Optimization of Ion-Exchange Protein Separations Using a Vector Quantizing Neural Network

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In this work, a previously proposed methodology for the optimization of analytical scale protein separations using ion-exchange chromatography is subjected to two challenging case studies. The optimization methodology uses a Doehlert shell design for design of experiments and a novel criteria function to rank chromatograms in order of desirability. This chromatographic optimization function (COF) accounts for the separation between neighboring peaks, the total number of peaks eluted, and total analysis time. The COF is penalized when undesirable peak geometries (i.e., skewed and/or shouldered peaks) are present as determined by a vector quantizing neural network. Results of the COF analysis are fit to a quadratic response model, which is optimized with respect to the optimization variables using an advanced Nelder and Mead simplex algorithm. The optimization methodology is tested on two case study sample mixtures, the first of which is composed of equal parts of lysozyme, conalbumin, bovine serum albumin, and transferrin, and the second of which contains equal parts of conalbumin, bovine serum albumin, tranferrin, β-lactoglobulin, insulin, and α-chymotrypsinogen A. Mobile-phase pH and gradient length are optimized to achieve baseline resolution of all solutes for both case studies in acceptably short analysis times, thus demonstrating the usefulness of the empirical optimization methodology.

Introduction

High performance liquid chromatography (HPLC) is a separation technique commonly used in the pharmaceutical industry for both preparative and analytical scale separations. Optimal operating conditions for analytical scale separations, where analysis and quantification of the feed mixture is desired, result in baseline separation of each solute in the feed mixture in an acceptably short overall analysis time. Baseline separation is desired to maximize product purity and minimize downstream processing costs, while a short overall analysis time leads to increased productivity and decreased methods development time. HPLC methods development is traditionally accomplished through an exhaustive trial-and-error experimental procedure. This is extremely inefficient as a result of lengthy development times and the large amounts of potential product that are wasted in the search for acceptable, though not necessarily optimal, operating conditions.

Ion-exchange chromatography (IEC) is a mode of HPLC operation that is typically used at the clinical and preclinical analytical stages to separate small concentrations of proteins and other bioproducts. In IEC an ionically charged column packing facilitates the fractionation of the feed mixture based on the affinity of the individual solutes for the column packing. Methods development for analytical scale separations is difficult and complex in IEC because of the high degree of interaction among process variables, which can be categorized as either online or a priori variables. A priori variables include parameters such as the chemistry and size of the column packing and the size of the column itself and must be selected on the basis of past chromatographic experiences prior to online optimization. Online process variables such as the composition, pH, ionic strength, and flow rate of the mobile phase and the column temperature can be manipulated and optimized online.

The two most influential online process variables in IEC are the pH and ionic strength of the mobile phase. The pH of the mobile phase affects the formal charge of the protein molecules and thus their affinity for the charged column packing. The ionic strength of the mobile phase is indicative of the concentration of counterions present and controls the elution of bound proteins from the column. Elution is usually performed by introducing a linear salt gradient of increasing ionic strength to the column using a salt such as NaCl (Wankat, 1990). Simultaneous optimization of mobile phase pH and ionic strength is a challenging problem because of the complex interactions between these two process variables and the inherent nonlinearity of the ion-exchange purification of multicomponent protein mixtures.

Recently, success has been reported in predicting chromatograms with theoretical models (Gallant et al.,

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1995; Galushko, 1991; Galushko et al., 1994; Gu, 1995). However, these fundamental models, which require knowledge of the thermodynamic and kinetic properties of the system and the feed mixture, are of little use in analytical applications where the chromatographer has little, if any, a priori knowledge of the feed mixture composition. To accurately predict chromatograms with these models at the analytical stage, it is therefore necessary to estimate adsorption isotherm constants for each individual solute in the feed mixture at each mobile phase composition and ionic strength investigated. This leads to excessive experimentation and causes such an approach to the optimization problem to be costly and inefficient.

In the past, several academic researchers, more recently joined by industrial researchers, have applied empirical optimization methods to reversed-phase chromatographic separations. These empirical methods generally utilize a statistical factorial design, which uniformly samples the experimental domain, to generate an experimental test matrix and a response or criteria function to rank chromatograms in order of desirability (Cotton and Down, 1983; Hu and Massart, 1989; Lindberg et al., 1981; Jandera and Prokes, 1991). Several such criteria functions, also known as chromatographic response functions (CRFs), are reported in the literature, the most popular and basic of these being the resolution statistic, which quantifies the separation of neighboring peak pairs (Klein and Rivera, 2000). Results of criteria function analysis are typically fit to second-order polynomial response models (Bourguignon et al., 1993). Once a criteria function is chosen and the experimental data is regressed to a response model, the model is optimized in the experimental variables to maximize the criteria function. Techniques used previously to maximize the values of these chromatographic response models include computerized grid searches (Cotton and Down, 1983; Lundell and Markides, 1993), response surface modeling (Felinger and Guiochon, 1992; Felinger and Guiochon, 1994; Wang et al., 1991), and the sequential simplex method (Berridge, 1985; Palasota et al., 1992; Wang et al., 1993).

In this work, a previously developed methodology for IEC optimization that is based on the empirical strategy outlined above and requires a minimum of experimentation is reviewed (Klein and Rivera, 1998). The usefulness of the technique is demonstrated through two illustrative case studies. Specifically, the pH and ionic strength (slope of a linear salt gradient) are simultaneously optimized for the separation of two feed mixtures containing four and six proteins commonly found in the pharmaceutical industry, respectively, while requiring as few as seven experiments. As a result of the strictly empirical nature of the methodology, no previous knowledge of the feed mixture is necessary, and the method is applicable to separations in which the feed is partially or totally unknown, which is frequently the case in industrial applications. Furthermore, the optimization methodology is such that the inclusion of additional optimization variables such as mobile-phase flow rate and temperature is a straightforward task.

Optimization Methodology

The complete optimization methodology, which is presented elsewhere in full detail (Klein and Rivera, 1998), is outlined in Figure 1. To begin the optimization all a priori or fixed process variables such as column size and packing are selected on the basis of past chromatographic experiences and/or vendor recommendations. The

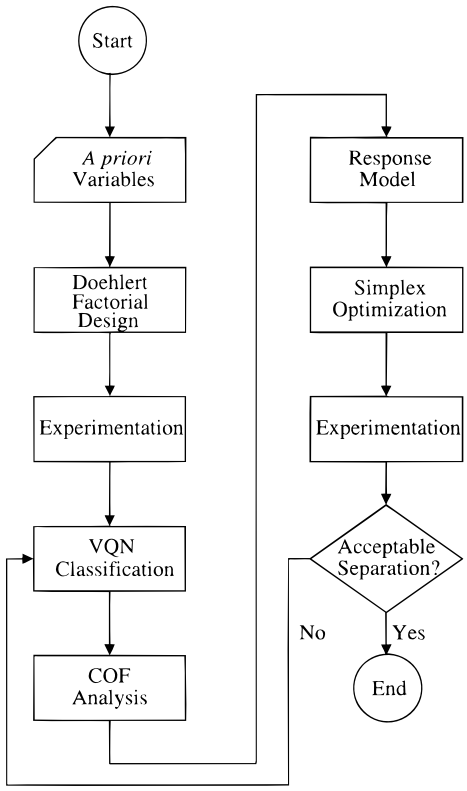

Figure 1. Flowchart of the optimization algorithm.

remaining variables such as mobile-phase composition, flow rate, and temperature are optimized online with the methodology described below. In this study, the online variables investigated are the pH and steepness of the linear ionic concentration gradient in the mobile phase.

The experimental domain is defined by placing bounds on the optimization parameters, and a Doehlert shell design (Doehlert, 1970) is used to generate the experimental matrix. The chromatographic peaks generated by these experimental conditions are classified by a vector quantizing network (VQN) on the basis of peak geometry. The VQN is trained to recognize the desirable class of Gaussian-shaped peaks, as well as peaks that exhibit undesirable tailing or shoulders, which are indicative of coeluting solutes.

To rank chromatograms in order of desirability, it is necessary to assign a numerical value to the result of each experimental separation using a criteria function (Klein and Rivera, 2000). The most commonly used of these response criteria is the resolution function:

$$R_{ij} = \frac{2(t_j - t_i)}{w_i + w_j} \tag{1}$$

where R_{ij} is the resolution between peaks i and j, t_i is the elution time of peak i, and w_i is the baseline bandwidth of peak i (Figure 2).

The individual peak resolutions can be summed to give an overall resolution for the chromatogram. However, the resolution does not account for total analysis time, nor does it include a penalty for chromatograms that exhibit fewer peaks than others. This information is extremely important when dealing with an unknown sample, and it is desirable to incorporate these data in the response function. That is, there must be a way to penalize chromatograms that do not contain as many peaks as other chromatograms in an attempt to attain complete

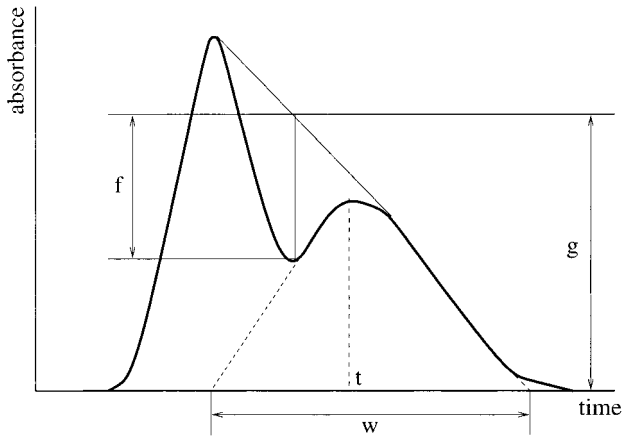

Figure 2. Definition of the resolution criteria (eq 1) and chromatographic optimization function (COF, eq 2) parameters *f*, *g*, *t*, and *w*.

resolution of the feed mixture, even if the number of solutes in the feed mixture is unknown.

The chromatographic optimization function (COF) used in this study has the form

$$\mathrm{COF} = \sum_{i=1}^{n_p}[\ln(f_i/g_i)] - A(M - N) + B(t_m - t) + \sum_{i=1}^{n} K_i \quad (2)$$

where f_i and g_i are parameters used to describe the separation of peak pair i (Figure 2), n_p is the number of peak pairs, M is the number of expected peaks, N is the number of peaks eluted, t_m is the maximum desirable total analysis time, t is the elution time of the last peak, K_i is the penalty based on neural network peak classification, n is the number of peaks, and A and B are user-adjustable weights.

The first term of the COF describes the degree of separation between neighboring peak pairs. The second term accounts for the fact that some chromatograms may contain more or fewer peaks than others and assigns a penalty to chromatograms that contain fewer than the expected number of peaks (i.e., chromatograms where peaks coelute). In cases where the expected number of peaks is unknown, the value of M can be set arbitrarily, thus assigning a penalty that is proportional to the number of peaks exhibited by each chromatogram. The third term accounts for total analysis time, which it is desirable to minimize in the optimization problem. The final term in the COF assesses a penalty based on neural network classification of the peak geometry so that the optimization is forced toward areas of the parameter space that produce Gaussian-shaped peaks.

To numerically optimize the chromatographic optimization function (COF, eq 2) in the experimental parameters it is necessary to represent the experimental results with an empirical model. The COF values resulting from the Doehlert matrix experiments are fit to a second-order polynomial model, which is dependent on both pH and gradient column volumes (CV), using a least-squares approach. The model is of the form

$$y = a_1 + a_2x_1 + a_3x_2 + a_4x_1^2 + a_5x_2^2 + a_6x_1x_2 \quad (3)$$

where y = COF, x_1 = pH, and x_2 = CV.

Finally, the response model is optimized using a constrained, variable-sized, sequential simplex algorithm to find the values of the experimental parameters (pH and slope of the salt gradient) at which the COF is maximum. An additional experiment is performed at these optimal conditions, and the resulting COF is used to update response model parameters. As is depicted in Figure 1, optimization continues in this iterative manner until the chromatographer is satisfied with the resulting separation.

It should be noted that it is straightforward to expand the optimization methodology to allow the optimization of additional process variables by adding appropriate terms to the response model of eq 3 and increasing the dimensionality of the Doehlert design. Moreover, this methodology is sufficiently generic that it can be applied to all modes of HPLC operation, including normal- and reversed-phase HPLC. In addition, the empirical nature of the methodology is such that no a priori knowledge of the sample mixture is required.

Experimental Method

Equipment. The chromatographic station consists of two Waters 510 HPLC pumps (Waters; Milford, MA), a Waters 590 programmable HPLC pump, a Waters automatic gradient controller, a Waters 440 UV detector operating at 280 nm, and a Waters 746 data module/integrator. The signal from the UV detector is also logged to a data file on a Gateway 2000 486 PC using PC-Lab Card's PCL-812PG data acquisition card (Advantech Co., LTD; Sunnyvale, CA) and VisSim software (Visual Solutions; Westford, MA). The column employed is a weak anion exchanger Toyopearl column with DEAE (diethylaminoethyl) chemistry (Supelco; Bellefonte, PA).

Mobile-Phase Preparation. The mobile phase consists of two buffers that are mixed to the desired mobile-phase ionic strength by controlling the flow rates of their respective pumps. Buffer A contains 10 mM tris[hydroxymethyl]aminomethane (Tris) and 10 mM 1,3-bis-[tris(hydroxymethyl)methyl-amino]propane (Bis) (Sigma, Ltd., St. Louis, MO). Buffer B contains 10 mM Tris, 10 mM Bis, and 1 M NaCl. Buffer pH is adjusted off-line to the desired pH of each run using 1 M HCl and 1 M NaOH. The initial and final concentrations of the salt gradient in all experiments are 0.0 and 0.5 M, respectively. Each experiment is followed by a 100% buffer B (1 M NaCl) wash to clean any remaining solutes from the column. All buffers are dissolved in deionized water, filtered through a 40 μm Millipore filter, (Bedford, MA) and degassed with helium before use. Mobile-phase flow rate is 1.5 mL/min.

Sample Preparation. The proteins included in the feed mixtures are bovine serum albumin (BSA), lysozyme (L), conalbumin (C), α-chymotrypsinogen A (A), transferrin (T), insulin (I), and β-lactoglobulin (β-L) (Sigma, St. Louis, MO). All proteins are dissolved in deionized water to concentrations of 6 mg/mL. Equal volumes of each standard protein solution are combined to give the final feed mixtures, yielding protein concentrations of 1.5 mg/mL and 1 mg/mL for the four- and six-protein mixtures, respectively. Sample size is 25 μL for all experiments.

Results and Discussion

The experimental domain is defined by placing constraints on the optimization variables such that the pH is bound between 6 and 9 and the salt gradient length is bound between 5 and 20 column volumes (CV). The limits on pH are imposed because of concerns of protein denaturation at extremely acidic or basic conditions. The upper limit on gradient length is set as a result of

Table 1. Doehlert Design Experimental Matrix

	experiment						
	1	2	3	4	5	6	7
pH	7.5	9.0	8.2	6.0	6.8	6.8	8.2
CV	12.5	12.5	19.0	12.5	6.0	19.0	6.0

Table 2. Experimental Results vs Model Predictions for Case Study 1

			COF		$\sum R_{ij}$	
expt	pH	CV	data	model	data	model
1	7.5	12.5	−1.67	−1.07	11.20	11.27
2	9.0	12.5	−2.95	−2.72	2.73	4.82
3	8.2	19.0	−0.91	−1.42	13.05	10.99
4	6.0	12.5	−1.18	−1.80	4.49	2.32
5	6.8	6.0	−0.34	−0.97	5.24	7.37
6	6.8	19.0	−1.19	−0.74	6.63	8.68
7	8.2	6.0	−1.05	−1.21	10.02	7.89
8	6.0	5.0	−2.24	−1.60	n/a	n/a

analysis time considerations, and the lower limit is set to ensure that a linear gradient rather than a step change in ionic strength is employed. On the basis of these imposed constraints, the Doehlert shell design is employed to generate the experimental matrix shown in Table 1.

The maximum desired analysis time, t_m, of the COF (eq 2) is chosen to be 20% of the full range of the constraints imposed on the length of the salt gradient (8 CV). The adjustable weights, A and B, are set at 2 and 0.1, respectively, for case study 1 and 3 and 0.1, respectively, for case study 2 so that each term in the chromatogram is of the same order of magnitude, namely, that of unity. The higher value of A is required for case study 2, which involves a six-protein mixture, because the first term in the COF (eq 2) becomes largely negative as a result of the large number of peaks in the summation. This higher value of A allows for chromatograms exhibiting more peaks, even though partially overlapping, to be ranked higher than chromatograms containing fewer peaks, which indicates coelution and is highly undesirable.

Case Study 1. The protein mixture investigated in the first case study is a quaternary protein mixture containing 1.5 mg/mL each of lysozyme (L), conalbumin (C), bovine serum albumin (BSA), and transferrin (T). The optimization of this mixture is complex since the pI values of BSA and T and thus their affinity for the column packing are similar (approximately 4.8 for BSA and 5.0 for transferrin (Budavari, 1989)). Therefore, achieving complete resolution of the BSA and T presents a challenge to the optimization methodology.

The Doehlert design experiments (Table 1) are performed, and values of the chromatographic optimization function (COF, eq 2) are determined for each experiment and are reported in Table 2. The sum of individual peak pair resolutions (eq 1) is also calculated for each experiment and reported in Table 2. These COF and resolution sum values are fit to the quadratic response model of eq 3. Response model coefficients are reported in Table 3, while model predictions of the COF and resolution sum are reported in Table 2. Values of the R^2 statistic are found to be 0.87 and 0.93 for the COF and resolution summation models, respectively. While additional experiments will result in a more accurate model and higher R^2 values, the goal of this work is to optimize the separation with the least possible amount of experimentation.

The conditions first found as optimum using the COF (pH of 6.0 and 5.0 CV) produce an undesirable chromatogram with unresolved peaks. The results of the COF analysis of this experimental point are added to the existing matrix of data, and a new optimum is calculated, so that in this case study eight experiments instead of seven are used to find the optimum operating conditions. The new optimum COF value is found to exist at a pH of 7.4 and 19.2 CV, which corresponds well with an inspection of the response model surface (Figure 3).

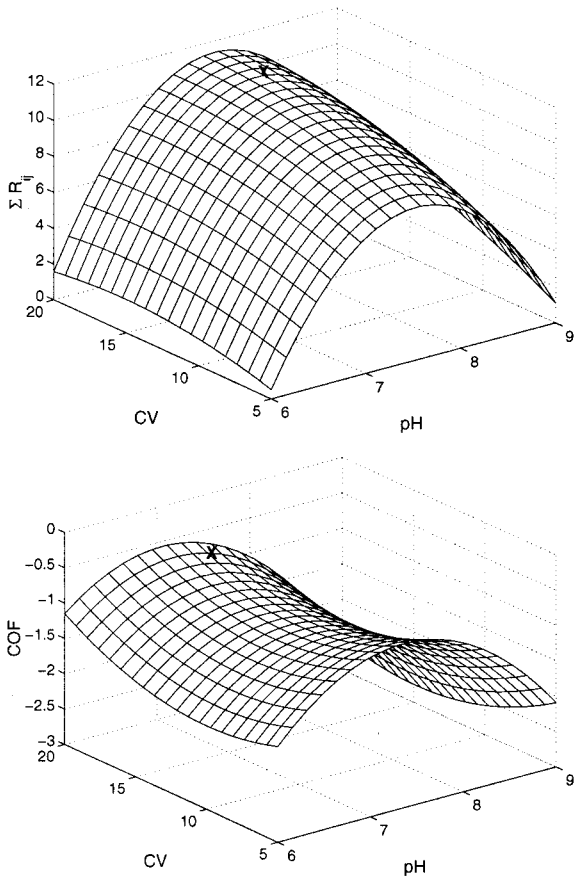

Figure 3. Resolution summation (top) and COF (bottom) response surfaces for case study 1. Optimal conditions found at **x** (pH 7.8, CV 17.7 for the resolution summation and pH 7.4, CV 19.2 for the COF).

Table 3. Model Parameters and R^2 Regression Statistics for Case Study 1

	COF	$\sum R_{ij}$
a_1	−2.94E+01	−1.85E+02
a_2	7.87E+00	5.12E+01
a_3	−2.05E−03	−6.09E−02
a_4	−5.27E−01	−3.42E+00
a_5	6.71E−03	−1.25E−02
a_6	−2.20E−02	7.42E−02
R^2	0.87	0.93

Note that there is a local COF optimum at a pH of approximately 7.5 and 5 CV, which is due to the third term in the COF. That is, total analysis time will be a minimum at low values of the gradient length. Thus the sensitivity of the response model to the weighting constants in the COF (eq 2) is a factor that must be considered, and the weights employed require careful selection based on the overall goals of the separation. The weights reported here have been found to be appropriate for a wide range of IEC protein purifications (Klein and Rivera, 1998).

A chromatogram run at these optimum conditions confirms that baseline separation of all solutes occurs at these conditions (Figure 4). In this case study, the

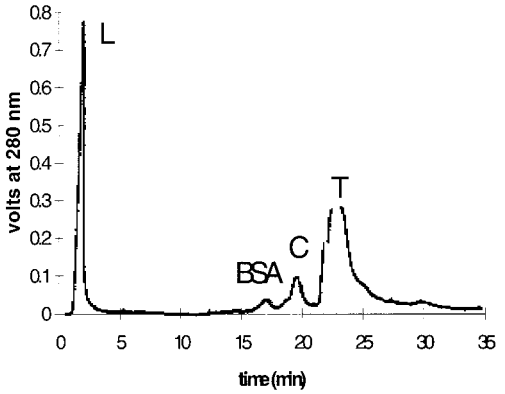

Figure 4. Case study 1: chromatogram at maximum COF with optimum pH 7.4, CV 19.2.

Table 4. Experimental Results vs Model Predictions for Case Study 2

			COF		$\sum R_{ij}$	
expt	pH	CV	data	model	data	model
1	7.5	12.5	−7.66	−7.66	8.91	8.91
2	9.0	12.5	−5.23	−6.30	5.23	4.20
3	8.2	19.0	−8.56	−7.49	6.01	7.03
4	6.0	12.5	−9.74	−8.67	4.28	5.30
5	6.8	6.0	−6.85	−7.92	7.99	6.96
6	6.8	19.0	−9.22	−10.28	10.40	9.38
7	8.2	6.0	−9.40	−8.33	7.17	8.20

Table 5. Model Parameters and R^2 Regression Statistics for Case Study 2

	COF	$\sum R_{ij}$
a_1	3.57E+00	−1.10E+02
a_2	−2.41E+00	2.96E+01
a_3	−7.66E−01	1.41E+00
a_4	7.64E−02	−1.85E+00
a_5	−2.11E−02	5.75E−04
a_6	1.65E−01	−1.83E−01
R^2	0.98	0.98

optimum value of the resolution summation is also found in approximately the same area of the experimental domain, at a pH of 7.8 and 17.7 CV (Figure 3). The unlikely good agreement found between the COF and resolution summations in this case study stems from the fact that the separation of solute peaks is the crucial term in the COF as a result of the complexity of the feed mixture.

Case Study 2. The protein mixture investigated in the second case study is a six-protein mixture containing 1 mg/mL each of conalbumin (C), β-lactoglobulin (β-L), insulin (I), α-chymotrypsinogen A (A), bovine serum albumin (BSA), and transferrin (T). This case study is extremely challenging as a result of the number of solutes in the feed mixture and the fact that many of the solutes exhibit similar retention behavior. COF and resolution summation values are reported for the Doehlert experiments in Table 4, and response model coefficients are reported in Table 5. The R^2 statistics for this case study are 0.98 for both the COF and the resolution summation models.

The optimum COF value is found to exist at a pH of 9.0 and 19.7 CV, which corresponds well to the response plot found in Figure 5. The chromatogram produced at these final conditions exhibits baseline separation of all of the solutes in the mixture, though the first peak exhibits extreme tailing and the second peak is shouldered (Figure 6). Though these peak geometries are less than desirable, they are tolerable and are expected since the adsorption is extremely nonlinear as a result of the large number of components in the feed mixture. If higher yield or purity is desired for these first two peaks, they can be collected and rechromatographed individually.

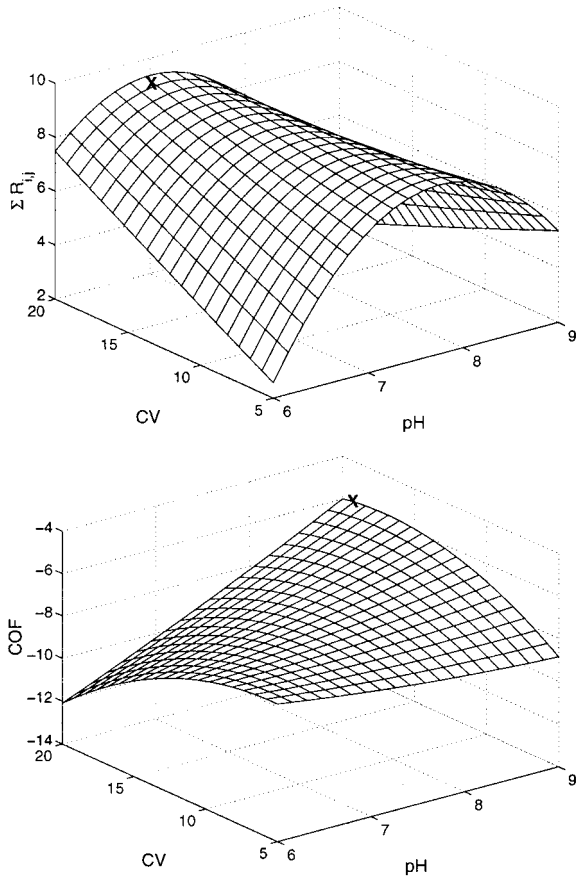

Figure 5. Resolution summation (top) and COF (bottom) response surfaces for case study 2. Optimal conditions found at **x** (pH 6.8, CV 19.0 for the resolution summation and pH 9.0, CV 19.7 for the COF).

The conditions of the optimum resolution summation are found to be a pH of 6.8 and 20.0 CV (Figure 5). Figure 6 contains a chromatogram from a Doehlert matrix experiment that was run at a pH of 6.8 and 19.0 CV. As can be seen, serious peak overlap exists at these conditions. Only two peaks are resolved at these conditions, which means that four of the six solutes in the feed mixture coeluted and are hidden. This is extremely undesirable and demonstrates the importance of the term in the COF that accounts for the number of peaks eluted. It can be concluded that in this particular case study the COF is far superior to the resolution summation for ranking chromatograms in order of desirability.

Conclusions

A novel approach to optimal HPLC methods development is proposed for the ion-exchange separation of protein mixtures. The methodology is composed of a Doehlert factorial design of experiments, a new criteria function to quantify and rank the quality of the separations, a vector quantizing neural network for automatic peak classification, and a variable-sized simplex algorithm for response surface optimization. The methodology is illustrated for the simultaneous optimization of pH and ionic strength (salt gradient length) of the mobile phase to maximize protein separation while decreasing total analysis time. However, this methodology can easily be extended to the optimization of a larger number of process variables.

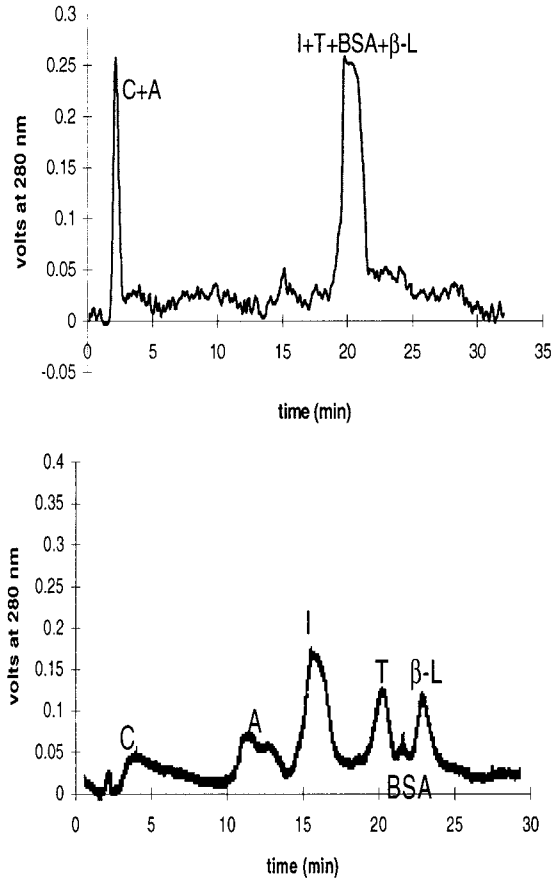

Figure 6. Case study 2: chromatogram at maximum resolution summation (top) with optimum pH 6.8, CV 19.0 and maximum COF (bottom) with optimum pH 9.0, CV 19.7.

The COF criteria function employed in this work is found to be useful and far superior to the resolution summation in representing the quality of the chromatograms. A variable-sized simplex algorithm is utilized in conjunction with a quadratic response model to determine the optimal operating conditions for feed mixtures containing four and six proteins that are commonly found in the pharmaceuticals industry. Results are acceptable, with baseline resolution of all solutes and a relatively short analysis time being realized for both of the test mixtures. Furthermore, because of the empirical nature of the optimization methodology, this method can be applied to separations where no prior knowledge of the feed mixture composition exists, which is frequently the case in industrial applications.

Nomenclature

A	user-adjustable weight
a_i	response model parameters
B	user-adjustable weight
COF	chromatographic optimization function
f_i	measure of separation of peak pair i (Figure 2)
g_i	measure of separation of peak pair i (Figure 2)
K_i	peak geometry penalty for peak i
M	number of expected peaks
N	actual number of eluted peaks
n_p	number of peaks exhibited on chromatogram
R_{ij}	resolution between peaks $i$and j
$\sum R_{ij}$	summation of individual peak pair resolutions over the entire chromatogram
t	total analysis time
t_i	elution time of peak i
t_m	maximum desirable total analysis time
w_i	baseline bandwidth of peak i
x_i	independent response model variables
y	dependent response model variable

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