INCREASING THE SCALE OF ELECTROPHORETIC TRUE MOVING BED
ENANTIOMER SEPARATIONS USING VOLTAGE GRADIENTS
AND FILTRATION ENHANCEMENT

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of BRIAN MATTHEW THOME find it satisfactory and recommend that it be accepted.

____________________________
Chair
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Abstract

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Chair: Cornelius F. Ivory

Over the past decade the moving bed process has become a common tool for the continuous separation of chiral compounds, and its recent adaptation to electrophoretic separations has provided a model system for determining the effects of gradients in electric field or velocity on the process. Several approaches were used to explore the addition of gradients to True Moving Bed (TMB) electrophoresis. A linear mathematical model of the TMB process was developed that allowed the dynamics of the technique to be studied in great detail, and this provided the foundation for experimental work using stepped electric field gradients and counterflow velocity gradients using filtration.

The modeling work showed that the addition of a stepped electric field gradient allowed a 7.66 fold increase in the maximum homatropine throughput when compared to the non-gradient case. Continuous electric field gradients were also explored, resulting in a maximum throughput of 7 fold over the non-gradient case. Continuous gradients resulted in a significant decrease in product concentrations within the unit, lowering the chance of precipitation and reducing the time required to reach steady state. Experimental work
using stepped field gradients in a three electrode Vortex Stabilized Electrophoresis Apparatus encountered problems with both dialysis and anion exchange membranes at the middle electrode during homatropine separations. Due to this, the electric field gradient process was tested using bovine serum albumin and hemoglobin. The results showed that a 63% increase in throughput was attainable using an electric field step gradient, and while this is nowhere near the 7.66 fold increase predicted by the homatropine model, it is still a significant enhancement.

The model was then adapted for use with velocity gradients created by the addition of a filtration unit. An increase in throughput of 3.14 fold was predicted due to a reversal of the unfavorable step in counterflow velocity in between sections II and III. Experimental results showed a 3 fold benefit in section II, but the tailing in section III was increased due to dispersion caused by flow oscillations stemming from the pump used in the membrane system preventing useful results to be obtained in that region.
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triangle area, which is greatly exaggerated in this figure, is the set of operating parameters where both compounds can be separated at a given purity. If the fluid velocities in regions II and III fall outside of the area between $\eta_s E$ and $\eta_f E$ then there will be contamination in the offtake streams. In actuality the $\eta$ values are not necessarily constant as they depend on the binding relationship between the enantiomer and the chiral selector which leads to skewing of the triangle.

**Figure 2.9:** A triangle diagram illustrating the effects of different stabilizing electric field gradients. The solid line denotes the region where the model predicts the purity of both homatropine enantiomers to be greater than 99% under a constant electric field of -50 V/cm. The dashed line shows the 99% purity region of the same separation but with a step gradient at the feed port from -45 V/cm in region II to -55 V/cm in region III. This small 10 V/cm step has a massive effect on the separation, greatly expanding the 99% purity region and allowing a 7.66 fold increase in the maximum processing rate of homatropine enantiomers. The dotted line denotes a continuous electric field gradient with focal points at (0.07 m, -45 V/cm) and (0.23 m, -55 V/cm). This continuous gradient also has a very large effect on the 99% purity region with a predicted maximum enantiomer throughput increase of 7 fold over the constant field separation but the concentrations in the unit were 40% less at the same throughput value as the step gradient separation.

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**Figure 3.5:** A plot of the measured pH of the samples at the end of the homatropine gradient run shown in Figure 3.4b. An anion exchange membrane was used at the center electrode to prevent the positively charged homatropine from leaving the separation chamber. The ion selective nature of the membrane required that all of the current carrying ions be negatively charged, and this caused a large pH shift from approximately 4.5 in section II to 7.5 in section III. The hydroxypropyl-β-cyclodextrin does not bind homatropine selectively above pH 5.5, thus in section III there was no functioning chiral selector. The purpose of electric field gradients is to reduce the size of the fast enantiomer tail in section III, but since the chiral discriminator was ineffective, there was no ability to selectively reduce the tailing of the fast enantiomer.

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**Figure 4.1:** An electrophoretic moving bed enantiomer separation. Electromigration forces the enantiomers downward as they are counteracted by a fluid flow upward. The fast enantiomer is able to overcome the fluid flow and migrate to the bottom offtake while the slower enantiomer is pushed upward towards the top offtake.

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<td>TMB</td>
<td>True Moving Bed</td>
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<td>EACA</td>
<td>Epsilon Amino Caproic Acid, 6-aminohexanoic acid</td>
</tr>
<tr>
<td>DFGF</td>
<td>Dynamic Field Gradient Focusing</td>
</tr>
<tr>
<td>ITP</td>
<td>Isotachophoresis</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut Off</td>
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<tr>
<td>FE-TMB</td>
<td>Filtration Enhanced-True Moving Bed</td>
</tr>
<tr>
<td>TFF</td>
<td>Tangential Flow Filtration</td>
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<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
</tbody>
</table>
LIST OF SYMBOLS

- $u_i$: Migration velocity of species $i$, ($\text{m/s}$)
- $\eta_i$: Electrophoretic mobility of species $i$, ($\text{m}^2/\text{V}\cdot\text{s}$)
- $\phi$: Electric potential, ($V$)
- $V$: Voltage, ($V$)
- $E$: Electric field, ($V/m$)
- $z_i$: Charge of species $i$
- $\mu_i$: Ionic mobility of species $i$, ($\text{s/m}$)
- $F$: Faraday’s constant, 96485 ($\text{C/mol}$)
- $N_i$: Flux of species $i$, ($\text{mol/m}^2\cdot\text{s}$)
- $D_i$: Diffusivity of species $i$, ($\text{m}^2/\text{s}$)
- $c_i$: Concentration of species $i$, ($\text{mol/m}^3$)
- $R_i$: Reaction rate of species $i$, ($\text{mol/m}^3\cdot\text{s}$)
- $K_S$: Slow component binding equilibrium constant, ($\text{mM}$)
- $K_F$: Fast component binding equilibrium constant, ($\text{mM}$)
- $\overrightarrow{k_i}$: Forward reaction rate of species $i$, ($\text{mM/s}$)
- $\overleftarrow{k_i}$: Reverse reaction rate of species $i$, ($\text{mM/s}$)
- $A_S$: Cross-sectional area, ($\text{m}^2$)
- $Q_{cf}$: Flowrate of the counterflow stream, ($\text{mL/min}$)
- $Q_{bo}$: Flowrate of the bottom offtake stream, ($\text{mL/min}$)
\( Q_{\text{feed}} \) Flowrate of the feed stream, \( (\text{mL/min}) \)

\( Q_{\text{filtrate}} \) Flowrate of the filtrate stream, \( (\text{mL/min}) \)

\( Q_{\text{to}} \) Flowrate of the top offtake, \( (\text{mL/min}) \)

\( Q_j \) Flowrate of inlet of offtake stream \( j \), \( (\text{mL/min}) \)

\( u_I \) Fluid velocity in region I, \( (\text{cm/min}) \)

\( u_{II} \) Fluid velocity in region II, \( (\text{cm/min}) \)

\( u_{III} \) Fluid velocity in region III, \( (\text{cm/min}) \)

\( u_{IV} \) Fluid velocity in region IV, \( (\text{cm/min}) \)

\( u_x \) Fluid velocity in region \( x \), \( (\text{cm/min}) \)

**Subscripts**

\( S \) Slow enantiomer

\( F \) Fast enantiomer

\( CD \) Chiral descriminator

\( SCD \) Slow enantiomer-Chiral descriminator complex

\( FCD \) Fast enantiomer-Chiral descriminator complex
1. INTRODUCTION

Whether it is the left and right hands of a human being, or the dextrorotatory and levorotatory stereoisomers of a drug, chirality is an extremely important natural phenomenon. In recent years, the pharmaceutical industry has been paying much more attention to stereochemistry due to the realization that the two enantiomers of the same drug can have drastically different effects on the body [1]. As a simple example of the differences possible, R-thalidomide has been shown to be an effective sleep aid, but S-thalidomide caused an epidemic of birth defects due to its teratogenic effects on unborn fetuses [2].

In the past, production of enantiomerically pure drugs or pharmaceutical intermediates required stereo-specific synthesis or crystallization of mixtures from racemic reactions. Although the crystallization process is a powerful separation tool, it often does not have the ability to resolve enantiomers, and until recently, stereospecific synthesis was the only other option. This can significantly increase drug development costs as the chiral precursors required for these reactions are often not available commercially and have to be produced in house, making the reaction sequence complicated and costly [3]. While stereospecific synthesis is still important, recent advances in chiral separations using chromatography have given pharmaceutical chemists and process engineers more options to save both capital and operating costs in their processes, and allow for a faster time to market by reducing the development time required for a drug [4].
This dissertation will focus on the electrophoretic True Moving Bed (TMB) process which is a direct analog of Simulated Moving Bed (SMB) chromatography. Though TMB electrophoresis has not been demonstrated as a viable process-scale enantiomer separation technique, its similarity to the common SMB chromatography process allows it to be used as a model system to predict the effects of stepwise and continuous gradient elution and velocity gradients using filtration on the SMB process. The remainder of this chapter will explain the principles of SMB chromatography and TMB electrophoresis in order to set the stage for the process improvements detailed in the following chapters.

1.1. Simulated moving bed chromatography

1.1.1. Chromatography

Before explaining SMB chromatography, it is important to explain the process of chromatography itself. Chromatography literally means “color writing” as it was originally used for the separation of plant pigments on filter paper in the mid 19th century [5]. The technique has come a long way since its humble beginnings, but the basic principle has remained the same. In any adsorptive chromatography technique there is a stationary phase on which there is a functional moiety that binds to the components being separated. A mobile phase, which is generally a liquid or a gas that sweeps across the packing, moves the less strongly bound components through the system faster than the more strongly bound components allowing a separation based on affinity to the stationary phase. Most liquid chromatography today is performed on a packed bed of particles inside a glass or metal tube with a pump forcing the mobile phase through the column.
The column is commonly followed by a detector and a fraction collector to allow the analysis and recovery of the products from the separation process.

1.1.2. Chiral Stationary Phases

The introduction of chiral stationary phases (CSP’s) [6] has allowed chromatography to become the dominant separation technique for resolving enantiomers. There are several different types of CSP’s, but the most common are based on chiral small molecules bonded to the stationary phase known as Pirkle packings [6]. The small nature of the chiral molecules on this packing provided a quantum leap in resolution over earlier protein-based CSP’s, as the small size allowed multiple chiral interactions with many binding sites on the stationary phase. There have also been CSP’s based on cellulose and amylose polymers [7] and macrocyclic glycopeptides which form cavities with many exposed chiral centers for stereoisomers to interact with [8]. Cyclodextrin based packings, in which the stationary phase moiety forms a truncated cone shape with a hydrophobic inner core and a hydrophilic outer surface as shown in Figure 1.1, can be functionalized with many different chemistries and are the most recently available CSP’s. The advantage to cyclodextrin packings is their ability to be used in the reversed phase as well as normal phase modes since there is no protein or macropeptide denaturation to contend with [9].

1.1.3. The SMB Process

Traditional chromatography is generally performed as a batch process where the sample is loaded onto the column and then eluted into fractions where the peaks are collected.
Figure 1.1: The unsubstituted β-cyclodextrin molecule contains seven glucose units connected in a ring, which forms a three dimensional truncated cone shape with a slightly hydrophobic inner core and a hydrophilic outer surface. The OH groups on the molecule can be functionalized with many different chemical moieties such as sulfates, quaternary amines and non-charged hydrophilic and hydrophobic side chains which allow the ligand to selectively bind enantiomers of different classes of drugs with differing strengths. The cyclodextrins have been used successfully as functional groups on chiral stationary phases as well as soluble enantioselective ligands in chiral electrophoresis. Copyright John Wiley & Sons Limited. Reproduced with permission [10].
Since the stationary phase is only being effectively used as the products move past the active sites, it takes a large number of theoretical plates to accomplish a high resolution separation. Simulated Moving Bed chromatography turns this batch elution process into a continuous system, mimicking movement of the stationary phase by periodically switching the columns counteracting the motion of the products due to the mobile phase as shown in Figure 1.2a. In moving bed chromatography, the active sites on the resin are constantly being used by product that is continually sweeping by, thereby making the resin efficiency much higher [3]. Furthermore, the number of theoretical plates required for a continuous moving bed separation is greatly reduced since the resin is being used so efficiently. The SMB technique was invented at UOP in the 1960’s for the large scale separation of xylenes [11], as it required far less energy than the inefficient distillation process used previously. The technique was adapted for the separation of carbohydrates [11], and with the advent of CSP’s, to chiral separations where it has become an indispensable process scale separation technique in the pharmaceutical industry [3].

The basic principle of the SMB process comes from a special case of True Moving Bed chromatography where there is an actual solid flow that counteracts the flow of the products induced by the mobile phase [12]. When the stationary phase counteracts the product migration at the average of their two component velocities, one component will be able to overcome the solid flow rate and the other will get carried along with it, allowing the two different products to elute at opposite ends of the unit [3]. Continuous movement of the stationary phase is impractical due to the way in which chromatography columns are packed, necessitating a periodic switching mechanism that simulates the true
Figure 1.2: The SMB chromatography process. (A) An idealized concentration profile during an SMB separation. The feed stream is input into the middle of the chamber. The less strongly bound enantiomer is able to move faster than the solid movement created by the switching, and it elutes from the raffinate offtake. The more strongly bound enantiomer is overcome by the solid flow, and migrates out the extract offtake. (B) A diagram of an SMB chromatography set up showing the 8 columns in series with the four fluid inlet and outlet streams and the switching which simulates the solid flowrate.
moving bed process. This is generally accomplished with between 6 and 12 columns as shown in Figure 1.2b.

In the SMB chromatography process, the desorbent or mobile phase is pumped into the train in between columns 1 and 8. Columns 1 and 2 form section I, which has the highest mobile phase velocity in the unit preventing any strongly adsorbed components from exiting the section with the solid "flow." In between columns 2 and 3 there is the extract, or strongly adsorbed component offtake. Columns 3 and 4 form section II, where the bulk of the strongly adsorbed component is separated, and the feed stream is added in between columns 4 and 5. Section III is the region where the bulk of the weakly adsorbed component is separated from the mixture and is comprised of columns 5 and 6. This leads to the raffinate, or weakly adsorbed component offtake between columns 6 and 7. Section IV is made up of columns 7 and 8, and due to the removal of the raffinate offtake, this section has the lowest mobile phase velocity in the unit, preventing any component from leaving the train at column 8 and contaminating column 1 which would short circuit raffinate into the extract offtake and ruin the separation. In order to create the stationary phase “flow,” the columns are switched on a regular interval, with column 2 becoming column 1, 3 becoming 2, etc. The switch time gives control over the solid flowrate, and this must be balanced with the mobile phase flowrate to operate the SMB unit in a region where there are two pure components at either end [12].
1.2. True moving bed electrophoresis

1.2.1. Electrophoresis

The first practical electrophoresis technique was developed in the 1930’s by Tiselius for the separation of blood proteins [13]. The process has come a long way since then, and electrophoresis is now an indispensable analytical tool in most biological laboratories [14]. The technique has been used for many different types of separations ranging from SDS-PAGE gels for the separation of proteins [15] to multiple parallel capillaries used for the sequencing of the human genome [16]. The technique works by applying an electric field across a usually aqueous media, and charged species in solution migrate towards the oppositely charged electrode. The rate at which the ions move is a complicated function of the sieving of the media and the charge of the species, represented by the electrophoretic mobility, which is the proportionality constant between the applied electric field and the velocity each species travels in the media [17].

The invention of capillary electrophoresis [18] allowed a great leap in the electric field strengths that could be applied using electrophoretic techniques. The small capillaries used had a very large surface area to volume ratio, allowing the Joule heat created during electrophoretic separations to be dissipated easily. The small cross sectional area of the capillary tubes reduced the amount of sample needed and prevented large currents from being drawn even at 600 V/cm electric fields. Other benefits of capillary electrophoresis are the ease of automating the technique, making the process less time consuming, and the optical transparency of the fused silica tubes, allowing absorbance detection to take place through the capillary without disturbing the separation inside.
1.2.2. Chiral electrophoresis

Around the same time that capillary electrophoresis was being developed [18], the first chiral stationary phases [6] were being experimented with, and the marriage of the two techniques has allowed chiral separations by capillary electrophoresis to become the dominant method for the analysis of enantiomers [19]. Many of the same classes of molecules used in chiral stationary phases have been adapted for use in free solution. Macropptides such as vancomycin [20] have been successfully used, but the most common chiral selectors are based on the functional derivatives of the crown ethers [21,22] and cyclodextrins [23] as seen in Figure 1.1. This large range of soluble chiral selectors along with the ease of automation and high resolution of capillary electrophoresis has made analytical method development for enantiomer separations a routine task.

1.2.3. The TMB electrophoresis process

The moving bed electrophoresis process is based on the principles of Flow Counterbalanced Capillary Electrophoresis (FCCE) [24], in which a fluid flow counteracts species electromigration. FCCE allows an increase in resolution, as the total velocity of the analytes can be slowed or individual components can be stopped, allowing them to spend a longer time under the influence of the electric field [24]. The technique can also be used to pass components by the detector multiple times by varying the fluid velocity or electric field. This allows monitoring of reactive species or other time dependent phenomena. In the case of a bi-component separation, if the fluid flow counteracts the analytes at a rate that is slower than the fast component and faster than the
slow component, the two peaks can be forced to migrate in opposite directions [25], and this can be used for small scale, continuous separations [25].

When the principles behind SMB chromatography and FCCE are combined, the result is true moving bed electrophoresis as shown in Figure 1.3. A feed stream containing a racemic mixture of the enantiomers is introduced into the middle of the apparatus. The electric field is oriented such that the electromigration pulls the enantiomers down towards the bottom electrode. A counterflow stream flows into the bottom of the unit, counteracting the electromigration of the enantiomers. If the flowrate of the counterflow is maintained such that the fast enantiomer is able to overcome the fluid flow and the slow enantiomer is not, the two components will move in opposite directions. Since the fast enantiomer is able to overcome the counterflow stream, it moves towards the bottom offtake of the apparatus. On the other hand, the slow enantiomer is overcome by the counteracting fluid flow and gets pushed upward towards the top offtake of the apparatus. Separations of up to 10 mg/h of racemic piperoxan have been demonstrated at 99% purity using the TMB electrophoresis process, and the major goal is increasing the throughput with the addition of process improvements such as electric field gradients and velocity gradients using filtration.

There are several advantages to the TMB electrophoresis separations when compared to SMB chromatography. The system does not require any packing, and the chiral selector is in solution with the enantiomers. This allows moving bed electrophoresis to be accomplished in one chamber, unlike the 6-12 columns required in SMB.
Figure 1.3: An enantiomer separation using the true moving bed electrophoresis process. Electromigration forces the enantiomers downward as they are counteracted by a fluid flow upward. The fast enantiomer is able to overcome the fluid flow and migrate to the bottom offtake while the slower enantiomer is pushed upward towards the top offtake. Copyright Elsevier B.V. Reproduced with permission [26].
chromatography. The major disadvantage of TMB electrophoresis is the lack of ability to conduct process scale separations, while SMB chromatography has been used at the industrial scale since its inception [11].

1.2.4. Scale-up of electrophoresis

While chromatography lends itself well to scaling from analytical to production throughputs, electrophoresis has never been successfully used above the preparative or bench scale. The dissipation of Joule heat due to the voltage applied to electrophoretic systems has prevented them from reaching similar scales as competing chromatographic techniques. Scale up of electrophoresis in gel media is impractical due to the poor thermal conductivity of the polymers, and free solution electrophoresis techniques suffer from natural convection effects that limit the technique to systems with a high surface area to volume ratio to prevent large temperature gradients.

In spite of these limitations on large scale electrophoretic processing, there have been many preparative scale electrophoresis systems used to successfully separate enantiomers. There are several flat bed apparatuses such as the Octopus [27] and the RF3 [28] that have been used to separate milligram quantities of enantiomers in continuous elution and continuous recycle operation. In these systems there are electrodes at the sides of the apparatus that pull the components perpendicular to the flow path so that they elute at different offtake ports at the end of the rectangular chamber [27]. These types of units can be operated as once-through or as recycle systems using multi-channel peristaltic pumps and have been used for isoelectric focusing [29-32], zone [33,34] and isotachophoresis [28] enantiomer separations.
Gel media has been used to conduct milligram scale separations of enantiomers as well. Units such as the Bio-Rad Mini Prep continuous gel elution cell use a column of gel that acts as a sieving medium which separates the enantiomer-ligand complexes in a time-resolved manner [35,36]. Multi-compartment membrane based devices such as the IsoPrime [37], GradiFlow [38], and Off-Gel [39] units can be used to separate amphoteric enantiomers using isoelectric focusing assisted with chiral selective ligands which bind to the enantiomers, slightly altering their isoelectric point [38,40-42]. The IsoPrime and GradiFlow apparatuses use isoelectric membranes stacked to form multiple chambers in parallel with electrodes at either end of the stack. A fluid is pumped through the chambers parallel to the membranes, which form a pH gradient thereby causing focusing perpendicular to the fluid flow through the unit. The buffer is eluted from the membrane chambers at the end of the unit allowing the focused components to be removed from the apparatus [40]. The Off-Gel isoelectric focusing technique uses pre-cast immobiline [43] strips with a pH gradient imbedded directly in the gel. A set of unconnected fluid compartments are pressed onto the strip and an electric field is applied causing the species to migrate to the well above the point on the gel where the pH is equal to the isoelectric points of the components.

The Vortex Stabilized Electrophoresis Apparatus [44] was originally developed for use with isoelectric focusing but has since been expanded for use in many different free-flow electrophoresis modes such as isotachophoresis [44] and zone electrophoresis [26]. This instrument is ideally suited for TMB electrophoresis and has previously been used to separate up to 10 mg/h of piperoxan and homatropine enantiomers at up to 99% purity in
both off takes [26]. The Vortex Stabilized Electrophoresis Apparatus design allows modification for use with stepwise, and in the future, continuous electric field gradients along the axial length of the separation chamber. This thesis contains modeling work and experiments that focus on the addition of continuous and stepwise electric field gradients to the TMB electrophoresis process in the Vortex Stabilized Electrophoresis Apparatus. Velocity gradients in the counterflow and their effects on TMB electrophoresis separations will also be explored by using filtration to selectively remove buffer from the system.

1.3. References


2. CONTINUOUS VOLTAGE GRADIENTS AND THEIR APPLICATION TO TRUE MOVING BED ELECTROPHORESIS

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Gradient Elution, Simulated Moving Bed, True Moving Bed, Preparative Electrophoresis, Chiral Separation, Enantiomer

2.1. Abstract
Gradient elution has been practiced in chromatographic separations for many years. The application of discontinuous “step” gradients in Simulated Moving Bed (SMB) chromatography has been very successful in increasing both processing rates and column productivity, resulting in a reduction in the number of SMB columns required. With the advent of the Field Gradient Focusing techniques, electrophoresis has gained the ability to apply a continuous electric field gradient to a True Moving Bed (TMB) electrophoretic separation. Application of a spatial gradient allows a large degree of control of the product concentrations inside the separation unit as well as a large increase in product throughput. A model of moving bed electrophoretic separations has been developed that demonstrates the potential advantages of applying a continuous gradient to the moving bed process. These advantages include the reduction of detrimental peak tailing and the ability to decrease the concentrations of the compounds being separated in comparison with commonly used step gradient elution.

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2.2. Introduction

Since its inception in the 1960’s, the moving bed technique has steadily transformed the field of continuous adsorptive separations [1]. The application of simulated moving bed chromatography (SMB) to the pharmaceutical and fine chemical industries has shown the power of this strategy for processing hard-to-separate mixtures such as chiral compounds [2], and this success has recently resulted in the application of the moving bed technique to other separations such as electrophoresis [3,4], solid-liquid extraction [5] and gas chromatography [6]. While the electrophoretic moving bed technique [3,4] uses a different mechanism of separation, it is an idiom of SMB chromatography in that the mathematics describing the two processes are the same. This similarity allows true moving bed (TMB) electrophoretic separations to be used as a model system for SMB chromatography.

Much of the recent work in SMB chromatography has revolved around increasing throughput and reducing the number of columns and the amount of solvent consumed in the process. Several different approaches have been utilized to reach these goals. The Novasep “Varicol” process [7-9] involves asynchronous switching of the SMB regions which allows for more efficient usage of the columns. Zhang et. al. [10] have developed the “Power Feed” operation which allows for modifying the column flowrates during different intervals of the separation. Finally the “ModiCon” process, developed by Schramm et. al. changes the feed concentration during different portions of the switch interval [11]. All of these processes allow increases in productivity and reduced solvent
consumption by changing the mechanical variables of the system, making the columns work more efficiently.

Another technique for increasing the effectiveness of an SMB separation is gradient elution, which operates by a change in mobile phase chemistry that affects the binding strength of the products to the stationary phase. Gradient elution SMB has been successfully implemented by many different groups, with a model first proposed by Jensen et. al. in 2000 [12], and additional work performed by Abel et. al. [13,14], Antos et. al. [15] and Houwing et. al. [16]. These papers all incorporate a step change in the mobile phase concentration at the feed port, allowing the columns upstream of the feed port to have different elution strengths than those below. The fact that there are only two input streams in SMB chromatography, the desorbent and feed streams, brings about a major limitation in the ability to form continuous gradients, as there are no other places to introduce changes in solvent strength. This limitation has been addressed by the addition of temperature gradients [17] which allow alteration of the stationary phase binding strengths in each column giving a higher degree of flexibility. Even with this advantage, the gradient formed is still not continuous and the temperature changes during switch times cannot occur instantaneously which limits the effectiveness of this technique. The only existing example of a true continuous gradient in SMB chromatography comes with the use of a supercritical fluid as the mobile phase (SF-SMB) [18-21] in which the pressure drop through the column due to viscous losses in the packing can change the properties of the supercritical fluid, thus allowing a continuous change in the mobile phase properties [22].
Electrophoretic analytical scale gradient separations have already been demonstrated by Huang and Ivory using a dynamically controlled voltage profile [23]. Since the electric field is the driving force for electrophoretic separations, applying a digitally controlled voltage gradient to a moving bed separation allows continuous gradient moving bed separations to be carried out. It should be noted that the term “gradient” refers to different separation mechanisms in chromatography and electrophoresis, but these two processes are mathematically equivalent. In chromatography a change in the mobile phase chemistry alters the strength of the binding interaction between the analyte and the stationary phase which in turn causes the bulk migration velocity of the compound to increase or decrease. In chiral electrophoresis the ligand – selector binding interaction is unaffected by the addition of a gradient, but the alteration of the electric field causes a change in bulk migration velocity of the compounds. Though the mechanisms are different, in both cases the term gradient translates to a change in the transport velocities of the components being separated [24]. In order to demonstrate continuous gradient moving bed electrophoresis and its feasibility for scale up, a mathematical model of the process has been developed which will allow the benefits of continuous gradient separations to be displayed, and this model will allow comparisons with SMB chromatography step gradient elution.

2.3. Theory

2.3.1. Introduction

The moving bed process has traditionally been defined in terms of chromatography where the stationary phase is continually moving against the flow of the mobile phase [2], but this process can be extended to other separation techniques as well. The most common
The application of the moving bed process is SMB where the mobile phase does not actually move, but the columns are periodically switched to simulate the desired movement. The application of the moving bed process to electrophoresis stems from "flow counterbalanced capillary electrophoresis [25,26] in which a pressure driven flow counterbalances electromigration allowing higher resolution in a shorter capillary. In moving bed electrophoresis the analog of the stationary phase is the carrier ligand which resides in solution with the analytes. Application of an electric field causes the migration of charged compounds and this is analogous to the mobile phase of a chromatographic separation. The preparative manifestation of a moving bed electrophoresis separation is shown in Figure 2.1 [3], which depicts the fluid flow and electromigration during a separation along with an idealized concentration profile.

2.3.2. Electrophoresis modeling

In an electrophoretic separation, charged molecules migrate in an electric field according to their electrophoretic mobility which is shown below in Equation 1,

\[ u_i = \eta_i (\nabla \phi) = \eta_i \cdot E \]  

where \( u_i \) is the velocity of component \( i \), \( \eta_i \) is the electrophoretic mobility of component \( i \), \( \phi \) is the electric potential and \( E \) is the electric field. It is difficult to find electrophoretic mobilities in the literature as ionic mobilities are generally reported and, as such, the electrophoretic mobility can be defined as follows

\[ \eta_i = z_i \mu_i F \]
Figure 2.1: An electrophoretic moving bed enantiomer separation. Electromigration forces the enantiomers downward as they are counteracted by a fluid flow upward. The fast enantiomer is able to overcome the fluid flow and migrate to the bottom offtake while the slower enantiomer is pushed upward towards the top offtake.
where $z_i$ is the charge of species i, $\mu_i$ is the ionic mobility of species i and F is Faraday’s constant [27]. This allows an equation for the flux of species i to be derived from the diffusive, convective and electrophoretic fluxes

$N_i = -D_i \nabla c_i - z_i \mu_i F c_i \nabla \phi + c_i u$

where $N_i$ is the flux of species i, $D_i$ is its diffusivity, and $c_i$ is the concentration. Using this constitutive equation for the flux, a mass balance can be formulated as

$\frac{\partial c_i}{\partial t} + \nabla \cdot (-D_i \nabla c_i - z_i \mu_i F c_i \nabla \phi + c_i u) = R_i$

where $R_i$ is a reaction term. Equation 4 is the root of this model as the integration of this mass balance for each species being modeled yields the concentration profiles which occur during an electrophoretic separation. All of the further equations developed for this model provide the variables needed to solve for these concentration profiles.

In order to make the modeling of electric field gradients easier, the electric potential is defined before the equations of this model are solved. This has the effect of linearizing the equations, as the electric field is not calculated as a function of the conductivities and concentrations of the ions in solution. This type of simplification is analogous to the use of Henry’s law in chromatography modeling where the adsorption isotherm is assumed to be linear and does not vary its slope with concentration. The linearized model should be accurate at low concentrations of analyte, but as these concentrations increase, the changes in conductivity can become more pronounced and may cause reduced accuracy preventing the model from predicting actual operating conditions. In order to confirm that
this linear assumption is valid, the model will be verified by comparison with experimental data.

2.3.3. Chiral ligand binding

In order to simulate a chiral separation where an enantioselective carrier molecule such as a cyclodextrin or crown ether is used, a mass action kinetic model was utilized to simulate this binding. This can be expressed mathematically as

\( S + CD \Leftrightarrow SCD \)  
\( F + CD \Leftrightarrow FCD \)

where \( S \) and \( F \) are the slow and fast enantiomers, \( CD \) is a cyclodextrin chiral selector and \( SCD \) and \( FCD \) are the stereoisomer ligand complexes formed [28]. An equilibrium model can be derived for these reactions,

\[ K_S = \frac{c_{SCD}}{c_SC c_{CD}} \]

\[ K_F = \frac{c_{FCD}}{c_FC c_{CD}} \]

where \( K_S \) and \( K_F \) are the equilibrium constants for the slow and fast enantiomer complex reactions. In order to allow the model to investigate the effects of complexation kinetics, mass action equations are derived

\[ R_{SCD} = -R_S = \dot{c}_S c_{CD} - \dot{c}_{SCD} \]

\[ R_{FCD} = -R_F = \dot{c}_F c_{CD} - \dot{c}_{FCD} \]

\[ R_{CD} = R_S + R_F = \dot{c}_S c_{CD} + \dot{c}_{SCD} - \dot{c}_F c_{CD} + \dot{c}_{FCD} \]
where $R_i$ is a reaction rate of component $i$, and $k_i^\rightarrow$ and $k_i^\leftarrow$ are the forward and reverse reaction coefficients of component $i$. These reaction rate constants are related to the equilibrium constant

$$K_i = \frac{k_i^\rightarrow}{k_i^\leftarrow}$$

which allows modeling of the interconversion of the five species in this model, and since the complexed and uncomplexed forms of the enantiomers have different mobilities, this allows the calculation of their migration speeds. It should be noted that the fast enantiomer has a lower $K_i$ than the slow enantiomer and as such, it spends less time bound to the bulky cyclodextrin and this provides the difference in electrophoretic velocities between the fast and slow enantiomers.

### 2.3.4. Moving bed electrophoresis model

Traditionally, moving bed separations have been modeled using four regions as shown in Figure 2.2 [4,29]. Due to the flowrates of the inlet and outlet streams, the fluid velocities in each of the four regions is different. In region I, the fluid velocity $u_t$ flowing through the cross sectional area $A_s$ is

$$u_t = \frac{Q_o}{A_s}$$

and this velocity is able to overcome any electromigration, forcing the compounds being separated up towards the fast offtake of the apparatus preventing any accumulation in region I. The bottom offtake $Q_{bo}$ is situated in between regions I and II and this reduces the fluid velocity in region II to
Figure 2.2: The four regions of an electrophoretic moving bed separator. The hydrodynamic velocities, as denoted by the arrows $u_f - u_{iv}$, are determined by the flowrates entering and exiting the chamber in between each region. The bulk of the separation is carried out in regions II and III, as these are the regions that frame the feed port. Due to the offtakes, the velocities in regions I and IV are very fast and slow respectively when compared to regions II and III, and this keeps the compounds being separated out of these regions, keeping them retained in the vicinity of the offtake ports.
which is the region where the bulk of the faster component is separated from the slower molecules. The feed stream $Q_{\text{feed}}$ is generally small in comparison to the offtake flowrates, but this affects the fluid velocity in region III,

$$u_{III} = \frac{Q_{cf} - Q_{to} + Q_{\text{feed}}}{A_s}$$

making it slightly larger where the bulk of the slower component is separated from the faster molecules. In between regions III and IV is the top offtake port $Q_{to}$ which slows the hydrodynamic velocity in region IV,

$$u_{IV} = \frac{Q_{cf} - Q_{to} + Q_{\text{feed}} - Q_{to}}{A_s}$$

such that the compounds being separated are pushed back towards the slow offtake by the electrophoretic force, preventing loss of product from the chamber through the purge stream outlet at the top. The integration of this hydrodynamic model with the electrophoretic and ligand binding models derived above allows for a complete full differential representation of a moving bed enantiomer separation to be obtained.

2.3.5. Boundary conditions

In order to test the model under conditions that accurately reflect experimental data taken in the Vortex Stabilized Electrophoresis Apparatus as shown in Figure 2.3, Equation 4 was integrated numerically in four subdomains using Femlab 3.1i (Comsol, Stockholm, Sweden) resulting in the concentration profiles of the fast and slow enantiomers, the cyclodextrin and the fast and slow enantiomer-ligand complexes. Subdomain I which
Figure 2.3: The Vortex Stabilized Electrophoresis Apparatus. The separation chamber is situated in the annular gap between two grooved cylinders that form stable Taylor-like vortices when rotated. The peristaltic pumps at the front of the apparatus control the counterflow, purge stream and offtake flowrates. The syringes along the back of the chamber are used to sample the concentration profile within the apparatus at the end of each experiment. There are only two electrodes on this unit which are situated at the top and bottom of the apparatus separated from the annular chamber with dialysis membranes. Purge buffers stored in the cylindrical tanks at the back are pumped through the electrode chambers to remove the electrolysis gases that would otherwise build up. In order to allow gradient electric field separations many computer controlled electrodes will have to be added along the axis of the cylinder.
corresponds with region I as described above, the region below the bottom offtake, spanned from 0 to 7 cm reflecting the actual region’s size on the Vortex Stabilized Electrophoresis Apparatus. Similarly subdomain II spanned from 7 to 15 cm, subdomain III from 15 to 23 cm and subdomain IV from 23 to 30 cm. The concentrations of the components were all set to zero at the left end of subdomain I and the right end of subdomain IV with the exception of the carrier ligand which was fixed at its inlet concentration to simulate the buffer reservoirs at either end of the chamber. The internal boundaries of the four subdomains were modeled using a flux discontinuity

\[
\Delta Flux = N_i = \frac{Q_j}{A_s} c_i
\]

where \( Q_j \) is the input or offtake flowrate and \( c_i \) is the concentration of species \( i \) in the stream. This flux discontinuity was applied to each of the five species within the unit which allowed the model to replicate the effects that the input and offtake streams have on the separation. These boundary conditions and the initial conditions used in solving the model are shown in Table 2.1. The boundary conditions are listed in vector format with each of the five numbers designating a boundary, starting at the bottom of the apparatus and continuing through the boundaries of all four regions. The initial conditions are listed in each subdomain and are again listed in vector format starting with region I.
Table 2.1: Boundary and initial conditions used with Femlab in the solution of the model

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$c_S$</td>
<td>Slow enantiomer concentration (mM)</td>
<td>{0, NA, NA, NA, 0}</td>
</tr>
<tr>
<td>$c_F$</td>
<td>Fast enantiomer concentration</td>
<td>{0, NA, NA, NA, 0}</td>
</tr>
<tr>
<td>$c_{CD}$</td>
<td>Ligand concentration</td>
<td>{20, NA, NA, NA, 20}</td>
</tr>
<tr>
<td>$c_{SCD}$</td>
<td>Slow complex concentration</td>
<td>{0, NA, NA, NA, 0}</td>
</tr>
<tr>
<td>$c_{FCD}$</td>
<td>Fast complex concentration</td>
<td>{0, NA, NA, NA, 0}</td>
</tr>
</tbody>
</table>

**Concentration boundary conditions**

**Boundary †**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N_S$</td>
<td>Slow enantiomer flux</td>
<td>{NA, $-c_S \cdot Q_{ho}$, $c_S \cdot Q_{feed}$, $-c_S \cdot Q_{to}$, NA}</td>
</tr>
<tr>
<td>$N_F$</td>
<td>Fast enantiomer flux</td>
<td>{NA, $-c_F \cdot Q_{ho}$, $c_F \cdot Q_{feed}$, $-c_F \cdot Q_{to}$, NA}</td>
</tr>
<tr>
<td>$N_{CD}$</td>
<td>Ligand flux</td>
<td>{NA, $-c_{CD} \cdot Q_{ho}$, $c_{CD} \cdot Q_{feed}$, $-c_{CD} \cdot Q_{to}$, NA}</td>
</tr>
<tr>
<td>$N_{SCD}$</td>
<td>Slow complex flux</td>
<td>{NA, $-c_{SCD} \cdot Q_{ho}$, 0, $-c_{SCD} \cdot Q_{to}$, NA}</td>
</tr>
<tr>
<td>$N_{FCD}$</td>
<td>Fast complex flux</td>
<td>{NA, $-c_{FCD} \cdot Q_{ho}$, 0, $-c_{FCD} \cdot Q_{to}$, NA}</td>
</tr>
</tbody>
</table>

**Flux boundary conditions**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$c^0_S$, $c^0_F$</td>
<td>Initial Concentrations (mM)</td>
<td>[0, 0, 0, 0]</td>
</tr>
<tr>
<td>$c^0_{SCD}$, $c^0_{FCD}$</td>
<td>Initial Concentrations</td>
<td>[0, 0, 0, 0]</td>
</tr>
<tr>
<td>$c^0_{CD}$</td>
<td>Initial Concentration</td>
<td>[20, 20, 20, 20]</td>
</tr>
</tbody>
</table>

NA - This type of boundary condition was not used for this boundary
† - The boundaries are located at \{0, 7, 15, 23, 30\} cm
2.4. Experimental

2.4.1. Materials

In order to complete the binding constant and moving bed electrophoresis studies, chemicals were purchased from several suppliers. Trizma base and racemic Homatropine Hydrobromide, were purchased from Sigma (St. Louis, MO, USA). The hydroxypropyl-β-Cyclodextrin, HPβCD, carrier ligand was purchased from Cargill Food and Pharma Specialties (Cedar Rapids, IA, USA) under the trade name Cavitrion 82005. Citric, Glacial Acetic and Phosphoric Acids were purchased from JT Baker (Phillipsburg, NJ, USA).

2.4.2. Analysis

A Hewlett Packard (Waldbonn, Germany) 3DCE capillary electrophoresis unit was used to execute the binding constant studies and quantify the results from the moving bed experiments. A 50 cm, 365 µm outer diameter 50 µm inner diameter capillary was used from Polymicro Technologies LLC (Phoenix, AZ, USA) with the detection window at 42 cm. The system was run at 20 kV with positive inlet polarity for 20 minutes and was kept thermostated at 24 degrees Celsius.

2.4.3. Vortex stabilized electrophoresis apparatus

A Vortex Stabilized Electrophoresis Apparatus as shown in Figure 2.3 was used for the moving bed electrophoresis studies and has been described in detail previously [3,30]. It consists of a grooved rotor and stator that form an annular gap where the electrophoretic separation takes place. When rotating, the grooves on the rotor and stator create Taylor-
like vortices that are stable at all angular velocities allowing for better cooling efficiency to remove the Joule heat dissipated by the electrophoretic separation. The vortices also serve to inhibit the detrimental natural convection which causes mixing in the axial direction that reduces the effectiveness of the electrophoretic separation. There are four external Instech (Plymouth Meeting, PA, USA) peristaltic pumps that control the counterflow, offtake flowrate and purge streams which are shown in Figure 2.2. A 300 W Spellman (Hauppauge, NY, USA) 2 kV power supply is used to apply the voltage which drives the electrophoretic separation.

2.5. Results and Discussion

2.5.1. Validation of the model

In order to use the proposed model to explore the range of operating conditions in a moving bed electrophoretic separation, the physical constants of the system first needed to be measured. The binding constants were calculated as 0.06 mmol\(^{-1}\) and 0.03 mmol\(^{-1}\) for \(K_s\) and \(K_f\) respectively using capillary electrophoresis data and the method of Lemesle-Lamache et. al [31]. The only other constants in the model that needed to be defined were the binding reaction kinetic rate constants, and due to the difficulty of measuring these parameters, they were left to estimate by comparing the model with experimental data. The constants and expressions used in the validation and solution of the model throughout this work are shown in Table 2.2.

The moving bed electrophoresis model was solved using the electrokinetic flow equations in the chemical engineering package of Femlab 3.1i (Comsol, Stockholm,
Table 2.2: Constants and expressions used in the solution of the model

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_F, D_S$</td>
<td>Diffusion + Dispersion constants</td>
<td>$2.1 \cdot 10^{-8} , \text{m}^2/\text{s}$</td>
</tr>
<tr>
<td>$D_{CD}, D_{SCD}, D_{FCD}$</td>
<td></td>
<td>$2.05 \cdot 10^{-8} , \text{m}^2/\text{s}$</td>
</tr>
<tr>
<td>$K_F$</td>
<td>Fast complex equilibrium constant</td>
<td>$0.03 , \text{mM}^{-1}$</td>
</tr>
<tr>
<td>$K_S$</td>
<td>Slow complex equilibrium constant</td>
<td>$0.06 , \text{mM}^{-1}$</td>
</tr>
<tr>
<td>$k_F, k_S$</td>
<td>Complex formation rate constant</td>
<td>$0.0003 , \text{mM}^{-1}\text{s}^{-1}$</td>
</tr>
<tr>
<td>$c_{CD}$</td>
<td>Cyclodextrin inlet concentration</td>
<td>$20 , \text{mM}$</td>
</tr>
<tr>
<td>$c_F, c_S$</td>
<td>Enantiomer inlet concentration</td>
<td>$28.1 , \text{mM}$</td>
</tr>
<tr>
<td>$\eta_F, \eta_S$</td>
<td>Electrophoretic mobilities</td>
<td>$1.77 \cdot 10^{-8} , \text{m}^2/\text{V} \cdot \text{s}$</td>
</tr>
<tr>
<td>$\eta_{CD}$</td>
<td></td>
<td>$0 , \text{m}^2/\text{V} \cdot \text{s}$</td>
</tr>
<tr>
<td>$\eta_{FCD}, \eta_{SCD}$</td>
<td></td>
<td>$6.5 \cdot 10^{-9} , \text{m}^2/\text{V} \cdot \text{s}$</td>
</tr>
<tr>
<td>$A_s$</td>
<td>Cross sectional area of chamber</td>
<td>$0.685 , \text{cm}^2$</td>
</tr>
<tr>
<td>$Q_{to}, Q_{bo}$</td>
<td>Offtake flowrates</td>
<td>$0.1 , \text{mL/min}$</td>
</tr>
<tr>
<td>V (constant field)</td>
<td>Constant field voltage profile</td>
<td>$5000 , \text{V}$</td>
</tr>
<tr>
<td>V12 (step gradient)</td>
<td>Step gradient -45 V/cm in Regions I, II</td>
<td>$4500 , \text{V}$</td>
</tr>
<tr>
<td>V34 (step gradient)</td>
<td>Step gradient -55 V/cm in Regions III, IV</td>
<td>$5500 , \text{V} + 675 , \text{V}$</td>
</tr>
<tr>
<td>V (continuous)</td>
<td>Continuous gradient, focal points</td>
<td>$4062.5 , \text{V} + 3125 , \text{V}^2$</td>
</tr>
</tbody>
</table>
Sweden), the result of which is a set of concentration profiles for each of the five species modeled. The time dependent solver was run until a point beyond a steady state, usually 0.5 million seconds, was reached. This long solution time while not technically required for any useful separation, allowed the calculation of the moving bed model even under conditions where a steady state did not exist, and allowed for gradual convergence without requiring an accurate initial guess. In order to verify that the proposed model could accurately predict real conditions in a moving bed electrophoretic separation, the model was solved and compared to experimental data acquired under the same conditions. A separation using the Homatropine – HPβCD system was carried out in the Vortex Stabilized Electrophoresis Apparatus as described above with a counterflow rate of 0.353 mL/min, offtake flowrates of 0.1 mL/min, a feed flowrate of 0.05 mL/hr at a concentration of 10 mg/mL and a total applied voltage of 1500 V. The comparison between the model and experimental data is shown in Figure 2.4. This constituted a 0.5 mg/hr separation which was plagued by tailing effects of the slow enantiomer into the region below the feed.

After investigating a possible conductivity mismatch between the fast and slow enantiomer regions which turned out not to be the cause of the tailing [32], we tested the hypothesis that slow homatropine – HPβCD binding kinetics could be driving this tailing phenomenon. In comparing the model to the experimental data, it was found that the forward reaction rate constants, $k_f$ and $k_s$, provided a best fit when both values were 0.0003 mmol$^{-1}$s$^{-1}$. These slow reaction rate values were able to qualitatively reproduce
Figure 2.4: A comparison between the proposed model and experimental data taken from the Vortex Stabilized Electrophoresis Apparatus. The upward arrow at 0.15 meters denotes the feed stream location and the downward arrows at 0.07 and 0.23 meters represent the bottom and top offtake locations respectively. For the most part the predicted concentrations and tailing effects match the trends of the experimental data, showing that this linearized model neglecting electronic effects is able to predict operating conditions of a real moving bed electrophoretic separation. The large concentration mismatch between the fast enantiomer data and the model could possibly be due to the neglect of ionic coupling or to a transient in the experimental data.
the tailing phenomenon that was present in the experimental data thus suggesting that this model could be used to predict operating conditions and concentration profiles in a moving bed electrophoresis separation.

During the collection of the experimental data for the validation, voltage measurements were taken at 18 points along the chamber using platinum wire electrodes screwed into the back of the Vortex Stabilized Electrophoresis Apparatus. These voltage measurements were taken using a custom designed high impedance operational amplifier buffer circuit that interfaces with Labview 6 (National Instruments, Austin, TX, USA) on a PC. A deviation of less than 5% from an electric field of -50 V/cm was observed during these measurements for the duration of the experiment. This data suggests that nonlinear effects are not significant with this enantiomer-ligand system, thus justifying the use of a linear model.

It should be noted here that the very low 0.5 mg/hr maximum throughput is purely a function of the homatropine – HPβCD system and is not related to the Vortex Stabilized Electrophoresis Apparatus. The tailing effects from the low binding constant and slow binding kinetics greatly reduce the maximum processing rate of the homatropine enantiomers and this is not representative of the moving bed electrophoresis technique as a whole. However, the low throughput of the homatropine enantiomers makes this an excellent test case for the ability of gradient moving bed electrophoresis to improve the maximum throughput of the separation. Prior work with the Vortex Stabilized
Electrophoresis Apparatus has resulted in greater than 10 mg/hr throughput using piperoxan enantiomers and a sulfated β-cyclodextrin chiral selector [3].

2.5.2. Definition of focal points

By opposing the counterflow in a moving bed separation with an electric field gradient, depending on conditions there can be a point in the unit where the electromigration of a compound is equal and opposite of the fluid flow. This point is called a focal point, as the compound has no net mobility at this position. This focal point is illustrated in Figure 2.5 where a band of analyte is being focused in a linear electric field gradient which produces a parabolic electric potential. This type of applied gradient, called Field Gradient Focusing [23], can be formed using a computer-regulated voltage controller with a plurality of electrodes that are able to generate a desired voltage profile along the axis of separation. The focal point can be determined analytically as the point where the electrophoretic velocity and the counterflow velocity balance each other, i.e.,

\[
0 = u_x + u_i = u_x + \eta_i \cdot (-\Delta\phi) \quad \text{or} \quad u_x = \eta_i \Delta\phi,
\]

where \( u_x \) is the fluid velocity in the region of interest. This definition allows a focal point map to be created for a moving bed electrophoretic separation. By setting the focal points of the two components, a linear electric field gradient can be defined as shown in Figure 2.6 which can be used to drive the solutes to two different positions within the apparatus.
**Figure 2.5:** Dynamic Field Gradient Focusing in a linear electric field gradient. Since the electrophoretic migration of a compound is proportional to the electric field, at some point along the gradient, the electric field will balance the fluid flow within the apparatus, thus creating a focal point.
Figure 2.6: A focal point map for a moving bed electrophoretic separation. The fluid velocity profile is shown as the solid line and the fast and slow component focal points are shown as a circle and triangle respectively. The resultant electric field is shown as a dashed line. Here are examples of two different linear electric field gradient profiles, line A with a positive slope and line B with a negative slope.
2.5.3. *Gradient moving bed separations*

The main limiting factor of the throughput of moving bed separations stems from the tailing effects of the fast enantiomer into Region III and likewise the contamination of the slow enantiomer in Region II. At sufficiently high throughput there will be unacceptable contamination in both offtakes and this point determines the largest amount that can be processed. The benefit of applying electric field gradients to moving bed separations is that these undesirable tailing effects can be reduced allowing larger amounts to be processed with the same purity. By increasing the force on the slow enantiomer (Region III) with a higher electric field, the compound is able to offset more of the counterflow velocity in that region thus slowing its migration velocity towards the slow offtake. This also aids the fast enantiomer in overcoming the counterflow in Region III where it is a contaminant, thus reducing the amount of tailing. In the case of the fast enantiomer, the electric field is reduced in Region II causing the compound to have a slower electrophoretic velocity which decreases its migration speed towards the fast offtake. The lower electric field also decreases the electrophoretic velocity of the slow enantiomer in Region II causing it to be more easily overcome by the counterflow velocity, thus reducing the tailing effect. One other result of the gradient slowing down the enantiomer migrations is the increase in enantiomer concentrations in the apparatus. The flux is the product of concentration and velocity and this must remain constant at a given throughput in order for the mass balance to be maintained. This requires the concentration to be inversely proportional to the change in migration velocities towards the offtakes.
Once the focal points have been determined, an electric potential function can be obtained by integrating the electric field. The moving bed electrophoresis model can then be solved using the resultant electric potential profile, and solutions for several different electric field cases are shown in Figure 2.7. The focal point map shows the existence of both stabilizing and destabilizing electric fields. The negative-sloped “stabilizing” gradient case shows that the tailing effects of the fast enantiomer towards the slow offtake and vice versa are minimized even though the concentrations within the unit are increased approximately ten fold when compared to the constant field separation. This increasing concentration within the unit could possibly create problems with precipitation, but the control of the gradient allows this to be avoided by operating just at the solubility limit. The opposite case, the positively-sloped “destabilizing” gradient example made the tailing effects more pronounced causing nearly 25% contamination in each offtake. The concentrations within the unit were also decreased by about 50% from the constant field separation case.

The focal point map is a useful tool for showing how moving bed separations react to the changing gradient conditions. One important observation that came from this linear model is the fact that a step gradient at the feed location is the best possible case for decreasing tailing effects and increasing throughput in a moving bed separation. This special case where the magnitude of the electric field is larger in region III above the feed than below in region II suggests that the quest for continuous gradient separations in SMB chromatography might not yield any significant throughput increase over the already existing step gradient case commonly used in the SMB process. In order to make
Figure 2.7: The focal point map for homatropine – HPβCD moving bed enantiomer separations as well as resultant concentration profiles from these different electric field cases. In the case of a constant electric field, or non gradient operation, the focal points are located at plus or minus infinity. This leads to a straight line and Figure B is the result. As the gradient is shifted towards a negative slope, the separation is stabilized increasing the concentrations within the unit while reducing the tailing effects and allowing a larger amount to be processed. Figure C is an example of a separation using a stabilizing gradient; notice the 10 fold concentration increase over the constant electric field case. As the slope is shifted positive a destabilizing gradient results and the ensuing separation is shown in Figure D. Notice how the concentrations are very low in the apparatus and the tailing effects are pronounced. In all cases the electric field at the feed point was kept constant at -50 V/cm in order to keep the counterflow value from fluctuating.
sure that this was in fact the case, higher order electric fields were examined, up to fourth order polynomials, and in all cases these gradients yielded results that were inferior when compared with the simple step gradient at the feed port. This is not to say that gradient moving bed separations are not useful as they have the potential to limit the concentrations of the compounds within the unit, which may in fact allow a higher throughput to be practical while still avoiding precipitation, but this linear model cannot take solubility effects into account. Other benefits of continuous gradients include decreasing the time required to reach steady state and reducing the tailing effects that diminish resolution.

2.5.4. Operating parameter diagram

In order to better demonstrate the effects of gradient operation on a moving bed process, a common SMB chromatography analysis method is presented below which is colloquially referred to as “Morbidelli’s Triangle [12].” This diagram maps the relationships between the operating parameters in regions II and III, showing the regime where a complete separation can occur [29]. In order to adapt this triangle diagram to an electrophoretic separation, several of the parameters must be altered. In SMB chromatography, the flow rate ratios, $m_j$, are defined as the net fluid flow rate divided by the solid phase flow rate. Since there is no solid phase in an electrophoretic moving bed, these flow rate ratios can be recast as the fluid velocities in each region. The borders of the triangle are set by the electrophoretic velocities of the two components, $\eta_s \cdot E$ and $\eta_f \cdot E$ with the result shown in Figure 2.8. This simplified figure shows that there are several regions of operation in a moving bed electrophoretic separation and, in each case,
Figure 2.8: A simplified “Morbidelli’s Triangle” diagram showing the operating regions of an electrophoretic moving bed separation. The counterflow velocity in region II can be picked as a point on the 45 degree line. A feed line is then constructed based on the inlet fluid flow and the concentrations of the products. The end of this feed line determines the counterflow velocity in region III and the outcome of the separation. The triangle area, which is greatly exaggerated in this figure, is the set of operating parameters where both compounds can be separated at a given purity. If the fluid velocities in regions II and III fall outside of the area between $\eta_sE$ and $\eta_fE$ then there will be contamination in the offtake streams. In actuality the $\eta$ values are not necessarily constant as they depend on the binding relationship between the enantiomer and the chiral selector which leads to skewing of the triangle.
the purities of the two products are different. The triangle region in the operating parameter diagram shows the area in which the operation of the moving bed electrophoresis unit will result in two pure offtakes, which is the desired operating region, but the simplified figure does not accurately represent a true moving bed separation since the electrophoretic mobilities are a function of the concentrations and binding constants of the enantiomer and carrier ligand. This causes shifts in the triangle which require a more accurate model to be used to predict the actual desired operating parameters.

The moving bed electrophoresis model developed in this paper was used to prepare the triangle diagram shown in Figure 2.9. The model was solved under varying counterflow rates at increasing feed flowrates, and the resultant concentration profiles were examined for the points at which the slow enantiomer reached 1% contamination in the fast offtake and vice versa. Once the counterflow rates for a 99% pure separation were found, the fluid velocities in regions II and III were plotted at each throughput level resulting in the curves in Figure 2.9. At some feed flowrate, the 99% contamination of the offtakes happened at the same counterflow rate. This is the maximum throughput at which this separation can be operated and it is designated by the point farthest from the $u_{II} = u_{III}$ line.

In order to show the benefits of gradient separations, a homatropine-HPβCD separation with a constant electric field of -50 V/cm was compared with a continuous gradient separation and a step gradient separation as shown in Figure 2.9. The results show a massive increase in the area of the 99% purity region for both of the gradient cases,
Figure 2.9: A triangle diagram illustrating the effects of different stabilizing electric field gradients. The solid line denotes the region where the model predicts the purity of both homatropine enantiomers to be greater than 99% under a constant electric field of -50 V/cm. The dashed line shows the 99% purity region of the same separation but with a step gradient at the feed port from -45 V/cm in region II to -55 V/cm in region III. This small 10 V/cm step has a massive effect on the separation, greatly expanding the 99% purity region and allowing a 7.66 fold increase in the maximum processing rate of homatropine enantiomers. The dotted line denotes a continuous electric field gradient with focal points at (0.07 m, -45 V/cm) and (0.23 m, -55 V/cm). This continuous gradient also has a very large effect on the 99% purity region with a predicted maximum enantiomer throughput increase of 7 fold over the constant field separation but the concentrations in the unit were 40% less at the same throughput value as the step gradient separation.
suggesting that this separation is much easier with a gradient applied. In fact a 7.66 fold increase in the amount of homatropine processed for the step gradient case and a 7 fold increase for the continuous gradient case is predicted by the model. These large increases are probably exaggerated due to the fact that this model neglects nonlinear ionic effects that would alter the electric field depending on the concentrations within the unit, but it is still a staggering increase in the amount of throughput at 99% purity. The continuous gradient separation showed a 40% reduction in homatropine concentration within the unit at the same throughput, suggesting that continuous gradient separations could be used with some advantage in situations where the solubility of the compound is low. The maximum throughput in the gradient case was only 9% lower than that of the step gradient separation which further demonstrates the fact that continuous gradient moving bed separations hold a lot of promise for increasing throughput and purity.

2.6. Conclusions

The model developed in this paper has shown that applying continuous electric field gradients to moving bed electrophoresis processes has several beneficial effects on the separation. The manipulation of the electric field gradient allows focal points to be set at different places within the apparatus, and this allows a large degree of control of the concentration profiles during a separation. This focal point control can be used to reduce peak tailing, which is the major limiting factor on the maximum product throughput at a given purity. The maximum allowable processing rate was shown to be 7.66 fold greater using a 10 V/cm step gradient when compared to a constant -50 V/cm electric field
separation, and the “operating window” of counterflow rates for a 99% purity separation was greatly expanded.

The moving bed electrophoresis model developed in this work takes into account binding kinetics between enantiomers and a chiral ligand as well as the ability to control the electric field. The only major simplification was the uncoupling of the concentrations from the calculation of the electric field, thus linearizing the model, but this was shown to have a minimal effect on the concentration profiles through validation with experimental data. The linearized model showed that the step gradient case allows for the highest possible product throughput increase, but the concentrations within the unit can quickly rise to the point where precipitation becomes a problem. Continuous gradient separations allow a significant reduction of these concentrations without a large sacrifice in total throughput justifying their further study.

2.7. References


3. TRUE MOVING BED ELECTROPHORESIS USING STEPPED ELECTRIC FIELD GRADIENTS

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As submitted to Electrophoresis.

Keywords

Chiral Separation, Enantiomer, Gradient Elution, Simulated Moving Bed Chromatography, Preparative Electrophoresis

Abbreviations

Simulated Moving Bed: SMB
True Moving Bed: TMB
Hydroxypropyl-ß-Cyclodextrin: HPβCD
Fluorescein Isothiocyanate: FITC
Hemoglobin: Hb
Bovine Serum Albumin: BSA
Epsilon Amino Caproic Acid, 6-aminohexanoic acid: EACA
Dynamic Field Gradient Focusing: DFGF
Isotachophoresis: ITP
MWCO: Molecular Weight Cut Off

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3.1. Abstract

True moving bed electrophoresis has been shown to be an effective technique for the bench scale separation of enantiomers, and it is desired to increase the maximum possible throughput attainable with the process by using electric field gradients. Homatropine enantiomer separations were performed and results using a stepped electric field gradient were compared to those using a traditional non-gradient separation. In order to accomplish this, a newly designed stator was constructed for use with the Vortex Stabilized Electrophoresis Apparatus that has three sets of electrode housings, one set at both ends and one in the middle of the chamber. There were several problems related to the membranes used at the middle electrode. The dialysis membranes were permeable to the homatropine enantiomers, and while a switch to anion exchange membranes prevented the permeation of the homatropine, this caused a pH shift that interrupted binding to the hydroxypropyl-β-cyclodextrin chiral selector. These problems prevented any meaningful data from being collected using homatropine enantiomers, and due to this, a proof of concept study was conducted using two bovine proteins. The separations using fluorescein labeled BSA and bovine hemoglobin showed that a 63% increase in the maximum processing rate was attainable. The maximum throughput using the non-gradient process was 30.6 mg/h and the maximum was 50.0 mg/h using an electric field gradient that was 10% lower than the non-gradient field in section II and 10% higher in section III.
3.2. Introduction

The simulated moving bed (SMB) chromatography process was originally invented by UOP the 1960’s as a less energy intensive alternative to distillation for the separation of xylenes [1]. The SMB process generally uses between 6 and 16 identical columns in series and the addition of the desorbent stream moves the components through the train while the simulated “movement” of the stationary phase, mimicked by occasional switching of the columns in a periodic fashion, counteracts the migration of the components through the unit [2]. This creates a system where feed is continuously introduced while extract and raffinate offtakes are continuously removed as demonstrated previously by Juza et. al. [1]. SMB has completely revolutionized the process of chromatography, providing an economy of scale that was not previously achievable using batchwise techniques, and it has fundamentally changed the way that continuous adsorptive separations are performed [1].

The adoption of SMB by the pharmaceutical and fine chemical industries spurred major advances in the technique over the last decade, especially in the area of chiral separations [1]. Analogies of SMB have recently emerged in solid-liquid extraction [3], gas chromatography [4] and true moving bed (TMB) electrophoresis [5,6]. While electrophoretic processes cannot currently reach the scale of chromatography, TMB electrophoresis has been shown to be an effective model system for the application of process improvements such as counterflow velocity alterations using filtration [7] and the addition of stepped and continuous electric field gradients [8] which potentially benefit the SMB process as well.
The ability to conduct electrophoretic chiral separations was derived from the discovery of several classes of molecules such as the cyclodextrins and crown ethers [9,10]. The conical shapes of these ligands provide a binding cavity which can be functionalized to promote stereospecific binding interactions that allow the separation of enantiomers in an electric field [11,12]. The fact that these chiral selective ligands are soluble allows the adsorptive discrimination to take place entirely in the liquid phase [13], and this greatly simplifies the electrophoretic true moving bed process. In comparison with the multiple columns required in SMB chromatography, TMB electrophoresis does not require a packing and only requires one chamber with multiple inlet and outlet streams to accomplish a moving bed separation as shown in Figure 3.1.

There have been many preparative scale electrophoresis systems used to successfully separate enantiomers. There are several flat bed apparatuses such as the Octopus [14] and the RF3 [15] that have been used to separate milligram quantities of enantiomers in continuous elution and continuous recycle operation. In these systems there are electrodes at the sides of the apparatus that pull the components perpendicular to the flow path so that they elute at different offtake ports at the end of the rectangular chamber [14]. These types of units can be operated as once-through or as recycle systems using multi-channel peristaltic pumps and have been used for isoelectric focusing [16-19], zone [20,21] and ITP [15] enantiomer separations.
Figure 3.1: A true moving bed electrophoresis separation. Electromigration forces the components downward as they are counteracted by a fluid flow upward. The fast component is able to overcome the fluid flow and migrate to the bottom offtake while the slower component is overcome by the counterflow and pushed upward towards the top offtake. An idealized concentration profile of a two component separation is shown on the right. One important item to notice is the tailing of the fast component into the region above the feed and vice-versa. At a certain throughput these tails become so large that they contaminate the top and bottom offtakes with the undesired product, and this limits the processing rate of the separation.
Gel media has been used to conduct milligram scale separations of enantiomers as well. Units such as the Bio-Rad Mini Prep continuous gel elution cell use a column of gel that acts as a sieving medium which separates the enantiomer-ligand complexes in a time-resolved manner [22,23]. Multi-compartment membrane based devices such as the IsoPrime [24] and GradiFlow [25] units can be used to separate amphoteric enantiomers using isoelectric focusing assisted with chiral selective ligands which bind to the enantiomers, slightly altering their isoelectric point [25-28]. These apparatuses use isoelectric membranes stacked to form multiple chambers in parallel with electrodes at either end of the stack. A fluid is pumped through the chambers parallel to the membranes, which form a pH gradient thereby causing focusing perpendicular to the fluid flow through the unit. The buffer is eluted from the membrane chambers at the end of the unit allowing the focused components to be removed from the apparatus [26].

The Vortex Stabilized Electrophoresis Apparatus [29] was originally developed for use with isoelectric focusing but has since been expanded for use in many different free-flow electrophoresis modes such as isotachophoresis [29] and zone electrophoresis [5]. This instrument is ideally suited for TMB electrophoresis and has previously been used to separate up to 10 mg/h of piperoxan and homatropine enantiomers at up to 99% purity in both offtakes [5]. The Vortex Stabilized Electrophoresis Apparatus design allows modification for use with stepwise, and in the future, continuous electric field gradients along the axial length of the separation chamber.
Solvent gradients have been commonly used in chromatography for many years, and the application of stepwise gradient elution to the SMB process has allowed large increases in throughput and separation efficiency [30-34]. It has recently been shown that electric field gradients in TMB electrophoresis are analogous to solvent gradients in SMB chromatography, allowing electrophoresis to be used for predicting gradient behavior in the generalized moving bed process [8]. Prior mathematical modeling of a homatropine chiral separation has shown that step gradients in the electric field can have up to a 7.66 fold increase in throughput at a constant purity level in both offtakes [8]. This work will demonstrate the electric field gradient concept with a modified experimental set up using the Vortex Stabilized Electrophoresis Apparatus.

In the future, continuous electric field gradients will be applied to TMB electrophoresis using a preparative scale Dynamic Field Gradient Focusing (DFGF) [35] apparatus based on the Vortex Stabilized Electrophoresis Apparatus design [29]. Modeling has shown that continuous electric field gradients provide substantial benefits in process throughput in comparison with non-gradient moving bed separations while reducing the concentrations within the apparatus and decreasing the time required to reach steady state when compared to stepwise field gradients [8]. The instrument is currently being developed, but in order to prove the concept of electric field gradient TMB electrophoresis and corroborate the modeling work done previously, a stepwise electric field gradient will be examined experimentally in this work.
3.3. Theory

The TMB electrophoresis process is based on the principles of Flow Counterbalanced Capillary Electrophoresis [36] in which a fluid flow counteracts the electromigration of the compounds allowing peaks to separate at much higher resolution before they pass by the detector [36,37]. In the limiting case of a two component separation, the counterflow stream opposes the migrating species at a rate between the electromigration velocities of the two compounds. This allows the two species to move in opposite directions, with the faster of the species able to overcome the fluid flow while the slower component is carried along with the bulk fluid motion. TMB electrophoresis uses this concept to perform a continuous separation where the compounds are continuously fed into the middle of the apparatus and migrate in opposite directions toward the fast and slow component offtakes [5] as shown in Figure 3.1.

In order to model the TMB electrophoresis process, the system can be broken down into smaller regions or sections [6] as shown in Figure 3.2. These four regions are the portions of the separation chamber between the input and offtake streams and are differentiated from each other by the fluid velocity profile in each section. The counterflow buffer stream is introduced into the chamber at the bottom of the unit, resulting in a fluid velocity in region I. The bottom offtake forms the border between section I and II, and because some of the counterflow exits the separation chamber at this point, the fluid velocity in region II is lower than in region I. The feed stream is the means by which the compounds are introduced into the separation chamber and this fluid input, while generally small compared to the counterflow and offtake flowrates, causes a
**Figure 3.2:** The four regions of a moving bed separation, shown here for the TMB electrophoresis process. These four regions are separated by inlet and offtake streams which cause differences in the fluid velocity in each region, shown as $u_I - u_{IV}$. The bulk of the separation occurs in regions II and III, below and above the feed respectively. Regions I and IV serve to keep the products from leaving the ends of the separation since the counterflow velocities are too fast and too slow respectively for the products to overcome. An important thing to notice is an increase of the counterflow velocity between regions II and III due to the introduction of the feed stream which has a detrimental effect on the separation efficiency.
small increase in the fluid velocity in region III over section II. The top or slow component offtake forms the border between sections III and IV and this stream reduces the velocity in region IV to the lowest counterflow velocity in the chamber before it exits out the purge stream at the top.

The changes in fluid velocities between the four regions have a significant impact on the purity and throughput of the separation. The bulk of the separation occurs in sections II and III as can be seen in the idealized concentration profiles of Figure 3.1, with region II containing the majority of the fast component and region III containing the majority of the slow component. The increase in counterflow velocity between sections II and III has a detrimental effect on the separation due to the difference between the fast and slow component migration rates [7]. Under proper separation conditions in region III, the slow component is overcome by the counterflow and moves towards the top offtake. The step change in counterflow velocity in between regions II and III due to the feed introduction causes an increase in the counterflow velocity which increases the rate that the slow component is pushed towards the top offtake in section III. This also makes it more difficult for the fast component to overcome the counterflow velocity in section III, causing more of the fast component to migrate into region III, increasing the size of its contaminating tail towards the top offtake. This contaminating effect at the top offtake requires that the counterflow velocity be reduced in the entire unit to compensate for the section II/III velocity step, and this causes a similar effect at the bottom offtake. The resulting reduction in counterflow causes the section II velocity to be lower, and this
makes it easier for the slow component to overcome the counterflow in region II, increasing the size of its contaminating tail towards the bottom offtake.

The detrimental effect caused by the introduction of the feed can be reduced by maximizing the concentration of the feed stream, but this method can never completely eliminate the unfavorable II/III velocity step gradient. One method that has been used to reverse this phenomenon is the addition of a filtration unit outside the system that recycles fluid from the separation chamber at the section II/III boundary and pumps it through a membrane system that selectively removes buffer from the stream before it is pumped back into the apparatus. Known as filtration-enhanced TMB electrophoresis (FE-TMB) this has been shown to increase the maximum theoretical processing rate by 3.1 fold over the base TMB electrophoresis case [7].

Another method that can be used to counteract the effects of the addition of the feed stream is the use of an electric field step gradient [8]. The above analysis of the increase in tailing phenomena due to the feed addition assumed that the electric field was equal in both sections II and III. The theory of gradient TMB electrophoretic separations has already been thoroughly explored [8], and it has been shown that the addition of a step electric field gradient can increase the possible throughput of the separation by 7.66 fold [8]. The reason that the electric field gradient yields such a large theoretical increase in the maximum processing rate arises from a similar analysis to the unfavorable velocity step discussed above. Decreasing the field in section II causes both the fast and slow components to electromigrate slower, and this makes it more difficult for the slow
component to infiltrate region II reducing the tailing effect. Similarly in section III, the electric field is increased and this raises the electrophoretic velocities of the components, making it more difficult for the fast enantiomer to enter region III, reducing its tailing effect. Thus the step electric field gradient reduces the unwanted tailing effects, and increases the amount that can be processed before the tailing reaches an unacceptable contamination level in either offtake.

3.4. Materials and Methods

3.4.1. Vortex Stabilized Electrophoresis Apparatus

The apparatus used for the experiments conducted in this work is a modified version of the Vortex Stabilized Electrophoresis Apparatus which has been described in detail previously [29]. The external peristaltic pumps that control the counterflow and offtake flowrates as well as the set up of the peripheral devices such as the chiller and syringe pump for the feed stream are the same as reported in [5,8]. In order to allow the electric field to undergo a step change within the apparatus, a new stator had to be designed and manufactured. In the new design, a pair of electrode housings were added at the middle of the chamber allowing separate power supplies to control the electric field in the top half of the chamber above the feed stream and the bottom half below the feed.

The three electrode stator was manufactured from Plexiglas in the same way as the previous stator, with the vortex generating tines and axial clearances the same as the prior design [29]. There was a slight change in the number of syringe offtake and measurement electrode ports as a result of the new design. There are now 25 ports
spaced along the front and back of the stator every 1.2 cm with 1.9 cm gaps between the three middle ports due to mechanical constraints arising from the new center electrode housings. In order to measure the concentration profile along the separation chamber at the end of each run, 1 ml syringes were used to remove 0.4 ml of fluid from the innermost 21 ports on the stator for analysis. The top and bottom offtakes are located on the front of the stator, 8 ports (10.3 cm) from the middle of the chamber, and the voltage measurement electrodes used for monitoring the electric field in sections II and III are located at the same heights along the back of the unit. A picture of the Vortex Stabilized Electrophoresis Apparatus with the new stator design is shown in Figure 3.3.

The electric field was applied using two different power supplies with the electrodes at the middle of the chamber grounded. This allowed both power supplies to be referenced to ground, preventing any condition where a power supply case could potentially reach unsafe voltages. Another benefit of grounding the middle electrodes is that the power supplies can be completely decoupled from each other, preventing any cross talk between the two voltage controllers and allowing for a more stable electric field. Since the middle electrode is grounded, in order to allow the electric field to have the same sign in both the top and bottom halves of the chamber one of the power supplies was operated in reverse polarity. The positive voltage was supplied by a 2 kV EC 600 power supply (E-C Apparatus, St. Petersburg, FL, USA) and the negative voltage was supplied by a 2 kV Spellman SL300 power supply (Spellman High Voltage, Hauppage, NY, USA) operated in reverse polarity mode.
Figure 3.3: The Vortex Stabilized Electrophoresis Apparatus with a three electrode stator to allow different electric fields in the top and bottom halves of the chamber. A 2 mm annular gap between the stator and the boron nitride rotor in the center of the apparatus forms the separation chamber. The electrode housings are divided from the separation chamber with membranes and flushed with electrode buffer solution that is circulated through the tanks at the back of the apparatus. The syringes along the chamber are used to remove samples to measure the concentration profile at the end of each run. At the same heights as the syringes along the back are voltage measurement electrodes that allow monitoring of the electric field during separation experiments. The counterflow, pumparound and offtake streams are controlled using the peristaltic pumps shown at the front of the apparatus.
The electric field was measured in sections II and III using the electrodes on the back of the stator with Radio Shack 22-812 Digital Multimeter (Radio Shack, Fort Worth, TX, USA). During the stepped field gradient experiments, the voltage between the middle of the chamber and the top and bottom offtake electrodes were measured every half hour. The power supplies were adjusted over the course of the runs to ensure that the electric fields set in sections II and III remained constant during the entire separation. For the non-gradient TMB runs, measurements were taken between the top and bottom offtake electrodes to keep the voltage across sections II and III constant throughout all of the experiments. Only one power supply was necessary during the non-gradient runs, and this was adjusted accordingly.

3.4.2. Homatropine enantiomer separations

The conditions for the non-gradient homatropine enantiomer separations and the CE analytical method used in the experiments have been previously published [8]. The electric field used in the non-gradient case was -50 V/cm, and in order to attempt to verify the benefits shown in the prior modeling work [8], a step gradient of plus and minus ten percent was used. This resulted in electric fields of -45 V/cm in section II and -55 V/cm in section III and this was applied to the three electrode stator apparatus using the two power supplies as described above and analyzed using the same method as the non-gradient work [8].
3.4.3. Bovine blood protein separations

In order to show the advantages of the stepped electric field gradient [8], TMB electrophoresis separations using bovine serum albumin (BSA) (98% by electrophoresis, Sigma-Aldrich, St. Louis, MO, USA) and bovine hemoglobin (Hb) (Sigma-Aldrich, St. Louis, MO, USA) were performed. The BSA was labeled with fluorescein isothiocyanate (FTIC) dye (Sigma-Aldrich, St. Louis, MO, USA) using the Molecular Probes (Invitrogen, Carlsbad, CA, USA) FluoReporter FITC labeling protocol. The buffer system used for all of the bovine protein separations was 10 mM tris base titrated to pH 9.04 with epsilon amino caproic acid (EACA), and both chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The Vortex Stabilized Electrophoresis Apparatus with the three electrode stator as described above was used for all of the bovine protein separations. The top and bottom offtake flowrates were set at 0.152 ml/min, the pumparound stream was maintained at 0.133 ml/min and the counterflow stream flowrate was varied in order to find the upper and lower bounds at which 5% contamination occurred in either offtake. In the non-gradient experiments, the voltage between the top and bottom offtake electrodes was held constant at 537 V, giving an average electric field in sections II and III of 25 V/cm. The middle electrode housing was disconnected from the recirculating electrode buffer circuit and power supply and the membrane partitioning the electrode from the separation chamber was replaced with a piece cut from a non-conductive Ziploc bag (S.C. Johnson, Racine, WI, USA) to prevent any alterations of the electric field.
The electric field gradient bovine protein separations were conducted in a similar manner to the non-gradient process described above. The middle electrode housings were reattached to the circulating buffer and the membranes were cut from 500 molecular weight cut off (MWCO) cellulose acetate dialysis membrane tubing (Spectrum Labs, Rancho Dominguez, CA, USA). The electrode was reconnected to both power supplies and grounded as described above. The electric field in section II was maintained at 22.5 V/cm and in section III at 27.5 V/cm for a plus and minus 10% step in the electric field. The flowrates were the same as the non-gradient case with the counterflow stream flowrate varied to find the upper and lower bounds where 5% contamination occurred in either offtake at each different feed flowrate until the maximum throughput was reached.

In all the bovine protein separations the unit was run for 6 hours, far longer than is necessary to reach a steady state, and the samples were removed from the syringes and analyzed to create a concentration profile along the length of the apparatus. The analysis was conducted using a Wallac Victor³ 1420 multilabel plate reader (Perkin Elmer, Wellesley, MA, USA) measuring both the absorbance of the sample at 405 nm and the 535 nm fluorescence emitted from the sample when excited with 485 nm light. The FITC-BSA concentration was determined by comparing the signal against a log-log calibration curve. Since the FITC molecule absorbs slightly at 405 nm, the absorbance was predicted based on a linear absorbance curve and subtracted from the total absorbance signal at 405 nm. The corrected 405 nm signal was then used with a linear calibration curve to determine the Hb concentration. The plates used for analysis were a Costar 3788 round bottom 96 well assay plate (Corning Inc., Corning, NY, USA). For
the analysis, 100 µl samples from each of the syringes and offtakes were used in each well, and 1/10 and 1/100 dilutions were performed to keep the absorbance and fluorescence signals in the linear range.

### 3.5. Results and Discussion

#### 3.5.1. Homatropine enantiomer separations

In order to attempt to verify the modeling work done previously [8], homatropine enantiomer separations were carried out in the Vortex Stabilized Electrophoresis Apparatus. The non-gradient separations have been published previously in order to estimate the parameters and confirm the accuracy of a linear model of the separation [8], and Table 3.1 gives details of the conditions of the separation shown in Figure 3.4a. The homatropine enantiomer separation, due to the slow binding kinetics with the hydroxypropyl-ß-cyclodextrin chiral selector, suffers from a large amount of fast enantiomer tailing into section III causing contamination of the top offtake. This tailing effect significantly reduces the amount of homatropine that can be processed.

Experiments conducted to attempt to find the maximum throughput of the separation showed that, with a processing rate of 1 mg/h racemic homatropine, the top offtake was contaminated with 4.4% fast enantiomer and the bottom offtake contained 16.4% contamination with the slow enantiomer. These experiments concluded that in order to achieve less than 1% contamination in both offtakes, a processing rate of less than 0.5 mg/h racemic homatropine must be used. The low throughput of this separation is unimpressive compared to previous work with piperoxan enantiomers where up to 10
Table 3.1: Conditions used for homatropine enantiomer separations

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<thead>
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<th>Description</th>
<th>Value</th>
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<td>$Q_{Feed}$</td>
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</tr>
<tr>
<td>$C_{Feed}$</td>
<td>Feed concentration homatropine</td>
<td>$10 \frac{mg}{ml}$</td>
</tr>
</tbody>
</table>

Non-gradient experiment

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<tr>
<td>II</td>
<td></td>
<td>$0.378 \frac{cm}{h}$</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td>$0.379 \frac{cm}{h}$</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>$0.238 \frac{cm}{h}$</td>
</tr>
<tr>
<td>$E$</td>
<td>Electric Field</td>
<td>$-50 \frac{V}{cm}$</td>
</tr>
</tbody>
</table>

Gradient experiment

<table>
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</tr>
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<tr>
<td>III</td>
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</tr>
<tr>
<td>IV</td>
<td></td>
<td>$0.157 \frac{cm}{h}$</td>
</tr>
<tr>
<td>$E_{Top}$</td>
<td>Electric Field, top half</td>
<td>$-55 \frac{V}{cm}$</td>
</tr>
<tr>
<td>$E_{Bot}$</td>
<td>Electric Field, bottom half</td>
<td>$-45 \frac{V}{cm}$</td>
</tr>
</tbody>
</table>
Figure 3.4: A comparison between (A) 0.5 mg/h non-gradient and (B) +/-10% electric field gradient homatropine separations. The non-gradient separation used an electric field of -50 V/cm and there is significant tailing of the fast enantiomer into region III, leading to 5.6% contamination in the top offtake. The addition of a +/-10% electric field gradient in fact appears to make the separation much worse, as the tails of both enantiomers are contaminating the top and bottom offtakes. This is due to a change in pH in the separation chamber because of the anion exchange membrane used in the middle electrode housing.
mg/h could be processed [5], so it was desired to improve the maximum throughput by the use of electric field gradients.

The addition of an electric field gradient to the homatropine enantiomer separation should significantly improve the detrimental tailing effect described above. In order to test stepped electric field gradient separations, a three electrode Vortex Stabilized Electrophoresis Apparatus stator was used, and the membranes in the top and bottom sets of electrode housings of the unit were pieces cut from 500 MWCO cellulose acetate dialysis membrane tubing (Spectrum Labs, Rancho Dominguez, CA, USA). Although this membrane pore size is larger than the 356 g/mole molecular weight of homatropine, under proper separation conditions the enantiomers never reach the ends of the column due to the very fast and slow counterflow velocities in sections I and IV respectively.

The membranes at the middle set of electrodes were initially cut from 100 MWCO cellulose acetate dialysis tubing (Spectrum Labs, Rancho Dominguez, CA, USA), but it was very quickly realized that the membrane was almost completely permeable to the homatropine enantiomers. Due to the electric fields of -55 V/cm and -45 V/cm being maintained in the top and bottom halves of the chamber respectively, the middle electrode must act as a cathode. This negative electrode creates a large electrophoretic driving force for the positively charged homatropine enantiomers, which allows them to permeate the membrane. This caused more than 90% of the injected homatropine to be lost through the membrane to the recirculating electrode buffer, which prevented the separation from reaching usable throughputs.
Since membranes with a pore size smaller than 100 MWCO were not available, AMH anion exchange membranes (Electrosynthesis Corp., Lancaster, NY, USA) were substituted in the middle electrode housings. Since anion exchange membranes cannot pass cations, the homatropine enantiomers were prevented from passing through to the electrode buffer. Several field gradient experiments were run at 0.5 mg/h to attempt to find a region where both the top and bottom offtakes produced pure enantiomers, but the best result required 80 h of run time to reach steady state and is shown in Figure 3.4b with the experimental conditions shown in Table 3.1. The results show contaminations of 3.6 % slow enantiomer in the bottom offtake and 2.5% fast enantiomer in the top offtake. Merely looking at the contamination numbers at both offtakes does not tell the entire story, as the concentration profile of the gradient separation in Figure 3.4b shows large contaminating tails of both fast and slow enantiomers near the top and bottom offtakes respectively.

The contamination tails near each offtake were of great concern, as the modeling data clearly showed that the electric field step gradient should greatly reduce the amount of undesirable tailing. After the samples were examined by CE to determine the amounts of the enantiomers in each fraction, the pH of each was measured and this data is plotted in Figure 3.5. The results show that the pH in sections I and II varies between 4 and 4.5, which is similar to the initial pH of 4.03 used in the separation buffer. The more alarming observation was the sharp step at the middle electrode to pH 7.5 in sections III and IV. Initial experiments done to optimize the homatropine and hydroxypropyl-β-
Figure 3.5: A plot of the measured pH of the samples at the end of the homatropine gradient run shown in Figure 3.4b. An anion exchange membrane was used at the center electrode to prevent the positively charged homatropine from leaving the separation chamber. The ion selective nature of the membrane required that all of the current carrying ions be negatively charged, and this caused a large pH shift from approximately 4.5 in section II to 7.5 in section III. The hydroxypropyl-β-cyclodextrin does not bind homatropine selectively above pH 5.5, thus in section III there was no functioning chiral selector. The purpose of electric field gradients is to reduce the size of the fast enantiomer tail in section III, but since the chiral discriminator was ineffective, there was no ability to selectively reduce the tailing of the fast enantiomer.
cycloextrin enantiomer-selector system showed that the chiral discriminator does not preferential bind to either enantiomer above pH 5.5, with binding activity significantly curtailed above pH 5.0. In effect, the data in Figure 3.5 shows that there is only a difference in electrophoretic mobility between the fast and slow enantiomers in the bottom half of the chamber, and the electrophoresis in the top half is completely non-specific for either enantiomer.

The lack of enantiomer-selector interaction in the top half of the chamber completely undermines the ability of the electric field gradient to reduce tailing in the homatropine separation. The top half of the chamber, sections III and IV, is the portion in which the electric field needs to be higher in order to reduce the ability of the fast enantiomer to enter section III. Instead, the pH shift caused by the anion exchange membrane causes the enantiomers to both migrate at the same rate, which significantly increases the size of the fast enantiomer tail towards the top offtake. Even with this lack of specificity in the top half of the chamber, the separation in the bottom half should function normally. The problem is that since the fast enantiomer is free to tail towards the top offtake, in order to accomplish a high purity separation at both ends the counterflow velocity in the entire chamber must be reduced. This causes the concentration of fast enantiomer in the middle of the chamber to be reduced while the slow enantiomer concentration is increased at the middle, since less is pushed toward the top offtake. Since both enantiomers travel at the same rate in the top half of the separation chamber, this improves the purity of the slow enantiomer at the top offtake, but causes the tailing of the slow enantiomer towards the bottom offtake to significantly increase. These undesired increases in tailing due to the
addition of the middle electrode prevent electric field gradients from being studied successfully with the homatropine system. In spite of these setbacks, the theory behind the technique is sound, and in the next section this will be verified using bovine proteins.

3.5.2. Bovine protein separations

The permeability of the dialysis membranes and the pH shift caused by the ion exchange membranes were both phenomena that were not predicted by the linear model which showed a significant benefit from the addition of electric field gradients to the TMB electrophoresis process. There are several possible solutions to the problems caused by the middle electrode, e.g., finding membranes that have significantly smaller MWCO values, but the easiest possible way to demonstrate the benefits of using the electric field gradient technique is by separating components that are much larger, so the permeability of the dialysis membranes is no longer an issue. Since TMB electrophoresis is a technique that can be used for compounds other than enantiomers, demonstrating the benefit of the electric field gradient on a separation of protein mixtures will prove the concepts postulated in earlier work, even though the previous modeling work cannot be directly validated.

Electric field gradient TMB separations using bovine FITC labeled serum albumin and hemoglobin were conducted in the Vortex Stabilized Electrophoresis Apparatus. The membranes used on the middle electrodes were cut from the same 500 MWCO dialysis tubing as the top and bottom electrode membranes used in the homatropine experiments. The molecular weights of both the albumin and hemoglobin are several orders of
magnitude greater than the 500 Dalton cut off of the membrane, so permeability was not an important issue in the protein TMB separations.

During the initial gradient TMB separations using FITC-BSA and Hb, it was noticed that the Hb did not actually reach the top offtake, and focused into an isotachophoresis (ITP) like band that was in direct contact with the FITC-BSA. The buffer system used was 10 mM tris-acetate at pH 9.0, and it was determined that the acetate was forming a system or eigenpeak behind the Hb preventing it from eluting out the top offtake [38,39]. Several other acids were used to titrate the system, with phosphate and citrate showing effects similar to acetate. In order to prevent the system zones from interfering with the separation, the ionic mobility needed to be either significantly faster or slower than the compounds being separated, with slower ions being preferred due to lower conductivities reducing the required power density for the separation. Epsilon amino caproic acid (EACA), which has an isoelectric point of approximately 8.5, was used due to its very low negative charge at pH 9.0, leading to a very low electrophoretic mobility. After the eigenpeak issue was resolved, 10 mM tris-EACA at pH 9.04 was used for the remainder of the bovine protein separations.

With all of the issues relating to the addition of the middle electrode resolved, comparisons between gradient and non-gradient separations could finally be performed. For both separations, the counterflow velocity was kept constant in all four regions, and a throughput of 8 mg/h of total protein was used. The electric field conditions of 25 V/cm for the non-gradient work as measured across sections II and III was maintained, while
for the electric field gradient separation 22.5 V/cm was maintained in section II and 27.5 V/cm in section III. These are the same electric fields that were used during all of the bovine protein separations, and the conditions for these two experiments are shown in Table 3.2.

A comparison of the non-gradient and gradient separations is shown in Figure 3.6. The non-gradient separation shown in Figure 3.6a shows a significant level of Hb tailing towards the bottom offtake, and the addition of the electric field gradient shown in Figure 3.6b greatly reduces this undesired tailing. This reduction in tailing confirms the results of the mathematical modeling done previously [8], and shows that the addition of stepped electric field gradients to the TMB electrophoresis process has a beneficial effect on the separation.

Another important thing to notice is the increase in the concentration of the FITC-BSA in section II, due to the decreased electrophoretic driving force below the feed. Since the separation is nonlinear at this throughput, this increase in concentration also aids in reducing the tailing of the Hb into section II. Due to the nonlinear nature of the separation, there was never any significant observed tailing of the FITC-BSA towards the top offtake as can be seen with both separations in Figure 3.6. The interface between the FITC-BSA and the hemoglobin is nonlinear due to an isotachophoretic effect between the two proteins, with the slower Hb displacing the FITC-BSA due to the higher electric field created by the electrically slower hemoglobin.
### Table 3.2: Conditions used for binary bovine protein separations

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$u_I$</td>
<td>Section I fluid velocity</td>
<td>0.636 cm h</td>
</tr>
<tr>
<td>$u_{II}$</td>
<td>Section II fluid velocity</td>
<td>0.414 cm h</td>
</tr>
<tr>
<td>$u_{III}$</td>
<td>Section III fluid velocity</td>
<td>0.463 cm h</td>
</tr>
<tr>
<td>$u_{IV}$</td>
<td>Section IV fluid velocity</td>
<td>0.241 cm h</td>
</tr>
<tr>
<td>$Q_{Feed}$</td>
<td>Feed flowrate</td>
<td>2.0 ml h</td>
</tr>
<tr>
<td>$C_{Alb,Feed}$</td>
<td>Feed concentration albumin</td>
<td>2.0 mg ml</td>
</tr>
<tr>
<td>$C_{Hb,Feed}$</td>
<td>Feed concentration hemoglobin</td>
<td>2.0 mg ml</td>
</tr>
</tbody>
</table>

#### Non-gradient experiment

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E$</td>
<td>Electric Field</td>
<td>$-25 \frac{V}{cm}$</td>
</tr>
</tbody>
</table>

#### Gradient experiment

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{Top}$</td>
<td>Electric Field, top half</td>
<td>$-22.5 \frac{V}{cm}$</td>
</tr>
<tr>
<td>$E_{Bot}$</td>
<td>Electric Field, bottom half</td>
<td>$-27.5 \frac{V}{cm}$</td>
</tr>
</tbody>
</table>
**Figure 3.6:** A comparison between (A) an 8 mg/h non-gradient and (B) a +/-10% gradient FITC-Albumin/Hemoglobin separation. In both cases the counterflow is the same as shown in Table 2. The addition of the electric field gradient significantly reduces the size of the large hemoglobin tail that contaminated the bottom offtake in the non-gradient separation. Another effect of the gradient is the increase in the albumin concentration in section II, due to the lower electrophoretic driving force in that region.
Separations performed under the same experimental conditions are one way to compare the gradient and non-gradient TMB electrophoresis processes, but in order to show the bigger picture of how electric field gradients affect the separation a triangle diagram will be used [40]. The triangle diagram, colloquially known as “Morbidelli’s Triangle,” [30] shows the region of operation where the conditions result in offtake contaminations below a certain threshold, and an idealized triangle diagram is shown in Figure 3.7.

The counterflow velocity in section II is represented by the x-axis of the graph and the section III counterflow velocity is plotted on the y-axis in Figure 3.7. In order to perform a separation there must be a feed flowrate, and this requires that the velocity in region III be higher than in region II, thus the only possible operating conditions for the separation lie above the x=y line in the figure. The feed vector is perpendicular to the x=y line, and the endpoint represents the feed flowrate that is being injected into the separation chamber. For each length of feed vector, there is an upper bound on the counterflow in region III that causes the fast component tail to be pushed towards the top offtake, contaminating the slow component stream. At the same feed level, there is also a lower bound on the counterflow in region II, where the slow component tail reaches the threshold concentration in the bottom offtake contaminating the fast product. At the vertex of the triangle is the maximum length of the feed vector, representing the maximum amount that can be processed by the TMB electrophoresis separation. If the feed vector falls outside of the operating triangle, the purity in one or both of the offtakes will be at an unacceptable level, causing the product to be out of specification.
Figure 3.7: An idealized triangle diagram showing the effect of the counterflow velocities in sections II and III on the separation. The x-axis is the velocity in section II ($u_{II}$) and the y-axis is the velocity in section III ($u_{III}$). The amount being processed is shown on the graph as the distance perpendicular to the $u_{II}=u_{III}$ line, and this can be represented by the feed vector resulting in the dotted line parallel to the $x=y$ line shown above. At each feed flowrate there is an upper counterflow rate where the fast enantiomer tail breaks through and contaminates the top offtake (black circle) as well as a lower counterflow rate where the slow enantiomer tail contaminates the bottom offtake (hollow circle). These upper and lower bounds on the counterflow velocity form the edges of the triangle shown above leading to the point of maximum throughput at the vertex of the triangle. In an ideal separation, when the section II velocity falls below the slow component electromigration velocity ($\eta_{S}E$, or the slow component mobility times the electric field) the slow component can then contaminate the bottom offtake. The same is true when the region III velocity exceeds the fast component electromigration velocity ($\eta_{F}E$) as this allows the fast component to contaminate the top offtake. As long as the separation is operated within the triangle formed by these boundaries, both offtakes will be pure.
In the ideal separation shown in Figure 3.7 the edges of the triangle are simply the points at which the fluid velocity in section II equals the slow component electromigration velocity or the counterflow in section III equals the fast component electromigration velocity. In practice, phenomena such as diffusion, nonlinear electrophoresis and undesired product interactions can cause the edges of the triangle to be complicated functions of these parameters, significantly skewing the shape of the triangle region. This type of diagram is an important tool which shows the effect of adding electric field gradients to a TMB electrophoresis separation, as the size and shape of the triangle regions for both non-gradient and gradient separations can be compared.

In order to present the bovine protein separations for both the gradient and non-gradient cases in the form of a triangle diagram as described above, the data first had to be collected to describe these regions. In both the gradient and non-gradient cases, the boundary between the FITC-BSA and Hb was very sharp on the hemoglobin side, and the strong fluorescence signal of the FITC-BSA allowed the 5% contamination point to be easily estimated visually. In all cases though, a quantitative analysis using the plate reader was also done to confirm the visual results. To find the top boundary of the triangle region, separations were run at various different throughputs and the counterflow in section III was increased until the sharp FITC-BSA Hb interface was stable approximately 1 mm below the top offtake of the chamber at which point the run was stopped and the samples analyzed.
Finding the bottom boundary of the triangle region proved to be slightly more difficult, since the FITC-BSA side of the boundary with Hb is considerably more diffuse, and it is impossible to visually assess any brown color from the Hb with the large fluorescent background of the much more highly concentrated FITC labeled BSA. For the bottom boundary separations, several experiments had to be conducted for each throughput level, until two runs were found that bracketed the 5% Hb contamination point of the bottom offtake. Once separations above and below the desired contamination level were found, linear interpolation was used to calculate the crossflow necessary to cause 5% contamination and these points were plotted on the triangle diagram.

Triangle diagrams for both the non-gradient and gradient bovine protein TMB separations are shown in Figure 3.8. The most important thing to notice is that the +/- 10% electric field step gradient increases the maximum processing rate of the TMB separation from 30.6 mg/h for the non-gradient case to 50.0 mg/h for the gradient case. This represents a 63% increase, and while this is not as impressive as the 7.66 fold increase shown in the homatropine modeling work [8], it is still a significant benefit to the overall throughput of the process. The nonlinear nature of the FITC-BSA, Hb system limits the beneficial effect that the electric field gradient provides because of the sharp ITP effect that already limits the FITC-BSA tailing towards the top offtake.

Another interesting effect shown in Figure 3.8 is the transition from linear to nonlinear electrophoresis, represented by the upward skew in the data when the feed vector length, or the distance perpendicular to the x=y line as defined in Figure 3.7, is small. As the
Figure 3.8: A triangle diagram comparing the 95% purity operating regions of a non-gradient and a +/-10% electric field gradient separation of bovine FITC-BSA and Hb. Separations in the Vortex Stabilized Electrophoresis Apparatus were run to find the upper and lower bounds at each throughput where the contamination reached 5% of the undesired component in either offtake. The vertexes of each triangle are the maximum processing rates in each case, 30.6 mg/h for the non-gradient and 50.0 mg/h for the gradient separation which gives a 63% increase in maximum throughput by the addition of a +/-10% gradient.
feed vector length increases, the concentrations in the apparatus increase causing the
aforementioned ITP effect that reduces the tailing of the contaminant proteins toward the
offtakes. The results of this transition were quite surprising, as evidenced by the bottom
half of the non-gradient separation, which turns a very sharp corner when the nonlinearity
becomes fully developed.

It was expected that the gradient separation triangle would completely envelope the non-
gradient separation triangle, but this is clearly not the case seen in Figure 3.8. This is also
believed to be a result of nonlinear electrophoresis, as the voltage in the non-gradient
separations was regulated at 537 V from the bottom of section II to the top of section III,
while the voltages for the gradient separations were regulated individually with 191 V
across section II and 234 V across section III. While the average electric field across the
column was 25 V/cm in both cases, the non-gradient case had a larger voltage difference
between any two controlled electrodes, and the electric field was allowed to float in that
region. Clearly any separation with an ITP like boundary does not maintain a constant
electric field, and this resulted in higher fields in the less conductive, pure Hb area near
the top offtake of the apparatus. The net outcome is that a larger counterflow in section
III was required to overcome the faster electrophoretic velocity due to these higher fields.
The maximum throughput of the non-gradient triangle is 63% less than the triangle of the
gradient separation though, so even though the floating electric field effect benefited the
top half of the triangle, this lowered the electric field in the predominantly FITC-BSA
area of the separation chamber. This increased the tailing of Hb into section II and caused
5% concentration breakthrough in the bottom offtake at a lower throughput than the
gradient separation.

The bovine protein separations performed in this work used an average electric field of
25 V/cm, and this resulted in power dissipations of less than 6 W in all cases using the 10
mM tris EACA buffer system. The steady state design wattage of the Vortex Stabilized
Electrophoresis Apparatus is 100 W, which suggests that the electric field could be
increased by at least an order of magnitude and still be within the power dissipation limits
of the system. This should greatly increase the difference in electrophoretic velocity
between the two proteins, theoretically allowing approximately an order of magnitude
higher throughput than the maximum of 50.0 mg/h reported here. While this needs to be
confirmed experimentally, the reason for the use of 25 V/cm electric fields was due to the
large amounts of protein required to complete the triangle diagram shown in Figure 3.8.
The main goal of this work was to show the effects of adding stepped electric field
gradients to the TMB electrophoresis process, not to find the absolute maximum
throughput attainable with the Vortex Stabilized Electrophoresis Apparatus, and in that
regard these experiments were successful.

3.6. Concluding remarks

In the quest for further process improvements in the TMB electrophoresis technique,
electric field gradients have been an important area of study as they allow greater control
over the separation and allow significant throughput increases. The addition of a stepped
electric field gradient between sections II and III of the TMB electrophoresis process has
been shown to increase the maximum throughput of a FITC-BSA, Hb separation by 63%. While this is far less than prior modeling work based on the homatropine/hydroxypropyl-β-cyclodextrin system suggested, it is still a significant increase in the processing rate justifying further study of gradients.

Separations using homatropine enantiomers were attempted using dialysis and anion exchange membranes, but there were insurmountable problems that occurred in both cases. The permeability of the dialysis membranes and the pH shift caused by the anion exchange membranes both prevented successful TMB electrophoretic homatropine separations from being performed, and this prohibited the direct comparison of these separations to the electric field gradient modeling work. While there was no suitable membrane found that allowed a successful homatropine enantiomer separation in this work, there should be a sized based membrane that would allow this process to function as predicted. This may require the synthesis of specialized membranes with smaller pore sizes or other specialized chemistry, but this is an engineering problem that can eventually be solved leading to successful enantiomer separations using the TMB electrophoresis process.

3.7. References


4. INCREASING THE SCALE OF TRUE MOVING BED ELECTROPHORETIC SEPARATIONS USING FILTRATION TO REDUCE SOLVENT VOLUMETRIC FLOWS BETWEEN SECTIONS II AND III

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Keywords
Simulated Moving Bed, True Moving Bed, Preparative Electrophoresis, Chiral Separation, Enantiomer, Filtration

4.1. Abstract

Over the past decade the moving bed process has become a commonly used tool for the continuous separation of chiral compounds, and its recent application to electrophoretic separations allows the technique to be used as a model system for moving bed method improvements. Much of the recent research on moving bed separations has focused on improving the technique’s efficiency and increasing the maximum attainable throughput. This paper presents a novel method for reducing or reversing the increases in tailing that stem from the addition of the feed stream in a moving bed process by adding a filtration unit which retains the products while removing fluid from the boundary between the sections above and below the feed stream. This filtration-enhanced moving bed process was applied to a true moving bed (TMB) electrophoresis separation in the Vortex Stabilized Electrophoresis Apparatus, and its effect on a homatropine enantiomer separation was studied. Experiments showed that there is a 2.4 fold increase in the

1 To whom correspondence should be addressed
homatropine processing rate when 0.5 ml/h of water is removed through a reverse osmosis filter at the boundary between the sections above and below the feed stream. In order to further understand the process, filtration-enhanced TMB (FE-TMB) was also analyzed using a linear model of the system which shows that the 99% purity operating region of the separation is greatly increased even with moderate permeate flowrates.

4.2. Introduction

Chiral systems permeate through all known biological systems [1], and the interactions of these life forms with chiral chemicals have become an important part of pharmaceutical science. The different stereochemical interactions of drug molecules require either stereo-specific synthesis or enantiomer separations to purify the racemic product [2]. Due to the increasing complexity of modern drug candidates, stereo-specific synthesis can be very complicated, requiring a large number of process steps with expensive chiral precursors in order to yield reactions with an acceptable enantiomeric excess. In many cases it is more economical to use large scale purification schemes such as crystallization or simulated moving bed (SMB) chromatography for the separation of these enantiomers, and there has been considerable research in the development of these techniques for this purpose [3]. Although the technique will likely never reach the scale of SMB chromatography, moving bed electrophoresis can also be a useful enantiomer separation technique for providing bench scale quantities of enantiomers for use in the laboratory setting [4-6].
The SMB process has received a large amount of development attention in the last decade. SMB was originally invented in the 1960’s for the separation of petrochemicals [7], but has since been adapted for use in enantiomer separations in the pharmaceutical industry [8]. While the SMB process is inherently between one and two orders of magnitude more efficient than batch chromatography [9], there has been a large amount of work involved in further increasing the throughput capacity per unit mass of resin used in the process. These improvements have focused on two distinct pathways, one involving altering the mechanical variables of the system and the other involving gradient elution of the products. The true moving bed (TMB) electrophoresis technique, as shown in Figure 4.1, is also able to benefit from these two improvements as it is a direct analog of SMB chromatography [4]. In this paper, TMB electrophoresis will be used as a model system to demonstrate the tailing effect caused by the introduction of the feed stream. TMB electrophoresis experiments as well as a mathematical model of the process will be used to demonstrate a new strategy to reduce or reverse this tailing effect by adding a filtration system to the unit.

4.3. Prior work

Both continuous and stepwise gradient elution TMB electrophoresis have been explored by the authors previously [5], and these techniques have been shown to provide up to a 7.66 fold increase in the processing capacity of a homatropine-hydroxypropyl β cyclodextrin separation. In the case of gradient elution, the use of continuous electric field gradients has been shown to give greater control over the product concentrations within the separation chamber, allowing nearly all the processing benefits of a step
Figure 4.1: An electrophoretic moving bed enantiomer separation. Electromigration forces the enantiomers downward as they are counteracted by a fluid flow upward. The fast enantiomer is able to overcome the fluid flow and migrate to the bottom offtake while the slower enantiomer is pushed upward towards the top offtake.
gradient separation while reducing concentrations within the unit by 40% [5].

Gradient elution has also been extensively explored in the SMB chromatography process, where generally between 6 and 12 columns are placed in series and periodically switched in order to simulate a moving bed of the stationary phase as shown in Figure 4.2 [9]. Due to the nature of the SMB process there is no simple way to apply continuous solvent gradients, as this would require changes in mobile phase composition inside each column which is impractical [10]. Due to this limitation, most of the research done on gradient elution in SMB chromatography has been performed on step gradients in the mobile phase composition at the feed port [11].

In spite of the fact that solvent gradients are limited to step functions, there are several SMB chromatography systems that allow continuous gradients and piecewise approximations of these continuous gradients to be applied. There has been a significant amount of research done using supercritical fluid SMB chromatography in which the pressure in each of the sections is regulated or the pressure drop of the mobile phase traveling through the packing is used as the gradient in elution strength [12-17], but this has been limited to special cases in which a supercritical desorbent can be used. Temperature gradient SMB has also been explored, using different temperatures in each of the sections in the separation train, although the temperatures cannot be changed instantaneously with switch time which limits the effectiveness of the technique [18].

The other category of SMB chromatography process improvements involves alteration of
Figure 4.2: A schematic of an 8 column SMB chromatography process. There are two columns in each of the four sections (2,2,2,2) in between the inlet and offtake streams as shown above. The desorbent is pumped in to the system between columns 1 and 8 and this moves the compounds through the unit. The feed mixture is introduced opposite the desorbent in between columns 4 and 5. The column switching produces a solid movement (analogous to the counterflow in Figure 4.1) which is able to overcome the extract (strongly adsorbed component) migration and move it towards the extract offtake between columns 2 and 3. The raffinate (the weakly adsorbed component) is able to migrate faster than the “solid” flow rate and moves toward the raffinate offtake between columns 6 and 7. The switch time is determined by the migration velocities between the extract and raffinate components and after the switch column 1 becomes column 2, 2 becomes 3 etc.
the mechanical variables of the system such as the switch interval, flowrates and feed concentration. Several approaches have been developed such as the Novasep “Varicol” process, which involves asynchronous switching of the SMB regions [19-21] instead of the constant switch interval of the typical SMB process. Switching the regions at different times allows the columns in the process to be used more effectively for a longer or shorter period of time based on their operating characteristics in each region. This allows a normal 8 column SMB separation with 2 columns in each of four regions (2,2,2,2) to be accomplished with fewer columns, and these columns can be partially shared between the regions. An example of this process is a 6 column (1.75,1.25,1.25,1.75) separation that uses less columns, less solvent per unit product and which operates with comparable purity to the (2,2,2,2) SMB separation [19]. Another approach to improving the SMB chromatography process involves “Power Feed” operation [22,23] in which the fluid flow rates of the feed and desorbent streams along with the extract and raffinate offtakes are changed over the course of each switch interval. Though the mechanism of the improvement of the Power Feed operation is different, it provides similar results compared with the Varicol process in that it allows more efficient use of the stationary phase, requiring fewer columns and less solvent to be used at similar product purities.

The final mechanical variable advancement in SMB chromatography is known as the “ModiCon” process [24] in which the feed concentration is changed throughout each switch interval, causing changes in the shapes of the adsorptive fronts within the unit. Again this process shows improvements that are inline with the Varicol and PowerFeed
processes, but it uses gradient pumps instead of switching algorithms or flowrate variations [24]. Although these mechanically improved processes use different methods to increase the productivity of SMB chromatography, they all address shortcomings in the SMB process and attempt to use the columns more efficiently. One of these shortcomings is caused by the change in desorbent fluid velocity resulting from the feed stream introduction. The filtration-enhanced TMB electrophoresis separation presented in this work will allow the effect caused by the feed stream to be completely reversed, benefiting the efficiency of the process instead of simply reducing the tailing due to the feed stream introduction.

4.4. Theory

4.4.1. The moving bed process

The true moving bed process has traditionally been described in terms of countercurrent adsorptive separations, as this is where the technique got its name, but any moving bed separation can be defined in terms of the four regions as shown in Figure 4.3. These four regions come about because of the differences in the fluid velocities in each section due to the feed and product offtake streams at the region boundaries. In the terms of an electrophoretic TMB separation, the counterflow stream is introduced at the bottom of region I, which imparts an upward fluid velocity in the apparatus counteracting the downward product migration imparted by the electric field. At the boundary between regions I and II, the bottom product offtake causes a reduction in the counterflow velocity in section II and this is followed by the addition of the feed stream in between regions II and III which increases the fluid velocity in the third section. The top offtake in between
Figure 4.3: The four regions of a moving bed separation, shown here as a TMB electrophoresis process. These four regions are separated by inlet and offtake streams which cause differences in the fluid velocity in each region, shown as $u_I - u_IV$. The bulk of the separation occurs in regions II and III, below and above the feed respectively. Regions I and IV serve to keep the products from leaving the ends of the separation since the counterflow velocities are too fast and too slow respectively for the products to overcome. An important thing to notice is an increase of the counterflow velocity between regions II and III due to the introduction of the feed stream. This velocity change at the feed port affects the separation, because the counterflow pushes harder on the slow component than it does on the fast one. This increases the tailing effects because as the faster component enters region III, the higher velocity makes it harder for it to overcome the counterflow and conversely when the slower component enters region II the reduced counterflow is less able to push the slow component back towards region III. Adding a membrane filtration at the feed location counteracts this phenomenon by reducing the counterflow velocity in region III.
regions III and IV further reduces the velocity in section IV, and at the top of the unit, the
purge stream carries the remaining fluid out of the apparatus. The bulk of the separation
is carried out in regions II and III. At the bottom and top of the unit, sections I and IV
respectively, the velocities are such that any product excursion into these regions is
overcome by the counterflow (region I) or the electromigration (region IV) returning the
product to the bottom or top offtake stream, preventing the loss of solute out the ends of
the apparatus.

One important effect resulting from the traditional moving bed process is the increase in
counterflow velocity between sections II and III due to the feed stream. This in effect
pushes harder on the components in region III which makes it more difficult for the fast
cOMPONENT to overcome the counterflow velocity in this section, thus increasing the
tailing of the fast component towards the top offtake. This requires that the counterflow
in the entire apparatus be reduced to combat this top offtake tailing effect, which makes it
harder for the counterflow in section II to overcome the electromigration of the slow
enantiomer causing tailing towards the bottom offtake. Though this process involves the
fluid variables of the system, it is analogous to an unfavorable stepwise electric field
gradient at the feed port as has been previously demonstrated [5]. The simplest way to
mitigate this effect is by using the highest concentration possible in the feed stream,
which reduces the feed flowrate at a given product throughput, but this cannot completely
reverse the effect.
4.4.2. Filtration-enhanced TMB

The addition of a filtration system between regions II and III that is able to remove buffer or mobile phase without removing products is one way to completely reverse the tailing effect that the feed stream imparts on a moving bed separation. Another method that could have a similar effect is evaporation to remove the solvent, but this would only be useful for separations that do not involve heat sensitive chemicals such as proteins or volatile components such as many common small molecule drugs. It should be noted that the filtration system does not have to be placed at the section II/III boundary, but this is the location where it provides the largest benefit according to a similar analysis of electric field gradients done previously [5]. Since the TMB electrophoresis process is carried out in one chamber, it would theoretically be possible to have multiple filtration systems at any point along the apparatus, as the only requirement is that inlet and outlet ports be drilled into the Plexiglas stator for each filtration unit. The separation could also be carried out in a hollow fiber membrane apparatus, allowing the counterflow velocity to vary continuously along the length of the chamber, but it would be difficult to control a TMB separation with these extra variables.

A comparison between the traditional TMB process and the filtration-enhanced TMB process is shown in Figure 4.4. The use of filtration at the section II/III boundary allows the counterflow velocity in region III to be lower than region II, which causes the fluid flow equivalent of a favorable step gradient in the electric field [5]. The lower counterflow velocity in region III makes it much easier for the fast component to overcome the fluid flow thus reducing the size of the contaminating tail in this region.
Figure 4.4: A comparison of the concentration profiles between a traditional TMB and an FE-TMB electrophoresis separation. The fluid velocity in each of the four regions, labeled I-IV, is represented by the bold line. The important thing to notice is the change in the direction of the velocity step in between sections II and III in the two figures. In the normal TMB separation on the left, the addition of the feed causes the velocity in region III to be higher than that in region II. For the filtration enhanced TMB case on the right, the process is able reduce the velocity in region III when compared to region II, because the filtration unit can remove buffer from the section II/III boundary at a rate higher than the feed flowrate. The enantiomers travel away from the center at the rate indicated by the arrows, and these rates are different in the two figures because of the counterflow velocity differences. These changes in migration rate cause the concentrations in the unit to increase in the FE-TMB, but more importantly, the amount of tailing is markedly reduced. The decreased velocity in section III in the FE-TMB case makes it more difficult for the fast enantiomer to get pushed towards the top offtake at the section III/IV boundary, reducing the length of undesirable tailing. A similar effect happens for the slow enantiomer tail into region II.
The reduction in fluid velocity in region III allows the overall counterflow velocity in the apparatus to be increased, causing the fluid velocity in region II to more easily overcome the electromigration of the slow component in this section, reducing its contaminating tail as well.

The net result of adding a filtration system at the section II/III boundary is that the operating triangle of the separation becomes larger, and the process can now operate below the $u_{II}=u_{III}$ line of the triangle diagram as shown in Figure 4.5. In effect this allows significantly larger amounts of product to be processed when compared to the standard TMB electrophoresis separation. Another important benefit of adding filtration to the system is that permeate can be recycled, reducing the overall amount of solvent required for the separation, which can significantly reduce operating costs.

4.4.3. TMB electrophoresis modeling

In order to be able to look at the effects of adding filtration to the section II/III boundary, an accurate model of the TMB electrophoresis process is required. A linear model of this process has been developed and validated with experimental data previously, and has been used to predict the effects of adding electric field gradient elution to the TMB process [5]. This model required only a slight adaptation in order to be useful in predicting the operating conditions of the filtration-enhanced TMB system, and as such, an abbreviated explanation of the model will be presented detailing those changes.

An equation for the concentration of species $i$, $c_i$, can be derived by integrating the time
**Figure 4.5:** An idealized triangle diagram showing the effect of adding a filtration system at the section II/III boundary. On the left is a standard TMB electrophoresis triangle diagram where $u_{II}$ and $u_{III}$ are the section II and III fluid flow velocities, $\eta$ signifies the electrophoretic mobility and $E$ is the electric field. If the operating point denoted by the feed vector falls within the triangle, both offtakes will contain pure components. Increasing the processing rate requires raising the feed flowrate, and this longer vector becomes constrained by the operating triangle, such that at the maximum throughput only one counterflow velocity allows pure components at both offtakes. A TMB separation with filtration at the section II/III boundary is shown on the right. The filtrate stream offsets the feed vector, allowing higher feed flowrates while still operating in the triangle region. The area below the $u_{II}=u_{III}$ line is also accessible, in effect making the triangle region a much larger square. If the filtration vector becomes too long, the products cannot leave the separation chamber, and they will eventually precipitate.
dependent mass balance of the system

\[
\frac{\partial c_i}{\partial t} + \nabla \cdot \left( -D_i \nabla c_i - z_i \mu_i F c_i \nabla \phi + c_i u \right) = R_i
\]

which takes into account the diffusive, electrophoretic and convective fluxes in the apparatus. In the above equation for species i, \( D_i \) is the diffusivity, \( z_i \) the molecular charge, and \( \mu_i \) represents the ionic mobility. The other variables in Equation 1 are the velocity \( u \) which changes in each of the four sections, the electric potential \( \phi \) and Faraday’s constant \( F \). In order to model the binding reactions between the fast and slow enantiomers and the chiral selector which results in fast and slow enantiomer ligand complexes, the reaction term \( R_i \) is used for each of these five species in solution. Mass action kinetic formulations of the reaction equations for each of the five species as well as the boundary conditions used in the integration of Equation 1 have been derived previously [5] and the constants used in the solution of the model are shown in Table 4.1.

The addition of the filtration process at the section II/III boundary changes the counterflow velocity profile within the apparatus, requiring a change in the velocity equations in each of the four regions. The counterflow stream enters the apparatus at the bottom of section I and the velocity in this region can be derived as

\[
u_I = \frac{Q_{cf}}{A_S}
\]

where \( u_I \) is the velocity in region I, \( Q_{cf} \) is the flowrate of the counterflow stream and \( A_S \) is the cross sectional area of the apparatus. The bottom offtake forms the boundary between sections I and II, and consequently the velocity in region II is
<table>
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<td>$D_F, D_S$</td>
<td>Diffusion + Dispersion constants</td>
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</tr>
<tr>
<td>$D_{CD}, D_{SCD}, D_{FCD}$</td>
<td></td>
<td>$2.05 \cdot 10^{-8} \frac{m^2}{s}$</td>
</tr>
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<td>$K_F$</td>
<td>Fast complex equilibrium constant</td>
<td>$0.03 \frac{1}{mM}$</td>
</tr>
<tr>
<td>$K_S$</td>
<td>Slow complex equilibrium constant</td>
<td>$0.06 \frac{1}{mM}$</td>
</tr>
<tr>
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<td>Complex formation rate constant</td>
<td>$0.0003 \frac{1}{mM \cdot s}$</td>
</tr>
<tr>
<td>$c_{CD}$</td>
<td>Cyclodextrin inlet concentration</td>
<td>$20 \ mM$</td>
</tr>
<tr>
<td>$c_F, c_S$</td>
<td>Enantiomer inlet concentration</td>
<td>$28.1 \ mM$</td>
</tr>
<tr>
<td>$\eta_F, \eta_S$</td>
<td>Electrophoretic mobilities</td>
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</tr>
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<td>$\eta_{CD}$</td>
<td></td>
<td>$0 \frac{m^2}{V \cdot s}$</td>
</tr>
<tr>
<td>$\eta_{FCD}, \eta_{SCD}$</td>
<td></td>
<td>$6.5 \cdot 10^{-9} \frac{m^2}{V \cdot s}$</td>
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<tr>
<td>$A_s$</td>
<td>Cross sectional area of chamber</td>
<td>$0.685 \ cm^2$</td>
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<tr>
<td>$Q_{to}, Q_{bo}$</td>
<td>Offtake flowrates</td>
<td>$0.1 \frac{mL}{min}$</td>
</tr>
<tr>
<td>$\phi$</td>
<td>Electric potential function</td>
<td>$5000 \times$</td>
</tr>
</tbody>
</table>
where $Q_{bo}$ is the bottom offtake flowrate. The section II/III boundary is the point where this model begins to differ from the previously published derivation, as in addition to the feed stream entering at this boundary, the newly added filtrate offtake stream is present as well. The velocity in region III can be derived as

$$u_{III} = \frac{Q_{cf} - Q_{bo} + Q_{feed} - Q_{filtrate}}{A_s}$$

where $Q_{feed}$ is the feed flowrate and $Q_{filtrate}$ is the newly added filtrate offtake stream flowrate. Finally the top offtake is located at the section III/IV boundary and the velocity in this region can be calculated as

$$u_{IV} = \frac{Q_{cf} - Q_{bo} + Q_{feed} - Q_{filtrate} - Q_{to}}{A_s}$$

where $Q_{to}$ is the top offtake flowrate. The velocities in each of the four sections of the apparatus derived above in Equations 2-5 can be used to solve Equation 1 in each of the four regions. Solving these equations provides the concentration profiles of all five species in the apparatus, namely the fast and slow enantiomers along with the chiral selective ligand and the fast and slow enantiomer-ligand complexes.

4.5. Experimental

4.5.1. Materials

In order to complete the TMB electrophoresis experiments, chemicals were purchased from several suppliers. Trizma base and racemic homatropine hydrobromide, were purchased from Sigma (St. Louis, MO, USA). The hydroxypropyl-β-cyclodextrin
(HPβCD) carrier ligand was purchased from Cargill Food and Pharma Specialties (Cedar Rapids, IA, USA) under the trade name Cavitron 82005. Citric, glacial acetic and phosphoric acids were purchased from JT Baker (Phillipsburg, NJ, USA).

4.5.2. Analysis

A Hewlett Packard (Walldbronn, Germany) 3DCE capillary electrophoresis unit was used to quantify the results from the TMB electrophoresis experiments. A 50 cm, 365 µm outer diameter 50 µm inner diameter capillary from Polymicro Technologies LLC (Phoenix, AZ, USA) was used, with the detection window at 42 cm from the inlet. The system was run at 20 kV with positive inlet polarity for 20 minutes and was kept thermostated at 24°C. The buffer used for analysis contained 15 mM HPβCD chiral selector with 50 mM Trizma base titrated to pH 3.0 with concentrated phosphoric acid.

4.5.3. Vortex Stabilized Electrophoresis Apparatus

The Vortex Stabilized Electrophoresis Apparatus shown in Figure 4.6 was used for the moving bed electrophoresis studies and has been described in detail previously [4,25]. It consists of a grooved rotor and stator that form an annular gap where the electrophoretic separation takes place. When rotating, the grooves on the rotor and stator create Taylor-like vortices that are stable at all angular velocities allowing for better cooling efficiency to remove up to 100 W of Joule heat dissipated by the electrophoretic separation. The vortices also serve to inhibit detrimental natural convection which causes mixing in the axial direction that reduces the effectiveness of the electrophoretic separation. There are four external Instech (Plymouth Meeting, PA, USA) peristaltic pumps that control the
Figure 4.6: A schematic of the Vortex Stabilized Electrophoresis Apparatus shown with the new reverse osmosis system. The separation chamber consists of a 2 mm annular gap between a grooved 1” diameter rotor and stator. The rotation forms Taylor-like vortices that are stable at all rotational velocities. Electrode housings at the top and bottom of the unit allow buffers to circulate and remove any electrolysis gases that form during a separation. The electrodes are separated from the separation chamber with 500 MWCO dialysis membranes. Peristaltic pumps control the counterflow, offtake and purge or pumparound streams during the course of the experiments and the feed is accomplished with a syringe pump. The reverse osmosis system removes fluid from the separation chamber at the same height as the feed port, runs it through an HPLC pump and into a custom designed tangential flow RO system. The backpressure of the retentate stream is controlled with an adjustable backpressure regulator that can operate between 345 and 1034 kPa (50 and 150 psi). The retentate is then recycled back into the separation chamber at a reduced volume.
counterflow, offtake flowrate and purge streams shown in Figure 4.3. A 300 W Spellman (Hauppauge, NY, USA) 2 kV power supply is used to apply the voltage which drives the electrophoretic separation.

A filtration system was added to the apparatus at the middle of the chamber at the same height as the feed stream. Fluid was removed from the chamber using a single piston Accuflo range I HPLC pump (Fisher Scientific, Hampton, NH, USA) and forced through a custom built tangential flow filtration (TFF) device with 8.5 cm\(^2\) of membrane contact area. The TFF unit housed an Osmonics (Minnetonka, MN, USA) YMAKSP3001 polyamide reverse osmosis membrane which allowed the removal of water from the buffer stream. After the filtration unit, the retentate stream passed through an adjustable backpressure regulator (Upchurch Scientific, Oak Harbor, WA, USA) which allowed precise control of the pressure in the TFF system between 345 and 1034 kPa (50 and 150 lbs/in\(^2\)) and then the retentate was returned to the separation chamber.

4.6. Results and discussion

4.6.1. Solution of the model

Since the model derived for the TMB electrophoresis process has been validated previously with the homatropine-HPβCD system [5], it can be extended to predict the operating conditions of filtration-enhanced TMB electrophoresis. The appropriate reaction constants, diffusion parameters and ionic mobilities of the components in Table 4.1 are all substituted into Equation 1 which can be solved to produce the concentration profiles of the fast and slow enantiomer, the chiral selector and the enantiomer ligand
complexes under varying process conditions. In order to solve Equation 1, a Femlab 3.1i (Comsol, Stockholm, Sweden) model was created using the electrokinetic flow equations in the chemical engineering package. In each case, the time dependent solver was used for 0.5 million seconds, which is far longer than required to reach a steady state for the TMB electrophoresis process, but this allowed prediction of operating conditions even where operation of an actual TMB separation is impractical.

Since the output of the model is a set of concentration profiles for each condition, a way to present the trends in the data is needed. In order to demonstrate the effects of the filtration-enhanced TMB process, the data will be presented in the framework of the commonly accepted “triangle theory” as developed by Mazzotti et. al. [26]. For each set of filtrate stream flowrates, an operating envelope can be defined based on the region where the TMB separation produces top and bottom offtake purities greater than 99%. In order to find this region, the model was solved at varying counterflow rates, and the purity in both offtakes was monitored. The counterflow rate at which the top offtake was exactly 99% pure provided an upper bound for the velocity in the operating envelope. The upper bound at each feed flowrate forms the data set for the right hand side of a triangle diagram, and the counterflow velocities in sections II and III are plotted in each case. Similarly, the velocities where the purity of the bottom offtake is exactly 99% form the left hand side of the operating envelope, signifying the lower bound of the operating triangle.
4.6.2. Effect of permeate flowrate on filtration-enhanced TMB

The process described above was used to create Figure 4.7, which is the triangle diagram of a filtration-enhanced TMB electrophoresis separation using the homatropine-HPβCD system. The solid triangle is the area where a traditional TMB electrophoresis process can operate with 99% purity in both offtakes, and the maximum processing rate under these conditions is approximately 3 mg/h of racemic homatropine. The removal of filtrate at the section II/III boundary causes the baseline of the operating triangle to shift below the $u_{II}=u_{III}$ line, as there is a net negative flowrate when filtrate is removed and no feed is being added to the system. As explained in section 3.2, the filtrate removal process causes the triangle to expand as the membrane permeate flow increases, and this allows the separation to maintain 99% purity in both offtakes over a much larger operating envelope. By connecting the vertexes of the triangles in Figure 4.7, a new region can be drawn where at least one set of operating conditions will achieve greater than 99% purity in both offtakes. This much larger accessible area where a 99% pure separation takes place at both ends shows the power of the filtration-enhanced TMB technique.

The results of the filtration-enhanced TMB model show that by removing permeate at a rate of 0.25 ml/h, as shown by the middle triangle of Figure 4.7, the maximum processing rate is 2.3 times greater than the rate attainable without filtration enhancement. The maximum feed flowrate in this case, denoted by the upper vertex of the triangle, is 0.345 ml/h for a total of 6.9 mg/h of racemic homatropine as compared to 0.15 ml/h and 3 mg/h for the non-enhanced TMB process. The beneficial effect is further magnified with a 0.5
Figure 4.7: A triangle diagram of the homatropine/hydroxypropyl-ß-cyclodextrin system at -50 V/cm with reverse osmosis added to the system. The model was solved for the upper and lower counterflow boundaries where greater than 99% purity was achieved at both offtakes for a variety of different feedrates and RO conditions. The solid triangle is the region of operation with no RO, and the maximum processing throughput in this case is 3 mg/h with a rather small operating envelope. By adding RO, the 0.25 ml/h and 0.5 ml/h operating triangles can be calculated, resulting in much larger area and larger maximum throughputs of 2.3 and 3.14 times the no RO processing limit respectively. The faint dotted line that connects the ends of each of the operating triangles signifies the increase in possible operational area due to the addition of filtration to the TMB separation. For any point within this new boundary, there will be at least one set of conditions that allows 99% purity. This newly accessible area is extended significantly both above and below the $u_{\parallel}=u_{\perp}$ line indicating that the filtration-enhanced TMB process makes difficult separations easier to perform and allows for significant throughput increases.
ml/h permeate stream which allows 0.471 ml/h maximum feed flowrate, or 9.4 mg/h of racemic homatropine to be processed which is a 3.1 fold increase over the base case. This shows the potential advantage of applying the filtration technique to a moving bed process.

One other important effect of the filtration-enhanced process is the increase in product concentrations within the apparatus as demonstrated in Figure 4.4. This phenomenon occurs because the section III velocity is reduced which makes it easier for the fast component to leave the region, but it also retards the migration of the slow component towards the top offtake of the apparatus. A similar effect happens in section II, as the counterflow rate is generally increased to compensate for the slower fluid velocity in section III. This increase in region II velocity decreases the tailing of the slow component in the section, but it also slows the migration of the fast component towards the bottom offtake. Since the flux of a species can be defined as the concentration of that species multiplied by its velocity, in order for the flux of a component to remain constant, the concentration must increase proportionally as the transport velocity decreases. At a given throughput, the flux must remain constant in order for a mass balance to close in the apparatus, and this dictates that as the filtration effect is increased, the concentration will increase within the unit. At a certain level of permeate flow, the product concentrations within the apparatus will reach the saturation point and start to precipitate or crystallize, thus providing an upper limit on the maximum processing rate. The model predicts the filtration process will increase the product concentrations by 10% for every 0.25 ml/h of permeate flow taken from the section II/III boundary. When this is
combined with the increased product processing rate, which causes the concentration to increase proportionally, the effect becomes magnified considerably, but filtration enhancement can still provide a large gain in the processing rate when compared to the standard TMB process.

It should be noted that the model derived in this work is linear which causes it to become less accurate as the concentrations of the products increase. The linear approximation has been shown not to cause significant inaccuracies at the low concentrations used in the base case [5], but as the processing rate increases, the ratio of product to background electrolyte increases, eventually causing significant nonlinear effects in the electric field. This should not materially change the analysis of the filtration-enhanced TMB process, but it may tend to overestimate the triangle region and maximum processing rate in cases of very high permeate flowrates, nonlinear separation conditions and high enantiomer processing rates.

4.6.3. Filtration-enhanced TMB in the Vortex Stabilized Electrophoresis Apparatus

Since the filtration-enhanced TMB process model showed a significant benefit over standard TMB electrophoresis, experiments were conducted in order to demonstrate its effects on an actual electrophoretic separation. The Vortex Stabilized Electrophoresis Apparatus was filled with 15 mM HPβCD in a 20 mM Tris-acetate buffer at pH 4.0 and run at 1500 V for 24 hours to allow the system to come to steady state. The concentration profile results of both a standard electrophoretic TMB and a filtration-enhanced TMB process are shown in Figure 4.8. The standard process shown in Figure
Figure 4.8: The results of two different homatropine/hydroxypropyl-ß-cyclodextrin separations. Conditions used for data collection in both cases are shown in Table 4.2. (A) A 0.5 mg/h homatropine enantiomer separation in which there was a significant amount of peak tailing due to the slow binding kinetics of the chiral selector. The countercurrent rate was 0.356 ml/min during the run. (B) A 1.5 mg/h separation at a crossflow rate of 0.371 ml/min using similar conditions to Figure (A), but using reverse osmosis to remove 0.5 ml/h at the feed port. The RO allowed a much higher amount of fast enantiomer to be purified at >99.3% purity, but the tailing of the fast enantiomer into the slow offtake was much worse under these conditions. This is most likely due to pulsing from the single piston HPLC pump used, as this added a noticeable periodic flow effect on the outlet at the top of the apparatus dramatically increasing dispersion in regions III and IV. Note that the concentrations have increased 2.4 fold in relation to Figure (A) instead of more than tripling as predicted by the model. This is due to the fact that the RO membrane was somewhat permeable to the homatropine enantiomers, and measurements show between 28% and 35% was removed from the apparatus in the permeate stream at different time points.
4.8a is a 0.5 mg/h homatropine enantiomer separation in which significant peak tailing of the fast enantiomer into region III is present. In Figure 4.8b a 1.5 mg/h homatropine enantiomer separation is shown, where 0.5 ml/h of RO filter permeate was removed from the section II/III boundary. The conditions for both separations in Figure 4.8 are detailed in Table 4.2.

At first glance, it appears that the tailing of the fast enantiomer into section III becomes much worse with the addition of the filtration process. This is believed to be due to the HPLC pump that was used to cycle the fluid through the RO membrane unit and generate the high pressures required to maintain the permeate flowrate. The pump used was single acting, and it only had one piston that moved at a controlled rate during the forward pumping cycle. In order to reduce pressure fluctuations on the high pressure side of the pump, the piston retracts at a much faster rate than that of the forward cycle, allowing the pump to fill quickly and resume the forward cycle at a controlled flowrate. While this decreases pressure and flowrate fluctuations in liquid chromatography, this pulsating action of the piston causes the low pressure, inlet side of the pump to have a violent oscillatory flow pattern that resembles a saw tooth wave. The rapid inlet cycle of the pump removes nearly 1 ml of liquid from the separation chamber at the middle of the Vortex Stabilized Electrophoresis Apparatus in less than half a second, which represents a significant fraction of the 25 ml volume of the chamber. Due to the vertical design of the instrument, the top outlet of the chamber is the only uncontrolled fluid stream, and this caused the counterflow velocities between the feed port and the top of the chamber to mimic the saw tooth function from the pump. This created a periodic back and forth
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<td>Feed concentration homatropine</td>
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<tr>
<td>$E$</td>
<td>Electric Field</td>
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<tr>
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<td>Section I fluid velocity</td>
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<td>Section II fluid velocity</td>
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<td>$u_{III}$</td>
<td>Section III fluid velocity</td>
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<td>Section IV fluid velocity</td>
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<td>$u_I$</td>
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<td>Feed flowrate</td>
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motion several times per minute in sections III and IV which was visible in the outlet tubing at the top of the chamber.

This violent back and forth motion of the counterflow in regions III and IV severely increases dispersion, causing the tailing of the fast enantiomer in section III to increase dramatically. Another effect from this pulsatile dispersion is a decrease in the slow enantiomer concentration in region III, as this reduces the capacity of the electromigration to overcome the fluid flow in this section. One way to correct this periodic oscillation would be to use a higher quality HPLC pump that maintains both constant inlet and outlet flowrates, as this would allow a steady permeate recycle stream velocity and mitigate these dispersion effects. HPLC pump designs that maintain a constant inlet flowrate are not common, as the inlet side of the pump is rarely important in chromatography. Although there are high quality pump designs that meet the pressure and inlet flowrate criteria, one was not available during the course of these experiments.

Even with the periodic dispersion phenomenon mentioned above, the filtration-enhanced TMB process does show some positive results. The separation in section II maintains the same low level of slow enantiomer tailing even though the processing rate is 3 times higher than the 0.5 mg/h traditional TMB separation. This shows that the filtration process did have a beneficial effect on the separation, supporting the conclusions derived from the modeling work above, demonstrating that the technique can have a significant impact on throughput in the true moving bed process.
One other important effect that was noticed during the course of the filtration-enhanced TMB runs is the membrane permeability to the homatropine enantiomers. This effect can be seen in Figure 4.8b, as the model suggests a 300% processing rate increase would result in greater than a 300% increase in product concentrations within the unit, but the separation shows only a 2.4 times increase in the fast enantiomer concentration. This is due to the fact that the polyamide reverse osmosis membrane used during the experiments was somewhat permeable to the homatropine enantiomers, thus reducing the effective processing rate. The total homatropine flux across the membrane was measured at between 0.42 and 0.525 mg/h which represents 28-35% of the 1.5 mg/h of total feed. This permeation problem could most likely be solved by using different membrane chemistry or possibly using dialysis instead of a high pressure reverse osmosis membrane, but even with the membrane permeability the experiments were able to demonstrate the potential positive effects of the filtration-enhanced moving bed process.

4.7. Conclusions

Whether it is a bench scale TMB electrophoresis process or a commercial scale SMB system, there is a continuing emphasis on increasing the throughput and processing efficiency of the moving bed technique. This work has shown that the addition of a filtration system that removes fluid from the section II/III boundary can reduce or reverse the inherent tailing effect that results from the addition of the feed stream, and this allows greater than three-fold increases in the maximum throughput rate attainable with a TMB separation. A previously developed linear model was modified to allow the prediction of operating parameters and benefits of filtration-enhanced TMB electrophoresis, and
experimental data was taken to corroborate the model results. While the experimental data showed significant tailing of the fast enantiomer into region III, it is believed that this was entirely due to the pump system used to recycle the filtration stream from the separation chamber to the reverse osmosis unit. Even though there was an increase in tailing in section III, region II showed a 2.4 fold increase in the processing rate while maintaining the same level of peak tailing, demonstrating that the filtration-enhanced TMB process can have significant advantages over standard moving bed separations.

4.8. References


5. CONCLUSIONS

This thesis has demonstrated two techniques for the improvement of the TMB electrophoresis process: the use of electric field gradients and the formation of velocity gradients using filtration. In both cases, a linear mathematical model was used to predict the effects of the technique on a homatropine enantiomer separation. Experiments were also conducted to test both process improvements, and although both electric field and velocity gradient techniques were plagued by several problems, the data presented in this work has shown the potential benefit of using these methods to improve throughput in moving bed separations.

The mathematical model developed in Chapter 2 for the electric field gradient work was linear, and included the binding reactions between the homatropine enantiomers and the hydroxypropyl-ß-cyclodextrin (HPßCD). The binding equilibrium relationships between the enantiomer and selector were measured using capillary electrophoresis. By observing changes in the electrophoretic mobility between the bound and unbound state of each enantiomer, the fraction of bound and unbound enantiomer was calculated. The results showed a remarkably low binding affinity of 0.06 and 0.03 mmol\(^{-1}\) for the slow and fast homatropine respectively. This indicates that the unbound state is highly favored, and thus in order to perform effective enantiomer separations, the concentration of HPßCD had to be maintained at a very high level of 20 mM.

This model was verified by comparing it to experimental data obtained using the Vortex Stabilized Electrophoresis Apparatus. Since the kinetics of homatropine binding to
HPßCD could not be measured experimentally using capillary electrophoresis, the kinetic rate constants for the fast and slow enantiomer binding were used as fitting parameters. The rate constant that gave the best fit for both enantiomers was 0.0003 mmol\(^{-1}\) s\(^{-1}\), and this was much slower than expected. This slow reaction rate was believed to be the cause of the large amount of tailing of fast enantiomer into section III seen during the experimental TMB electrophoresis separations. The model was able to predict the concentration profiles in the apparatus, and although there were some important discrepancies in absolute concentration, the tailing phenomenon was estimated very accurately, and this accuracy is paramount when attempting to model the upper and lower bounds of the separation at 99% purity.

The theory of the application of electric field gradients was demonstrated in Chapter 2 using the focal point map, and this showed that a step gradient at the section II/III boundary resulted in the best possible improvement. While this analysis is only strictly valid for linear separations, it was surprising nonetheless. Even though the best possible throughput improvement resulted from the step gradient case, it was shown that the concentration levels in the unit were decreased significantly when a continuous gradient electric field was used. This concentration decrease has a significant impact on the time required to reach steady state, showing that there are indeed significant benefits stemming from the ability to apply continuous gradients to the TMB electrophoresis process.
The application of electric field gradients to TMB electrophoresis was also analyzed in Chapter 2 using the framework of the triangle diagram which was altered slightly for use with electrophoretic separations. The results showed that there were large increases in the area of the triangle for both step and continuous gradients, indicating that they both provide significant improvements over the standard TMB electrophoresis process. The result of adding a continuous gradient with focal points at (7 cm, -45 V/cm) and (21 cm, -55 V/cm) was a 7 fold increase in throughput. The application of a step gradient with -45 V/cm in section II and -55 V/cm in section III was a 7.66 fold increase in maximum processing rate at the vertex of the triangle. In both cases, massive throughput increases were shown, and this demonstrates the power of gradient elution in the moving bed process.

In order to verify the modeling work done using electric field gradients, a three electrode stator was manufactured for use with the Vortex Stabilized Electrophoresis Apparatus as shown in Chapter 3. This allowed the application of step gradients in the electric field, and several homatropine enantiomer separations were performed. The initial experiments used 100 Molecular Weight Cut Off (MWCO) dialysis membranes in the middle electrode housings, as these were shown to be impermeable to homatropine in a small diffusion test cell. When an electric field was applied however, the permeability increased to unacceptable levels due to the increased driving force through the membrane caused by the electrostatic attraction of the positively charged homatropine to the cathode. This caused greater than 90% of the homatropine entering the separation chamber in the feed stream to be lost through the membrane to the recirculating buffer.
that removes electrolysis gases from the electrodes, and this prevented any useful separation.

In order to eliminate the dialysis membrane permeability, anion exchange membranes were placed in the middle electrode housing. These did not allow the positively charged homatropine enantiomers through, but they ended up causing a pH shift in the chamber due to the fact that only negatively charged ions could pass through the membrane. This pH shift in the apparatus caused the loss of binding between the homatropine and the HPßCD chiral selector, and this actually made the tailing phenomenon worse in both sections II and III.

After these two unsuccessful attempts to demonstrate the benefits of electric field gradients using homatropine enantiomers, it was decided to switch to bovine proteins, as these are significantly larger than the dialysis membrane MWCO. Separations using FITC labeled BSA and hemoglobin were performed to find the upper and lower bounds of the triangle region in both a non gradient case, and a +/- 10% electric field gradient case. The results showed that the maximum processing rate using a step gradient was 50.0 mg/h total protein, while the non-gradient case was only able to process 30.6 mg/h. Although this was nowhere near the 7.66 fold increase predicted by the model, this separation was nonlinear, and a 63% increase in processing rate is a significant improvement attainable with a step gradient in the electric field.
The other gradient method used to improve the TMB electrophoresis process was filtration-enhanced TMB (FE-TMB). This technique used a reverse osmosis (RO) membrane to remove buffer from the separation chamber between sections II and III, causing a favorable step in the counterflow velocity in the chamber instead of the typical unfavorable step resulting from the feed stream. Experiments and mathematical modeling were conducted to show the benefits of FE-TMB as detailed in Chapter 4.

The model that was previously developed in Chapter 2 for the electric field gradient TMB separations was modified to use the counterflow velocity profile resulting from the removal of filtrate from the section II/III boundary. The same process was used to generate a triangle diagram showing the result of removing different levels of filtrate from the apparatus. The model shows that RO permeate flowrates of 0.25 ml/hr and 0.5 ml/hr result in 2.3 and 3.14 fold increases in the maximum homatropine throughput respectively, and this resulted in a much larger accessible area of operation with greater than 99% purity in both offtakes on the triangle diagram.

In order to confirm the model results, experimental data was collected using the Vortex Stabilized Electrophoresis Apparatus with a modified experimental setup and the RO filtration system installed. The results of adding the reverse osmosis were not as positive as expected. The single piston HPLC pump caused a violent oscillation in the counterflow rate in the sections above the feed, leading to significant levels of dispersion in these regions, which prevented meaningful data from being gleaned in the top half of the apparatus. The bottom half of the chamber did show improvement, as the level of
tailing was kept the same even though the throughput was increased by more than 2 fold. In order to prevent this dispersion from happening, a higher quality HPLC pump was needed, but no pump that had a constant inlet flowrate was found that would reach the high pressures needed.

The other problem that occurred was the permeability of the RO membrane to the homatropine enantiomers. Due to the high pressure differential that was maintained across the RO system, some of the homatropine was forced through the membrane, resulting in between a 28 and 35% loss of product in the permeate stream. This could possibly be solved using different membrane chemistry or by using a different method of removing fluid from the section II/III boundary in the future.

The addition of these gradient techniques to the TMB electrophoresis process showed some significant benefits in maximum throughput demonstrating the large reductions in tailing possible. The modeling work performed showed large improvements of up to 7.66 fold using a step electric field gradient and 3.14 fold using a filtration induced counterflow velocity gradient. Though both of the homatropine enantiomer separations were plagued by problems with the membranes used in the processes, the fundamental theory behind the gradient concepts are sound, and this was shown conclusively using bovine proteins, as a 63% increase in throughput was observed with the addition of a +/- 10% step gradient in the electric field.