are involved since it is difficult to visualize fourth- and higher-order surface plots. Furthermore, since this technique involves the chromatographer reading numbers off of a plot, the technique cannot be automated and there is a possibility for the introduction of human error.

Nevertheless, several researchers report the use of this optimization technique. Bourguignon et al.[4] discuss the use of minimum resolution (Equation 3) surface plots to find the optimum pH and organic modifier content of the mobile phase for the reversed-phase separation of a chlorophenol mixture. In addition, Nyiredy et al.[22] report the use of CRF response surfaces in the optimization of the mobile phase composition for the reversed-phase separation of furocoumarin isomers.

Though graphical solutions to the optimization problem are usually applied only in situations where there are two or fewer factors to optimize and the separation can be described by a single criteria function, they can be extended to larger systems. Lindberg et al.[6] describe using topographical plots of the capacity factors (Equation 1) of each of the solutes in the feed mixture. The optimum operating conditions for the reversed-phase separation of opiates are then found by superimposing these plots and finding the conditions at which the capacity factors are simultaneously sufficiently high for each solute. One last example of the use of graphical optimization techniques is described by Bergqvist and Kaufmann.[28] They discuss the determination of optimum conditions for the analysis of triacylglycerol by using surface plots, despite the fact that more than two experimental variables are involved. To accomplish this, they employ linear combinations of the variables to reduce the dimensional order of the surface plots.

Though all of the above researchers report success in using graphical approaches to the optimization problem, the automation of methods development optimizations is becoming increasingly desirable. Since graphical methods cannot easily be translated to a computer algorithm, numerical optimization techniques are necessary. The simplest numerical technique reported in the literature is the computerized grid search. In these studies, criteria values are often fit to response models of the type found in Equation 15. These models are then optimized in a trial-and-error fashion by a computer. A computer program evaluates the model starting with an initial set of operating conditions. It then systematically samples the entire experimental domain and keeps track of the optimum value of the criteria function found along with the corresponding values of the optimization variables. Selecting the step size in a grid search is a key issue. Too large of a step size will lead to a loss of information, while too small of a step size will lead to excessive computational burden.

Several researchers discuss the use of a grid search for the optimization of chromatographic separations. Wester et al.[8] report the use of a grid search to maximize the resolution of the separation of monoamine neurotransmitters. In addition, Otto and Wegscheider[25] discuss the use of a computerized grid search for the optimization of the separation of dipeptides. Djordjevic et al.[17] report using a variation of a computerized grid search. In their work, a computerized grid search is used to find the optimum mobile phase conditions as predicted by a response model. Experiments are then run at these optimum conditions as well as at the conditions corresponding to the point in the experimental domain where the least information was known (i.e., where no previous experiments have been performed). In this manner, the optimization algorithm is forced to find global rather than local optima.

It should be noted that the traditional experimental grid searches for the optimization of HPLC separations are still widely used.[29] Like a computerized grid search, an experimental grid search is a trial-and-error search for the optimum value of the chosen criteria function. However, an experimental grid search does not make use of a response model but requires an actual experiment to be performed at each point of interest. Experimental grid searches lead to excessive experimentation, which should be avoided. Extensive experimentation becomes overly costly due to both the significant number of man-hours involved and to the large amount of potential product which is wasted during these experiments. Nonetheless, experimental grid searches are still used today. Lundell and Markides[11] describe the use of a primitive grid search for the optimization of the mobile phase for the reversed-phase separation of peptides. They report performing twenty-five experiments in the course of this optimization.

Of the more sophisticated numerical optimization techniques that exist, the simplex method is perhaps the most widely used for the optimization of liquid chromatography. The simplex algorithm can be used as either a numerical or an experimental technique. This algorithm makes use of a simplex, a geometric figure having $d+1$ sides in d-space. Thus in two factor space the simplex contains three sides and is a triangle. The three vertices of this triangle represent three different sets of the two experimental factors, or three different sets of operating conditions in the optimization problem. The response model is evaluated (or an experiment is performed) at each of these sets of conditions, the value of the chosen criteria function is determined for each point, and the points are listed in order from most to least desirable.[30] Through a series of logical moves the simplex then "climbs" the response surface in search of the optimum. The main drawback of the simplex algorithm is that it often finds local rather than global optima. Starting the simplex from more than one point in the factor space and checking that the same optimum point is found can circumvent this problem. If the same optimum is found when the algorithm is started from various points in the experimental domain, the optimum can be considered global.[29]

In the past, the simplex technique has been used as an experimental optimization technique. The use of the simplex in this manner requires extensive experimentation, since experiments must be performed at each newly calculated simplex vertex. Palasota et al.[16] describe the use of the simplex technique for the experimental optimization of the mobile phase in reversed-phase liquid chromatography.

More recently, however, the simplex technique has been used numerically to optimize chromatographic response surface models. When used numerically, the value of the model at each simplex vertex is evaluated by direct substitution of the experimental conditions into the response model (or fundamental model) rather than through experimentation. This significantly reduces the number of experiments that must be performed in the optimization procedure.

Guillaume and Guinchard[12] discuss the use of a numerical simplex technique to optimize the flow rate and composition of the mobile phase as well as the column temperature. Wang et al.[15,27] also employ a numerical simplex algorithm, and report that over fifty experiments can be eliminated from the optimization process when the simplex is used numerically rather than as an experimental technique. In addition, Klein and Rivera[23] have investigated the use of a numerical simplex algorithm for the optimization of a quadratic response model during the methods development of ion-exchange protein separations.

In closing, a numerical optimization technique is recommended so that the optimization scheme can be automated. Of the optimization techniques reported on in the HPLC optimization literature, the computerized grid search and the simplex technique seem to be the most popular due to their roots in the experimental optimization of chromatographic systems. While both are highly accurate algorithms, the grid search is much more computationally intensive than is the simplex algorithm. However, the grid search samples the entire experimental domain whereas the simplex algorithm does not. Therefore, if a simplex method is employed, it should be initialized at several different starting points in order to be sure that the global optimum is found.

An Illustrative Example

As an example of the benefits to be gained through optimization of a chromatographic system, consider the following case study in which a ternary protein sample consisting of equal concentrations of lysozyme (L), conalbumin (C), and bovine serum albumin (BSA) was separated using anion-exchange chromatography. The available optimization parameters were the pH of the mobile phase and the slope of the salt gradient. Optimization was performed using a Doehlert design and a numerical simplex optimization algorithm. The COF of Equation 14 was employed as the criteria function, which was modeled

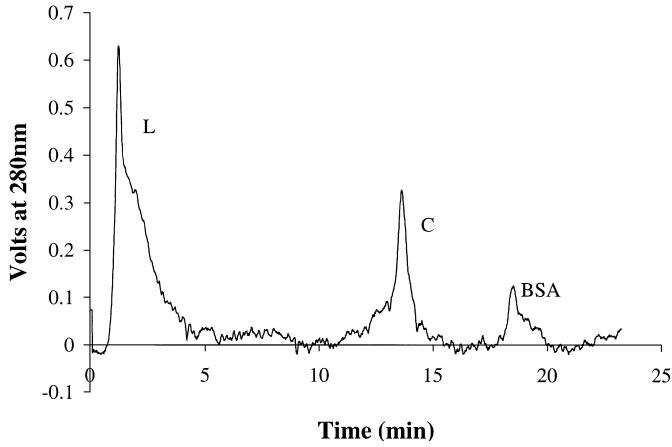

Figure 3. Ternary protein mixture containing equal concentrations of lysozyme (L), conalbumin (C) and bovine serum albumin (BSA) chromatographed at optimum operating conditions.

within the experimental domain using a quadratic response model of the form found in Equation 15.[24]

Figure 3 contains a chromatogram at the optimum conditions, while Figure 4 contains a chromatogram of the same system at non-optimum operating conditions. Note that the non-optimum conditions depicted in Figure 4 lead to a total analysis time of almost twice that of the optimized case. In addition, lysozyme and conalbumin co-elute at the non-optimum conditions which is highly undesirable, as the overall goal of the separation was the baseline resolution of all solutes in the feed mixture. It should be further noted that the chromatogram of Figure 4 gives a much higher value of the resolution function (Equation 3) than does Figure 3, although, it is clear that the chromatogram of Figure 3 is far superior to that of Figure 4. This further illustrates the importance of implementing one of the more detailed criteria functions which is able to account for the total number of peaks eluted and the final analysis time.

SUMMARY

HPLC methods development is a complex optimization problem due to both the lack of a robust process model and the high degree of interaction amongst process variables. Traditionally, HPLC methods development is performed through tedious, inefficient trial-and-error experimental grid search

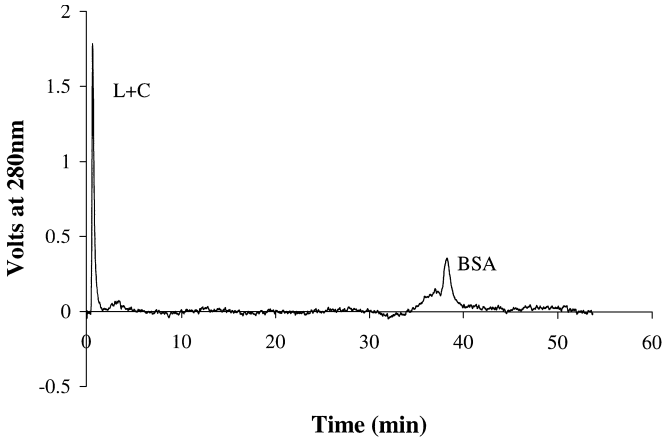

Figure 4. Ternary protein mixture containing equal concentrations of lysozyme (L), conalbumin (C) and bovine serum albumin (BSA) chromatographed at non-optimum conditions. Note the excessive analysis time and co-elution of L and C.

methods. These trial-and-error methods are undesirable due to the excessive amount of experimentation required. This excessive experimentation causes the approach to be overly costly due to increased development time and the large amount of potential product that is wasted while performing the necessary experiments.

A promising alternative to the trial-and-error methods is the use of an empirical approach using a response model. This numerical approach keeps experimentation to a minimum while allowing the optimization algorithm to be computerized. Several researchers have reported the use of various empirical approaches to the optimization problem.

The general empirical approach includes a statistical design of experiments and a criteria function to quantify and rank the results of these experiments in order of desirability. Factorial designs commonly used in the optimization of chromatographic processes include the central composite design, the Box and Benhken design and the Doehlert shell design. Of these designs, we find the Doehlert design to be the most attractive due to its efficiency (number of experiments generated per experimental variable) and the fact that it is multi-level.

By far, the most commonly used criteria function is the resolution criteria. However, this criteria function accounts only for the separation of neighboring

peaks. During the optimization it is desirable to minimize total analysis time and to account for the number of peaks eluted to assure against the presence of co-eluting peaks. More sophisticated chromatographic response functions (CRFs) have been developed to account for this information. The applications of several of these CRFs have been discussed in this review and an example of an optimization in which the powerful COF of Equation 14 outperformed the standard resolution function of Equation 3 has been presented.

Finally, once the experimental matrix has been generated, the experiments have been performed and the results quantified using an appropriate criteria function, a response model must be chosen to map the resulting values of the criteria functions to the experimental variables. This response model then becomes the objective function of the optimization problem. Several researchers have investigated the use of second-order polynomial response models and have found that in most cases these models are sufficient for predicting criteria function values within the optimization domain. Once the coefficients of these models have been found through data regression, the model can be optimized in the experimental variables through the use of an appropriate optimization algorithm.

This review paper contains several examples of applications of the above empirical approach, which suggest that this methodology is valid and consistently produces acceptable results. This empirical approach is far superior to the trial-and-error grid search method in practical applications due to the relatively small number of experiments that are needed to successfully optimize the HPLC separation.

APPENDIX A: NOMENCLATURE

b_i, b_{ij}	response surface model parameters
c	cost of an individual analysis
c_{max}	maximum acceptable analysis cost
CRF	chromatographic response function
d	size of experimental domain
d_i	discrimination factor for peak pair i
D_i	desirability function for peak pair i
f_i	measurement of separation for peak pair i (Figure 1)
F_{obj}	objective function
g_i	measurement of separation for peak pair i (Figure 1)
$h_{p,i}$	height of the shorter of the two peaks in peak pair i
h_{vi}	depth between peaks in peak pair i
I_k	Boolean expression, value of 1 indicates desired separation achieved
K_i	peak geometry penalty for peak i

k_i'	capacity factor for solute *i*
M	number of expected peaks
N	number of actual peaks
N_i	number of theoretical plates for solute *i*
n	measure of baseline noise in the system
np	number of peak pairs on a given chromatogram
$R_{i,j}$	resolution statistic for peaks *i* and *i+1*
R_{avg}	average of peak pair resolutions
R_{min}	minimum acceptable resolution
R_{opt}	optimum or desired resolution
R_p	resolution product
R_s	resolution sum
t_i	retention time of peak *i* (Figure 1)
t_{max}	maximum desired retention time
t_{min}	minimum desired retention time
t_0	void volume retention time
w_i	bandwidth of peak *i* (Figure 1)
x_i	independent optimization variables
y	response value (dependent variable in optimization)
δi	user adjustable weights

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