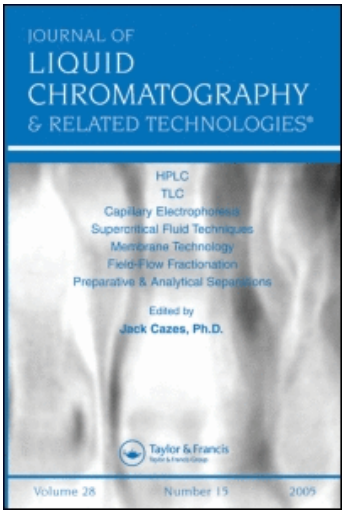

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Thomas E. Wheat[a]; Fadwah M. Chiklis[a]; Kim A. Lilley[a]
[a] ATI Separations Group, The Schrafft Center, Boston, Massachusetts

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METHODS DEVELOPMENT IN CAPILLARY ELECTROPHORESIS WITH AUTOMATED PEAK TRACKING BY CHEMOMETRIC ANALYSIS OF DIODE ARRAY DETECTION DATA

THOMAS E. WHEAT, FADWAH M. CHIKLIS, AND KIM A. LILLEY

ATI Separations Group
The Schrafft Center
529 Main Street
Boston, Massachusetts 02129

ABSTRACT

Developing methods for capillary electrophoresis is complicated by the complex array of interacting variables that affect resolution. Optimizing a separation is, therefore, time-consuming. This process can be minimized and simplified if the individual sample components can be tracked through a minimal structured experimental set. We have adapted an HPLC methods development system, Unicam Diamond Optimization Software, for this purpose. An experimental plane of ten different conditions is executed, and the separations are monitored with a diode array detector. Each three-dimensional data file is subjected to Principal Component Analysis and Iterative Target Transform Factor Analysis to test the spectral homogeneity of each peak and to deconvolute comigrations. The resulting pure spectra are used to track the individual sample components across the experimental plane. In this way, a migration model is calculated for each analyte, and, in turn, a resolution map is constructed. The optimal CE separation is obtained with a minimal number of experiments and without prior knowledge of the sample or the use of standards. This approach is illustrated and tested with over-the-counter pharmaceutical preparations.

INTRODUCTION

The exploitation of the selectivity inherent in electrophoresis as a high resolution technique has been contingent on minimizing dispersion during the separation. Professor

S. Hjertén recognized that this dispersion would be minimized in small diameter tubes and demonstrated this concept in the first capillary electrophoresis system in 1967(1). The extension of this principal to smaller tubes by Virtanen(2) culminated in the use of fused silica capillaries(3) that give extraordinarily high plate counts. This high separation efficiency made it relatively easy to develop separations for a variety of applications, leading to the exponential growth of high performance capillary electrophoresis (HPCE) as an analytical technique. Since high plate counts are so readily achieved, techniques for optimizing chemical selectivity have not been thoroughly explored.

The principles of HPCE separations have been reviewed(4-5). There are many operating parameters that can be manipulated to improve the resolution for a particular sample, including pH, ionic strength, voltage, capillary surface chemistry, etc. The number of variables is even larger when the analytes include both charged and uncharged species that are to be separated by micellar electrokinetic capillary chromatography (MECC)(6-7). The complexities of developing separation methods with so many interacting variables often leads to large numbers of experimental runs. It should be possible to simplify this process by applying some of the principles of automated methods development as applied to HPLC(8).

In both HPLC and HPCE, methods are most commonly developed by either trial-and-error techniques relying on the expertise of the analyst or by a sequential optimization where each result suggests the next experiment. Both approaches are time-consuming and seldom reach true optimum conditions. While the sequential approach can be systematically formalized using Simplex algorithms, it is still sensitive to the conditions selected for the first experiment and prone to identifying local rather than global optima(9). Modeling, or interpretive, methods do not suffer from these disadvantages. In these approaches, a predictive mathematical model of the behavior of each analyte is derived from the observed changes across a limited number of experiments systematically spanning significant variables. These methods, however, are often based on the analysis of standards for each compound so that they can be tracked across the experimental set. The need for standards can be, in part, obviated if spectroscopic data can be used to match peaks among the experiments. Such data can be obtained by monitoring the separations with a diode array detector. For matching, overlapping peaks must be identified and deconvoluted so that pure spectra are available for comparison. A commercially available software package, Unicam Diamond Optimization Software, includes chemometric tools for such spectral analysis. The same system also has algorithms for peak tracking and for calculation of separation models. This software was selected for adaptation to HPCE methods development.

The mathematics underlying this software have been fully documented(10), but they should be briefly described. The migration modeling is based on a triangular, ten experiment plane. The vertices represent any three selected extremes of selectivity variables, *e.g.*, pH, ionic strength, *etc.*, with the seven interstitial points as evenly spaced combinations of these extremes. These ten experiments are monitored with a diode array detector to give the usual three-dimensional data file. Each separation is divided into well-defined peaks or clusters, and the number of analytes in each segment is determined by principal component analysis (PCA) (11,12). All segments are analyzed with Iterative Target Transform Factor Analysis (ITTFA) and overlapping peaks are deconvoluted(13). The result is a set of pure spectra for each experimental separation (14). Each pure spectrum is associated with a time and an amount expressed as the integral volume under the peak across the wavelength range. Each component is then matched across the ten experimental sets based on all three factors (15,16). From the observed time as a function of experimental conditions for each analyte, a model is calculated by a piece-wise quadratic fit (17). The separation models for each analyte are combined into a resolution model and evaluated for quality of resolution(18). In this way, the conditions for optimal resolution are identified in a minimal number of experiments without the use of pure standards or prior knowledge of the sample.

MATERIALS

All separations were performed on an HPCE system including a Crystal 310 Capillary Electrophoresis System, a Crystal 240 Diode Array Detector, Chromascan 3 software, and Diamond Optimization Software(ATI Unicam; Boston, MA).

The capillary was unmodified fused silica, 75μ x 92cm with the window at 60cm (Polymicro Technologies, Phoenix, AZ). Buffers were prepared from the highest commercially available grade salts and 18mΩ water (Barnstead Nanopure, Dubuque, IA). Buffers were prepared by adjusting the pH of a 50mM Borate stock with either phosphoric acid or tribasic sodium phosphate after adding the required amount of sodium dodecyl sulfate as listed below for each experiment. Samples were prepared by mixing commercially available cough and cold syrups to obtain the desired mix of active ingredients as described below.

METHODS

All sample mixtures were diluted with an equal volume of half-strength running buffer for each run condition. Injections were made by positive pressure at 25mbar for

0.25min. All separations were all performed at 25kV. Spectra were collected at one point per second at full (1.3nm) spectral resolution.

RESULTS

Deconvolution

A sample containing acetaminophen, pseudoephedrine, dextromethorphan, pyrilamine, and various excipients was analyzed in 100mM SDS, pH 9.0, as shown in Figure 1. The last peak, near 22min, is of particular interest. It appears as a single, symmetrical peak with the spectrum as shown. Principal component analysis of a pure, or spectrally homogeneous, peak yields two eigenvalues, one for the baseline and one for the analyte(11-13).However, when this peak is subjected to PCA, it has three eigenvalues. The extra eigenvalue indicates the presence of at least one additional component in this peak. With the application of Iterative Target Transform Factor Analysis, two pure components are reconstructed as overlapping peaks with the spectra as shown(Figure 2). These spectra correspond to dextromethorphan and pyrilamine. This application of PCA and ITTFA can, therefore, deconvolute comigrating peaks in HPCE and reconstruct pure spectra of sufficient quality for peak identification during methods development.

Migration Modeling for Methods Development

For the methods development experiments, a sample was prepared containing dextromethorphan, pseudoephedrine, guaifenesin, phenylpropanolamine, and several unidentified inactive ingredients found in the commercial formulations. Since this mixture includes both charged and uncharged analytes, it is best separated by MECC. Many variables can influence the selectivity of such a separation, including pH, detergent type and concentration, ionic strength, organic modifiers, and so on(4,7). This sample was separated over a range of increasing pH with the SDS concentration held constant at 100mM(Figure 3). Under these conditions, the last three peaks show substantial changes in relative migration, but the first three are largely unaffected. In contrast, increasing the SDS concentration from 20 to 107mM at a constant pH of 7.5 alters the selectivity for the first three peaks, but does not affect the last three(Figure 4) This pattern of interacting variables affecting analytes to different extents is appropriate for the experimental design and migration modeling embodied in Diamond software.

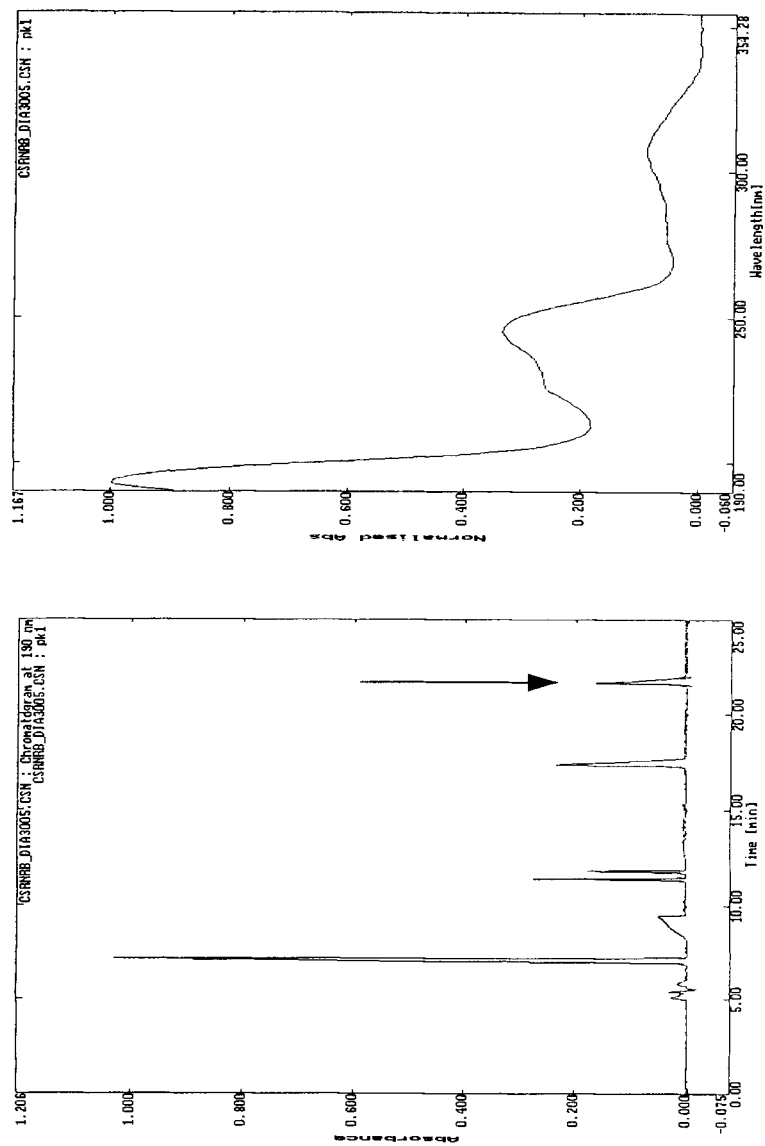


FIGURE 1 Analysis of OTC cough and cold relief formulation by MECC. The left panel shows an electropherogram monitored at 190nm of a sample containing guaifenesin, pseudoephedrine, dextromethorphan, pyrilamine, and excipients. The spectrum of the last peak (arrow) is shown in the right panel. This peak is impure as judged by PCA.

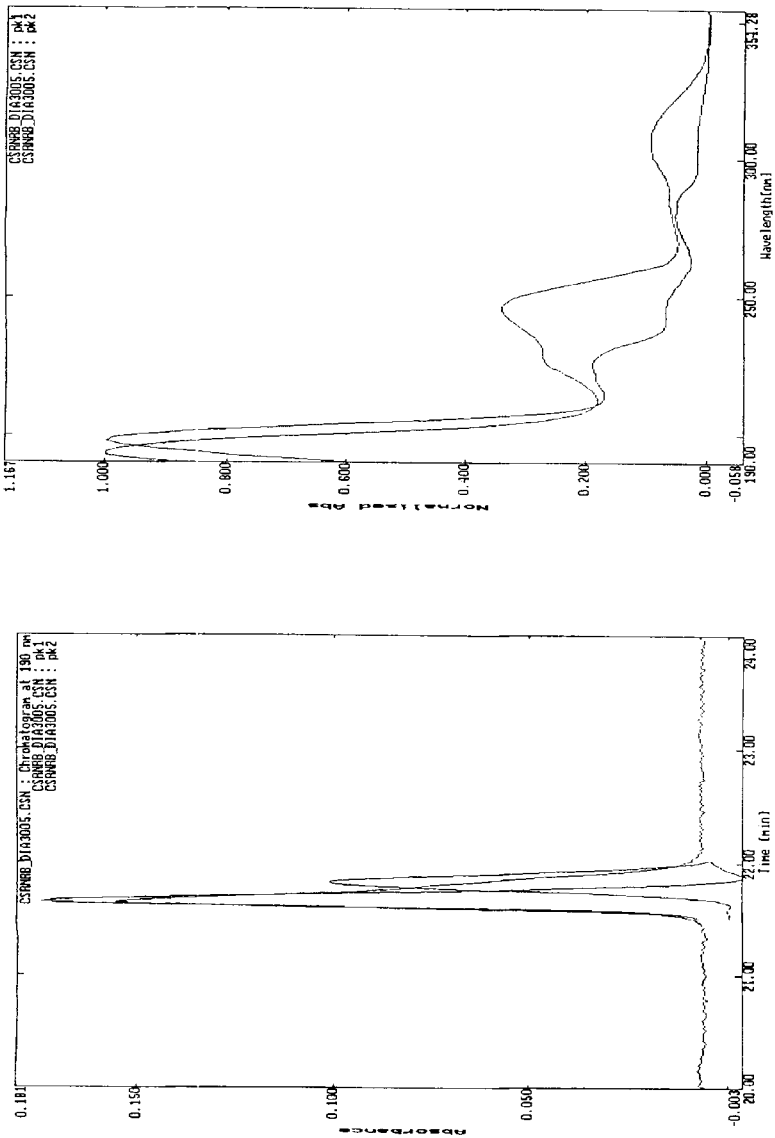


FIGURE 2. Reconstruction with ITTFA. The last peak shown in Figure 1 was deconvoluted with PCA and reconstructed with ITTFA. The two overlapping pure peaks are shown superimposed on the observed single mixed peak in the left panel. The reconstructed pure spectra are shown in the right panel.

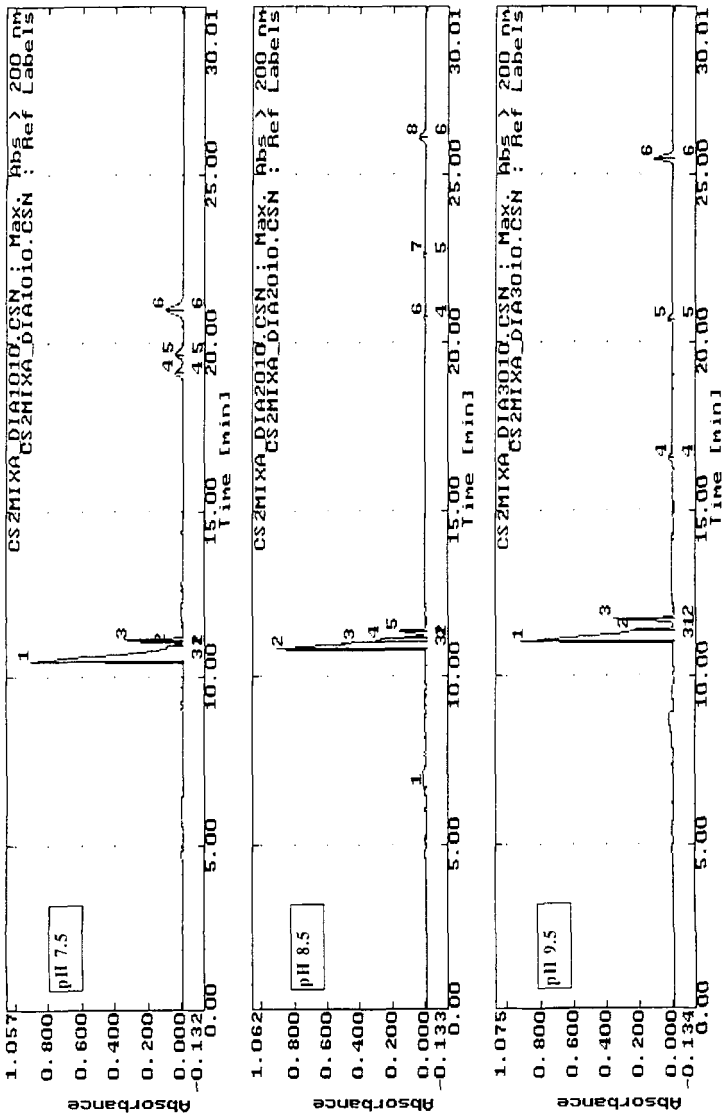


FIGURE 3. Effect of pH on selectivity. A sample containing guaifenesin, pseudoephedrine, phenylpropanolamine, dextromethorphan and excipients was separated in 100mM SDS at pH's of 7.5, 8.5, and 9.5. The peaks in these electropherograms are numbered twice. Numbers above the baseline indicate peaks recognized by the integration algorithm in that particular electropherogram while those below are specific analyte labels common to all three.

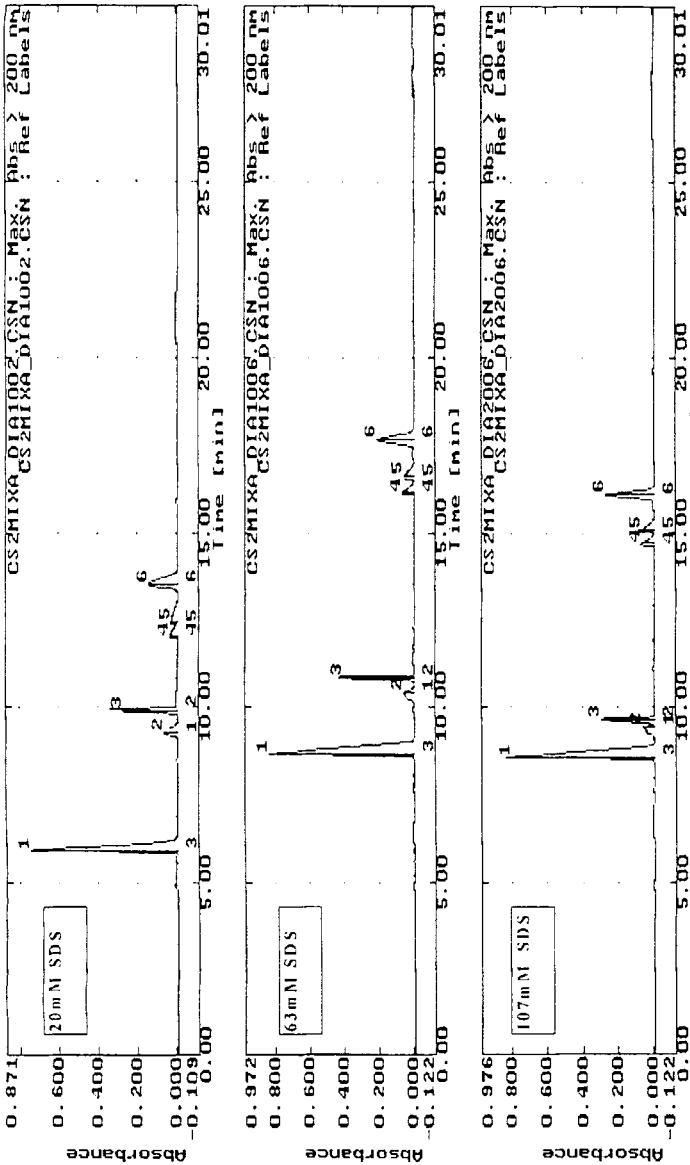


FIGURE 4. Effect of SDS concentration on selectivity. A sample containing guaifenesin, pseudoephedrine, phenylpropanolamine, dextromethorphan and excipients was separated in 20, 67, and 107mM SDS at a pH of 7.5. The peaks in these electropherograms are numbered twice. Numbers above the baseline indicate peaks recognized by the integration algorithm in that particular electropherogram while those below are specific analyte labels common to all three.

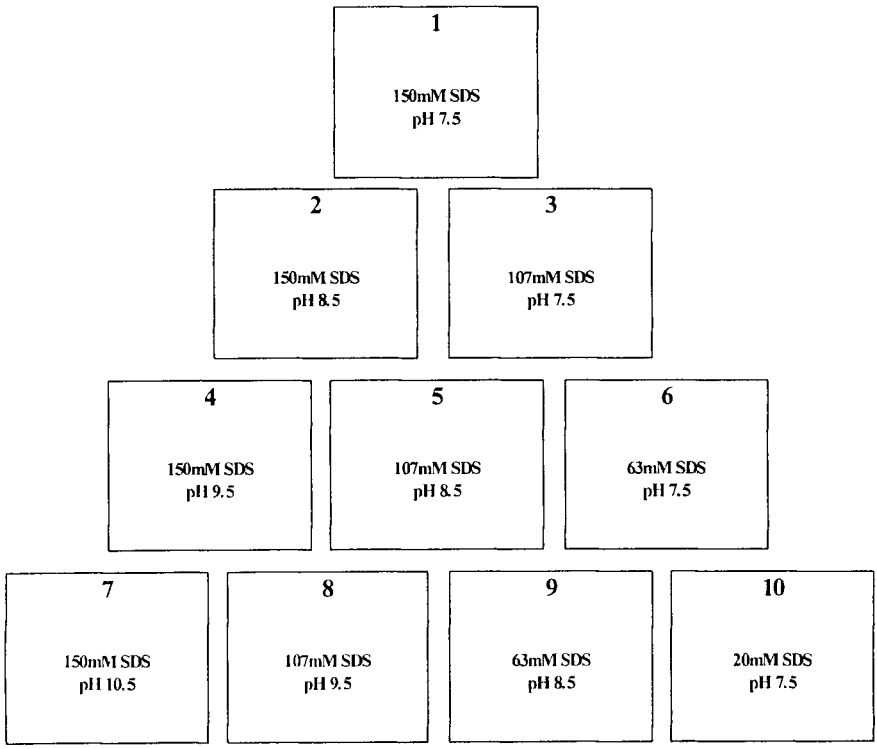


FIGURE 5. Experimental design for methods optimization. The pH and SDS concentrations for the 10 point experimental plane are shown. Each electrolyte was prepared by titrating 50mM borate to the required pH after adjusting the SDS concentration.

An experimental plane was designed to bracket the pH and SDS combinations shown in Figures 3 and 4. The extreme conditions at the vertices define the seven evenly spaced interstitial points (Figure 5). The sample mixture was separated with each electrolyte, and the resulting diode array data files were analyzed with PCA and ITTFA. The result of this process is, for each electropherogram, a table of spectra, migration times, and amounts. These three parameters are used to match the peaks among the several runs of the experimental set(15,16).

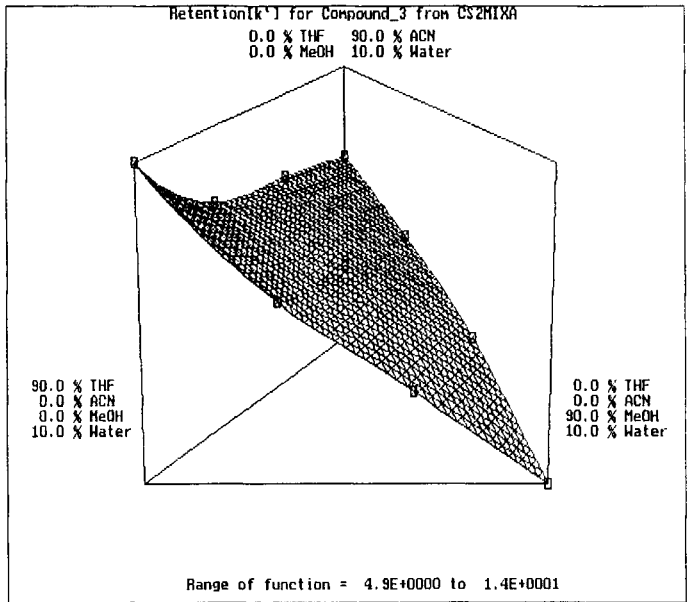
The matching process yields a table showing the migration time of each analyte in each experimental run. It is, therefore, possible to describe the migration behavior of a

given analyte as a function of electrolyte composition. For such a description to be useful, it must accurately predict migration for compositions between the experimental points. First-order linear interpolations are not sufficient so Diamond Software incorporates a piece-wise quadratic fit(17). This function calculates a three-dimensional migration surface for each analyte. Two examples are shown in Figure 6. When examining this figure and those that follow, it must be noted that this software was developed for HPLC, thus the THF, ACN, and MeOH labeling on the printout. In fact, no organic solvents were used. The 90% ACN corner is Experiment 1 in Figure 5. Similarly, 90% THF is Experiment 7 and 90% MeOH is Experiment 10. The plot is designated retention expressed as k'. For these experiments, electrophoretic migration times are equivalent to k' since the calculations are based on a t_0 of 1min. The shapes of these migration surfaces are quite different indicating that substantial changes in separation selectivity are expected over this experimental plane.

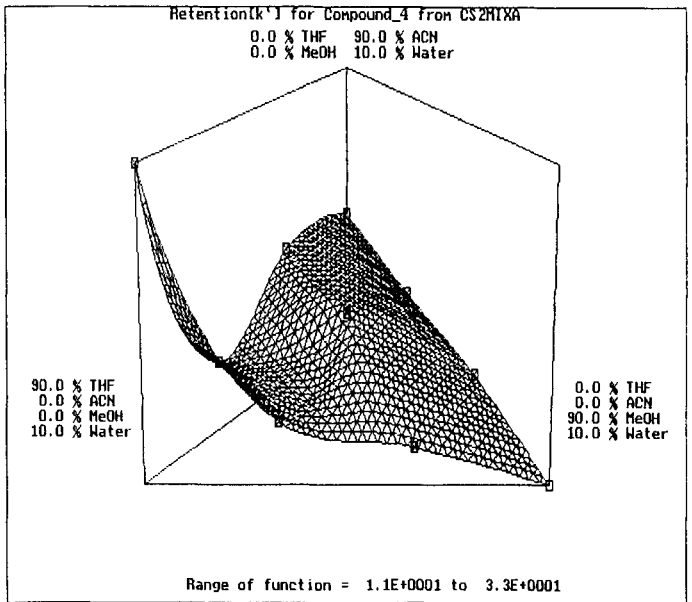
Given that a migration surface has been calculated for each analyte in the mixture, it is straightforward to combine these functions to predict resolution across the experimental plane. This is calculated automatically and expressed as quality of resolution as judged by any of the common evaluation functions(18). The results for this experimental set, judged by the r* or even spacing function, is shown in Figure 7. A clear optimum is apparent. This three-dimensional plot can also be viewed as a contour plot as shown in Figure 8. Note the cursor placed at the resolution maximum. The composition at this point is shown as well as the predicted electropherogram for these conditions. Although the composition is expressed as percentages of organic solvents, it is straightforward to calculate from Figure 5 that the optimal selectivity for this separation is obtained at 150mM SDS, pH 8.98. Since the cursor on the contour plot can be moved with the mouse, it is possible to examine the experimental plane for other useful separation patterns. For example, as shown in Figure 9, separation at higher pH and slightly lower SDS concentration changes the migration order such that Peak 3 is earlier than Peaks 1 and 2. At the optimum, Peak 3 appeared after Peaks 1 and 2. This predicted behavior matches that observed within the experimental set, confirming the accuracy of this approach to migration modeling.

DISCUSSION

These experiments were designed to test the adaptation of an automated HPLC methods development system for use with capillary electrophoresis. The chemometric



Guaifenesin



Phenylpropanolamine

FIGURE 6. Migration Models. Two of the six migration models calculated from the methods development experiments are shown. The surface represents migration time as a function of electrolyte composition. The labeling of the corners as organic solvents results from the original development of this software for HPLC. The actual conditions correspond to those in Figure 5. The analytes associated with these models were identified by testing standards after the method was developed. No pure standards were required to calculate the surfaces.

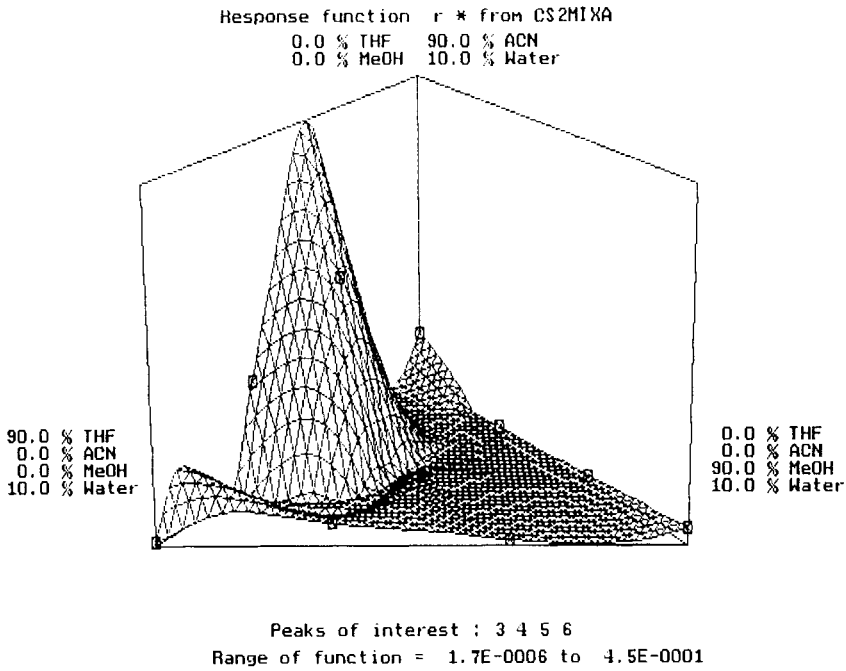


FIGURE 7. Response Function. The response function is a three-dimensional plot reflecting the quality of the separation. Resolution can be calculated in several ways. The function r*, biased toward equal peak spacing (18), is plotted here as a function of electrolyte composition. The labeling of the corners as organic solvents results from the original development of this software for HPLC. The actual conditions correspond to those in Figure 5.

algorithms proved useful in detecting spectral inhomogeneity in a peak, in deconvoluting the comigration, and in reconstructing the pure spectra contributing to the observed peak. The experimental framework was suitable for minimizing the experiments required to optimize selectivity in MECC. The matching algorithms were successful in tracking the individual analytes across the experimental plane without reference to standards. Migration models were calculated, and an accurate resolution map was constructed. These results confirm that the mathematical tools of Diamond Optimization Software can be applied in HPCE. Further, since these algorithms require high quality spectra, the successful deconvolution and peak tracking results confirm the high spectral sensitivity of this diode array detector.

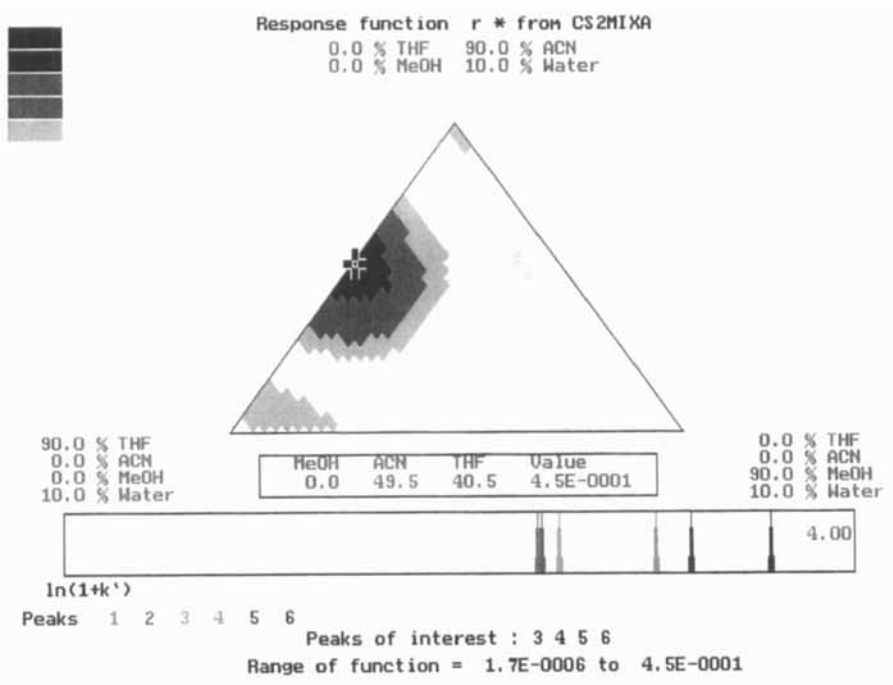


FIGURE 8. Contour plot of response function. The three-dimensional representation of separation quality (Figure 3) is drawn here as a contour plot. The cursor is placed at the point of optimum resolution. The conditions are presented in the frame immediately below the triangle, and the predicted electropherogram is shown in the bottom frame. The labeling of the corners and the optimum conditions as organic solvents results from the original development of this software for HPLC. The actual conditions correspond to those in Figure 5. The actual optimum conditions calculated from the organic percentages are 150mM SDS, pH 8.98.

This software was selected for evaluation for several reasons. It embodies a general approach, interpretive retention modeling, that has proven useful in other separation techniques. It automatically applies PCA and ITTFA to the experimental data to test peak purity and to extract pure spectra. The results are used by peak tracking and migration modeling functions to map resolution without requiring pure standards or prior knowledge of the sample composition. Perhaps most importantly, this process of methods development does not require that the analytes behave in accordance with a well-developed theory of the separation mechanism. It is firmly grounded in an efficient and systematic empirical analysis of raw data.

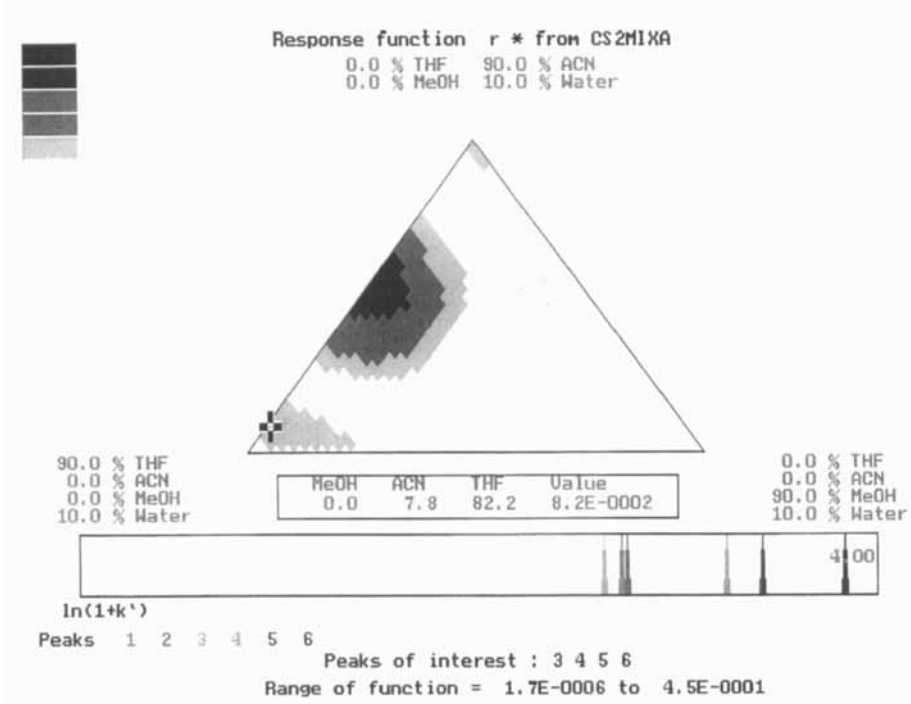


FIGURE 9. Contour plot of response function. The three-dimensional representation of separation quality (Figure 3) is drawn here as a contour plot. The cursor is placed at a point of non-optimum but useful resolution. The conditions are presented in the frame immediately below the triangle, and the predicted electropherogram is shown in the bottom frame. The labeling of the corners and the optimum conditions as organic solvents results from the original development of this software for HPLC. The actual conditions correspond to those in Figure 5. The predicted electropherogram should be compared to that shown for the optimum in Figure 8. Note the change in position of Peak 3 relative to Peaks 1 and 2.

The tools in this software could be used to approach other problems in HPCE. Any variables that affect selectivity can be used as the vertices of the experimental plane. This might include the use of different detergents and mixed micelles in MECC(19) or pH and ionic strength in free-solution capillary electrophoresis(20). The migration and resolution modeling functions should be useful in optimizing chiral separations, although for this application peak tracking cannot be based on spectral discrimination. The chemometric analysis can be used to solve problems outside the realm of methods development. Principal component analysis, iterative target transform factor analysis, and

peak matching can be used to confirm the purity of peaks and their identification in the analysis of complex samples, such as foods and beverages(21) or drugs in biological fluids(20). It may, therefore, be expected that the mathematical approaches embodied in Unicam Diamond Optimization software will be used in a variety of HPCE applications and will serve as a basis for future development of methods development approaches.

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