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Recent Advances in the Theory and Practice of Displacement Chromatography

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RECENT ADVANCES IN THE THEORY AND PRACTICE OF DISPLACEMENT CHROMATOGRAPHY

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ABSTRACT

Displacement chromatography has been shown to be a powerful technique for the simultaneous concentration and purification of biomolecules. Despite its distinct advantages, the displacement mode of chromatography has remained a relatively unknown technique. This is rapidly changing, however, as evidenced by the increasing number of papers on displacement chromatography in the literature and presentations at major conferences during the past few years. Much of this work has been reviewed by Frenz and Horvath[1]. In fact, there has been a quiet revolution in the perception of the preparative chromatographic community with respect to displacement chromatography. In this report, we will present some of the more recent advances, post 1987, in the theory and practice of the technique. Theoretical work on ideal

and non-ideal displacement chromatography will be presented as will experimental work on the displacement purification of biomolecules on a variety of stationary phase materials. Preliminary work on the scale-up of the process will be examined along with novel hybrid techniques such as continuous displacement chromatography. Finally, we will offer some perspective on future research needs for displacement chromatography.

INTRODUCTION

The development of efficient bioseparation processes for the production of high-purity biopharmaceutical products is a significant challenge facing the biotechnology industry today. While many new technologies are presently under active investigation for various stages of the downstream processing scheme, chromatography remains the major player in industrial bioseparations[2]. High performance liquid chromatography (HPLC) has proven eminently suitable for the analysis of complex mixtures[3], however, the direct scale-up of analytical separations is generally not sufficient for process-scale chromatography. Whereas emphasis is placed on resolution and analysis time in analytical separations, the critical parameters in preparative chromatography are the amount of material isolated per unit time at a specified level of purity (throughput) and the economics of the process[1, 4-7]. Since the objectives and constraints of analytical and preparative chromatography are quite different, it is critical that novel engineering approaches be employed to fully exploit the high

selectivity obtained with analytical chromatography for process-scale bioseparations.

To date, preparative chromatography has been conducted primarily in the elution mode under mass and/or volume overloaded conditions in order to increase product throughput[4,5,8-10]. In volume overloading, the sample concentration is maintained in the linear region of the isotherm and the volume is increased until the throughput is optimized[4,5,10]. A fundamental problem with this technique is the under-utilization of the column and the corresponding low throughputs. In mass overloading, the sample concentration is increased beyond the linear adsorption region[4,5,10,11]. In both volume and mass overloading, the peak shapes deviate from the gaussian profiles obtained in linear chromatography. Under conditions of volume overloading, the peaks have a flat top and are symmetrical in shape[6,10]. On the other hand, with mass overloading, the concavity of typical adsorption isotherms encountered in liquid chromatography results in a "self-sharpening" boundary at the front and a diffuse boundary at the rear of the substance peak[10,12-14]. Thus, the overloaded condition results in significant tailing of the peaks with a concomitant loss of separation efficiency. Furthermore, elution chromatography results in significant dilution of the feed components during the separation process.

Displacement chromatography is rapidly emerging as a powerful preparative bioseparation technique due to the high throughput and purity associated with the process[1,5,15-16]. This technique offers distinct advantages in preparative chromatography

as compared to the conventional elution mode[16-18]. The process takes advantage of the non-linearity of the isotherms such that more feed can be separated on a given column with the purified components recovered at significantly higher concentrations[17,19]. Furthermore, the tailing observed in elution chromatography is greatly reduced in displacement chromatography due to self-sharpening boundaries formed in the process[17,19]. Whereas in elution chromatography the feed components are diluted during the separation, the feed components are often concentrated during displacement chromatography[16,17,20]. These advantages are particulary significant for the isolation of biopolymers from dilute solutions such as those encountered in biotechnology processes.

Displacement chromatography can be carried out using existing chromatographic systems with minor modifications to enable the sequential perfusion of the column with the carrier, feed, displacer, and regenerant solutions. A schematic of the displacement chromatographic apparatus is depicted in Figure 1[20]. In displacement chromatography, a front of displacer solution traveling behind the feed, drives the separation of the feed components into adjacent pure zones. The column is first equilibrated with a carrier in which the components to be separated have a relatively high affinity for the stationary phase. A feed mixture is then pumped into the column followed by a displacer solution. During the introduction of the feed, the components saturate the stationary phase at the top of the column and frontal chromatography occurs. The displacer is selected such that it has a higher affinity for the stationary phase than any of the feed components[1,17,19]. As the displacer front

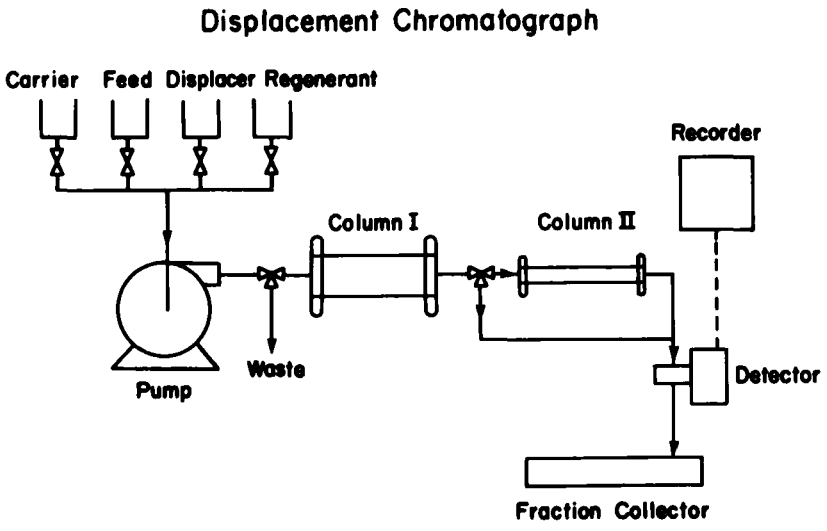


FIGURE 1. Schematic of a displacement chromatograph. (Reproduced with permission from ref. #20, published by Gordon & Breach)

traverses the column, the feed components are displaced and separated as they compete for the adsorption sites on the stationary phase. Under appropriate conditions, a displacement train is formed containing the feed components as adjacent bands all moving with the velocity of the displacer front which can be determined from a material balance to be[17,21]:

$$u_D = u_o/[1 + \Phi (q_D/c_D)] \quad (1)$$

where u_0 is the mobile phase velocity, Φ is the phase ratio, q_D and c_D are the stationary phase and mobile phase concentration of the displacer, respectively.

The order of the zones corresponds to the increasing affinity of the components for the stationary phase. The concentration of each component in the final displacement train is determined solely by its adsorption isotherm and the concentration and isotherm of the displacer[17,22,23] as shown in Figure 2. The operating line depicted in Figure 2 defines the final isotachic condition which is given by the expression:

$$q_1/c_1 = q_2/c_2 = \ldots = q_n/c_n \qquad (2)$$

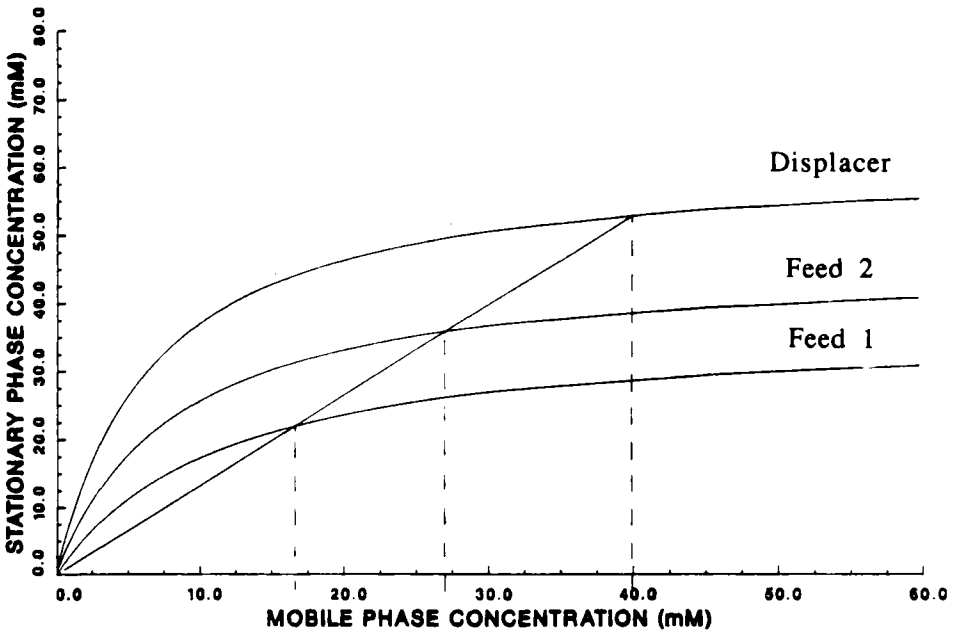
Thus, when the concentration of the inlet feed components are less then those dictated by the final isotachic conditions, the displacement chromatographic process results in significant concentration of the feed components during the separation process. The width of each zone is proportional to the amount of the component present in the feed. Upon the emergence of displacer, the column is regenerated by removal of the displacer with an appropriate solvent followed by re-equilibration with the carrier.

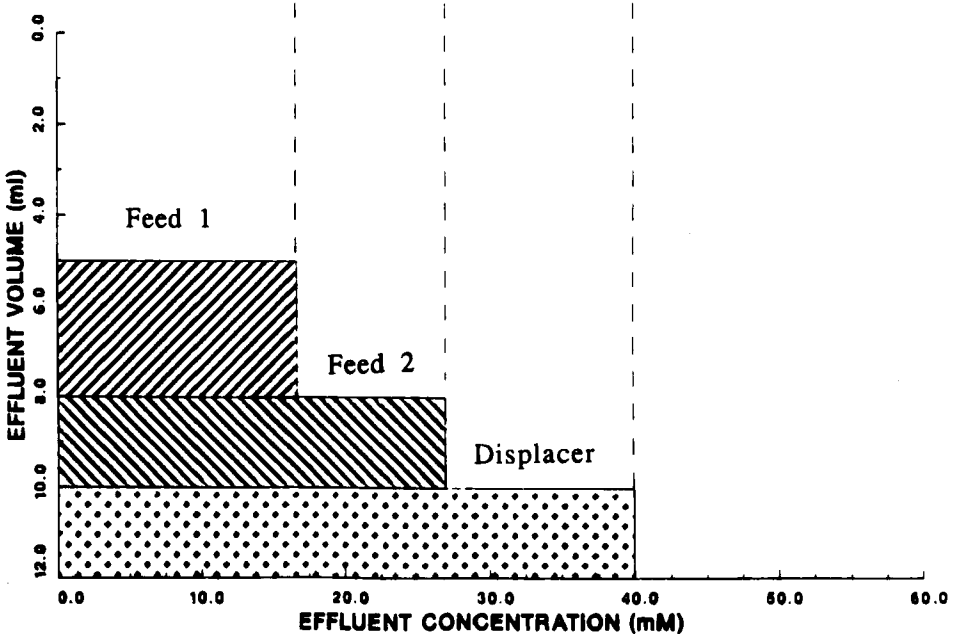
Although the physico-chemical basis of the displacement mode of chromatography was established by Tiselius[24] in 1943, it is only recently that displacement chromatography has been actively investigated for the simultaneous concentration and purification of biomolecules. Research into the theory and practice of displacement chromatography has been rapidly accelerating during the 80's. Much of this work has been reviewed in the excellent review paper by Frenz and Horvath[1]. In the present manuscript, we will complement that paper by focusing on advances in displacement chromatography post 1987. Experimental work on the purification of amino acids,

peptides, antibiotics, and proteins by displacement chromatography as well as theoretical work on modeling non-ideal displacement chromatography will be presented. The effect of flow rate, crossing isotherms, and particle diameter on displacement behavior will also be examined. Finally, we will offer some perspective on future research needs for displacement chromatography.

BACKGROUND

The displacement mode of chromatography was first recognized in 1906 by Tswett[25] who noted that sample displacement occurs under conditions of overloaded elution chromatography. In 1943, Tiselius[24] developed the classifications of frontal, elution, and displacement chromatography. The forties and fifties saw early development and applications of displacement chromatography in a variety of areas. Claesson[26] used displacement chromatography for the separation of sugars in 1948. Tiselius et al[27] introduced the concept of "carrier displacement chromatography" in which spacer substances are eluted between the isotachic zones of the feed components. Spedding et al[28] used displacement chromatography for the separation of rare earth complexes. In fact, the separation of isotopes by ion exchange displacement chromatography has continued to be an active area of research to the present day. Separation of proteins and protein hydrolyzates by displacement chromatography was carried out by Partridge[29]. However, these early attempts to develop the displacement mode into a practical bioseparation process were significantly limited by the relatively inefficient chromatographic systems of the time[16]. Progress in HPLC





with the availability of sorbents exhibiting rapid kinetics and mass transfer stimulated work on displacement chromatography using analytical HPLC equipment[19,20,30-36]. In this background section we will briefly review some of the advances in modern displacement chromatography occuring prior to 1987.

Horvath and co-workers have made significant contributions to the development of the technique for the simultaneous purification and concentration of biomolecules. Horvath et al[30] separated 50 mg of the amino acids histidine and arginine on a cation exchange column using benzyltributlyammonium chloride (BTBA) as the displacer. They also reported on the purification of peptides and nucleotides by displacement chromatography. 50 mg of a four component peptide mixture containing valine-valine, glycine-leucine, leucine-valine, and phenylalanine-valine were purified using tetrabutylammonium bromide (TBA) to displace the peptides from a reversed-phase analytical column. Nucleotides and nucleosides were also purified by displacement chromatography using butanol and BTBA, respectively, as the displacers on a reversed-phase system. In fact, the majority of displacements carried out on small biomolecules to date have employed reversed-phase adsorbent materials.

FIGURE 2. Schematic representation of isotherms of the feed components and the displacer along with the operating line and the corresponding fully developed displacement train. The two component feed mixture (components 1 and 2) are displaced by the displacer D. (Reproduced with permission from ref. #74, published by American Institute of Chemical Engineers.)

Displacement chromatography was employed to purify peptide diastereomers[35]. Butanol was used as the displacer in the purification of N-benzoyl-D-phenylalanyl-L-alanyl-L-proline and N-benzoyl-L-phenylalanyl-L-alanyl-L-proline on a reversed-phase column. An experimental optimization of the throughput was also carried out for the purification of hydrolysis products of methylfurylbutyrolactone, a potential anticancer drug.

An automated recycle enzyme reactor-displacement chromatograph system has been developed by El Rassi and Horvath[33] for the synthesis and purification of dinucleotides. Cramer et al[37] used a similiar system for the simultaneous synthesis and purification of di-peptides. These tandem reactor-separator systems may have significant potential for the synthesis and purification of complex biochemical substances.

Displacement chromatography was also employed for the purification of several other classes of compounds. Kalasz and Horvath[34] investigated the displacement of polymyxins using octaldecyldimethyl-ammonium-chloride as the displacer on reverse-phase columns. Torres et al have successfully employed displacement chromatography for the resolution of ten polyethyleneglycol (PEG) oligomers present in carbowax PEG-400[35]. Torres et al[38-42] also purified a variety of proteins by carrier displacement chromatography on an anion exchange column using carboxymethyldextrans as the displacers.

For a complete treatment of the history of displacement chromatography, the reader is referred to the review by Frenz and Horvath[17]. In this report we will present recent advances (post

1987) in the theory and practice of displacement chromatography and discuss future research needs in this area.

THEORETICAL ASPECTS OF DISPLACEMENT CHROMATOGRAPHY

The theory of linear chromatography is well established and analytical expressions can be readily obtained for the effluent profiles under a variety of operating conditions. However, at elevated concentrations the adsorption process becomes non-linear and competition of the feed components for the adsorption sites occurs. In fact, displacement chromatography takes advantage of the non-linear competitive adsorption process to produce self-sharpening concentration waves characteristic of the process. Under non-linear competitive adsorption conditions an analytical solution of the complete chromatographic process is not possible at present.

Helfferich and Klein[43-45] developed a model of displacement chromatography which assumes equilibrium adsorption, plug flow and constant separation factors. In their ideal treatment, a mathematical transformation was employed to convert the system of coupled partial differential equations describing the displacement chromatographic process to a set of algebraic equations. This greatly facilitates the calculation of boundary velocities and the trajectories of concentration boundaries in the column. Rhee and co-workers[46,47] developed a similiar approach to solve the mass balance equations.

Frenz and Horvath[19] have employed this technique to construct development graphs which can be used to predict the effluent concentration profiles in ideal displacement chromatography. The resulting simulations accurately predicted the displacement behavior

of the purification of resorcinol and catechol using phenol as the displacer. Yu and Wang[48] and Geldart et al[49] have also employed this h-transform technique to study the dynamics of ideal elution and displacement ion-exchange chromatographic systems.

The ideal theory of displacement chromatography neglects the effects of mass transport and non-equilibrium adsorption. However, the optimization and scale-up of the process requires an accurate accounting of non-ideal effects in displacement systems. Katti and Guiochon[50] have used the numerical dispersion of a finite difference technique to simulate the effects of mass transfer and axial dispersion in displacement chromatography. Their "semi-ideal" model was used to examine the effects of displacer concentration and column length on the process. Figure 3 shows the effect of column length on displacement development in non-ideal displacement chromatography. As seen in the figure, once displacement development is achieved, additional column length does not result in any change in the displacement profile due to the formation of a constant pattern.

Phillips et al[51] have developed a mathematical model for the simulation of non-ideal displacement chromatography. The model incorporates finite mass transport to the solid adsorbent by using a linear driving force approximation with a coupled external film and internal pore mass transfer coefficient. The model assumes equilibrium adsorption at the fluid-solid interface and employs an average concentration of the adsorbed species in the stationary phase.

The material balance for species i in the fluid phase can be written as:

$$\frac{\partial c_i}{\partial t} + u_o \frac{\partial c_i}{\partial z} + \frac{1-\varepsilon}{\varepsilon} \frac{\partial q_i}{\partial t} - D_i \frac{\partial^2 c_i}{\partial z^2} = 0 \quad i = 1,2,...,N \qquad (3)$$

$$\frac{\partial \bar{q}_i}{\partial t} = k_i(q_i^* - \bar{q}_i) \qquad i = 1,2,...,N \qquad (4)$$

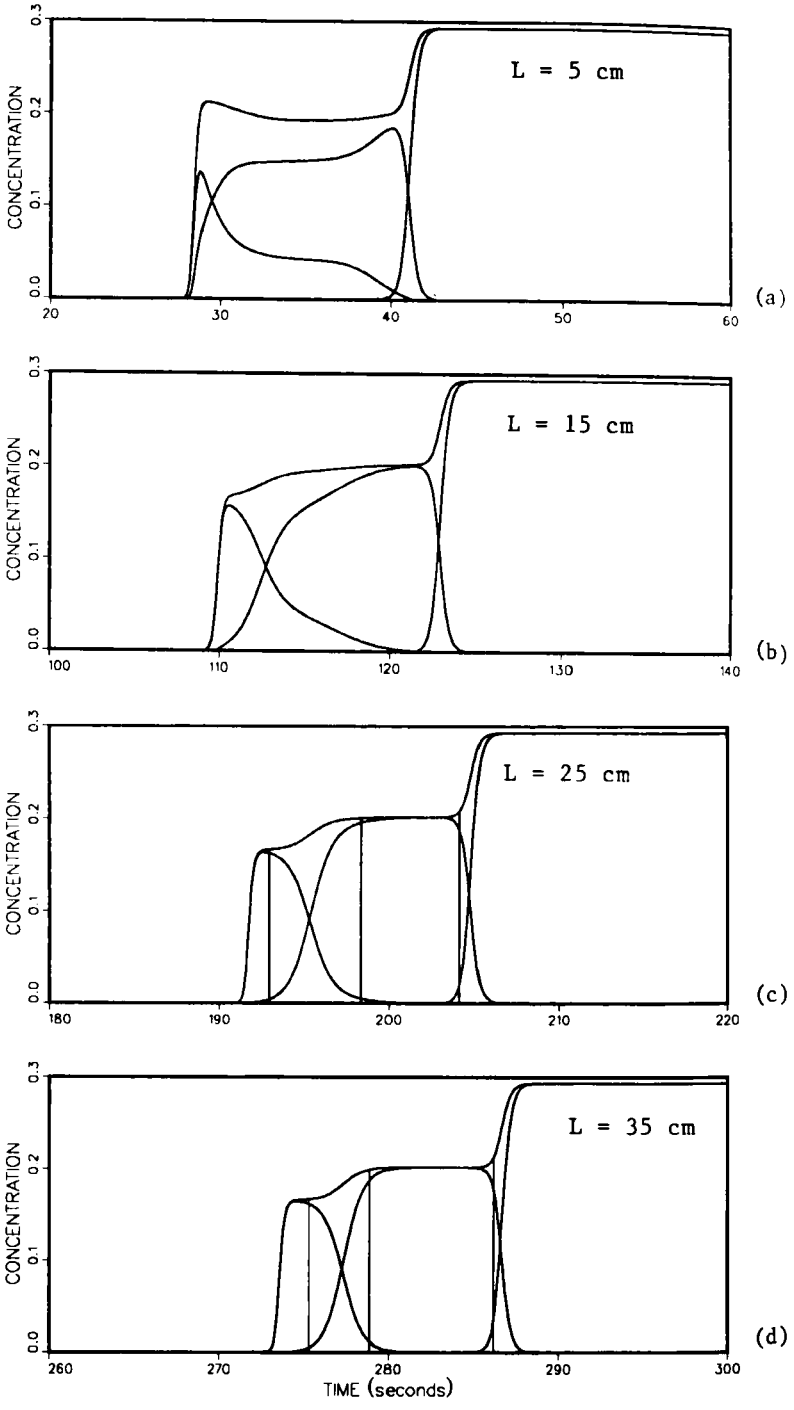
where u_0 represents the interstitial velocity and q_i^* represents the stationary phase concentration of species i in the absence of mass transport limitations. The axial dispersion coefficient for species i, D_i, includes longitudinal spreading due to both molecuar diffusion and eddy dispersion[52,53]. The stationary phase accumulation term, $\frac{\partial \bar{q}_i}{\partial t}$, is approximated using a linear driving force model which employs an overall effective mass transfer coefficient, k_i, to describe both the film and intraparticular mass transport.

The equilibrium adsorption of the multicomponent mixture to the chromatographic surface was represented by the multicomponent Langmuir isotherm[54-57]:

$$q_i^* = \frac{a_i c_i}{1 + \sum_{j=1}^{N} b_j c_j} \qquad (5)$$

where a_i and b_i are the Langmuir parameters for species i and N is the number of components in the mixture. The system of equations described by equations 1 and 2 are coupled through the multicomponent adsorption isotherms.

The material balance equations were rewritten in dimensionless form and the following dimensionless variables were



employed to facilitate the study of dispersion and mass transport effects in displacement systems:

$$t = \frac{u_o t}{L}, \quad x = \frac{z}{L}, \quad Pe_i = \frac{u_o L}{D_i}, \quad St_i = \frac{k_i L}{u_o}$$

where t, x, Pe, and St represent dimensionless time, axial position, Peclet and Stanton number, respectively.

A finite difference numerical technique was employed to approximate the system of coupled non-linear partial differential equations and the model was used to simulate the effluent concentration profiles under various displacement chromatographic conditions. While the displacement profile was fairly insensitive to axial dispersion, slow mass transfer rates had a significant dispersive effect on the self-sharpening boundaries generated in displacement chromatography. For rapid mass transport, St=1600, the displacement effluent profile approached that obtained under ideal chromatographic conditions as shown in Figure 4a. Under limiting conditions of mass transport, St=200, the concentration shock waves separating the displacement zones become more diffuse, resulting in increased zone overlap as illustrated in Figure 4b. Under these conditions, the mass transport limitations result in excessive zone overlap, significantly decreasing the amount of purified material obtained during the displacement process.

FIGURE 3. Simulations of displacement profiles as a function of column length for displacer concentration of 0.3 mM. (a) 5; (b)15; (c) 25; (d) 35 cm column. (Reproduced with permission from ref. #50, published by Elsevier)

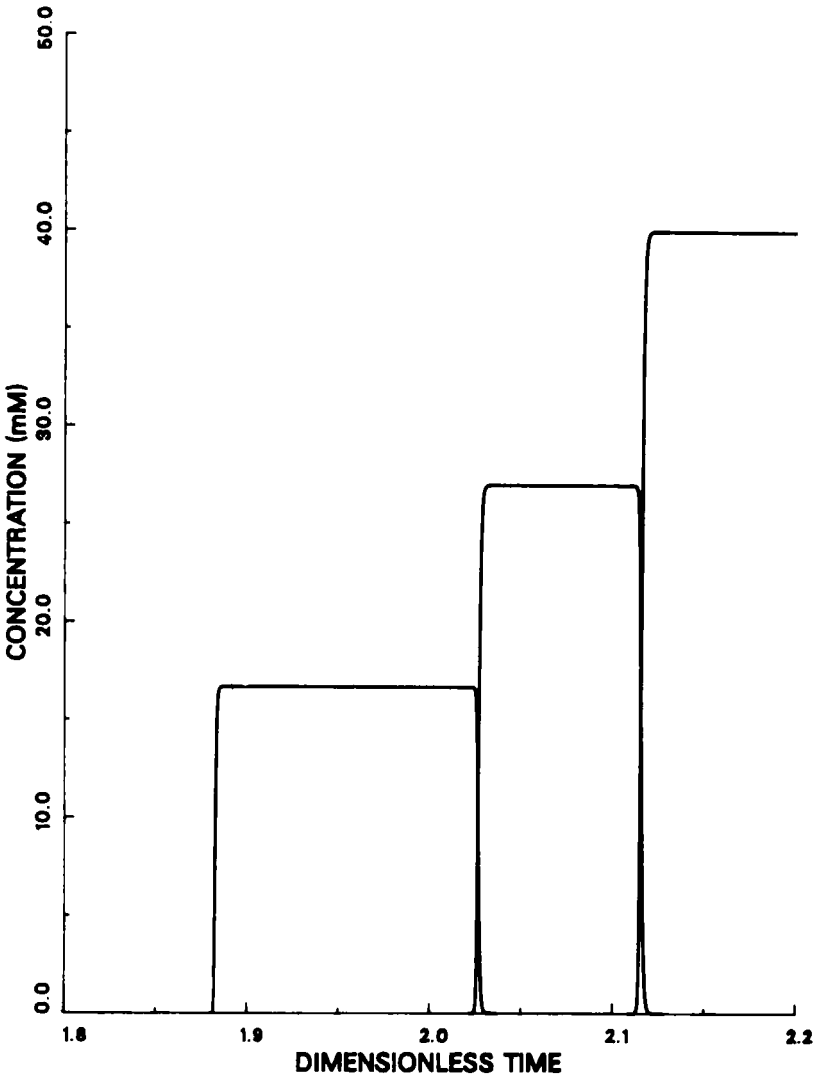


FIGURE 4. Effluent displacement profile obtained with a Stanton number of a) 1600 and b) 200 respectively. Simulation conditions are given in ref. #51. (Reproduced with permission from ref. #51, published by Elsevier)

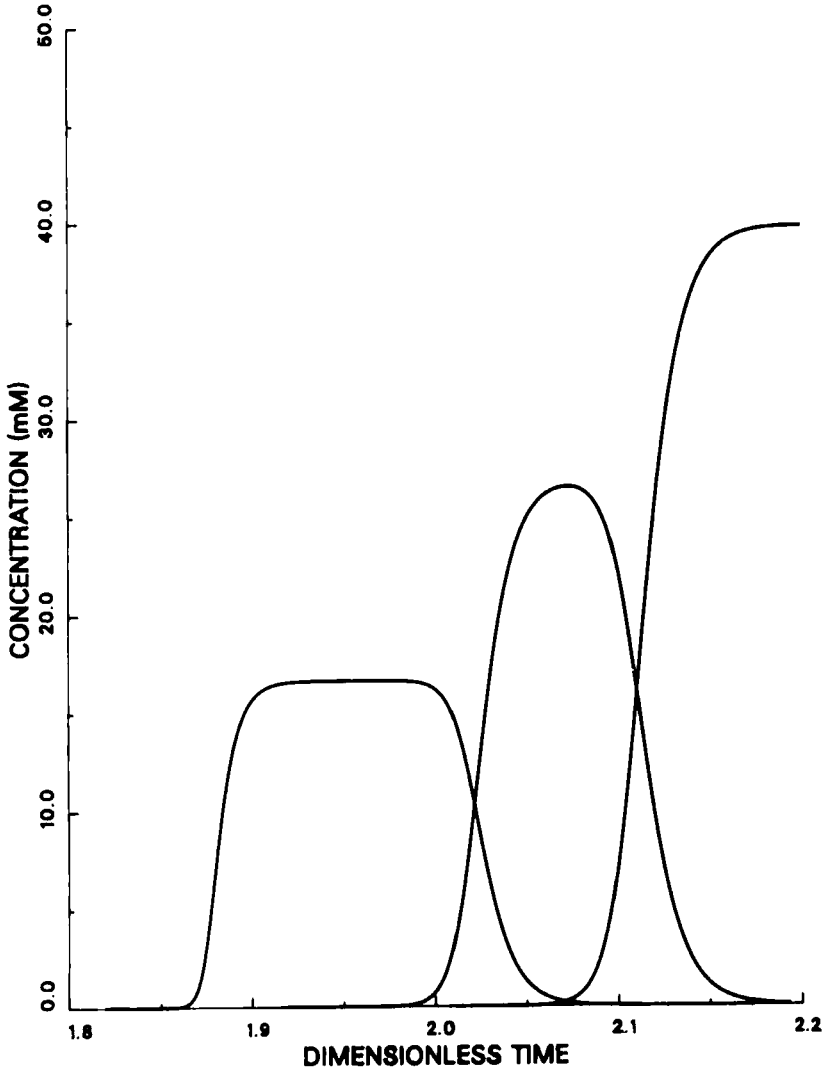


FIGURE 4B.

The model was employed to examine the throughput of these systems as a function of feed load, displacer concentration, and

interstitial velocity. Under non-ideal conditions, a unique optimum value of these operating parameters existed which maximized the product throughput.

The influence of mass transfer limitations on product throughput were also investigated as a function of particle diameter, interstitial velocity and solute diffusivity. The effects of interstitial velocity and particle diameter on the throughput of a small biomolecule are illustrated in Figure 5a. At low velocities, the throughput is independent of particle diameter. For small particle diameter systems, these simulations indicate that the throughput of small biomolecules can be dramatically increased by operating at elevated velocities. As the particle diameter increases, however, the increased throughput at higher velocities is less pronounced. In fact, for large particle diameter systems, the throughput obtained at high velocities can be lower than that attained at lower velocities.

In Figure 5b, the effects of solute diffusivity and particle diameter on the throughput are examined. This analysis assumes that the column length is kept constant. Throughput is insensitive to the particle diameter for small solutes, indicating that large particles can be employed for these separations with an accompanying reduction in capital costs. On the other hand, as the molecular dimensions of the solute increase, the throughput becomes an increasingly stronger function of the particle diameter under these simulation conditions.

Carta et al[58] have employed a local-equilibrium stage model to investigate the dynamics of amino acid separations in ion exchange displacement chromatography. Their model was seen to accurately predict the behavior of these displacement systems as seen in Figure

6. Morbidelli et al[59,60] have modelled displacement chromatography in gas adsorption systems using both the equilibrium theory of multicomponent chromatography and a pore-diffusion model. Their predictions were in good agreement with experimental results obtained in the displacement of para- and ortho- chlorotoluene.

There is presently much activity in the area of modeling non-linear, non-ideal chromatographic systems[61-64]. The availability of efficient numerical techniques now enable researchers to readily solve complicated systems of equations describing various non-linear chromatographic processes. However, the accurate determination of parameters involved in these models as well as an understanding of the concentration dependence of these parameters is not well in hand at present. Clearly, much work remains to be done on the modelling and optimization of displacement as well as other non-linear chromatographic techniques.

EXPERIMENTAL

Displacement Chromatography of Amino Acids

Carta et al[58] have recently separated the amino acids glutamic acid, valine, and leucine by ion exchange displacement chromatography using NaOH as the displacer as shown in Figure 6. These ion-exchange displacements resulted in zones of pure material at a characteristic pH for each amino acid. Amino acids have also been recently purified by continuous displacement chromatography as described below. Vigh et al[66] have used displacement chromatography for the separation of a racemic mixture of dansyl leucine using 2,4-dinitrophenol as the displacer on a β-cyclodextrin support.

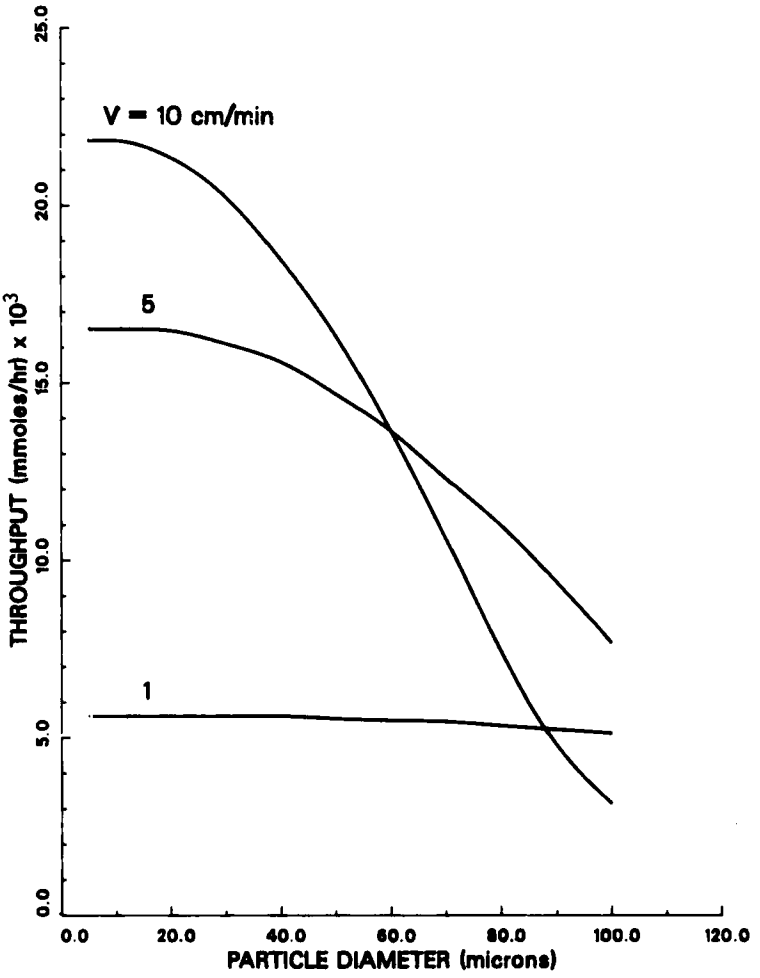
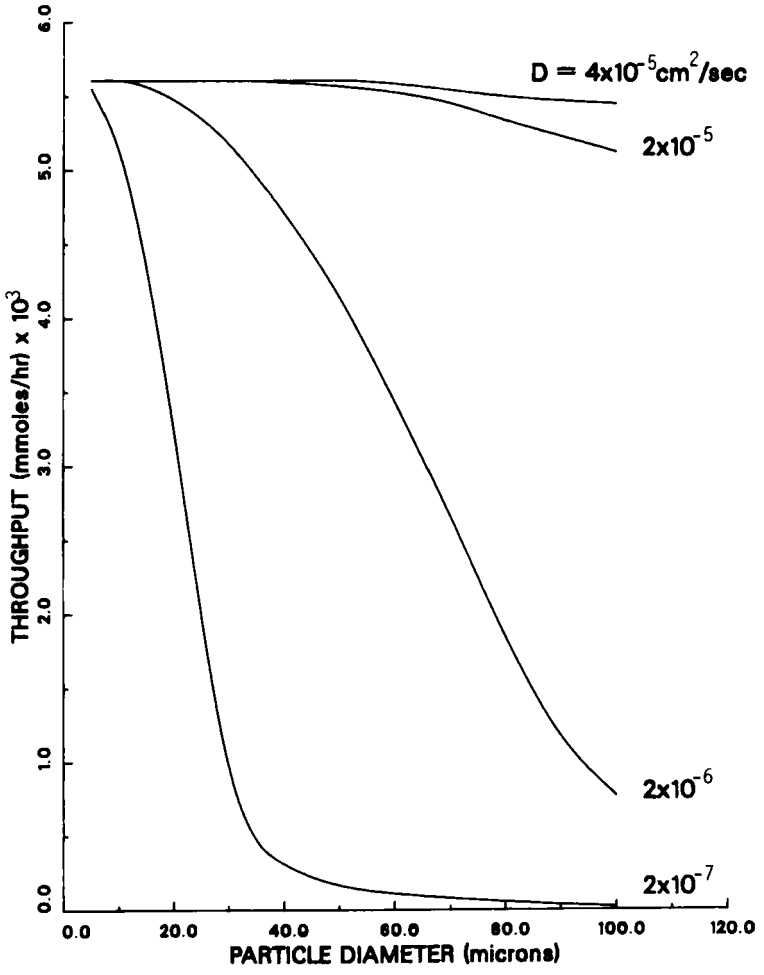


FIGURE 5. The effect of particle diameter on product throughput at: (a) a constant solute diffusivity of 2 x 10^{-5} cm^2/sec and (b) a constant intersitial velocity of 1 cm/min. (Reproduced with permission from ref. #51, published by Elsevier)



Displacement Chromatography of Peptides

Cramer and Horvath[20] have used displacement chromatography for the isolation of peptides synthesized with

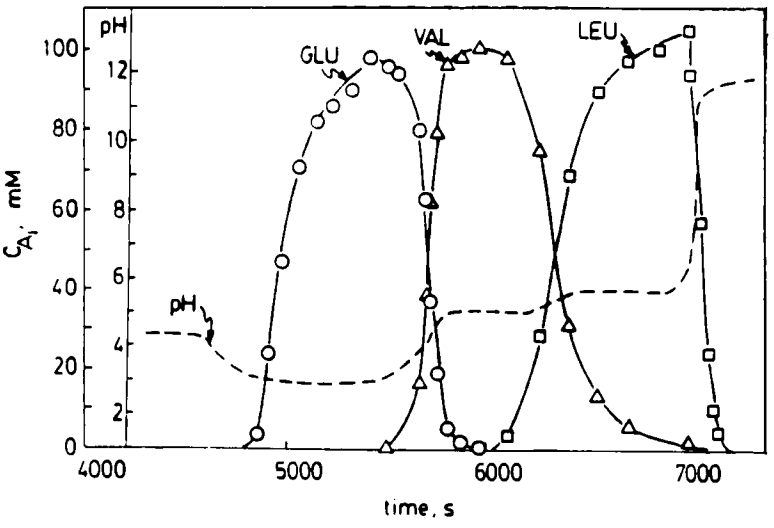


FIGURE 6. Displacement chromatography of a mixture of amino acids. Column: Dowex 50X8; flow velocity: 5.2 cm/s; displacer: 0.1 N NaOH; feed: 0.015 M each of Leu, Val and Glu. (Reproduced with permission from ref. #58, published by American Institute of Chemical Engineers.)

immobilized carboxypeptidase Y as shown in Figures 7 and 8. The displacers 2-(2-butoxyethoxy)ethanol (BEE) and decyltrimethyl-ammonium bromide were used for the purifications of the di- and tri-peptides, respectively. The earlier eluting components emerged ahead of the displaced feed components since their adsorption isotherms lied below the operating lines under the conditions of the experiment. These displacement separations also resulted in greater than 50-fold concentration of the displaced peptides. The di-peptide displacement was scaled-up to enable the purification of 5 grams in

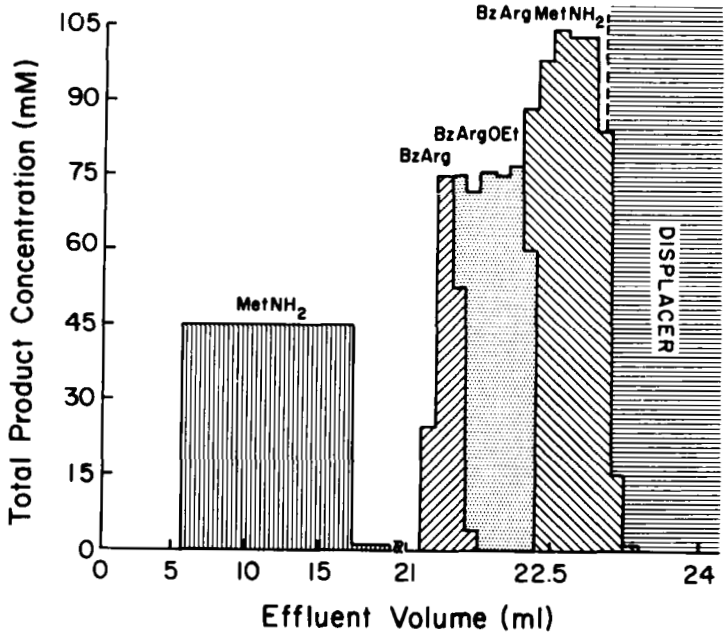


FIGURE 7. Displacement chromatogram obtained in the purification of Bz-Arg-Met-NH_2 from 15 ml of feed. Column: 250 x 4.6 mm I.D. packed with 10 μm C-18; carrier: 0.1 M phosphoric acid, pH, 2.2; displacer: 247 mM 2-(2-butoxyethoxy) ethanol (BEE) in carrier; temperature, 50 ^{0}C; feed: 5.2 mM $BzArgMetNH_2$, 1.3 mM BzArg, 5.1 mM BzArgOEt, 45 mM $MetNH_2$. (Reproduced with permission from ref. #20, published by Gordon & Breach)

a single run. Cetyltrimethylammonium bromide (cetramide) was also employed for the simultaneous purification and concentration of tetrapeptides on a reversed-phase system.

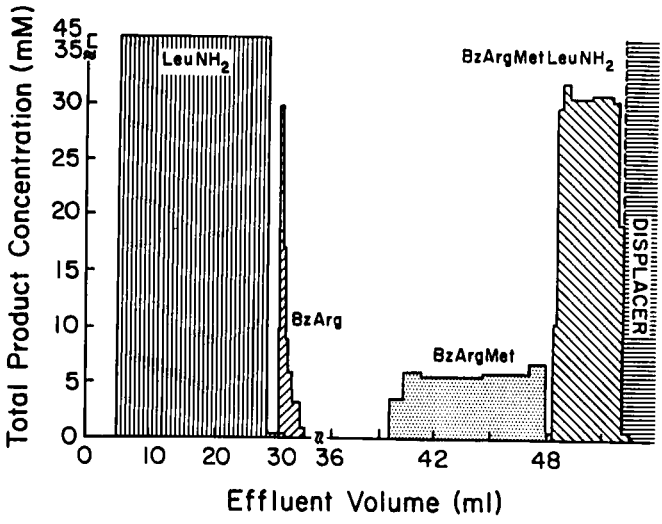
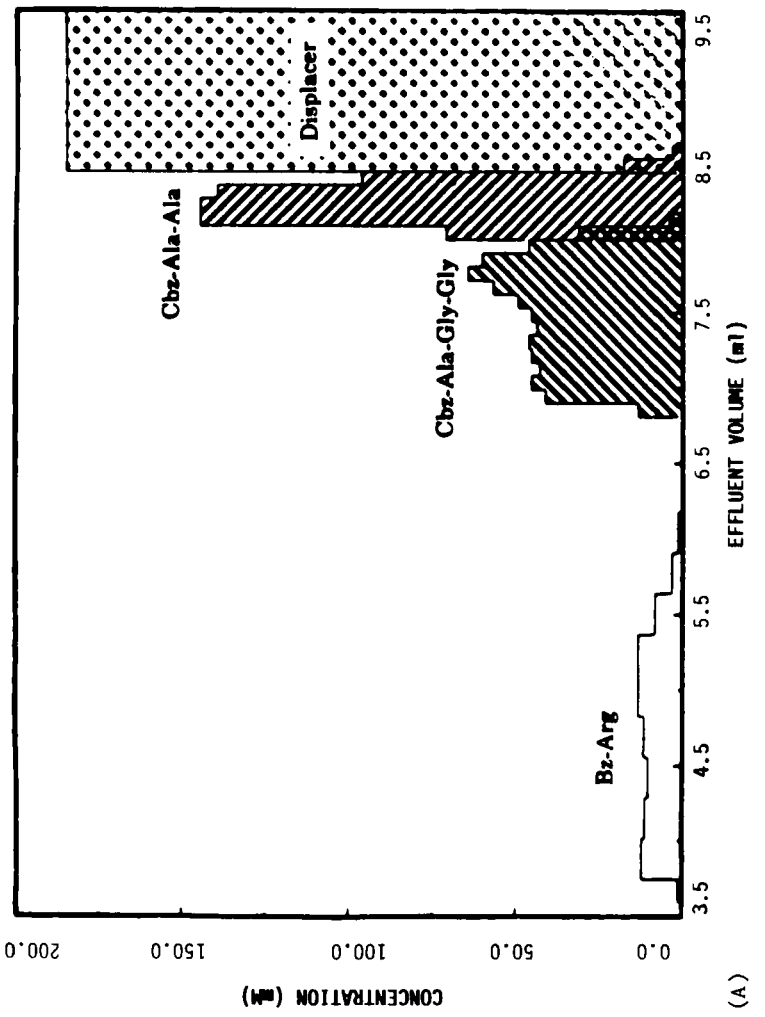


FIGURE 8. Displacement chromatogram obtained in the purification of Bz-Arg-Met-Leu-NH_2 from 30 ml of feed. Column: 400 x 4.6 mm I.D. packed with 5 μm C-18; carrier: 0.1 M phosphoric acid, pH 2.2; displacer: 40 mM decyltrimethylammonium bromide in a solution of 15% (v/v) methanol in carrier; feed temperature: 22 ^{0}C; displacer temperature: 50 ^{0}C; feed: 0.9 mM BzArgMet, 0.2 mM BzArg, 5.0 mM BzArgMetLeuNH_2 and 45 mM LeuNH_2. (Reproduced with permission from ref. #20, published by Gordon & Breach)

The use of organic modifiers in the carrier along with an elevated column temperature of 45^o C enabled the efficient displacement of relatively hydrophobic peptides using BEE as the displacer as shown in Figure 9a[16]. This displacement separation on

the analytical reversed-phase column was repeated at an elevated flow rate of 1.0 ml/min to increase the throughput of the system[16]. As shown in Figure 9b, the time required for the separation was reduced from 127 to 8.3 min with minimal adverse affect on the seperation efficiency. This corresponds to the simultaneous purification and concentration of 40 mg of peptides in 8.3 min on an analytical reversed-phase column. While this does not include the time required for regeneration and re-equilibration with the carrier, it is expected that total cycle time for this separation would be less than 30 min. Thus, under these conditions, the throughput of the displacement chromatographic process can be dramatically increased by operating at elevated flow-rates.

Viscomi et al[36] have purified biologically active α- and β-melanocyte stimulating hormones from complex mixtures by displacement chromatography. These separations employed benzyldimethyldodecylammonium bromide as the displacer on analytical reversed-phase columns. Hodges et al[67,68] have purified peptides using combinations of frontal, step gradient, and sample displacement chromatography as shown in Figure 10. Sample displacement naturally arises from the competitive binding of the feed components during non-linear elution chromatography. Under appropriate conditions, this displacement-like phenomena can be exploited to improve the separations obtained in non-linear elution systems. Newburger and Guiochon[69] have also employed sample self-displacement for the purification of a variety of biomolecules. While this technique is usually limited to the separation of binary mixtures, under appropriate conditions, a compound of interest can be purified from the other components in a complex mixture by sample displacement.



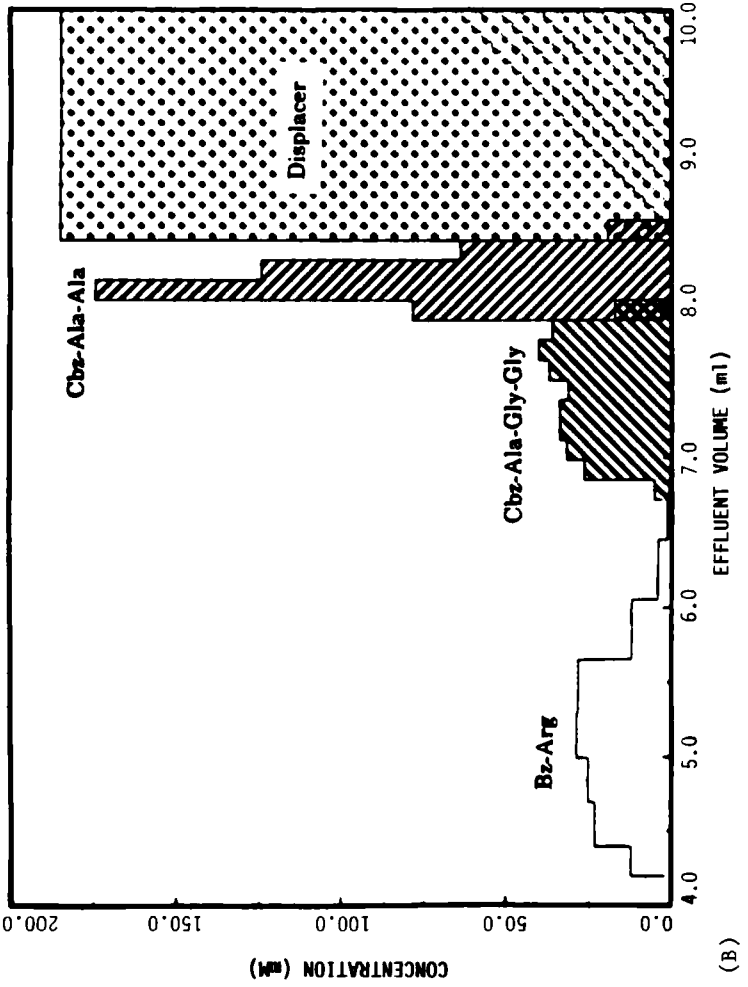
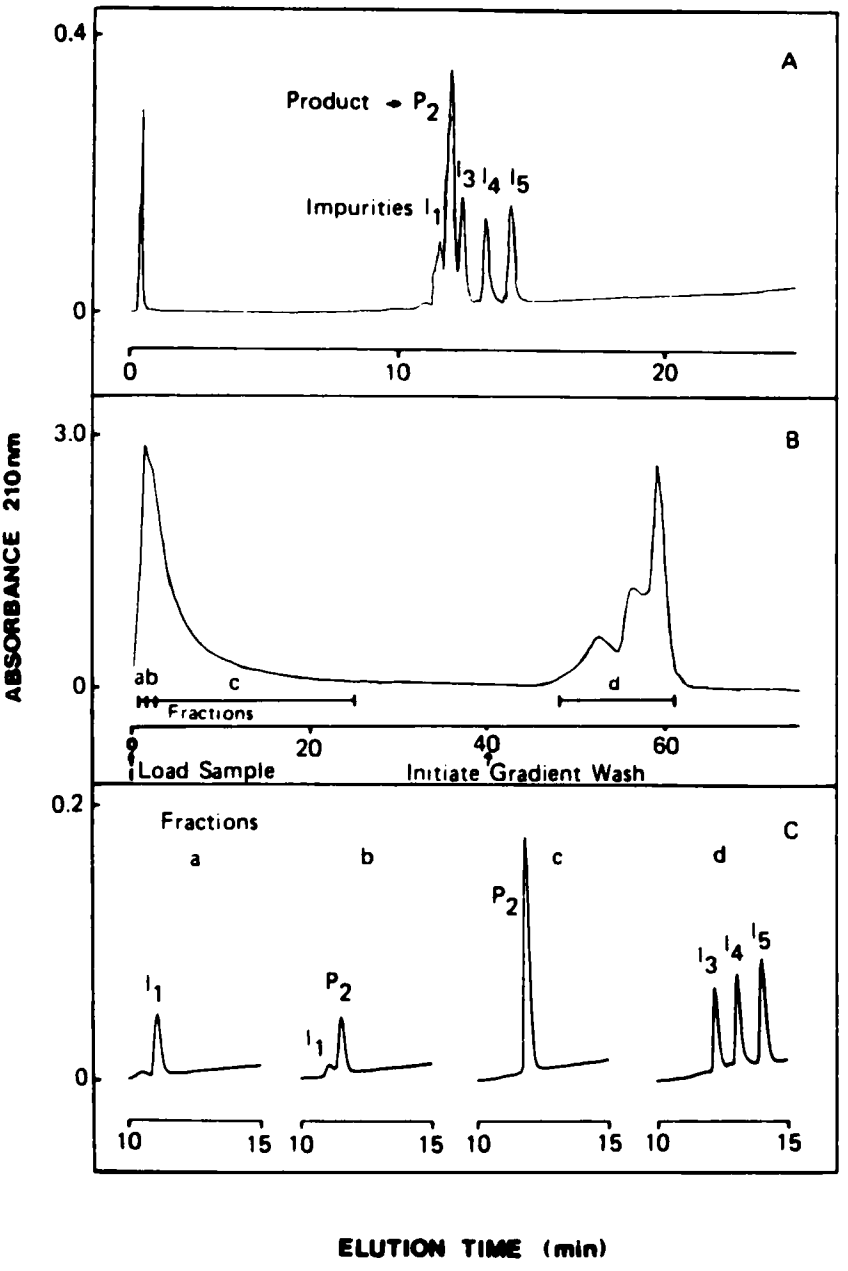


FIGURE 9. Displacement chromatogram of peptide mixture. Column: 250 x 4.6 mm Zorbax ODS; carrier: 40% (v/v) methanol in 50 mM phosphate buffer, pH 2.2; displacer: 30 mg/ml BEE in carrier; temperature: 45 C; flow rate: 0.06 ml/min and 1.0 ml/min for (a) and (b) respectively. (Reproduced with permission from ref. 16, published by Elsevier)



Displacement Chromatography of Antibiotics

For displacement chromatography to have utility for downstream bioprocessing, it must be able to separate complex mixtures of biomolecules obtained from actual processes. Valko et al.[70] have purified oligomycins on reversed-phase columns using palmitic acid as the displacer. The antibiotic cephalosporin C has been separated from the impurities present in a fermentation broth by displacement chromatography on a reversed-phase system as shown in Figure 11[16]. While the more polar impurities eluted ahead of the displacement train, cephalosporin C was well separated from the hydrophobic impurities and was concentrated during the displacement process. This separation illustrates that displacement chromatography can be used for the purification of a desired feed component from a relatively complex fermentation broth mixture. Furthermore, if there is a particular component of interest, the

FIGURE 10. Separation of peptide product (P2) from hydrophobic (I3,I4,I5) and hydrophilic (I1) impurities by sample displacement chromatography. Column: 30 x 4.6 mm I.D. C-8 silica. Panel A: analytical separation profile of peptide mixture. Panel B: preparative separation profile of peptide mixture. Conditions, isocratic elution for 40 minutes followed by linear gradient elution at 1% B/minute. Sample load, 3 mg of P2 and 1 mg each of I1, I3, I4 and I5. Panel C : analytical profiles of fractions a-d from Panel B. (Reproduced with permission from ref. #67, published by Elsevier)

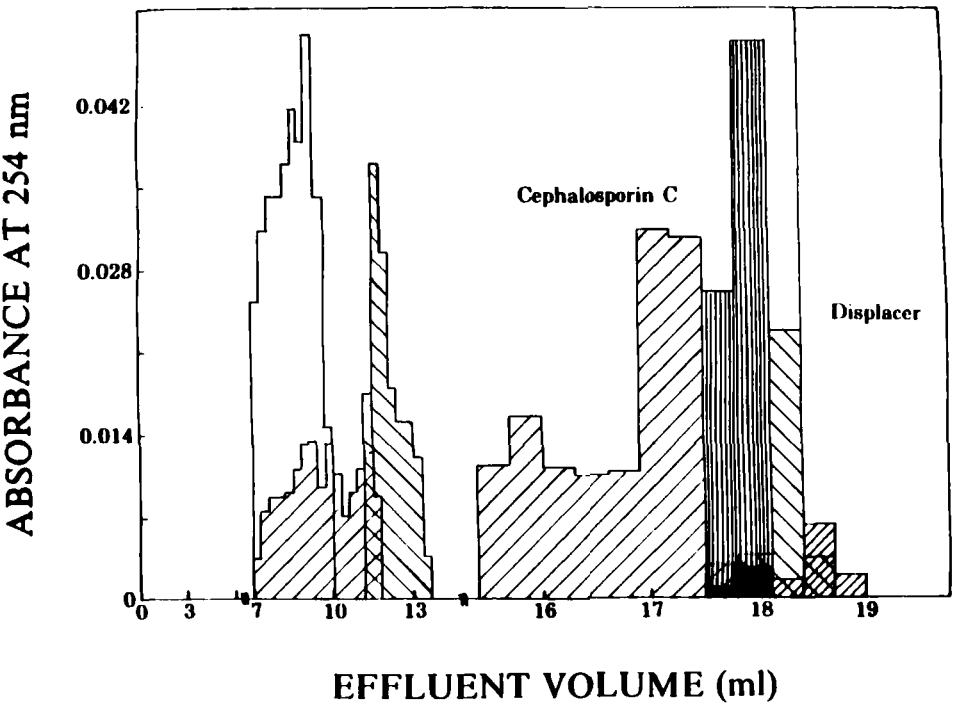


FIGURE 11. Displacement chromatogram of cephalosporin C fermentation broth. Column: 350 x 4.6 mm I.D. packed with 5 μm Zorbax ODS; carrier: 1% (v/v) acetonitrile in 20 mM sodium acetate, pH 5.2; displacer: 40 mg/ml BEE in carrier; flow rate: 1.0 ml/min and 0.1 ml/min for feed and displacer respectively; temperature: 22 ^{o}C and 35 ^{0}C for feed and displacer respectively; feed: 5 ml of fermentation broth. (Reproduced with permission from ref. #16, published by Elsevier)

displacement can be tailor-made to displace the desired compound while eluting many of the less adsorbing impurities. This method and

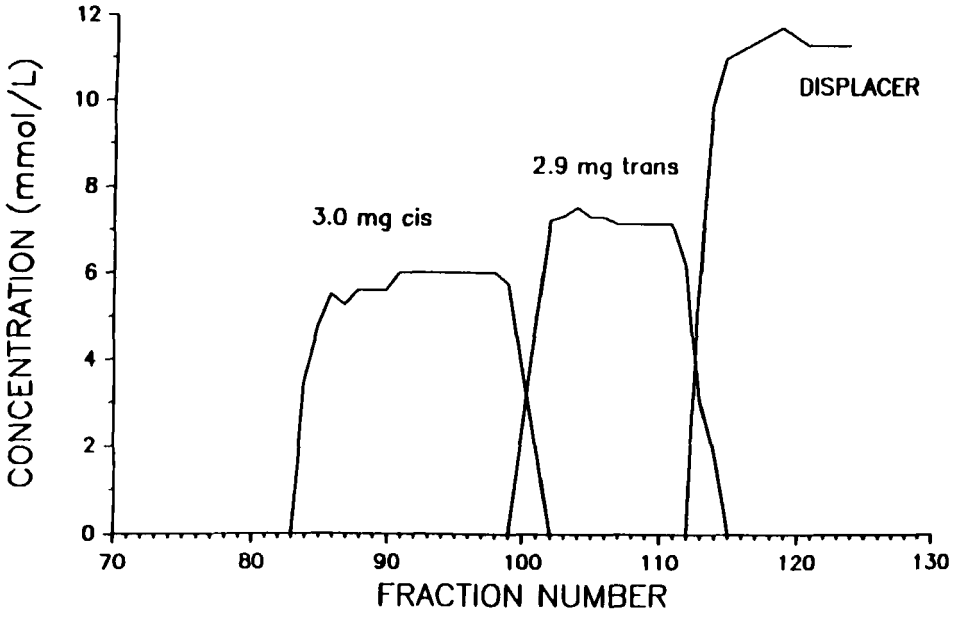


FIGURE 12. Displacement chromatogram of cis and trans isomers of a 6 mg sample of 3-hexen-1-ol. Column: 500 x 4.6 mm I.D. packed with alpha-cyclodextrin silica; carrier: water; displacer: 11.9 mM n-heptanol in carrier; flow rate: 1.0 ml/min; temperature: 30 °C. (Reproduced with permission from ref. #66, published by Elsevier)

other variants of the method can result in a significant improvement in the throughput of the process. Clearly, more case studies on the displacement purification of complex "real" mixtures are required to firmly establish displacement chromatography as a useful technique for difficult bioseparation problems.

Displacement Chromatography on Novel Supports

Vigh et al[65] have used cetramide to displace 1- and 2-napthol from a cyclodextrin-silica column. They also separated the cis and trans isomers of 3-hexen-1-ol on an α-cyclodextrin silica column using n-heptanol as the displacer as shown in Figure 12[66]. This purification of 5.9 mg of cis and trans isomers using a chiral stationary phase is important in that it indicates that the displacement mode has the significant advantage of combining the unique selectivites of these systems with the high throughput and purity obtained in displacement chromatography.

Displacement Chromatography of Proteins

One of the most pressing challenges to the biotechnology industry at present is the efficient purification of therapeutic proteins. Several research groups are presently involved in the development of protein displacement chromatographic systems. In fact, we believe that displacement chromatography may indeed have its greatest impact on the separation of these extremely valuable products.

Torres et al[71] have separated the proteins ovalbumin, α-lactalbumin, and soy bean trypsin inhibitor by carrier displacement on an anion exchange column using carboxymethyldextrans as the displacers. Liao et al[72] have purified the proteins β-lactoglobulin A and B by displacement chromatography using chondroitin sulfate as the displacer on a polymeric anion exchange column. Vigh et al.,[73] have used displacement chromatography to purify insulin on

reversed-phase columns using cetramide as the displacer. A four-component protein mixture containing α-chymotrypsinogen, ribonuclease, lysozyme, and cytochrome C was purified by displacement chromatography using a 20,000 molecular weight cationic polymer (Nalcolyte 7105) to displace the proteins from an analytical strong cation exchange (SCX) column[74]. The resulting displacement profile, shown in Figure 13, demonstrates that the proteins were well separated during the displacement process. Ribonuclease eluted from the column ahead of the well separated and concentrated displacement zones of α-chymotrypsinogen, cytochrome C, and lysozyme. This work demonstrates that displacement chromatography can be readily employed for the simultaneous purification and concentration of relatively complex protein mixtures.

A common perception is that in order for displacement chromatography to be successful, slow linear velocities in the range of 1-3 cm/min must be employed[67,75]. Our work has indicated that displacement systems can be operated at elevated flow rates, under appropriate conditions, with minimal adverse effect on the purity of the products. In addition to the work on peptide displacements described above, we have investigated the effect of flow rate on protein displacement systems[74]. Figure 14a shows the separation of a two-component protein mixture by displacement chromatography at a relatively slow flow rate of 0.1 ml/min (0.9 cm/min). The displacement experiment was repeated at an elevated flow rate of 1.0 ml/min as shown in Figure 14b. Under these conditions, the increased flow rate resulted in slightly increased tailing of the

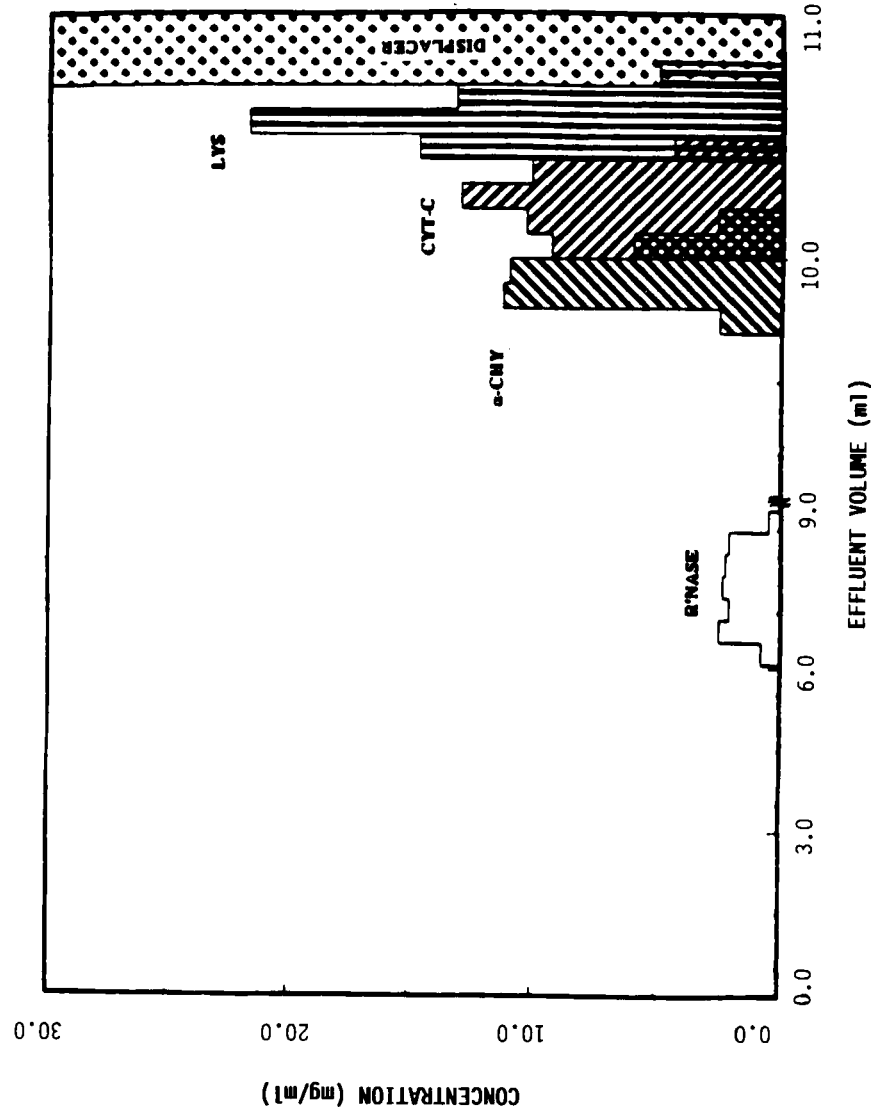
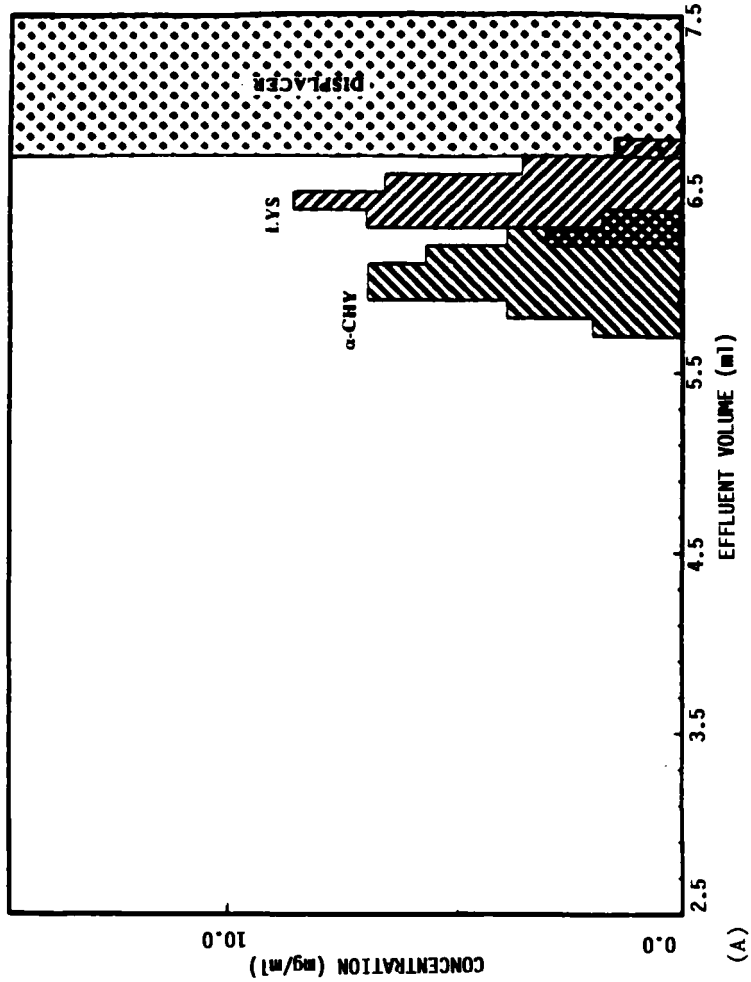


FIGURE 13. Displacement chromatogram of a four-component protein mixture. Column: 500 x 4.6 mm I.D. SCX; carrier: 0.1 M ammonium sulfate in 25 mM phosphate buffer, pH 7.5; displacer: 30 mg/ml Nalcolyte 7105 in carrier; flow rate: 0.1 ml/min; temperature: 37 °C; feed: 4.0 mg each of ribonuclease and chymotrypsinogen, 5.0 mg of cytochrome C and 5.5 mg lysozyme. (Reproduced with permission from ref. #74, published by American Institute of Chemical Engineers.)



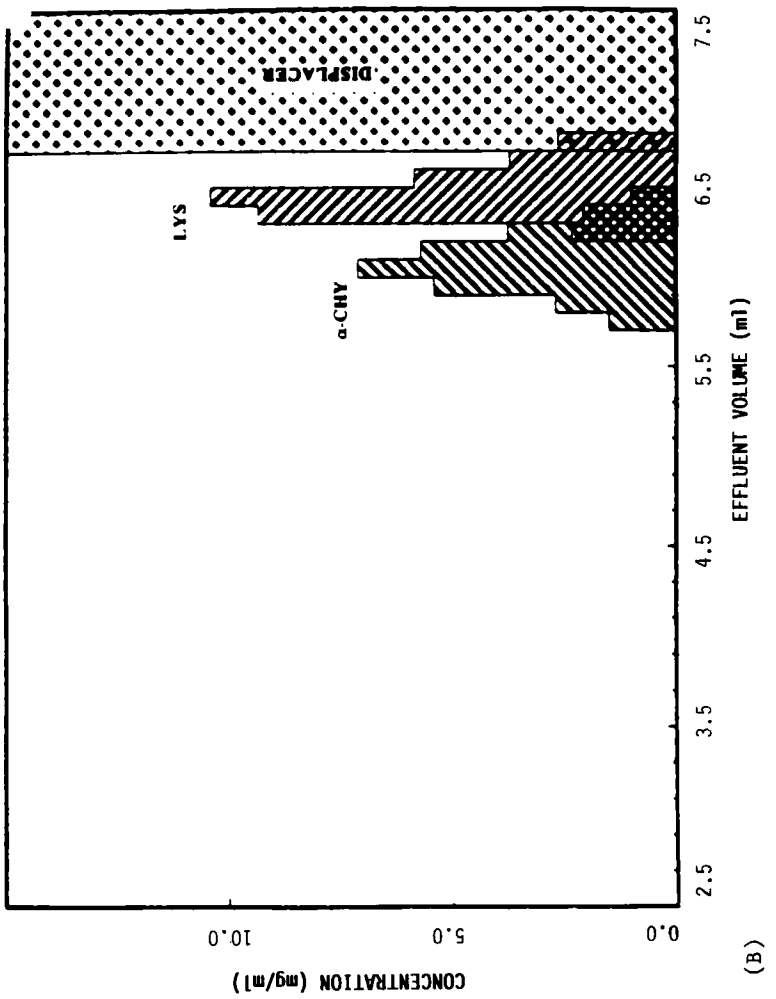


FIGURE 14. Effect of flow rate on displacement profile. Chromatographic conditions as stated in Figure 14, with the exception of: Colymn: 250 x 4.6 mm I.D. SCX; feed: 3.0 mg each of chymotrypsinogen and lysozome; flow rate: 0.1 and 1.0 ml/min for (a) and (b) respectively. (Reproduced with permission from ref. 74, published by American Institute of Chemical Engineers.)

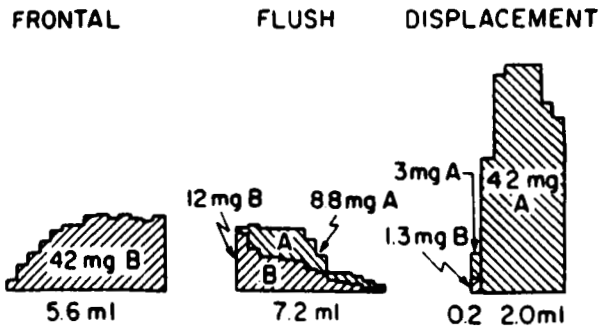


FIGURE 15. Separation of β-lactoglobulins by frontal chromatography followed by displacement. Column: 75 x 7.5 mm I.D. TSK DEAE 5-PW; carrier: 25 mM phosphate buffer, pH 7.0; displacer: 10 mg/ml chondroitin sulfate in carrier; flow rate: 0.1 ml/min; feed, 109 mg of LAC. (Reproduced with permission from ref. #76, published by Elsevier)

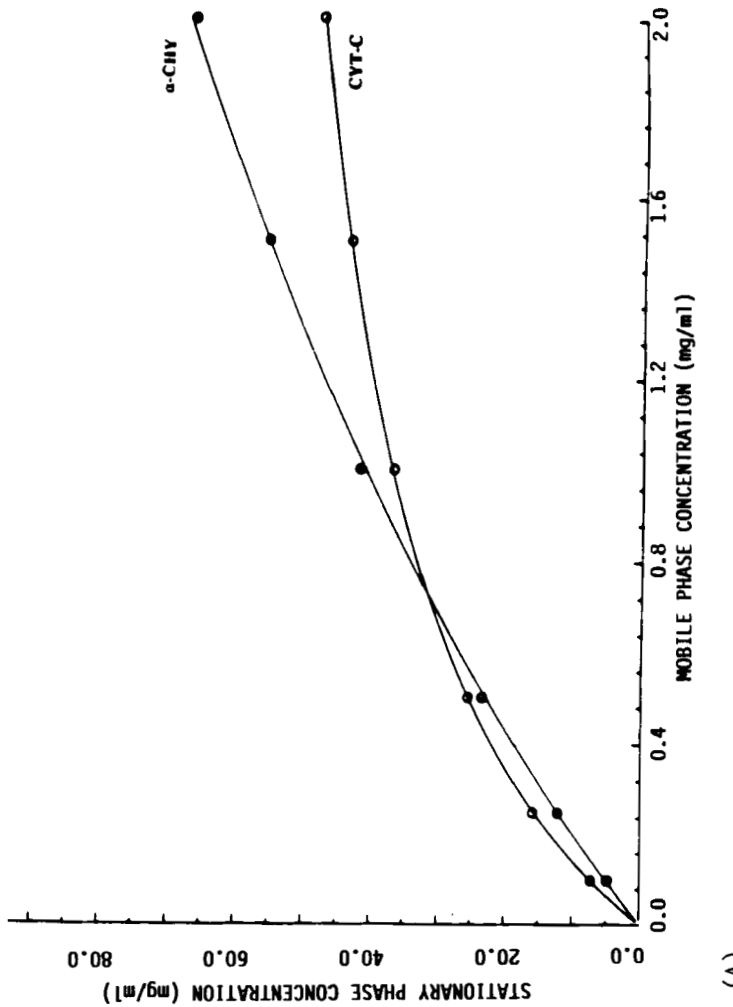
displacement zones. However, since the separation time is decreased an order of magnitude, the throughput in this displacement system is dramatically increased. This separation corresponds to the processing of 6 mg of protein in 15 minutes using an analytical SCX column.

Lee et al[76] have employed displacement in concert with frontal chromatography and an intermittent column flush to separate relatively large quantities of the proteins β-lactoglobulin A and B. Figure 15 demonstrates how these techniques can be used in series to further increase the high throughput and purity attained with displacement chromatography. The ability to conduct sequential chromatographic steps in a single column results in efficient use of the chromatographic bed, and can further simplify the downstream processing of biopharmaceutical products.

Effect of Crossing Isotherms on Displacement Behavior

While it has been shown that "concave-down" adsorption isotherms are necessary for displacement chromatography, the effect of crossing isotherms on the displacement profiles have not been established. Thermodynamic arguments indicate that when the isotherms cross, the order of the proteins emerging in the displacement train could be a function of the displacer concentration[77]. On the other hand, simulations of non-ideal displacement chromatography, which employ multicomponent langmuirian isotherms, predict that the order of the displacement train should be dependent only on the initial slopes of the isotherms and should be independent of displacer concentration. Clearly, when displacement chromatography is carried out on complex fermentation broth mixtures, it is likely that some of the feed components will exhibit crossing isotherms. Thus, it is critical to understand the effects of crossing isotherms on the displacement behavior of proteins.

We have experimentally investigated the displacement chromatographic behavior of crossing isotherm systems[74]. Figure 16a shows the adsorption isotherms of the proteins α-chymotrypsinogen and cytochrome C on a cation exchange material. Under these chromatographic conditions, the isotherms of the two proteins cross at a mobile phase concentration of approximately 0.7 mg/ml. Displacement chromatography under these carrier conditions using Nalcolyte 7105 as the displacer resulted in complete mixing of the feed components as shown in Figure 16b. This inability of the displacement system to differentiate between feed



(A)

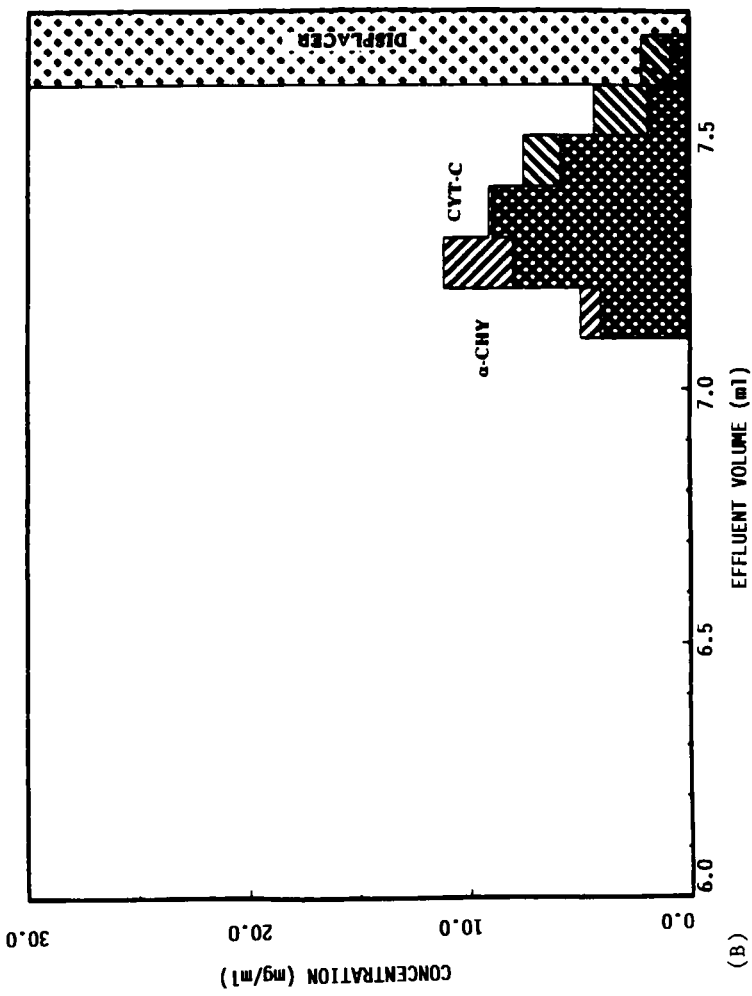
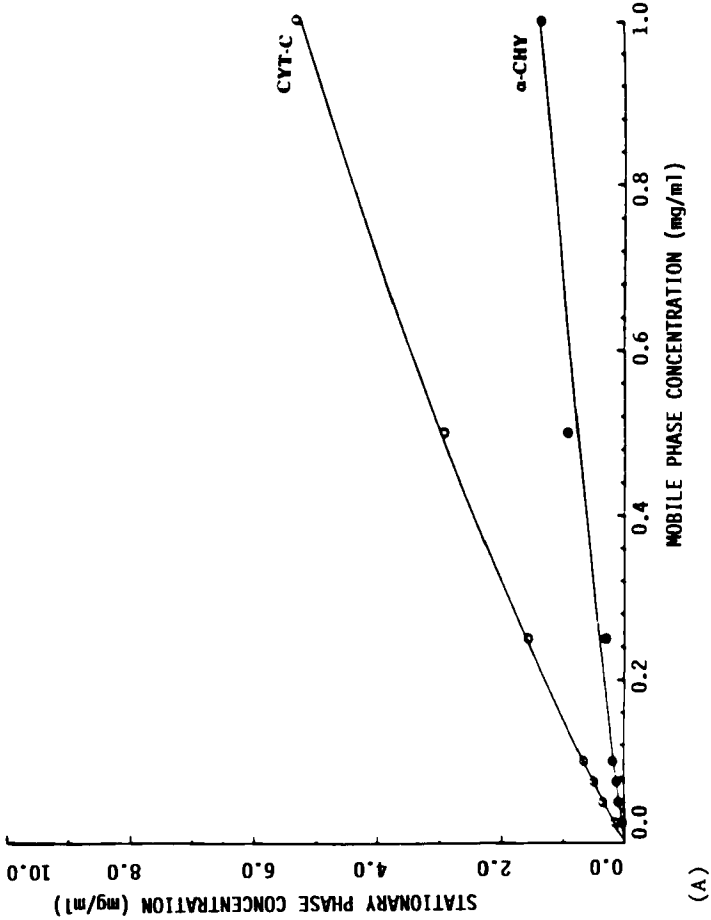


FIGURE 16. (a) Protein adsorption isotherms under crossing conditions. Column: 50 x 1 mm I.D. WCX; mobile phase: 0.2 M sodium acetate, pH 6.0: temperature: 25 C (b) Displacement chromatogram under crossing adsorption isotherm conditions. Column: 350 x 4.6 mm I.D. WCX; carrier: 0.2 M sodium acetate, pH 6.0; displacer: 40 mg/ml Nalcolyte 7105 in carrier; flow rate: 0.1 ml/min; temperature: 25 C; feed: 3.5 mg each of chymotrypsinogen and cytochrome C. (Reproduced with permission from ref. 54, published by American Institute of Chemical Engineers.)



(A)

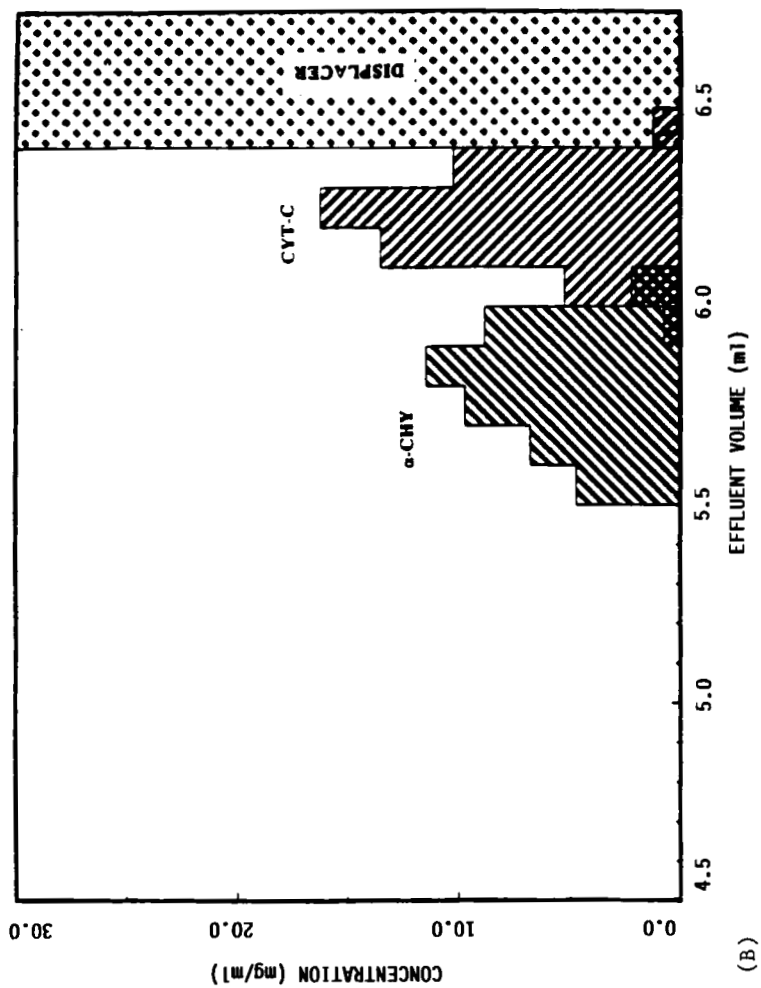


FIGURE 17. (a) Protein adsorption isotherms under "non-crossing" conditions. Column 50 x 1 mm I.D. SCX; mobile phase: 0.1 M ammonium sulfate in 25 mM phosphate buffer, pH 7.5, temperature: 25 C. (b) Displacement chromatogram under "non-crossing" conditions. Chromatographic conditions as stated in Figure 15a with the exception of: feed: 5.0 mg each of chymotrypsinogen and chytochrome C. (Reproduced with permission from ref. 74, published by American Institute of Chemical Engineers.)

components with crossing isotherms has also been observed by other workers in the field[78-80]. Vigh et al[80] have examined the displacement chromatographic separation of D and L enantiomers of dansyl phenylalanine using 3,5-dintrobenzoic acid as the displacer. In their experiment, the displacement zone of the more retained D-enantiomer was completely mixed with the displacer. Upon evaluation of the isotherms for this system, it was found that this displacement behavior could be attributed to the crossing isotherms of D-dansyl phenylalanine and the displacer. Cox et al[79] have reported that adsorption systems with crossing isotherms are unable to effect efficient sample displacement purification of the feed components. Furthermore, the affect of the relative "closeness" of adsorption isotherms on the ability of one compound to displace another slightly less adsorbed compound has not been established to date.

Since crossing isotherms adversely affect displacement systems, it is useful to establish adsorption conditions where the isotherms do not intersect. The conditions employed for the separation showed in Figure 16 were modified to eliminate crossing of the protein adsorption isotherms as shown in Figure 17a. Displacement chromatography under these conditions resulted in the well separated displacement zones as shown in Figure 17b. Thus, it appears that unsuccessful displacement separations due to crossing isotherms can be readily resolved by establishing alternative adsorption conditions. Horvath and co-workers[78] have postulated that the mixing behavior resulting from crossing isotherms is similiar to azeotropes often encountered in distillation. Several workers are presently developing theoretical models for the accurate simulation of these complex displacement systems.

Scale-Up of Displacement Chromatography

To date, most of the displacement separations of biomolecules reported in the literature have employed analytical columns packed with small particle diameter stationary phase materials. Clearly, for displacement chromatography to become a useful separation tool in the biotechnology industry, the economics of the process must be compelling. Since the cost of carrying out preparative chromatography decreases dramatically with increasing particle diameter, it is important to investigate the scale-up of displacement chromatography to large particle and column diameter systems.

The peptide displacement, shown in Figure 9a, was scaled-up[81] with respect to column diameter using a reversed-phase 19 mm I.D. column packed with 10 μm stationary phase materials. The resulting separation, shown in Figure 18, exhibited well separated and concentrated displacement zones. This separation corresponds to the purification of approximately one gram of peptide per displacement experiment.

The purification of proteins by displacement chromatography has recently been investigated with large particle diameter materials[81]. Figure 19 shows the displacement separation of proteins using a column packed with 30 μm cation exchange material. Under these conditions, the proteins were well separated and concentrated during the displacement process. These results are indeed dramatic in that they demonstrate that displacement chromatography of proteins need not be limited to small particle diameter systems, as previously thought.

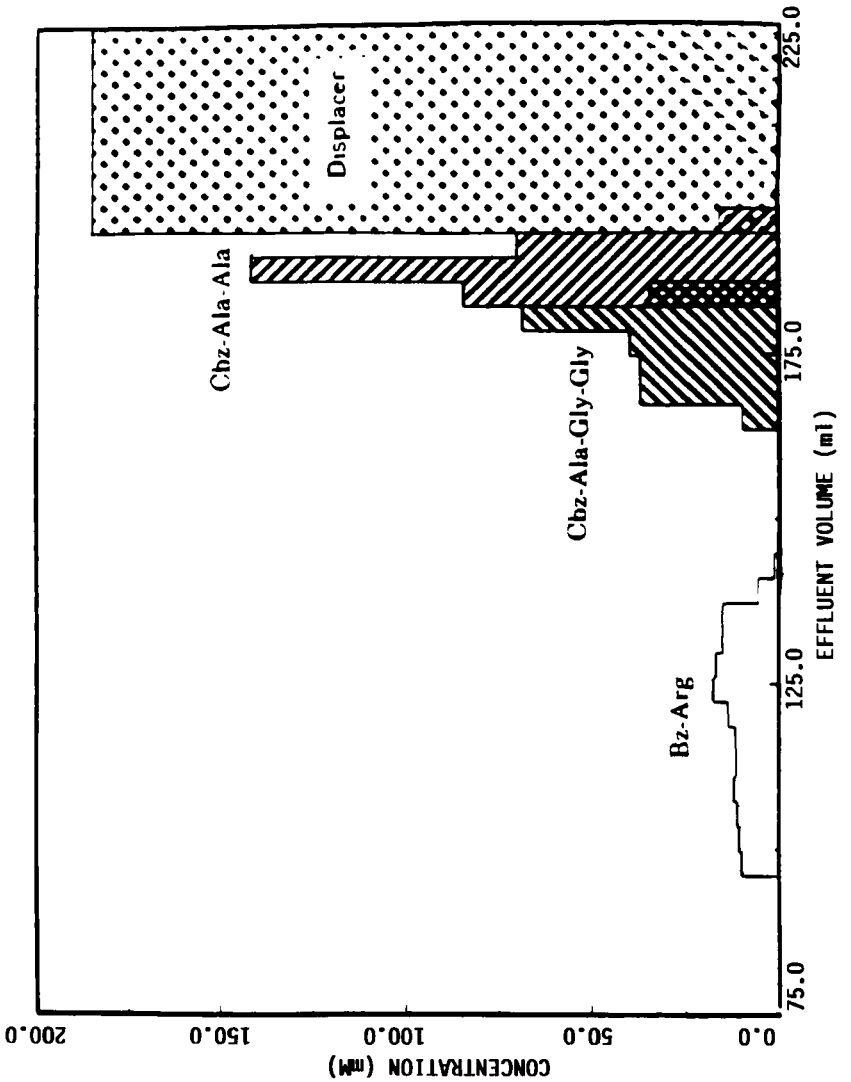


FIGURE 18. Preparative-scale displacement of a peptide mixture. Chromatographic conditions as stated in Figure 10 with the exception of: Column: 300 x 19 mm I.D. ODS; feed: 161.9 mg Bz-Arg, 303.1 mg Cbz-Ala-Gly-Gly, 412.5 mg Cbz-Ala-Ala; flow rate: 2.5 ml/min. (Reproduced with permission from ref. #81, published by Elsevier)

Displacement chromatography of proteins was further scaled-up to 90 μm agarose-based materials as shown in Figure 20. The displacement zones resulting from this separation exhibited relatively sharp boundaries, which is quite remarkable for such large particle diameter systems. It is postulated that the macroporosity of these materials facilitates the transport of the proteins, thus enabling efficient displacement separations. Preparative non-linear elution chromatography under the same carrier conditions resulted in extremely long elution times with significant dilution of the feed proteins. Thus, displacement chromatography can be readily carried out with large particle diameter systems which is expected to have a significant impact on the economics of the process.

Continuous Displacement Chromatography

Byers and co-workers[82,83] have modified annular chromatographic systems to enable operation in the displacement mode. This process combines the high throughput of displacement chromatography with the ability to operate annular chromatographic systems continuously. Figure 21 shows a two dimensional representation of the continuous displacement purification of the amino acids L-leucine, L-valine, and L-glutamic acid using NaOH as the displacer[82]. This work is a successful extension of the fixed-bed work on ion-exchange displacement of amino acids[58] to the continuous system. Taniguchi et al have also reported on the separation of a binary lanthanide (Nd/Pr) mixture using continuous displacement chromatography (CDC)[83]. Clearly, the ability to operate displacement systems in a continuous mode will significantly increase the processing capabilities of these chromatographic systems.

FUTURE DIRECTIONS

While much progress has been made in the theory and practice of displacement chromatography in the last decade, much work remains to be done before the process can be widely adopted for the downstream processing of biopharmaceuticals. We believe that there are several areas of research that will require intensive effort in the future to bring this technique to its maturity.

By far, the most important area will be the development of novel displacer compounds. The success of displacement separations using various chromatographic supports will clearly be dependent on the availability of appropriate displacer compounds. This will require fundamental studies on the relative importance of various displacer physico-chemical properties. Furthermore, the purification of bioproducts for eventual use in therapeutics will require the development of non-toxic displacers. Thus, it is critical that novel displacer compounds be identified and/or synthesized for various stationary phase materials.

Clearly, the regeneration step in displacement chromatography must be studied in more detail. An important aspect of the development of novel displacer compounds will be the development of displacers which can be rapidly removed from the column systems. Recent work by Chen and Pinto[84] has indicated that proteins can be displaced by relatively small molecular weight ionic polymers. It will be important to carry out a systematic study of the affect of various displacer properties on both the ability to displace biomolecules as well as ease of column regeneration.

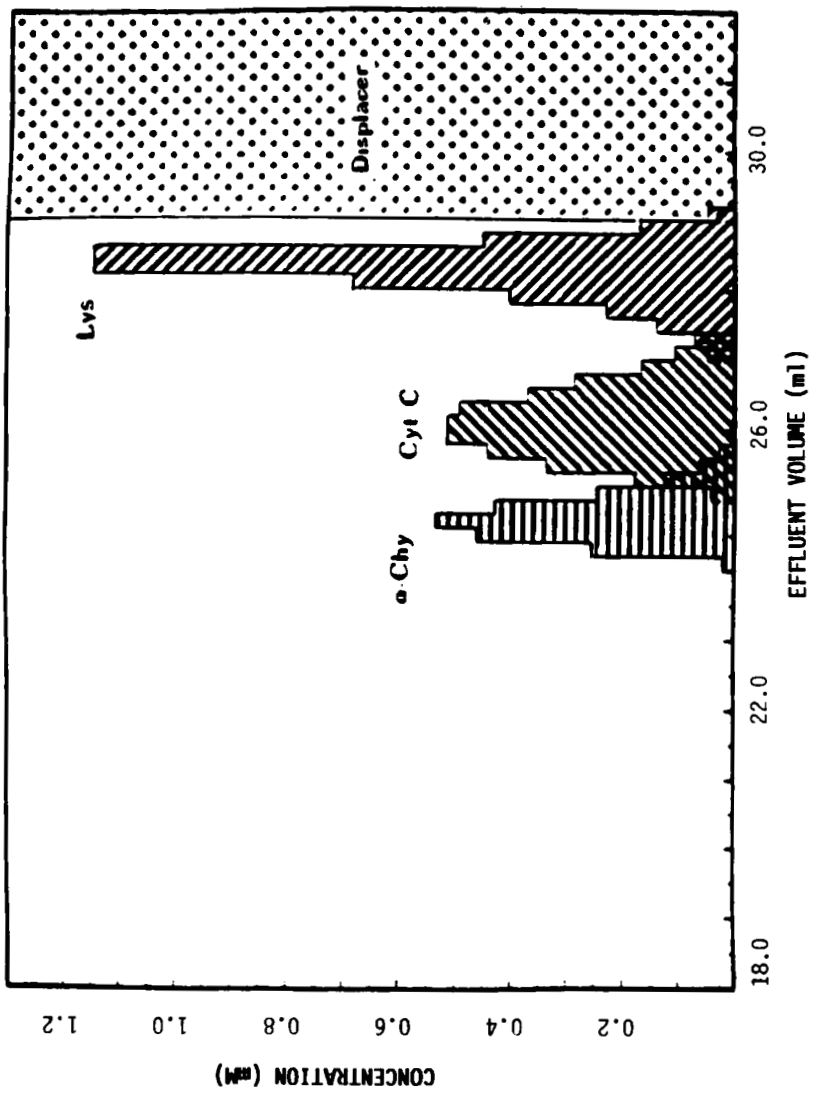


FIGURE 19. Displacement chromatogram of a three-component protein mixture on a 30 μm particle diameter system. Column: 300 x 10 mm I.D. Sepharose S cation exchanger (30 mm); carrier: 0.1 M ammonium sulfate in 25 mM phosphate buffer, pH 7.5; displacer: 75 mg/ml Nalcolyte 7105 in carrier; flow rate: 1.0 ml/min; temperature: 22 ^{0}C; feed: 6 ml of 1.67 mg/ml each of α-chymotryosinogen, cytochrome C and lysozyme.

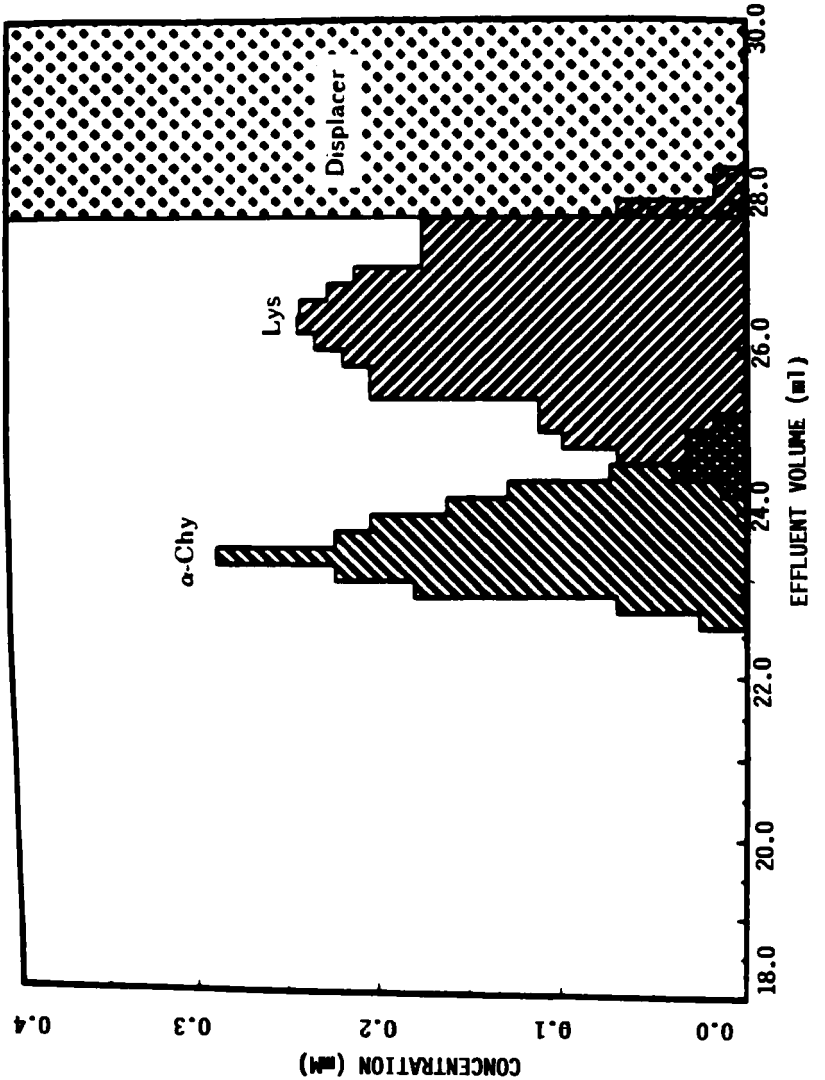


FIGURE 20. Displacement chromatogram of a two-component protein mixture on a 90 μm particle diameter system. Chromatographic conditions as stated in Figure 21 with the exception of: column: 300 x 10 mm I.D. Sepharose S cation exchanger (90 μm); feed: 6 ml of 1.67 mg.ml each of α-chymotrypsinogen and lysozyme. (Reproduced with permission from ref. #81, published by Elsevier)

It will also be important to establish several "case studies" which test the resolving power of this technique under "real-life" conditions. While displacement chromatography has been shown to have great utility for the simultaneous concentration and purification of biomolecules from relatively simple mixtures, the utility of the technique for purifications from complex mixtures remains to be proven. Furthermore, it will be important to establish where displacement chromatography is best employed in the multi-step downstream bioprocessing scheme. The ability of the technique to concentrate and purify bioproducts along with the introduction of the displacer compound into the system, dictates the use of displacement at early stages of the separation scheme. On the other hand, the unique resolving power of displacement suggests employing the technique at the final stages of the process. Again, "case studies" will be required to examine the utility of displacement at various stages of downstream bioprocessing.

We believe that a potentially important area of research will be the extension of displacement chromatography to alternative adsorptive systems. Although traditional stationary phase materials such as reversed-phase and ion-exchange have been successfully employed in the displacement mode, research on displacement chromatography with novel adsorbent materials is scarce at present. The extension of displacement chromatography to more specific adsorptive systems will combine the unique selectivites of these systems with the high throughput and purity obtained in displacement chromatography.

In the theoretical realm, much work remains to be done on the modeling and optimization of displacement as well as other non-

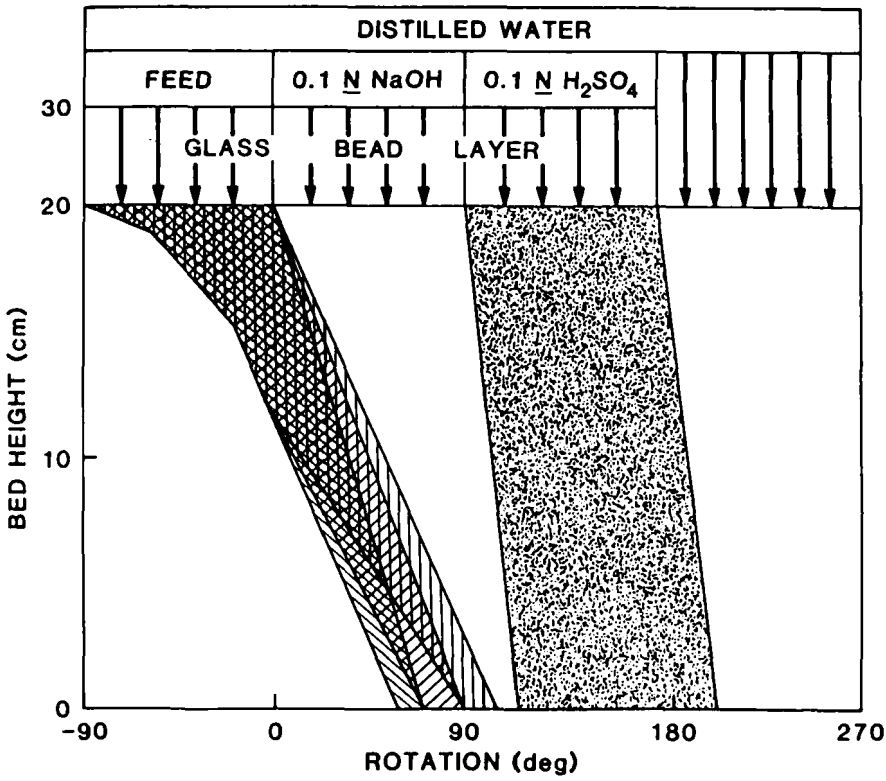


FIGURE 21. Two dimensional representation of the distribution of the various reagent sectors in the continuous displacement chromatographic separation of an amino acid mixture using NaOH as the displacer. (Reproduced with permission from ref. #82)

linear chromatographic techniques. At present the optimization of preparative chromatographic systems is generally carried out by extensive experimentation or by the use of "semi-quantitative" models which are only applicable for limited modes of operation. There is a need for theoretical models which carry out rigorous optimizations of these non-linear chromatographic processes.

Finally, the implementation of displacement chromatography along with other non-linear chromatographic techniques for purification of biopharmaceuticals will be dictated to a large extent by economic considerations. Accordingly, there is an urgent need for detailed economic evaluations of various preparative chromatographic schemes. In addition, it is necessary to consider how the chromatographic process of interest will affect the economics of other bioseparations processes in the multi-step downstream processing scheme.

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