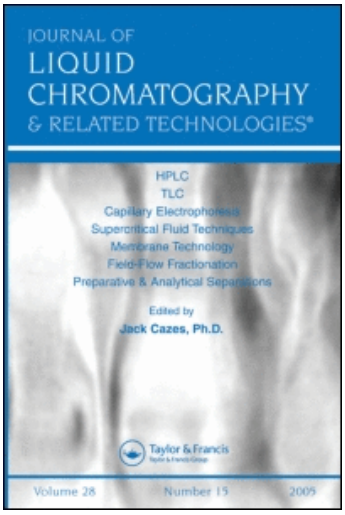

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EVALUATION OF LOT-TO-LOT CONSISTENCY IN ION EXCHANGE CHROMATOGRAPHY

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ABSTRACT

The reproducibility of ion exchange columns was investigated by rigorously evaluating critical chromatographic parameters as well as the performance of these ion exchange columns under various modes of operation. A detailed set of experiments were carried out to determine equilibrium Steric Mass Action parameters, HETP, column dead volume, total ion capacity, and porosity. In addition, the performance of these columns was examined under isocratic, analytical linear gradient, overloaded linear gradient, frontal, and displacement chromatographic conditions. While differences between the two lots were observed with isocratic experiments, the columns exhibited similar behavior under gradient, frontal, and displacement conditions. Furthermore, simulations obtained with the SMA model were shown to predict column performance under linear and non-linear conditions.

INTRODUCTION

Ion exchange chromatography is widely employed for the purification of biopharmaceuticals. A critical issue facing the industry today is the establishment of robust bioprocesses.

Clearly, this is strongly dependent on the availability of ion exchange materials which have minimal lot-to-lot variations. The reproducibility of reversed phase chromatographic materials has been the subject of investigation.[1-4] Stanley et al reported[1] on efficiency and capacity factor reproducibility as a function of column packing methods for a C_{18} stationary phase material. Guan and Guiochon[2,3] studied the performance of two C_{18} stationary phase materials by examining the variations in efficiency and capacity factors. Kirkland[4] demonstrated the consistent production of Zorbax Rx-C_{18} stationary phase material over a four year period. Johnson and coworkers[5] evaluated physical properties of 3M Emphaze AB 1 to show lot-to-lot consistency of the chromatographic material. Welinder et al.,[6] presented several case studies on column-to-column variations for separations of biopharmaceuticals. The authors showed variability in reverse phase, gel permeation and hydrophobic interaction chromatographic columns. To date, minimal attention has been provided to lot-to-lot variations with ion exchange chromatographic systems. However, since ion exchange chromatography is widely employed for the purification of biomolecules, there is an urgent need to evaluate the consistency of ion exchange systems.

The Steric Mass Action (SMA) model of ion exchange has been successfully employed for the prediction of linear gradient,[7] step gradient,[8] and displacement chromatography.[9] In this manuscript, the SMA model is used to evaluate lot-to-lot consistency in ion exchange systems. This work consists of a rigorous evaluation of critical chromatographic parameters as well as the performance of ion exchange columns under various modes of operation. In addition, simulations obtained with the SMA model are employed to predict column performance under linear and non-linear conditions as well as to examine differences between the lots.

EXPERIMENTAL

Materials

Four strong cation exchange (SCX) prepacked 1 mL columns (Source 15S, 15 μm, 30 X 6 mm I.D.) were donated by Pharmacia Biotech (Uppsala, Sweden). Columns 510546 and 510547 were Source 15S lot # 107727-01, columns 510108 and 510116 were from Source 15S lot # 106073-01. A strong cation exchange (SCX) (8 μm, 50 X 4.6 mm I.D.) column was donated by Waters Chromatography (Milford, MA, USA). Cytochrome C (horse heart), α-chymotrypsinogen A and neomycin sulfate were purchased from Sigma (St. Louis, MO, USA). NaCl was obtained from Aldrich (Milwaukee, WI, USA). Mono and dibasic sodium phosphate were from Fisher (Springfield, NJ, USA).

Equipment

Frontal, gradient and displacement runs were carried out using a modular chromatographic system containing a Waters 600E system controller, a Valco EC10W electrically actuated valve (Valco, Houston, TX, USA), a Kratos superflow 757 UV-Vis absorbance detector (Applied Biosystems, Foster City, CA, USA), a model 2212 Helirac fraction collector (LKB, Bromma, Sweden) and a NEC Power Mate 2 workstation with Waters Maxima 820 software. All other chromatographic experiments were carried out using a system containing a Waters 600 programmable HPLC pump, WISP 712 autoinjector, a Waters 484 tunable absorbance detector (Millipore, Milford, MA, USA), and a Bitwise workstation running Millenium software (Waters).

Procedures

Determination of extra-column volume, column void volume, and efficiency

The extra column volume for the equipment was determined by injecting 20 μL of a 2 mg/mL solution of sodium nitrate through the system (without the column) and monitoring the absorbance at 310 nm. The column void volume for each column was determined in the same manner as above only with the column on line. These experiments were carried out in triplicate for each column. The height equivalent to a theoretical plate for the columns were determined by injecting 20 μL of a 2 mg/mL solution of sodium nitrate through the system monitoring the effluent at 310 nm. These experiments were carried out in triplicate for each column.

Determination of total ion capacity

The column was initially regenerated with 2 N NaCl at pH 11 followed by a DI water wash. 25 mL of 1M HCl aqueous solution were then perfused into the column followed by a 25 mL wash with DI water. A 1N KNO_3 aqueous solution was consequently introduced into the column during which 25 mL of the effluent was collected. The collected volume was titrated with a 0.1N NaOH solution to determine the H+ ions displaced by the KNO_3 solution. These experiments were carried out in triplicate for each column.

Isocratic chromatography

Isocratic experiments were carried out in 50 mM sodium phosphate buffer, pH 6.0, at various salt concentrations and a flow rate of 1 mL/min. 20 μL injections were employed for these experiments.

The column effluent was monitored at 280 nm for α-chymotrypsinogen A and 408 nm for horse cytochrome C. These experiments were carried out in triplicate for each column.

Analytical linear gradient chromatography

Linear gradient experiments under low feed conditions were carried out in 50 mM sodium phosphate buffer, pH 6.0, using a fixed linear gradient of 15 mM Na per column dead volume and a flow rate of 1mL min. 20 μL injections were employed for these experiments and the effluent was monitored at 280 nm. These experiments were carried out in triplicate for each column. Similar linear gradient experiments were performed at a shallower fixed linear gradient slope of 5 mM Na per column dead volume.

Frontal chromatography

Frontal experiments were carried out using several protein concentrations (7-18 mg/mL) of α-chymotrypsinogen A and horse cytochrome C in 50 mM sodium phosphate buffer, pH 6.0, at various salt concentrations. The flow rate was 0.5 mL/min. The column effluent was monitored at 310 nm for α-chymotrypsinogen A and 600 nm for horse cytochrome C so that the detector was not saturated during breakthrough of the protein fronts.

Overloaded linear gradient chromatography

Linear gradient experiments under overloaded conditions were carried out at 50 mM sodium phosphate buffer, pH 6.0, using a fixed linear gradient of 5 mM Na per column dead volume and a flow rate of 1 mL/min. 1.6 mL of 7.5 mg/mL α-chymotrypsinogen A and 4 mg/mL horse cytochrome C in the carrier was employed as the feed. 200 μL fractions were collected for subsequent analysis.

Displacement chromatography

The displacement experiments were carried out by first equilibrating the column with carrier and then perfusing with the feed followed by the displacer solution.[10] The displacement experiments were carried out using 50 mM sodium phosphate buffer, pH 6 as the carrier, 1.6 mL of 11.7 mg/mL α-chymotrypsinogen A and 6 mg/mL horse cytochrome C as the feed and 10 mM neomycin sulfate as the displacer. The displacements were carried out at two flow rates: 0.5 and 0.2 mL/min. 200 μL fractions were collected for subsequent analysis. The columns were regenerated between displacement experiments using a regenerant containing 2 M NaCl at pH 11.

Protein analysis by HPLC

Protein analysis of the 200 μL fractions collected during overloaded gradient and displacement experiments was carried out by isocratic ion-exchange chromatography employing the SCX Waters column (50 x 5 mm I.D.). Fractions were diluted 5-100 fold with the mobile phase and 20 μL samples were injected at a flow rate of 0.5 mL/min. The mobile phase consisted of 50 mM sodium phosphate buffer, pH 6, and contained 90 mM NaCl. The effluent was monitored at 280 nm.

Displacer analysis

Neomycin sulfate, the compound employed in the displacement experiments, was analyzed according to the assay described by Taylor.[11]

Steric Mass Action (SMA) parameter estimation

The Steric Mass Action (SMA) parameters (i.e. characteristic charge (ν), equilibrium constant (K_{eq}) and steric factor (σ) for α-chymotrypsinogen A and horse cytochrome C were determined as reported by Gadam et al.[12] The linear Steric Mass Action parameters (i.e. ν and K_{eq}) were determined from log capacity factor versus log salt plots.[12]

The non-linear SMA parameter (i.e. steric factor) was determined through the fit of the non-linear isotherm data obtained from the frontal experiments. The SMA parameters for neomycin sulfate in the stationary phase examined were reported earlier by Gallant.[13]

Theoretical simulations

The steric mass action parameters were employed with the chromatographic model developed by Gallant et al.[7,8,13-15] to simulate the isocratic, gradient, and displacement experiments.

RESULTS AND DISCUSSION

The reproducibility of four pre-packed Pharmacia Source 15S cation exchange columns was evaluated by determining critical chromatographic parameters as well as the performance of these columns under various modes of operation. Columns 510546 and 510547 were from lot 107727-01 and columns 510108 and 510116 were from lot 106073-01. These four columns were selected at random within the lots of stationary phase materials.

Table 1

Total Ion Capacity for the Four Source 15S Pharmacia Columns

Lot No. **Column No.**	**107727-01** **510546**	**107727-01** **510547**	**106073-01** **510108**	**106073-01** **510116**
Bed Capacity Data	431	431	423	431
(mmoles/L	439	431	411	431
stationary phase)	431	439	423	431
Avg. Bed Capacity	434	434	419	431
(mmoles/L				
stationary phase)				
Avg void volume	0.733	0.733	0.717	0.733
(mL)				
Porosity	0.760	0.760	0.743	0.760
HETP (cm)	0.0056	0.0058	0.0056	0.0055

All four columns underwent identical procedures and were exposed to the same conditions at all times. These strict methods were required in order to rigorously compare the results obtained in all four columns. Furthermore, many of the experiments were carried out in triplicate for each column.

Column Void Volume, Porosity, and Efficiency

The experiments for the determination of column void volume were carried out in triplicate for each column and the results were seen to be very reproducible within each column (relative error of 0.7%). The average column void volume, porosity, and HETP for the columns are presented in Table 1. As seen in the Table, while the column void volume and the porosity were very similar for each of the four columns, column #510108 exhibited a slightly lower column void volume and porosity than the other three columns. The HETP results indicate that the efficiency of these columns were very similar. The relative error for each column for these triplicate experiments was 5%.

Determination of Total Ion Capacity

The total ion capacities of the columns were determined as described in the experimental section and the results are presented in Table 1. The reproducibility of the experimental technique can be clearly seen in these results. Furthermore, the results of the titrations yielded very similar mmoles of ion capacity in each column. The relative error for the total ion capacity in each

Table 2

Average Retention Times Under Isocratic Elution Chromatography

Column No.	Lot No.	Salt Conc. (mM)	Alpha-Chymo-trypsinogen A Retention Time (Min.)	Horse Cytochrome C Retention Time (Min.)
510546	107727	200	1.45	4.26
		175	1.92	8.16
		150	2.94	18.31
		125	6.66	67.11
		100	17.35	
510547	107727-01	200	1.45	4.23
		175	1.91	8.12
		150	2.96	18.60
		125	6.29	68.11
		100	17.08	
510108	106073-01	200	1.41	3.89
		175	1.82	7.28
		150	2.77	16.68
		125	6.01	59.81
		100	15.46	

column was 3%. However, the difference in porosity for column 510108 produced a slightly different ion capacity when expressed in the units of mmoles/L stationary phase. It is felt that this discrepancy is probably due to slight variations in the column packing.

Isocratic Chromatography

Isocratic experiments for α-chymotrypsinogen A and horse cytochrome C were carried out at various salt concentrations in triplicate at a protein concentration of 1mg/mL, 20 μL injection and 1mg min flow rate. The retention times for each column were found to be extremely reproducible (i.e. 0.1 %). The average retention times under isocratic elution conditions for columns 510546, 510547, 510108, and 510116 are presented in Table 2. As seen in the Table, the results within a specific lot (e.g. columns 510546 and 510547) were very similar for both proteins. However, when the lots are compared, it is seen

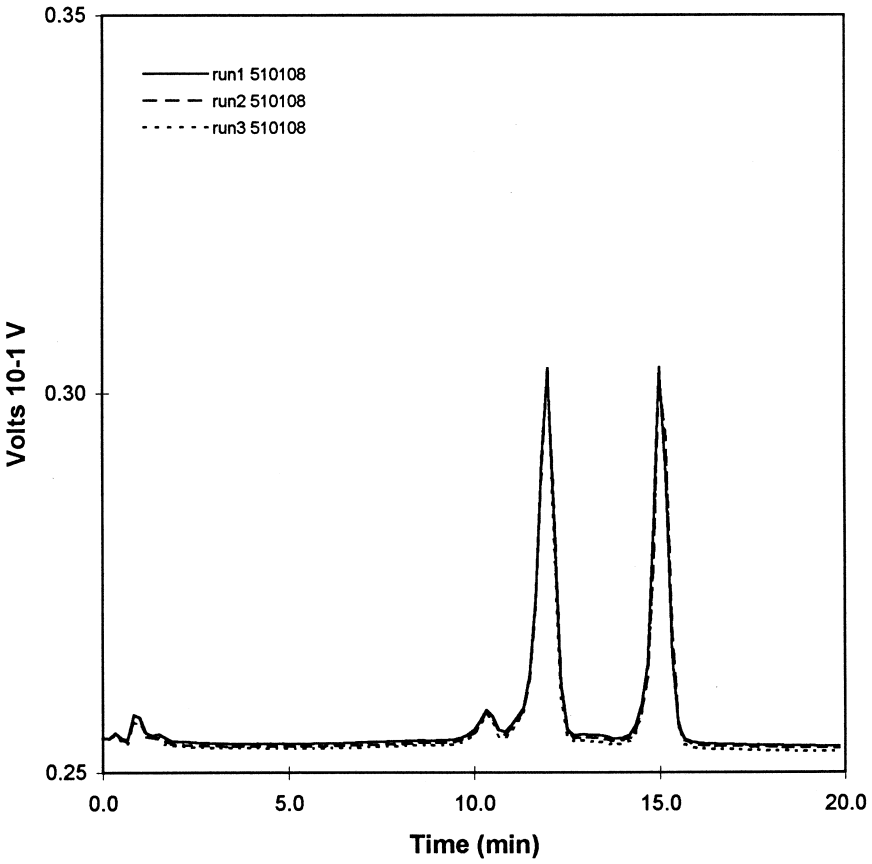


Figure 1. Triplicate linear gradient separation of α-chymotrypsinogen A and horse cytochrome C in Source 15S Pharmacia column #510108. Feed: 20μL of 1mg/mL each protein. Carrier: 50 mM sodium phosphate buffer, pH 6. Flow rate: 1mL/min. Gradient slope: 15mM Na gradient/column volume.

that there was a measurable difference in retention time under these isocratic conditions. For example, at 100 mM salt, the retention times for α-chymotrypsinogen A on lots # 107727-01 and lot # 106073-01 were approximately 17.3 and 15.5 minutes, respectively. While it is interesting that isocratic results indicated some lot-to-lot variability, protein chromatography is rarely carried out under isocratic conditions and is typically performed under gradient conditions.

Table 3

Comparison of Theory and Equipment

Isocratic Chromatography

Lot No.	Column No.	α-Chymotrypsinogen A 50 mM Sodium Phosphate Buffer in 100mM NaCl Elution Time (Min) Experiment	Theory	Cytochrome C 50 mM Sodium Phosphate Buffer in 125mM NaCl Elution Time (Min) Experiment	Theory
107727-01	510546	2.9	3.0	8.1	8.0
107727-01	510547	2.9	3.0	8.1	8.0
106073-01	51008	2.8	2.7	7.3	7.3
106073-1	510116	2.8	2.7	7.3	7.0

Gradient Chromatography

		15mM Na Gradient/Column Volume				5mM Na Gradient/Column Volume			
		Alpha-Chymo-trypsinogen A		Horse Cytochrome C		Alpha-Chymo-trypsinogen A		Horse Chytochrome C	
Lot No.	Column No.	Elution Time (Min) Experiment	Theory	Elution Time (Min) Experiment	Theory	Elution time Min Experiment	Theory	Elution Time (Min) Experiment	Theory
107727-01	510546	12.1	11.9	15.0	15.2	19.6	19.8	28.2	29.0
107727-01	510547	12.1	11.9	15.0	15.2	19.6	19.8	28.2	29.0
106073-01	510108	11.8	11.5	15.0	15.1	18.0	18.6	26.4	28.1
106073-01	510116	12.1	11.9	15.0	15.2	18.1	19.1	26.6	28.2

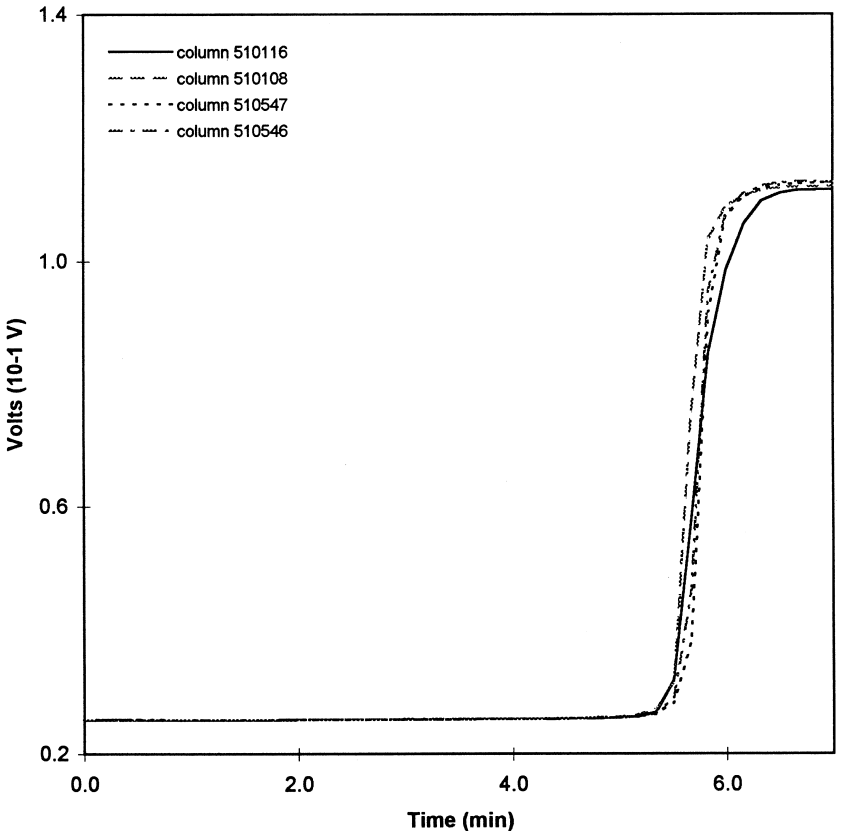


Figure 2. Frontal chromatography. Experiments were carried out in the four Source 15S Pharmacia columns. Feed: 10mg/mL horse cytochrome C. Carrier: 50 mM sodium phosphate buffer containing 75mM NaCl, pH 6. Flow rate: 0.5mL/min.

Analytical Linear Gradient Chromatography

Linear gradient chromatography was carried out in triplicate on each of the four columns with a feed mixture of 1mg/mL each protein, 20 μL injection and 50 mM sodium phosphate buffer for the initial condition. Representative chromatograms for a gradient of 15 mM Na/ columns volume of column 510108 is shown in Figure 1. As seen in the Figure, the triplicate runs were very reproducible. As seen in the Table 3, the data at 15 mM Na gradient/column volume indicated that the protein retention behavior was very similar in all the columns. In order to examine the chromatographic behavior under a more shallow gradient, experiments were carried out using a fixed linear gradient of 5

mM Na/column volume. The data for these conditions (Table 3) indicated that while the four columns exhibited similar chromatographic behavior under these conditions, there was a measurable difference between the two lots. Specifically, columns 510546 and 510547 (lot # 107727-01) exhibited slightly longer retention times than the other lot of material. Thus, when shallow gradients are employed, lot-to-lot variation may indeed result in slight changes in retention behavior.

Frontal Chromatography

As described in the experimental section, a variety of frontal chromatographic experiments were carried out using α-chymotrypsinogen A and horse cytochrome C on all four columns. The breakthrough fronts for experiments with 10 mg/mL cytochrome C at 125 mM salt concentration are presented in Figure 2.

As seen in the Figure, the breakthrough volumes were essentially the same for all four columns. Thus, although the isocratic data indicated lot-to-lot variation, these frontal experiments did not indicate any measurable difference.

Overloaded Linear Gradient Chromatography

The overloaded linear gradient chromatography experiments were carried out employing the shallow gradient slope of 5 mM Na/column volume with a feed mixture of α-chymotrypsinogen A and horse cytochrome C. The resulting histograms for columns 510546, 510547, 510108, and 510116 are presented in Figure 3.

As seen in the figure, both the breakthrough volume and the end of the non-linear peaks of the proteins were very similar in all four columns. Thus, when these columns were employed for preparative linear gradient chromatography, they exhibited very similar behavior with no measurable lot-to-lot variation.

Displacement Chromatography

Displacement experiments were carried out on the four columns using a feed mixture of α-chymotrypsinogen A and horse cytochrome C. Neomycin sulfate, which has been shown to be an effective low molecular weight displacer for cation exchange systems, was employed in these experiments.[16,17] Two representative displacements for the columns under investigation are presented

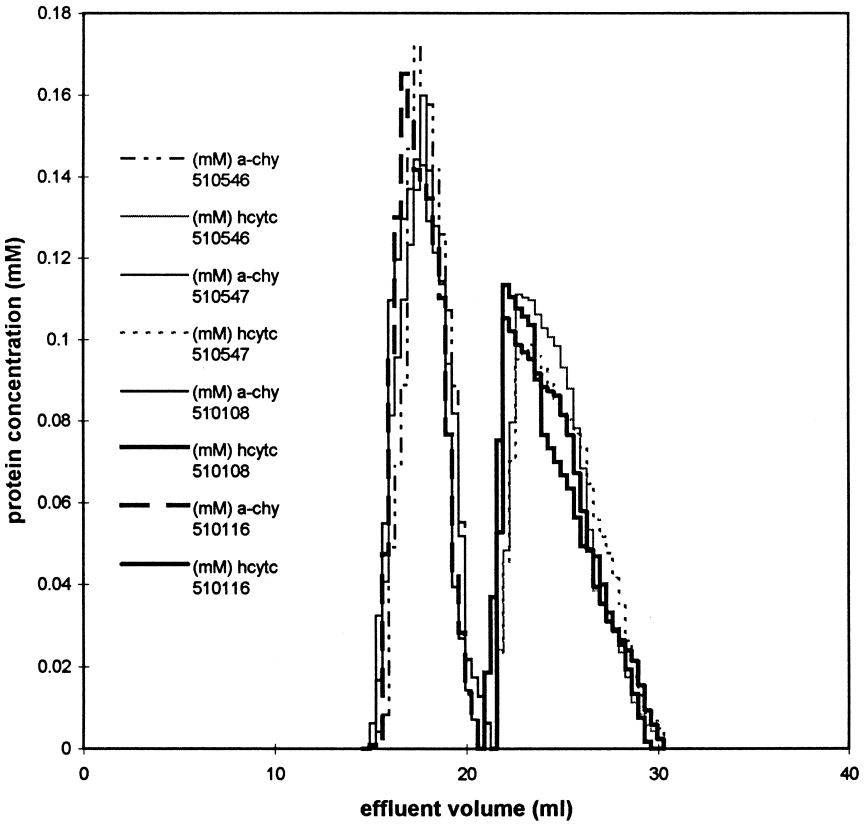


Figure 3. Overloaded linear gradient separation of α-chymotrypsinogen Aand horse cytochrome C. Experiments performed in the four Source 15S Pharmacia columns. Feed: 1.6 mL of 7.5 mg/mL α-chymotrypsinogen A and 4 mg/mL horse cytochrome C in the buffer. Carrier: 50mM sodium phosphate buffer, pH 6. Flow rate: 1mL/min. Fractions: 200 μL fractions. Gradient slope: 5 mM Na gradient/column volume.

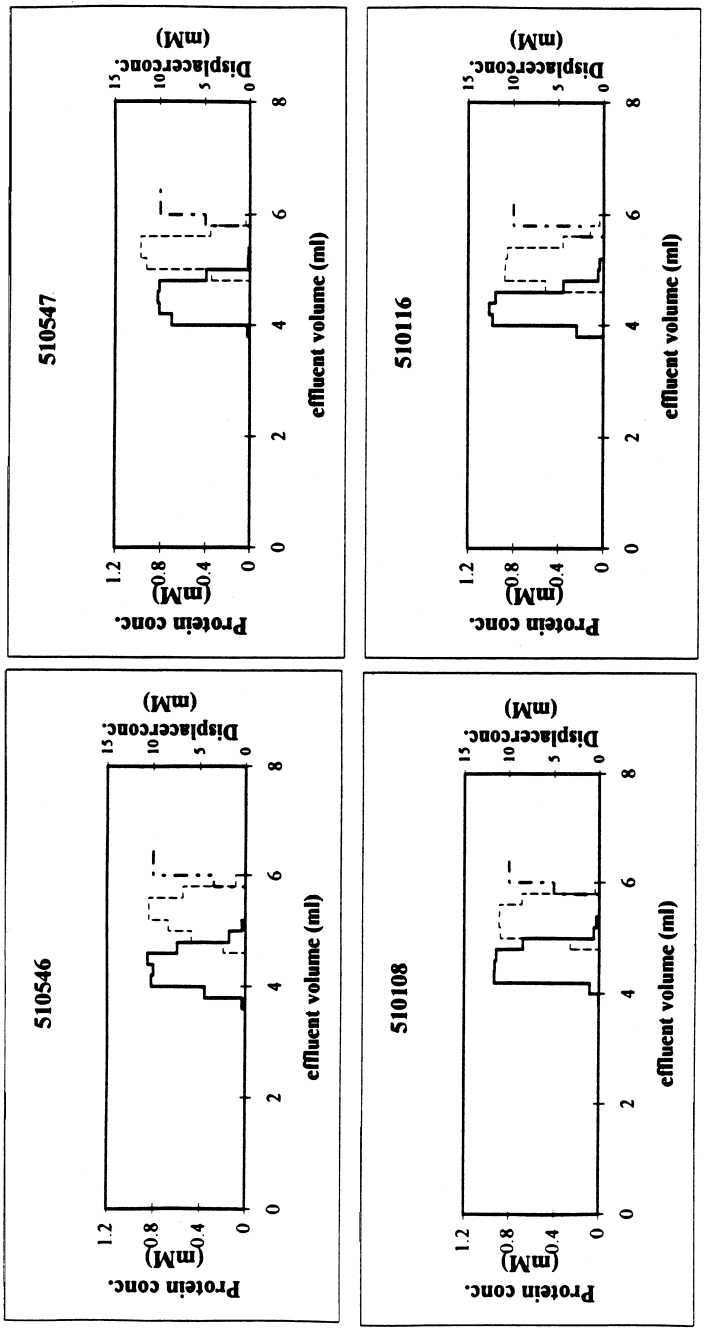


Figure 4. Displacement experiments and simulations of Source 15S Pharmacia columns # 510546 and 510116 for the separation of α-chymotrypsinogen A and horse cytochrome C using Neomycin sulfate as a displacer. Feed: 1.6 mL of 11.7 mg/mL α-chymotrypsinogen A and 6 mg/mL horse cytochrome C. Carrier: 50 mM sodium phosphate buffer, pH 6. Displacer: 10 mM. Flow rate: 0.2 mL/min. Fractions: 200 µL.

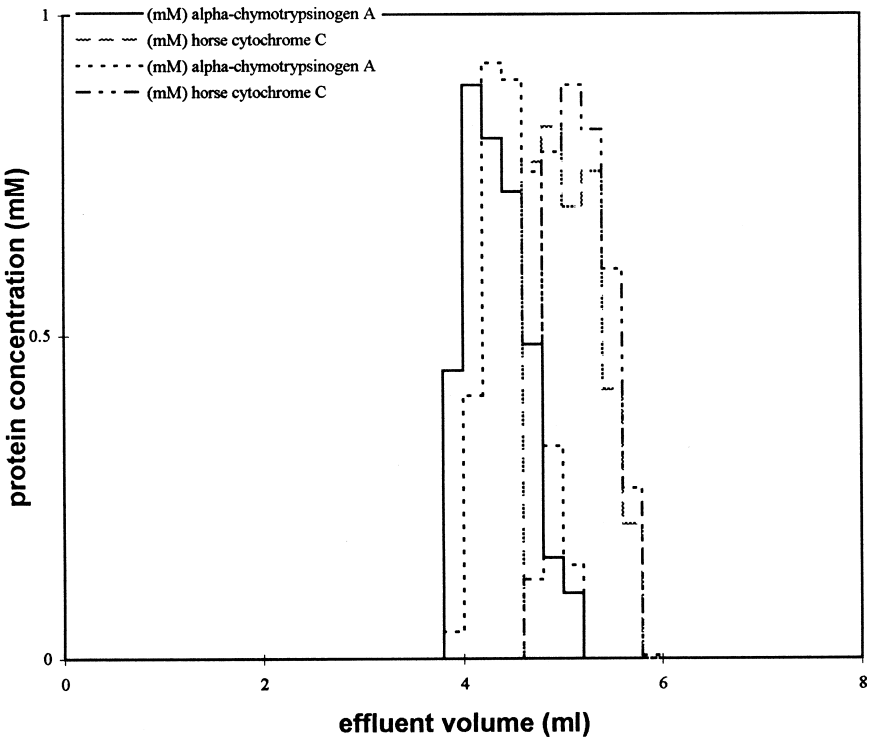


Figure 5. Two displacement separations of α-chymotrypsinogen A and horse cytochrome C using Neomycin sulfate as a displacer. Experiments shown for Source 15S Pharmacia column #510546. Feed: 1.6 mL of 11.7 mg/mL α-chymotrypsinogen A and 6 mg/mL horse cytochrome C. Carrier: 50 mM sodium phosphate buffer, pH 6. Displacer: 10mM. Flow rate: 0.5 mL/min. Fractions: 200 μL.

in Figure 4. As seen in the figure while the displacements had approximately the same displacer breakthrough volumes and protein elution times, there as some variations observed in the isocratic concentrations obtained . In order to examine whether this was true column to column variability, two displacements were carried out in the same column (#510546) to examine run to run variations. The displacement histograms corresponding to these two runs are shown in Figure 5. As seen in the figure, while the displacements presented identical protein breakthrough volumes the protein concentrations again varied slightly from run to run. Thus, the observed difference in protein concentrations obtained in the displacement experiments were mainly due to experimental variability (e.g. fraction collection).

Table 4

SMA Parameters in the Four Columns

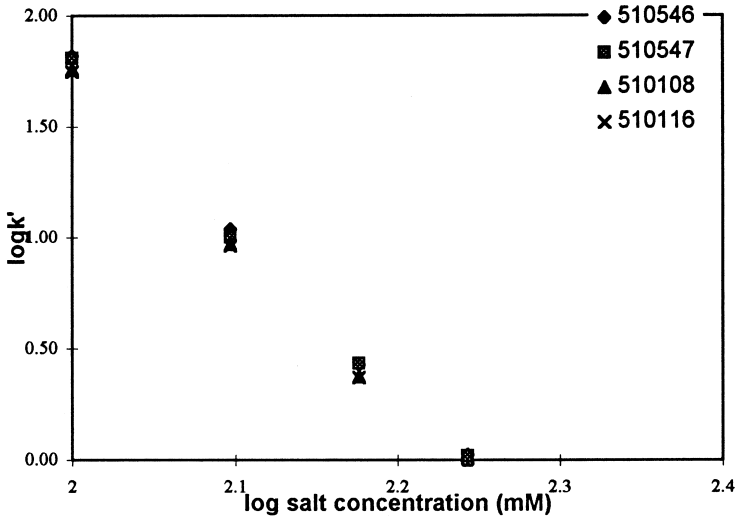
Lot Number	Column Number	Characteristic Charge	Equilibrium Constant	Steric Factor
a) Parameters for Alpha-Chymotrypsinogen A				
107727-01	510546	4.92	0.052	8.6
107727-01	510547	4.87	0.054	10.2
106073-01	510108	4.82	0.058	8.2
106073-01	510116	4.85	0.052	10.2
b) Parameters for Horse Cytochrome C				
107727-01	510546	6.299	0.105	21.7
107727-01	510547	6.35	0.099	21.9
106073-01	510108	6.30	0.121	22.0
106073-01	510116	6.29	0.098	24.2

Steric Mass Action Model (Parameters)

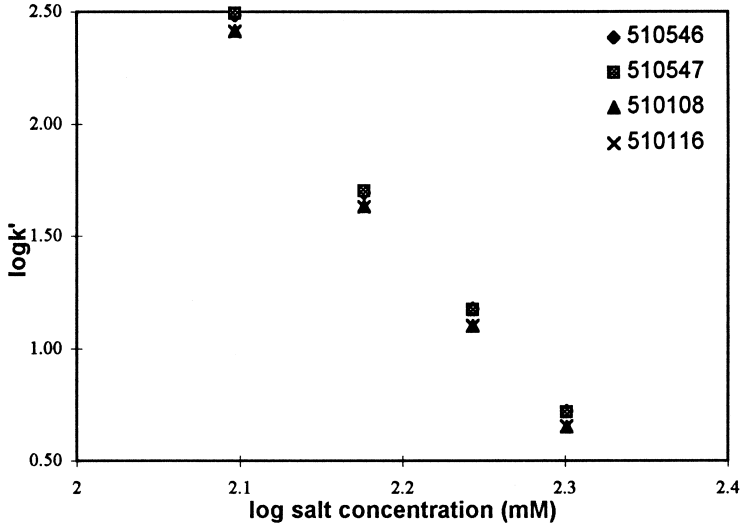
In order to further examine lot-to-lot consistency, both parameters and simulations obtained from the Steric Mass Action (SMA) model of ion exchange were employed.[9] The Steric Mass Action (SMA) model of ion exchange has been successfully employed for the prediction of linear gradient,[7] step gradient,[8] and displacement chromatography.[9,13-15] This equilibrium model has three parameters: characteristic charge, ν (the number of interaction sites of each molecule with the stationary phase material); the equilibrium constant, K_{eq}; and the steric factor, σ (the number of adsorption sites sterically inaccessible due to the presence of the adsorbed molecule). The retention data from the isocratic experiments were employed to construct a log k'- log salt plot as shown in Figure 6. The slope and intercepts of these plots were then used to determine the characteristic charge and equilibrium constant, respectively.[9] The characteristic charge and equilibrium constant for both proteins in all four columns are presented in Table 4.

As seen in the Table, the characteristic charge for the proteins employed on the four columns were quite similar (i.e. 5% for α-chymotrypsinogen A and 8% for horse cytochrome C). The equilibrium constants for these proteins were also similar and any differences from lot-to-lot were within the experimental uncertainty (i.e. 20% for α-chymotrypsinogen A and 28% for horse cytochrome C). While all columns presented similar linear SMA parameters, column

1 a) alpha-chymotrypsinogen A



1 b) horse cytochrome C



#510108 exhibited a higher equilibrium constant. The equilibrium constants were calculated using the average total ion capacity for each column in the units of mmoles/l stationary phase. The equilibrium constant is related to the total ion capacity through

$$K_{eq} = \frac{10^{(\log k' + \upsilon_i \log C_1)}}{\beta \Lambda^{\upsilon_i}} \quad (1)$$

where Λ is the total ion capacity, β is the phase ratio and C_1 the mobile phase salt concentration.

The total ion capacity is effected by the column porosity by:

$$\Lambda = \frac{MV}{V_o(1-\varepsilon)} \quad (2)$$

where M is the molarity of the titrating solution, V the volume of titrating solution required to achieve neutrality, V_o the column dead volume and ε the porosity of the column. Thus, it turns out that the elevated equilibrium constants obtained on column #510108 were due solely to its lower porosity.

Finally, the non-linear SMA parameter, the steric factor, was determined from the adsorption isotherms. The steric factor for α-chymotrypsinogen A and horse cytochrome C for the four columns are presented in Table 4. While the steric factors for cytochrome C were similar in the four columns, there was some measurable differences in the values for α-chymotrypsinogen A.

It should be noted that the steric factor was determined by frontal experiments and these experiments were not carried out in triplicate due to the amount of protein that the runs would have required. Therefore, the steric factor results had uncertainty due to the fact that each experiment were carried out once in addition to the uncertainties due to its calculation from the frontal chromatographic experiments. However, the steric factor results are relatively close for all four columns and no particular trends can be noticed among the columns from different lots of stationary phase material.

Figure 6 (left). Linear retention plot, log capacity factor as a function of log salt concentration employing the four Source 15S Pharmacia columns 30 X 6.4 mm I.D., 50 mM sodium phosphate buffer, pH 6, flow rate of 1 mL/min for a) α-chymotrypsinogen A and b) horse cytochrome C. The runs were conducted in triplicate for each column.

Comparison of Theory and Experiment

The SMA model was employed to simulate the isocratic, linear gradient and displacement experiments described above. Isocratic simulations were conducted for α-chymotrypsinogen A and horse cytochrome C at different salt concentrations in all four columns. The results of the simulations conducted for α-chymotrypsinogen A and horse cytochrome C at 150 and 175 mM salt respectively are presented in Table 3. As seen in the Table, theoretical and experimental results are in close agreement. Thus, the SMA model was capable of predicting subtle changes in the isocratic elution of proteins in columns derived from different lots of stationary phase material.

Linear gradient simulations at low feed loads were conducted using two gradient slopes: 15 and 5 mM Na gradient/column volume for all four columns. The results of the simulations for α-chymotrypsinogen A and horse cytochrome C in all four columns are also presented in Table 3. As seen in the Table, the model correctly predicted the trends in the data. For the 15 mM gradient, the theory predicts the consistency in the retention data for cytochrome C as well as the slight difference in the retention data for α-chymotrypsinogen on column 510108. For the 5 mM gradient, the theory qualitatively predicts the higher retention times with columns 510546 and 510547 (lot 107727-01) as compared to the other columns. While the theory slightly over-predicts the retention times under these shallow gradient conditions, it is well suited for predicting when lot-to-lot variations will be observed.

Linear gradient simulations were carried out at elevated feed loads of α-chymotrypsinogen A and horse cytochrome C using a 5 mM Na gradient/column volume. The simulations indicated that all four columns exhibited similar behavior under overloaded linear gradient conditions in agreement with the experimental trends. A comparison of theory and experiment is presented in Figure 7. As seen in the Figure, both the breakthrough volume and the end of the non-linear peaks of the proteins in column 510116 were well predicted by the theory. While the shape of the cytochrome C peak was well predicted, the α-chymotrypsinogen A peak exhibited less fronting in the experiment than expected from the theory. Nevertheless, under overloaded conditions, the four columns exhibited similar behavior and the SMA model was able to capture the salient features of the separations.

Displacements simulations of α-chymotrypsinogen A and horse cytochrome C were carried out for all four columns. The simulations indicated that these columns should have exhibited very similar displacement behavior. However, as was shown in Figure 5, experimental variability within a single column can have a significant effect on the displacement chromatograms

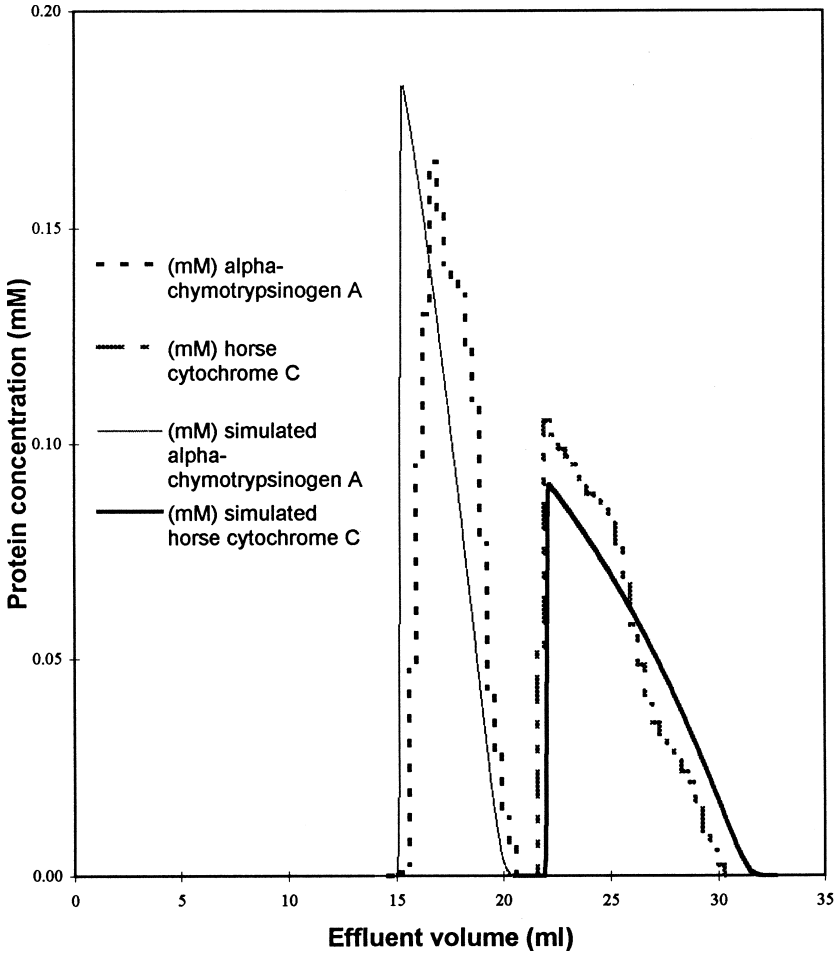


Figure 7. Comparison of theory and experiment in Source 15S Pharmacia column # 510116. Overloaded linear gradient separation of α-chymotrypsinogen A and horse cytochrome C. Feed: 1.6 mL of 7.5 mg/mL α-chymotrypsinogen A and 4 mg/mL horse cytochrome C in the buffer. Carrier: 50mM sodium phosphate buffer, pH 6. Flow rate: 1mL/min. Fractions: 200 μL fractions. Gradient slope: 5 mM Na gradient/column volume.

obtained. Thus, this experimental uncertainty makes it very difficult to determine any lot-to-lot variations in displacement performance when the columns are this consistent. Accordingly, simulations were not employed in examining lot to lot consistency for this displacement separation.

Sensitivity Analysis

The costs associated with the purification of a bioproduct depend among other aspects on lot-to-lot reproducibility. Therefore, as long as there is lot-to-lot reproducibility and the other process conditions are not altered, it is expected that the bioproduct of interest will exit the column within a predetermined effluent volume interval. The ability to predict the effluent volume at which the compounds exit the chromatographic column is, therefore, very relevant. In our case, the experimental results obtained under non-linear chromatography were similar for all four columns. In order to determine if there was a statistical difference among the experimental results, the mean of the breakthrough volume and the corresponding standard deviation were calculated for each protein under overloaded linear gradient. Considering each protein separately, it was found that the breakthrough volumes obtained in the overloaded linear gradient experiments were statistically the same (α= 99%). An identical analysis was conducted with the displacement experiments; the results indicated that the breakthrough volumes obtained in all four columns were statistically the same for each protein (α= 99%).

The same statistical analysis was conducted for simulations under each mode of non-linear chromatography. The statistical analysis indicated that the breakthrough volumes obtained in the overloaded linear gradient simulations were statistically the same (α = 99%). Similarly, the breakthrough volumes for α-chymotrypsinogen A and cytochrome C in the displacement simulations conducted for all four columns were statistically the same (α = 99%).

In order to determine how predictable the model is with respect to the overloaded experiments, the non-linear experimental and theoretical results were compared. This was accomplished by comparing the breakthrough volume means of the experiments and simulations. In particular, experimental and theoretical means were compared for each protein under overloaded linear gradient and displacement chromatography separately. The standard deviations for the breakthrough volumes of α-chymotrypsinogen A and cytochrome C under overloaded linear gradient chromatography were 0.3 mL. However, under displacement conditions, the standard deviation of the breakthrough volumes were lower for the two proteins, 0.1 mL for α-chymotrypsinogen A and 0.2 mL for cytochrome C. The lower standard deviation in displacement can be attributed to the sharpness of the protein front as the compounds exit the column. Nonetheless, the results for overloaded linear gradient and displacement chromatography indicated that the breakthrough volumes of the experiments and simulations were statistically the (α= 99%). Therefore, it is possible to expect that the SMA will accurately predict the experiments under overloaded conditions.

In addition to this analysis, there is a need to estimate the sensitivity of the model when the column parameters fall outside the uncertainty range. To assess the sensitivity of the model under these conditions, two fictitious cases were considered which could represent possible changes in a chromatographic column. The sensitivity studies were carried out employing the original data of column # 510546 and comparing it against the two cases. Both cases presented 10% higher linear retention data than the original.

In addition, the first case, Case 1, suited a chromatographic column that maintained the same pore structure and surface area as the base case, however, it had higher ligand density. Therefore, the first case consisted of a 10% increase in the total ion capacity and a 10% increase in the retention data maintaining the phase ratio, maximum binding concentration and the characteristic charge constant.

The second case, Case 2, included an increase in surface area, increasing the maximum binding concentration. Consequently, Case 2 consisted in a 10% increase in the maximum binding concentration with a 10% increase in the linear retention data maintaining the characteristic charge constant. It should be noted that for both cases the other SMA parameters were recalculated accordingly and Table 5 shows the data employed in these simulations.

The overloaded linear gradient simulations for the base case, Case 1 and 2 are presented in Figure 8 a. As seen in the figure, when the maximum binding concentration is increased (Case 2) the peaks eluted later affecting the breakthrough volume.

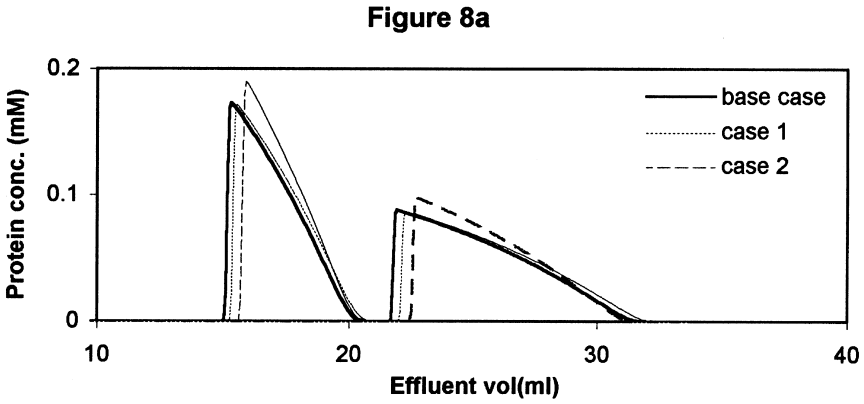
Also, an increase in the linear retention data affects the tail of the peak as the compounds take longer to elute from the column. Furthermore, comparing the three linear gradients, the gap between the proteins varied substantially for each case: 0.5 mL, 1.0 mL and 1.3 mL for the base case, Case 1 and Case 2, respectively.

The difference in the breakthrough volumes between Case 1 and the base case resulted in 0.3 mL and 0.4 mL for α-chymotrypsinogen A and cytochrome C, respectively. These differences do not fall outside the breakthrough volume interval calculated previously therefore a larger change in the parameters is required to obtain a significantly different result. On the other hand, the difference in the breakthrough volumes between Case 2 and the original data was 0.7 mL for α-chymotrypsinogen A and 1.1 mL for cytochrome C. These differences were greater than the breakthrough volume interval calculated for the simulations conducted with all four columns (α= 99%), indicating that the SMA model is sensitive to the changes of the same order or greater than Case 2.

Table 5

Parameters Used in the Sensitivity Analysis for 10% Change

Base Case	Alpha-Chymo-trypsinogen A	Cyctochrome C	Displacer
Porosity 0.76			
Total ion capacity (mM) 434			
Q max	32.1	15.5	115.73
Characteristic charge	4.92	6.29	3.75
Equilibrium constant	0.052	0.105	2
Steric factor	8.6	21.7	0.03
Case 1			
Porosity 0.76			
total ion capacity (mM) 477			
Q max	32.1	15.5	115.73
Characteristic charge	4.92	6.29	3.75
Equilibrium constant	0.035	0.064	0.77
Steric factor	9.9	24.5	0.40
Case 2			
Porosity 0.741			
Total ion capacity (mM) 434			
Q max	35.31	17.05	127.303
Characteristic charge	4.92	6.29	3.75
Equilibrium constant	0.050	0.106	1.98
Steric factor	7.37	19.16	0.00



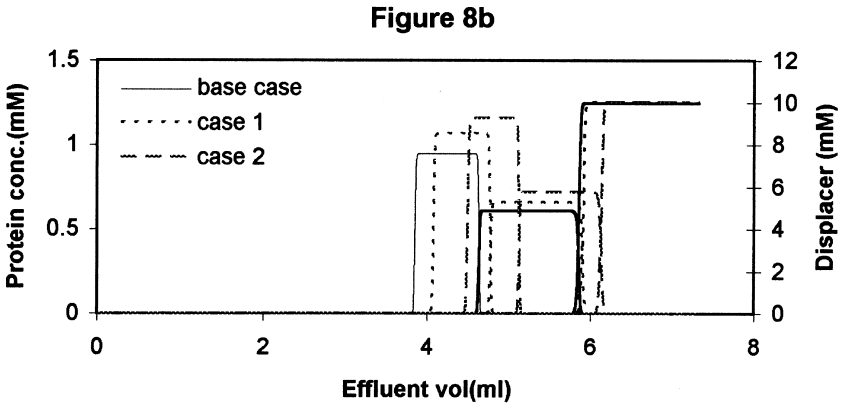


Figure 8. Sensitivity analysis of the SMA model for a) overloaded linear gradient and b) for displacement chromatography.

The displacement simulations for the base case, Case 1 and 2 are presented in Figure 8 b. As seen in the figure, the increase in the maximum binding concentration as well as linear retention data resulted in later eluting bands. The difference in the breakthrough volumes between Case 1 and the base case resulted in 0.3mL and 0.2mL for α-chymotrypsinogen A and cytochrome C, respectively. These differences are within the breakthrough volume interval, therefore, a larger change in the parameters is required to obtain a significantly different result. The difference in the breakthrough volumes between Case 2 and the base case were 0.7 mL for α-chymotrypsinogen A and 0.8 mL for cytochrome C. These differences were greater than the intervals calculated (α= 99%), indicating that the SMA model is sensitive to the column changes

performed for Case 2 and beyond. As the results indicated that 10% changes in Case 1 did not produce a significantly different overloaded linear gradient or displacement, simulations were conducted larger variations.

The simulations corresponding to a 20% change in Case 1 provided significantly different results compared to the base case(α= 99%). The difference in the breakthrough volumes for overloaded linear gradient between Case 1 and the base case resulted in 0.6 mL and 1.6 mL for α-chymotrypsinogen A and cytochrome C, respectively. These differences fall outside the breakthrough volume interval calculated for the simulations conducted with all four columns (α= 99%). In addition, the separation gap between the proteins increased to 1.1 mL for Case 1. The displacement simulations, the difference in the breakthrough volume between Case 1 and the base case for both proteins was 0.4 mL. These statistics indicate that for Case 1 parameter variations larger than 20% will yield a significantly different result. These results indicate that the SMA model is sensitive to the 20 % changes and beyond for Case 1.

CONCLUSIONS

This work employed the Steric Mass Action (SMA) model to examine lot-to-lot variability in four columns of Pharmacia Source 15S materials. This study was conducted using the exact same procedures for all four columns. Under isocratic conditions protein retention times were quite similar at moderate salt concentrations. At low salt concentrations the retention times were slightly different between the two lots of stationary phase materials. Under gradient chromatographic conditions with moderate gradient slope, the results for all four columns presented no measurable difference in the retention times of the two proteins. For shallow gradient conditions, a slight difference could be observed between the two lots of materials. Under frontal chromatographic conditions, the breakthrough volumes of the proteins were essentially the same at the same salt concentration and protein concentration for all four columns. Under overloaded linear gradient conditions, the non-linear peaks for both proteins were very similar in all four columns. In displacement chromatography, the displacer breakthrough volumes and the protein elution times were essentially the same in all four columns. However, experimental variability in these displacement experiments make it difficult to determine subtle differences in the displacement behavior between lots. Finally, these results indicate that the SMA model is well suited for predicting the performance of these systems as well as the impact of lot-to-lot variations. It is anticipated that the measurement of SMA parameters and use of the model to predict chromatographic behavior of proteins could become a useful methodology for assuring quality control of various lots of chromatographic materials.

In general, when large variations in the chromatographic columns parameters occur, it can be expected that the SMA model will be sensitive to these changes under overloaded conditions. Displacement simulations exhibited lower standard deviations than overloaded linear gradient. Eluting peaks in overloaded linear gradient are not as sharp as in displacement chromatography in which the different compounds exit the column as square zones, which can explain the larger standard deviations for non-linear gradient chromatography.

When more difficult problem mixtures undergo overloaded linear gradient, it is expected that the gap between the components will be small or nonexistent. Therefore, larger parameter variations will be needed to achieve similar results as the ones obtained in this work.

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